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Functional characterization of the 'PBX interacting protein' (HPIP) in normal and malignant human haematopoiesis.

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Funktionelle Charakterisierung des ‘PBX interagierenden Proteins’ (HPIP) in der normalen und malignen humanen Hämatopoese

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Dedicated to

My parents

Gagan, Ankur, Kashvi, Gulraj, Aman

And my loving husband

Emmanuel

Table of contents

3.2. Methods

1. Introduction

1.1. Haematopoiesis and development of blood cellular components

1.1.1. Human haematopoiesis

Haematopoiesis is the development of the blood cellular components. Vertebrate haematopoiesis occurs in two phases: The first phase is a transient embryonic (“primitive”) phase and this phase is followed by the definitive (“adult”) phase. These two phases of vertebrate haematopoiesis differ in their sites of blood cell production, the timing of haematopoiesis, the morphology of the cells produced, and even the type of globin genes used in the red blood cells (Fig.1.1.1).

Human haematopoiesis begins in the yolk sac during the first weeks of embryonic development. The embryonic phase of haematopoiesis is used to initiate the cell circulation that provides the embryo with its initial blood cells. This also helps to begin the flow of cells with its capillary network to the yolk sac. A dense population of blood cells is present and niched onto the ventral side of the aortic endothelium in human embryo. The studies in mammalian embryos demonstrate that the first complex multilineage lymphopoietic progenitors and HSC activities are autonomously generated within the intraembryonic para-aortic splanchnopleura /aorta–gonad–mesonephros region (PAS/AGM region) in the absence of the yolk sac.[1] This population of cells is specific for surface antigens and expression of genes that generally characterize the primitive progenitors.[2-4]

These intra-embryonic precursors have been implicated to play a fundamental role in the migration of the liver, enterprising of human definitive hematopoiesis[5,6] and initiation of a haematopoietic cell differentiation program.[7] The definitive phase of haematopoiesis is used to generate more differentiated and effecter cell types that loose the specific progenitor surface antigens with several courses of somatic cell divisions and differentiation.

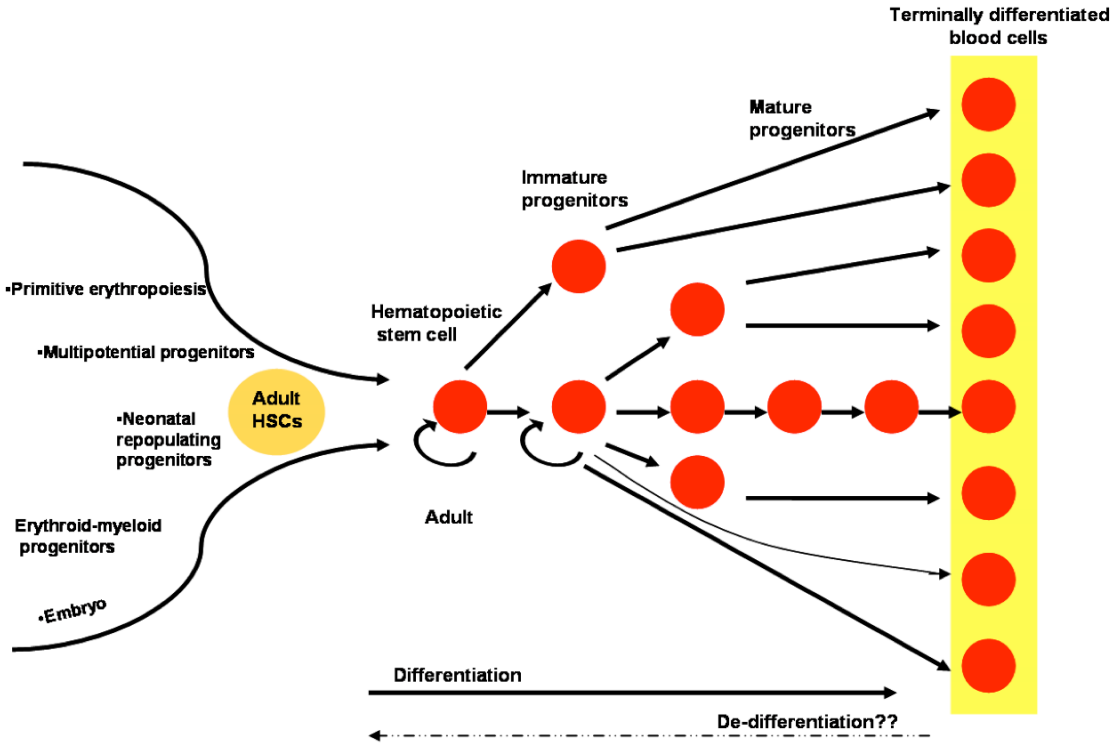

Figure 1.1.1: The figure illustrates the mammalian embryonic haematopoietic development and the adult haematopoietic hierarchy[1] (figure adapted from Dzierzak E *et al*, 2002). The progressive appearance of composite activities (left) leads to the appearance of HSCs with a long-term multilineage repopulation capacity of the entire haematopoietic system. The adult haematopoietic hierarchy (right), shows all differentiated haematopoietic lineages arising from HSCs. These HSCs self-renew (shown in the blue box) and differentiate in a unidirectional manner in a normal state individual. However, recent data in several experimental systems suggested that small dedifferentiation steps might occur in stem cells crossing lineage boundaries[8,9]

This phase of haematopoiesis also provides the pool of the stem cells with amazing self renewal capacity that lasts for the lifetime of the individual.[1] Different functionally specialized mature blood cells are derived from a common primitive progenitor cell. In haematopoietic system this population is called the stem cells (HSCs). It has been estimated that there is approximately 1 stem cell per 10^5 bone marrow cells.[10]

These stem cells represent a self-renewing population of cells (immature progenitors) and a potential to differentiate to become committed cells (mature progenitor), and finally to a short term lived and definitive effecter blood cell. HSCs self-renew and differentiate in a unidirectional manner in a normal state individual.

Recent data in several experimental systems has shown that small dedifferentiation steps can occur in stem cells and the lineage boundaries can be crossed.[8,9]

1.1.2. Classification of human haematopoietic stem cell

Human haematopoietic stem cells (hHSCs) are rare small mononuclear cells that tend to be noncycling or to have long cell cycles. They divide to form more hHSCs (self-generation or symmetric division) or commit to form the different haematopoietic lineages (asymmetric division)[11-16] Asymmetric cell division (ACD) in HSCs has been reported to make HSCs fate decisions. These immature progenitors are committing to lymphocyte progenitor formation (common lymphoid progenitors, CLPs) or to the formation of myeloid progenitors (common myeloid progenitors, CMPs). CMPs are large blast cells that can then form megakaryocyte-erythroid progenitors (MEPs) committed to the formation of erythroid and megakaryocytic cell lineages. CMPs can also form more restricted granulocyte-macrophage progenitors (GMPs) that are able to generate granulocytic, macrophage, and eosinophil progenitors. These lineage-restricted cells can generate respective mature lineage populations. [17-20]

In the haematopoietic system, the pluripotent HSC resides at the top of the haematopoietic hierarchy. Pluripotent stem cells are the descendants of totipotent cells and can differentiate into nearly all cells[21] i.e. the cells derived from any of the three germ layers (ectoderm, endoderm and mesoderm). A primitive haematopoietic cell that gives rise to erythrocytes, neutrophils, basophils, eosinophils, platelets, mast cells, monocytes, tissue macrophages, osteoclasts, and the T and B lymphocytes is coined as a “multipotential haematopoietic stem cell”. Because of the very short life span of most effecter-cells, mature blood cell production is an ongoing process, with the production of 1.5×10^6 blood cells every second in an adult human. This high turnover rate necessitates some homeostatic control mechanisms; the primary level of which resides with the HSCs.

Stem cells undergo two types of cell division: Symmetric and asymmetric. Symmetric division gives rise to two identical daughter cells both gifted with stem cell properties. Asymmetric division, produces two cells with one as stem cell and the second one as a progenitor cell with limited self-renewal potential.[22] In an alternative theory, the stem cells remain undifferentiated due to environmental prompt in their particular niche. Differentiation occurs only when they leave that niche or no longer receive environmental signals. Studies in Drosophila germarium have identified the signals dpp and adherens junctions that prevent germarium stem cells from differentiating.[23] [24]

Homeostasis of haematopoietic stem cells is a tightly regulated process, controlled by intrinsic and extrinsic signals. Although a variety of molecules involved in HSC maintenance and self-renewal are known, it remains unclear how robust HSC homeostasis is achieved. There is an enormous proliferative and developmental capacity of the more committed multipotent, oligo-potent and lineage-restricted progenitor cells within the haematopoietic hierarchy. A significant degree of homeostatic control of mature blood cells is also mediated at the level of these progenitors.[25]

1.1.3. The haematopoietic colony forming cell (CFC)

A single cell from the bone marrow is capable of forming many different kinds of colonies composed of erythrocytes (E), granulocytes (G), and platelet precursors. This cell is called the colony forming unit (CFU). This type of cell has also been called the CFU-GEMM (multipotent stem cell), the colony-forming unit for granulocytes, erythrocytes, macrophages, and megakaryocytes (Fig.1.1.3) and is able to self renew.

The CFU that can reside in the spleen and form a colony are called CFU-S. The colony-forming unit–spleen (CFU–S) depends on the ability of infused bone marrow cells to give rise to clones of maturing haematopoietic cells in the spleens of irradiated mice after 8 to 12 days. It is used to measure more mature progenitor or Transit Amplifying Cells rather than stem cells.[26] From HSC to mature blood

cells, extensive proliferation and expansion occurs that results in the production of millions of blood cells. Multi-potential progenitors (e.g. CFU-GEMM) and lineage-committed

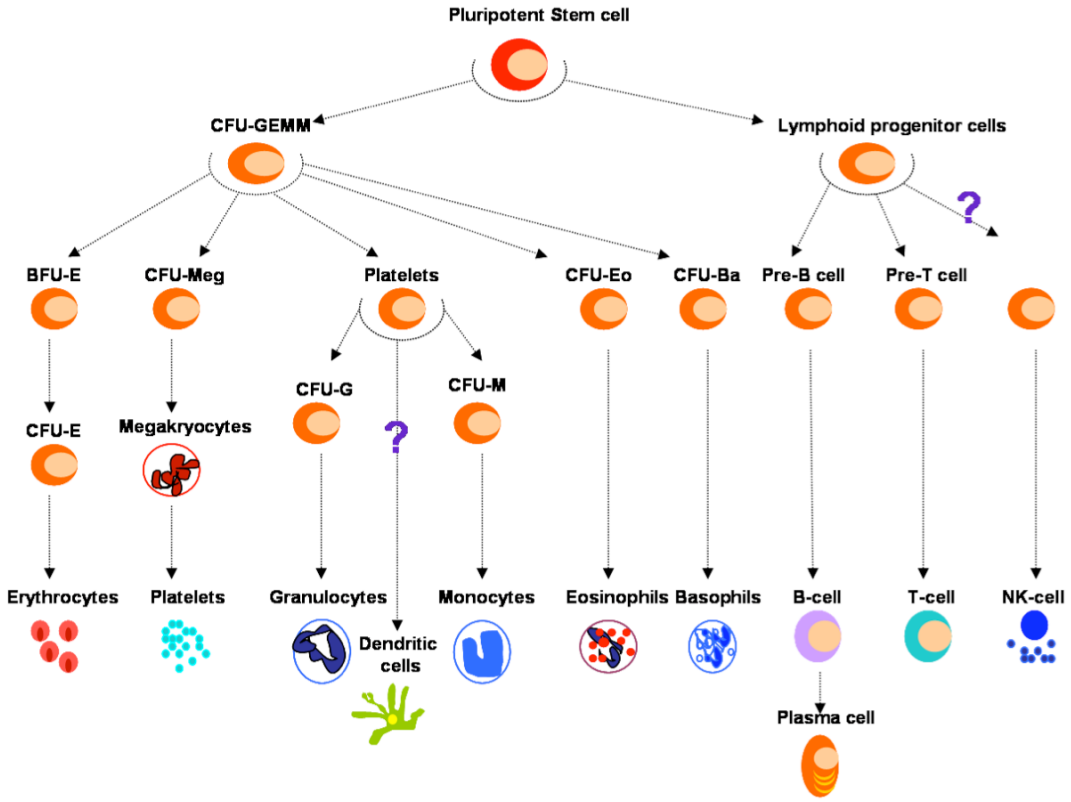

Figure 1.1.3: Hierarchal presentation of colony forming cells in hHSCs. The above figure illustrates the nature of colony forming cells in human haematopoiesis. Stem cell division and differentiation is a procedure that continuously takes place to replenish the various lymphoid, myeloid and erythroid-megakaryocyte lineages and also to maintain a small pool of HSC with the self-renewal capacity that is capable of carrying on haematopoiesis. From HSC to mature blood cells, extensive proliferation and expansion occurs that results in the production of millions of blood cells. Multi-potential progenitors (e.g. CFU-GEMM) and lineage-committed progenitors (e.g. CFU-E, CFU-GM), representing various stages along the differentiation pathway with various differentiation and proliferation potentials, can be identified by *in vitro* assays and by the expression of known surface antigens.

progenitors (e.g. CFU-E, CFU-GM), representing various stages along the differentiation pathway, with various differentiation and proliferation potentials, can be identified by *in vitro* assays and by the expression of known surface antigens.

The first multi-potential haematopoietic stem cell is the CFU-M, L. The CFU-M, L gives rise to the CFU-S and the several lymphocytic stem cell types. The immediate progeny of the CFU-S, however, are lineage- restricted stem cells. Each can produce only one type of cell in addition to renewing itself. The BFU-E (burst-forming unit, erythroid), is a lineage-restricted stem cell formed from the CFU-S. The above CFUs are based on the lineage in which they differentiate after plating in methylcellulose culture. The colony-forming unit–spleen (CFU–S) was the basis of an *in vivo* clonal colony formation, which depends on the ability of infused bone marrow cells to give rise to clones of maturing haematopoietic cells in the spleens of irradiated mice after 8 to 12 days. It is considered to be a measure of more mature progenitor or Transit Amplifying Cells rather than stem cells.

1.1.4. The haematopoietic inductive microenvironments.

Regions of determination of colony forming units (CFUs) such as spleen and bone marrow are referred to as haematopoietic inductive microenvironments (HIMs). In early blood cell formation (in the mesoderm surrounding the mammalian yolk sac), the endothelial cells of the blood islands appear to be heterogeneous HIMs, inducing the stem cells to form different blood and lymphocyte lineages.[27] Haematopoietic stem cells require the BM microenvironment, which regulates their migration, proliferation, and differentiation in order to maintain active haematopoiesis throughout life.[28,29]

1.1.5. Homing, engraftment and repopulation of hHSCs

Homing is a swift process in which circulating haematopoietic cells actively cross the blood/BM endothelium barrier and lodge transiently in the BM compartment by activation of adhesion interactions prior to their proliferation. Stem cells normally home to bone marrow, but can also migrate to spleen in case of alarm situations and do not require cell division, while engraftment does.

Many cell types, including long-term repopulating stem cells, expressing $hCD34^{+}CD38^{-}$ surface markers, short-term repopulating progenitors, expressing

hCD34^{+}CD38^{+} surface markers and mature, specialized T cells and neutrophils, can home to the BM. [30,31] The only stem cells, which can home to their endosteal niches, initiate long-term repopulation. The murine BM homing cells, which successfully adhere to the recipient stroma, are viable due to protection from apoptosis. Lineage negative immature cells (Lin^{-}) demonstrate stronger adhesion properties (30-fold) than lineage positive cells [32,33].

Engraftment and repopulation: Short-term engraftment (ranging from weeks to a few months) is initiated by differentiating progenitors. Durable long-term multilineage engraftment (many months in mice, and years in patients) is carried out by stem cells following their unique homing to their specialized niches.[34] Growth factors play an important role in initiating signal transduction pathways, altering transcription factors; these in turn activate genes determining the differentiation of blood cells. Prestimulation of human cord blood (CB) CD34^{+} cells with stem cell growth factor (SCF) induces surface CXC chemokine receptor 4 (CXCR4; CD184) expression and the appearance of cycling G_1 hCD34^{+}CD38^{+} cells (short term repopulating cells). This in turn creates an increased production of the proteolytic enzyme matrix metalloproteinase 9 (MMP9) and stromal cell derived factor-1(SDF-1) directed migration and homing to the bone marrow and spleen of NOD/SCID mice that have received the transplant .[35-37]

1.1.6. Sources of haematopoietic stem cells

Bone Marrow: The classic source of HSCs is the bone marrow. About 1 in every 100,000 cells in the marrow is a long-term, blood-forming stem cell; other cells present include stromal cells, stromal stem cells, blood progenitor cells, and mature and maturing white and red blood cells.

Peripheral Blood: For clinical transplantation of human HSCs, cells are preferably harvested from peripheral circulating blood. These are essential CD34^{+} enriched population of cells.

Umbilical cord blood cells: The blood within the umbilical cord, known as cord blood, is a rich and readily available source of primitive, undifferentiated stem

cells (of type CD34$^+$ and CD38$^-$). These cord blood cells can be used for bone marrow transplant. Embryonic stem cells: Embryonic Stem (ES) cells are pluripotent stem cells. They are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm.

1.1.7. Human cord blood CD34$^+$ cells

The majority of human cells with repopulating cell activities have been identified as CD34$^+$ cells. The hCD34 over expression experiments in haematopoietic cells have indicated its role in cell adhesion and inhibition of haematopoiesis. Haematopoiesis in CD34 knockout mice appears unaffected; however knock-out embryos contain 2-3 times fewer CFUs than normal controls.[38] Enriched human CB CD34$^+$CD38$^-$ cells, home successfully to the murine BM and spleen. They show long term repopulation capacity and have homing capabilities. The CD34$^+$ human bone marrow cells contain the vast majority of long term-HSCs, as well as primitive myeloid and lymphoid progenitors.[39]

1.2. Developmental pathways in human haematopoiesis

1.2.1. Long term-HSCs in xenotransplant models

The long-term reconstitution potential of human HSCs has been described in SCID mouse repopulating assays. In the most advanced xenotransplant models by utilizing the Rag2 and IL-2R$^{\gamma}$ double-deficient mouse[40] or the NOD/SCID IL2R$^{\gamma}$ deficient mouse.[41] The CD34$^+$CD38$^-$ or the CD34$^+$CD90$^+$ fractions of human bone marrow and cord blood have been shown to reconstitute all human hematolymphoid lineage cells for a long term, indicating that these CD34$^+$ fractions contains normal human HSCs. An injection of at least 1000 cells of the human CD34$^+$CD38$^-$ bone marrow cells is required to obtain multi-lineage, long-term reconstitution in xenotransplant models. The percent of hCD34$^+$CD38$^-$ or hCD34$^+$CD90$^+$ cells that are multipotent, long-term HSCs is still difficult to determine in a engraft model.

1.2.2. The subsets of human lymphoid and myeloid progenitor.

The existence of lymphoid-committed progenitors in the human bone marrow with the $hCD34^+CD38^+CD45RA^+CD10^+$ phenotype has been reported.[42] In human cord blood, common lymphoid progenitors (CLP) activity is found in the $CD7^+$ fraction of $CD34^+CD38^-CD45RA^+$ population. The $hCD34^+CD38^-CD45RA^+CD7^+$ population do not exist in the adult bone marrow.[43] IL-7Rα, a critical marker for the murine CLP, is expressed in human $hCD10^+$ CLPs in the bone marrow, but not in $hCD7^+$ CLPs in the cord blood. These data suggest that the CLP phenotype and the requirement of IL-7 signaling might have changed during the human ontogeny. In the myelo-erythroid pathway, CMP, GMP and MEP subsets can be isolated from the $hCD34^+CD38^+$ fraction in both the bone marrow and cord blood (Manz et al., 2002). All these fractions of cells are negative for the early lymphoid markers hCD10, hCD7 or hIL-7Rα. These myelo-erythroid progenitors can be prospectively isolated according to the expression of hCD45RA and hIL-3Rα. $CD45RA^-hIL\text{-}3R\alpha^{lo}$ (CMPs), $hCD45RA^+hIL\text{-}3R\alpha^{lo}$ (GMPs) and $hCD45RA^-hIL\text{-}3R\alpha^-$ (MEPs) efficiently form distinct myelo-erythroid colony types according to their definitions (Fig.1.1.2). CMPs give rise to MEPs and GMPs in vitro, and a significant proportion of CMPs possess clonal GM and MegE potentials.[44] Thus, the hierarchical myeloid progenitor relationships demonstrated in mice is well preserved in human haematopoiesis.

Phenotypic comparisons between mouse and human subsets show that CD34, a marker positive only for murine CMPs and GMPs, is uniformly expressed on all three human subsets, and that the FcγRII/III (CD16/CD32), marking murine CMPs and GMPs, was not detectable in any of the human myeloid progenitors [44] All haemato/lymphoid progenitors develop from $CD34^+CD38^-$ HSC population [41]but themselves have no self-renewal activity in xenogenic transplantation models [45], indicating that they are downstream of human LT-HSCs in both the bone marrow and the cord blood.

Flt3 shows a significant difference in its distribution in human and mouse hematopoiesis. This suggests a critical role of Flt3 signaling in hematopoietic

development in humans. hFlt3 is expressed in leukemic blasts in most cases with acute myelogenous leukemia (AML)[46,47] (Carow et al., 1996; Rosnet et al., 1996), and FLT3 is one of the most frequently mutated genes in AML.[48,49] The signal from FLT3 mutations should be controlled under the regulation of normal Flt3

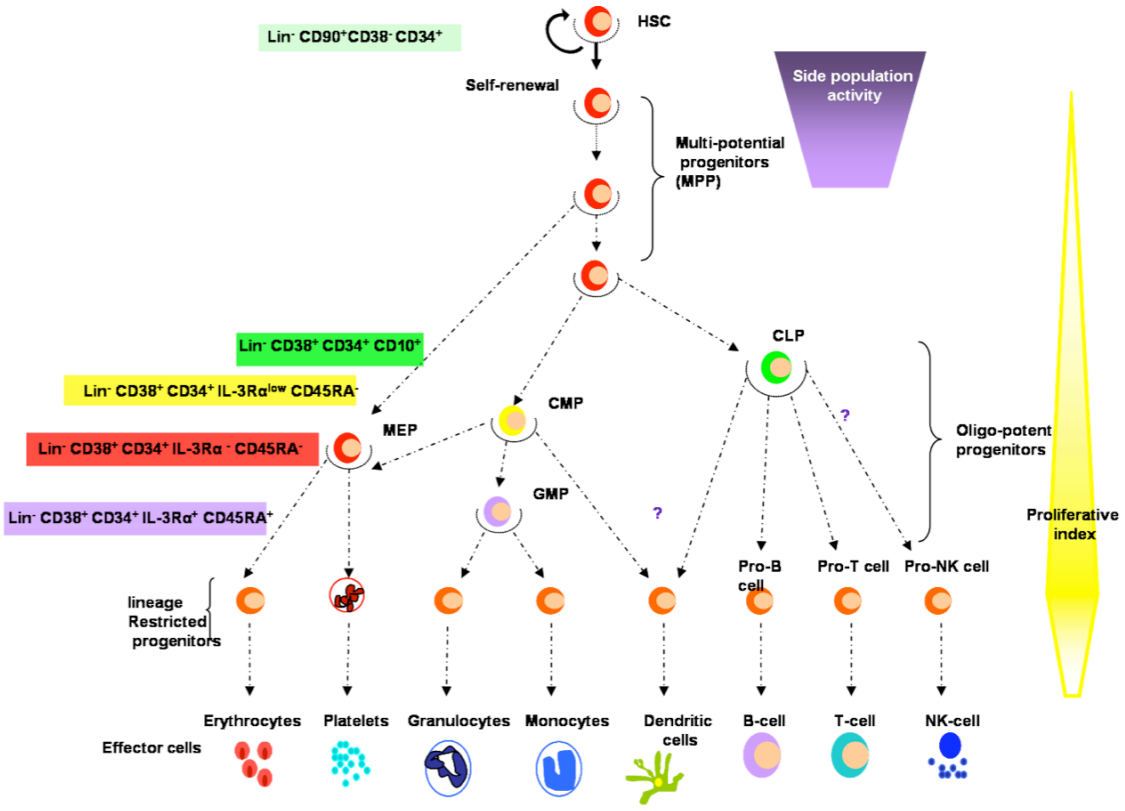

Figure 1.2.2: A model of the haematopoietic developmental hierarchy. Self-renewing HSCs reside at the top of the hierarchy, thereby giving rise to a number of multipotent progenitors. Multipotent progenitors give rise to oligo-potent progenitors including the CLP, which gives rise to mature B lymphocytes, T lymphocytes, and natural killer (NK) cells. The common myeloid progenitor (CMP) gives rise to granulocyte-macrophage progenitors, which differentiate into monocytes/macrophages and granulocytes, and megakaryocyte/erythrocyte progenitors, which differentiate into megakaryocytes/platelets and erythrocytes. The cell surface phenotype of many of these cell types is shown for the murine and human systems [50](Figure adapted from Bryder D et al., 2006).

expression machinery, signaling from FLT3 mutations should involve HSCs and GMPs, both of which are critical targets for leukemic transformation in mouse AML models.[51-54] Thus, special considerations are required in utilizing mouse models to understand the role of FLT3 mutations in human leukemogenesis.

Further phenotypic and functional characterization of human hematopoietic cells should be performed by utilizing improved efficient xenotransplant models. These approaches will eventually help to identify LSCs that should be a critical cellular target in leukemia treatment.[55]

1.2.2. Lineage-Bias in HSCs

Using limiting dilution strategies combined with other streamlined experimental and statistical methods for examining HSCs at the clonal level, HSCs fall into three distinct lineage-biased clusters.[56-58] These are quantitatively defined by the ratio "ρ" of lymphoid to myeloid cells that the HSC generates upon differentiation (which makes ñ a peripheral predictor for the clonal association of a reconstituted haematopoietic system). Balanced HSCs repopulate peripheral white blood cells in the same ratio of myeloid to lymphoid cells as seen in unmanipulated mice (on average about 15% myeloid and 85% lymphoid cells, or 3≤ñ≤10), in the NOD/SCID mice. Myeloid-biased (My-bi) HSC give rise to too few lymphocytes resulting in ratios 0<ρ<3, whereas lymphoid-biased (Ly-bi) HSC generate too few myeloid cells, which results in lymphoid-to-myeloid ratios of 10<ρ<00. All three types are normal HSCs in the sense that they have self-renewal capacity and can regenerate all haematopoietic lineages (pluripotency).

The lineage-bias is preserved through multiple rounds of serial transplantation. Balanced HSC self-renew to give rise to daughter HSC that are also balanced, My-bi HSC give rise to My-bi daughter HSC, and Ly-bi produce Ly-bi daughter HSC. There is no precursor-progeny relationship between the three types of HSC and they do not represent stages of differentiation. Rather, these are three classes of HSC, each with an epigenetically-fixed differentiation program.

1.3. Extrinsic and intrinsic factors effecting haematopoiesis

1.3.1. Cytokines

The production of haematopoietic cells is under the tight control of a group of haematopoietic cytokines. These cytokines are glycoprotein that are normally present in the human blood circulation in very low (pico-molar) doses. Some of the known cytokines that were validated by trail in humans are: erythropoietin

(EPO), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and interleukin-11(IL-11).[59,60]

Some of the cytokines for example, G-CSF, granulocyte-macrophage colony GM-CSF, interleukin-3 (IL-3), monocytic colony stimulating factor (MCSF), SCF and interleukin-6 (IL-6) are found to have proliferative effects on granulocyte colony formation[61] (Fig.1.3.1) Individual cytokines can be lineage specific or can regulate cells in multiple lineages, and for some cell types, such as stem cells or megakaryocyte progenitors, the simultaneous action of multiple cytokines is required for proliferative responses. Cytokines exert their action through high-affinity receptors on the cell surface that are linked to pathways of cellular activation, commitment, differentiation, survival, proliferation and differentiation.

Cross-linking of receptor subunits on the outside of the cell wall leads to adjoining of kinases associated with the intracellular receptor tails, either as intrinsic activities, or because of pre-association of secondary kinase molecules. This intracellular association of signaling molecules results in phosphorylation of tyrosine residues in the receptor tail and binding of further signaling molecules that have phospho-tyrosine-binding domains. Several aspects of the downstream intracellular pathways of cytokines are similar, because different activated receptor cytoplasmic domains often bind a common signaling molecule or family of signaling molecules. Thus stoichiometry and rate of reaction are important regulatory influences that allow discrimination between signaling processes with different outcomes (Molecular cell biology. Lodish, Harvey F. 5. ed.)

1.3.2. Cooperative Interaction of Cytokines

For efficient *in vitro* proliferation and differentiation, pluripotent and multi-potent progenitor cells require a combination of cytokines [e.g. SCF, IL-1, IL-3, IL-6, GM-CSF, and colony stimulating factor-1, (CSF-1)]. Immature haematopoietic cells have been shown to co-express a number of different lineage specific receptors at low levels. As these immature cells develop, they lose receptors for

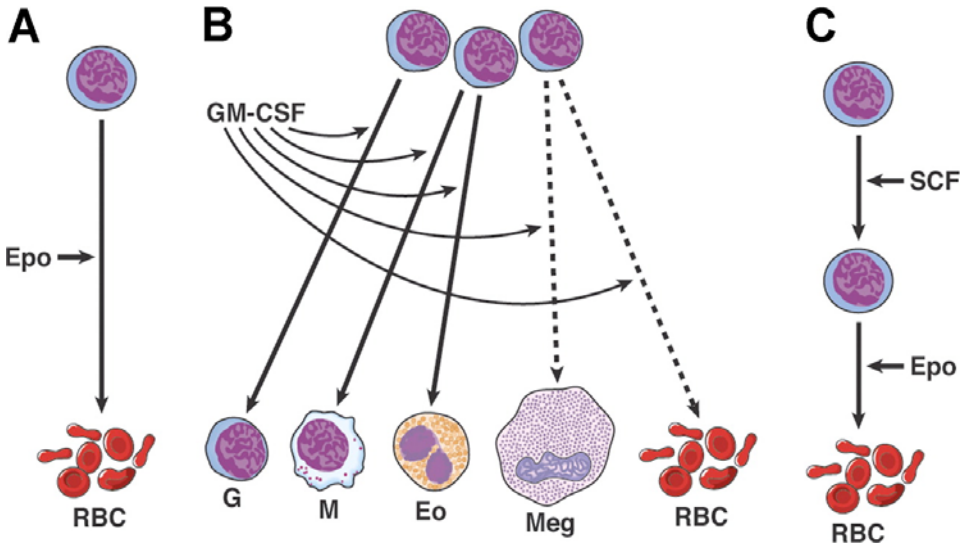

Figure 1.3.1: The above figure illustrates the three types of action of haematopoietic cytokines [62](Figure taken from Metcalf, D. Blood 2008). (A) Lineage restricted. (B) Action on multiple lineages; broken line shows actions only at high concentrations. (C) Sequential actions; SCF acts on stem and early erythroid progenitors, while EPO acts on more mature precursors. The notion of sequential actions was later found to be incorrect.

some cytokines e.g. SCF and IL-3, while retaining receptors for later acting cytokines such as CSF-1.

Eventually immature cells reach the stage of the committed progenitor cell, where their proliferation and differentiation are along one particular lineage, guided by the lineage-restricted cytokines. Synergy occurs between some late-acting lineages restricted cytokines such as CSF-1, EPO and G-CSF, with early acting cytokines such as SCF and IL-3, in stimulating the proliferation and differentiation of multi-potent cells. This provides a mechanism by which the tightly regulated changes in the level of a late acting cytokine can be coupled to the channelling of multi-potent progenitor cells into a lineage to satisfy the demand for differentiated cells. The underlying mechanism of synergy may lie at the level of either the receptors or at the level of post receptor signaling pathways (Molecular cell biology. Lodish, Harvey F. 5. ed.)

The action of cytokines may be autocrine, paracrine, and endocrine. Cytokines are critical to the development and functioning of both the innate and adaptive immune response, although not limited to just the immune system. They are often secreted by immune cells that have encountered a pathogen, thereby activating

and recruiting further immune cells to increase the system's response to the pathogen. Cytokines are also involved in several developmental processes during embryogenesis.

1.3.3. Tyrosine kinase receptor and cell signalling

Many cytokines such as CSF-1, SCF and FL are homodimeric that share some sequence homology and are structurally similar to one another. These three cytokines/ growth factors have complex patterns of expression due to alternative mRNA splicing. This allows them to be expressed as either membrane-spanning, cell-surface or secreted glycoproteins. All three growth factors are widely expressed in all tissues and two of them, SCF and CSF-1, also affect non-haematopoietic as well as haematopoietic cells.

The receptors for the above cytokines are members of the platelet derived growth factor (PDGF) receptor family. Each receptor possesses an extra-cellular domain, consisting of a five immunoglobulin (Ig) like repeat. These repeats are: a heavily glycosylated with N-linked sugars, a trans-membrane domain, intra-cellular domains containing a juxta-membrane region, a src-related tyrosine kinase domain that is interrupted by a kinase insert domain, and a carboxy-terminal tail. The three amino terminal Ig-like domains incorporate the ligand binding domains of the SCF and CSF-1 receptors. Binding of this type of dimeric receptor to its cognate ligand stabilizes the non-covalent association between the two chains of the receptor at the cell surface and permits the trans-phosphorylation of the intra-cellular domain of one chain by the other.

Tyrosine phosphorylation in response to cytokine binding is not restricted to those proteins that are stably associated with the receptor. Regions containing tyrosines that are phosphorylated as a consequence of receptor activation act as docking sites for src-homology region 2 (SH2) domains of signaling and adaptor proteins. These proteins in turn may interact with plasma membrane associated proteins. An example is the association of recruited Grb2/Sos with Ras, which leads to their activation. The associated proteins may themselves become tyrosine phosphorylated. Many of the signaling pathways activated by SCF, CSF-

1 and flat-3 ligand (FL) receptors, including the Ras/Raf-mitogen activated protein kinase cascade, the janus kinase (JAK)/ signal transducers and activators of transcription (STATs) pathway, Src family members and phosphatidylinositol-3-kinase (PI3K), are shared.

All three receptors are likely to exhibit ligand induced, Cbl-mediated decreases in receptor expression. The SCF and CSF-1 receptors are encoded by the proto-oncogenes c-kit and c-fms, respectively. The oncogenes derived from these two proto-oncogenes are present in mutated forms in retroviruses that cause sarcoma in cats. The mutations in the receptor genes cause constitutive activation of the receptors in the absence of cytokines, thus contributing to unregulated cell proliferation.

1.3.4. The role of transcription factors

Nuclear transcription factors are essential for stem cell lineage commitment. HSCs express a great diversity of transcripts, including a wide range of genes originally believed to be restricted to more mature and lineage-committed cell types. The single-cell polymerase chain reaction strategies had suggested that such loose transcription of lineage-associated transcripts was necessary to prime primitive progenitor cells for differentiation toward downstream fates.[63]

An alternative interpretation is that stem cells possess global transcriptional accessibility and that it is the step-wise restriction of locus accessibility that underlies lineage specification.[64,65] Cell fate can also be switched from one committed cell type to another on over expression of IL2R or GM-CSF receptors [17,18] GATA-1[66] or C/EBP /ß.[67] An additional mechanism of lineage specification has been revealed which attributes to the ablation of the transcription factors Pax5 or GATA-1 in lineage-restricted progenitors. This was sufficient to despecify their B-cell and erythroid fate, respectively, and allow a multilineage developmental potential.

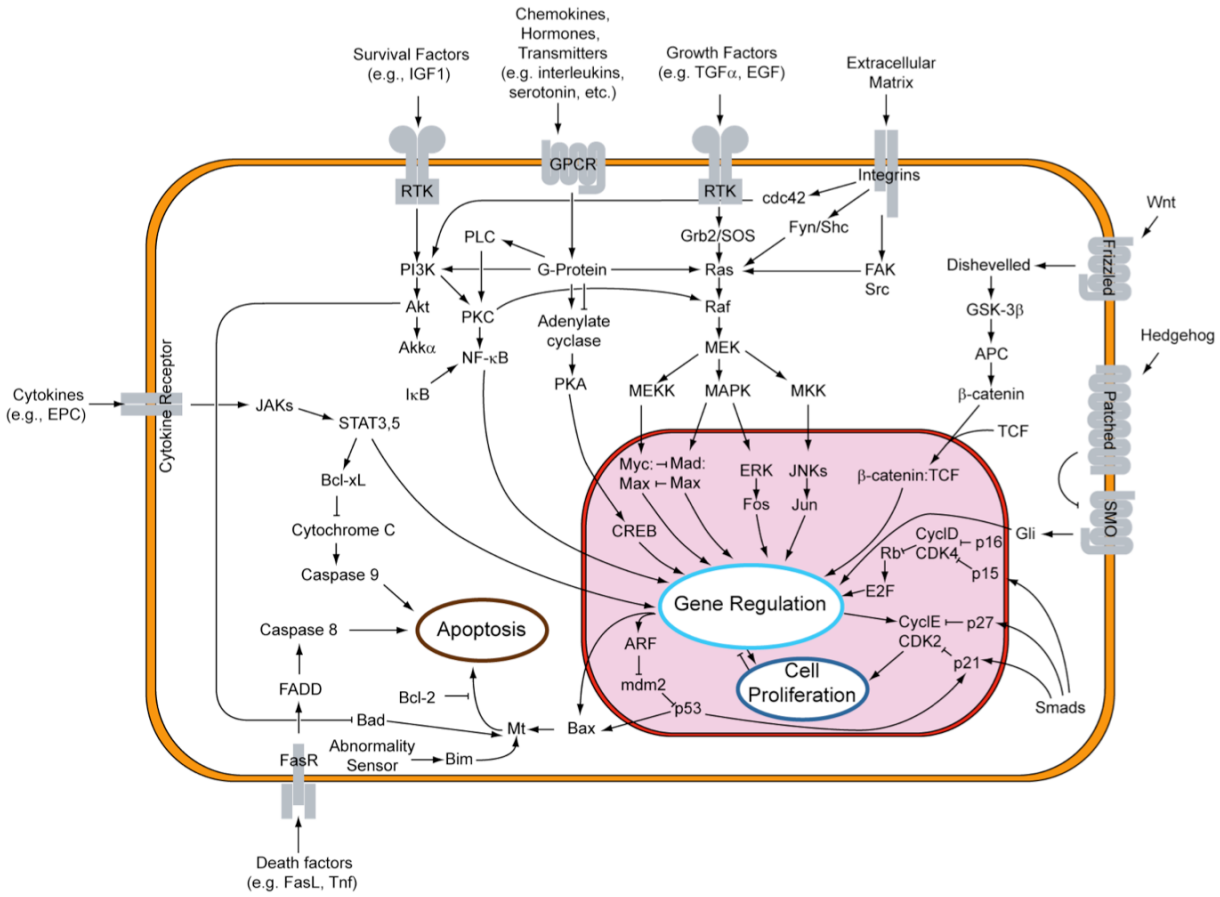

Figure 1.3.4a: A complex signal transduction pathway is shown in the picture above. This pathway involves changes of protein-protein interactions inside the cell, induced by an external signal (figure taken from Molecular cell biology. Lodish, Harvey 5 ed).The production of mature blood cells from HSC requires three distinct genetic programs. These include: (a) the specification of HSC, (b) their self-renewal and c)

their commitment/ proliferation/ differentiation. Most of the studies leading to the knowledge of genes involved in HSC genetic programs have been carried by assaying haematopoietic cells from animals deficient for the gene of interest. More recently, expression profiling strategies have been used to determine genetic and molecular signatures of HSC.[68,69] Genes involved in deciding fate of HSCs during early embryogenesis include: SCL and Rbtn2/Lmo-2, which are necessary for primitive and definitive haematopoiesis.[70,71] GATA-2 and AML1 are specifically required for definitive haematopoiesis.[72]

Some other factors appear to be more lineage-specific in action such as GATA-3, Ikaros, PU.1, GATA-1, CBP, Atf4, c-myb, and E2A, and their absence affects specific haematopoietic lineages. [73-77] The genetic factors involved in regulating fetal liver HSC are: *Meis1*, which is highly expressed in fetal liver Sca-1 $^{+}$ Lin^{-} cells that are enriched for HSC activity[78] and *Hoxb4*, which causes *in vivo* and *ex vivo* expansion of HSC when constitutively expressed. [79,80] Hox proteins interact with another transcription factor Pbx1, which itself interacts with Meis1 and forms a trimeric nuclear complex which is involved in target gene regulation[81,82] (Fig.1.3.4a).

HSC self- renewal maintenance in adult BM is regulated by a different set of genes. A number of recent studies point out to nuclear factors such as the *Polycomb (PcG*) genes *Bmi-1* and *Rae-28*, *GATA-2* and *TEL* for potentially regulating this process. It has been observed that *Bmi-1* levels decline during haematopoietic development, and that *Bmi-1* deficient mice develop hypocellular BM and die at less than 2 months of age. This led to the speculation that Bmi-1 is involved in maintenance of the HSC pool.[83] *Rae-28*, a known nuclear partner of Bmi-1, also plays a crucial role in maintaining the activity of HSCs during fetal haematopoiesis. [84]

The zinc-finger transcription factor GATA-2, a member of GATA family, plays a critical role in maintaining the pool of multipotent progenitors and HSCs, both during embryogenesis and in the adult.[85] The zinc-finger transcription factors GATA-1 and its transcriptional cofactor called Friend of GATA-1 (FOG-1), have been found to be essential for erythroid and megakaryocytic differentiation.[86,87] PU.1 is a member of the Ets family of transcription factors and is essential in the development of cells of the monocytic, granulocytic and lymphoid lineages.[88]

Many growth factors (CSF, GCSF, GMCSF, FLT3, TPO,IL-6, IL-3, IL-11 etc.) bind to receptors at the cell surface and stimulate cells to progress through the cell cycle and divide. Several of these receptors are kinases that start to phosphorylate themselves and other proteins when binding to a ligand. This phosphorylation can generate a binding site for a different protein and thus induce protein-protein interaction. In the figure, the ligand called epidermal growth factor (EGF) binds to

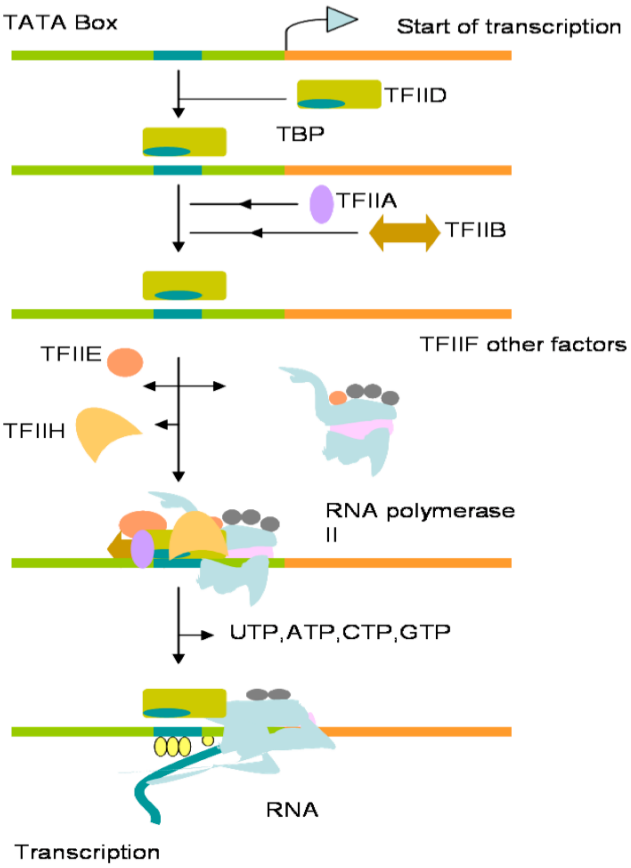

Figure 1.3.4b: Above figure is a simple illustration of basal transcription regulation mediated by general transcription factors (GTFs) in a eukarayotic cell (Figure adapted from Essential cell biology E-2). To begin transcription, eukaryotic RNA polymerase II requires the general transcription factors. These transcription factors are called TFIIA, TFIIB, and so on. (A) The promoter contains a DNA sequence called the TATA box, which are located 25 nucleotides away from the site where transcription is initiated. (B) The TATA box is recognized and bound by transcription factor TFIID, which then enables the adjacent binding of TFIIB. (C) For simplicity the DNA distortion produced by the binding of TFIID is not shown. (D) The rest of the general transcription factors as well as the RNA polymerase itself assemble at the promoter. (E) TFIIH uses ATP to pry apart the double helix at the transcription start point, allowing transcription to begin. TFIIH also phosphorylates RNA polymerase II, releasing it from the general factors so it can begin the elongation phase of transcription. As shown, the site of phosphorylation is a long polypeptide tail that extends from the polymerase molecule.

the receptor (called EGFR). This activates the receptor to phosphorylate itself. The phosphorylated receptor binds to an adaptor protein (GRB2), which couples the signal to further downstream signaling processes. One of the signal transduction

pathways that are activated is called the mitogen-activated protein kinase (MAPK) pathway.

The signal transduction component labelled as "MAPK" in the pathway was originally called "ERK," so the pathway is called the MAPK/ERK pathway. The MAPK protein is an enzyme, a protein kinase that can attach phosphate to target proteins such as the transcription factor MYC and, thus, alter gene transcription and, ultimately, cell cycle progression. Many cellular proteins are activated downstream of the growth factor receptors (such as EGFR) that initiate this signal transduction pathway (Fig.1.3.4b). Some signaling transduction pathways respond differently depending on the amount of signaling received by the cell. For instance, the hedgehog protein activates different genes, depending on the amount of hedgehog protein present. Complex multi-component signal transduction pathways provide opportunities for feedback, signal amplification, and interactions inside one cell between multiple signals and signaling pathways.

1.4. Role of Hox and non-homeobox genes in haematopoiesis

1.4.1. The structure of HOXB4 and its co factors.

The mammalian HOX genes are the master regulators of developmental processes such as embryogenesis and hematopoiesis. The complex arrangement, regulation and co-factor association of HOX has been an area of intense research, particularly in cancer biology. The mammalian class I homeobox (HOX) gene network consists of 39 genes organized in four clusters (AD) on four separate chromosomes (Human; 7p14-15, 17q21-22, 12q12-13 and 2q31-37 and Mouse; 6C2, 11B4, 15F2 and 2C3 respectively)

The canonical HOX gene consists of two exons with one intron that ranges in size from 200 bases to several kilobases. The 180 bp homeobox sequence is located within a GC-rich region of the second exon and encodes a 60 amino acid DNA-binding motif, the homeodomain. The best characterized co-factors of HOX are the mammalian pre-B cell leukaemia homeobox (Pbx)[89] and the myeloid

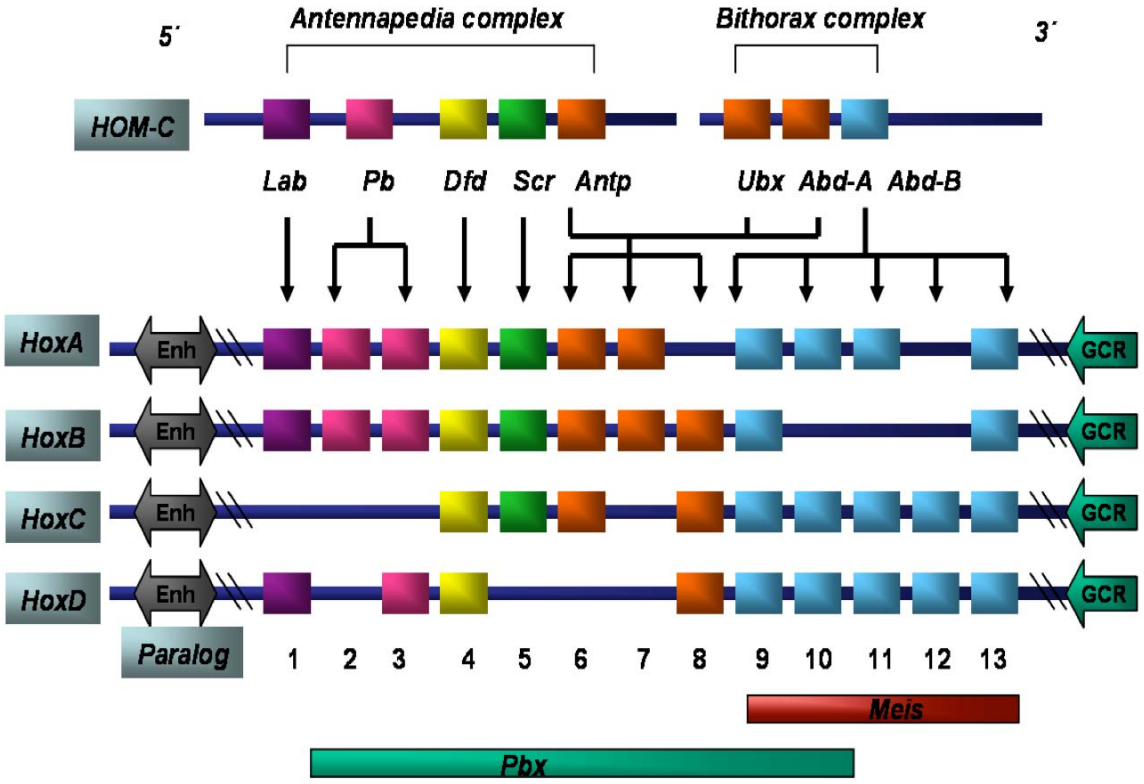

Figure 1.4.1: Schematic representation of conservation of the mammalian HOX gene network from *Drosophila Hom-C* depicting preferential binding of Pbx to paralog groups 1-10 and Meis to paralog groups 9-13 [90](Figure taken from McGonigle GJ *et al.*, 2008). Downstream Enh (Enhancer) sequences and upstream GCR (global control region) elements thought to control global expression of individual clusters are represented.

ecotropic viral insertion site (Meis)[91] family members (Fig.1.4.1). All of them are the members of the TALE (three amino acid loop extension) family of homeodomain proteins encoded by Class II non-clustered homeobox genes.[92]

The founding members of the two best characterised TALE subfamilies, namely Pbx1 and Meis1 interact preferentially with 3' or 5' Hox proteins respectively [93] trimeric Hox-Pbx-Meis complexes have also been reported [94] The. Pbx/Hox heterodimers bind to the consensus TGAT (T/G) NA (T/C) with the Hoxproteins binding to (T/G) NA (T/C) and the Pbx binding to the 5′ TGAT. The Meis1 consensus binding site (TGACAG) is followed by an AbdB-like Hox binding site TTA (C/T) GAC. It is predicted that Hox, Pbx and Meis1 proteins interact within

multiprotein complexes that have the highest level of specificity in terms of DNA binding and co-ordination of activation or repression events. [82]

1.4.2. The expression of HOX in haematopoietic cells

HOX genes are highly expressed in primary haematopoietic stem cells (HSCs) and in haematopoietic progenitors (HPCs) in a characteristic pattern. For example HOX genes such as HOXB4 or HOXA10 are highly expressed in primitive haematopoietic cells but silenced in more differentiated cells. Similar expression patterns have been observed in cells originating from both foetal and adult tissues and in an embryonic stem cell model of haematopoietic differentiation (References). Self-renewal of HSCs and HPCs may be HOX-dependent while inappropriate HOX expression may underlie leukaemia stem cell maintenance. [95-97]

Experimental murine transplantation models using retroviral gene transfer to over express the HOX gene of interest have provided detailed insight into the function of individual HOX genes: *HOXB3* over expression resulted in haematological anomalies that included reduced B and T-cell differentiation and a delayed increase in myeloid progenitor numbers.[98] *HOXA10* over expression also resulted in reduced B-cell differentiation but without affecting the T-cell compartment. In addition *HOXA10* over expression resulted in a marked increase in megakaryocyte blast colony-forming progenitor production at the expense of macrophage colony formation.[99,100]

The majority of *HOXA10* recipient mice succumbed to leukaemia after a long latency period (19-50 weeks). Perhaps the most surprising finding in this experimental system was that over expression of *HOXB4* did not induce overt haematological anomalies, but resulted in an enhanced HSC regeneration (~50-fold) compared to controls as demonstrated by serial transplantation. Increased HSC self-renewal is a common feature of several over expressed *HOX* genes; however lack of leukaemic transformation appears to be unique to HOXB4.[101]

1.4.3. The TALE homeodomain protein family PBX

PBX1 is a homeodomain protein that functions in complexes with other homeodomain-containing proteins to regulate gene expression during development and/or differentiation processes. PBX is a member of the PBC protein family. The human PBX1 protein was initially identified as the chromosome 1 participant of the t(1;19) translocation, which occurs in 25 % of paediatric pre-B cell acute lymphocytic leukaemia which creates a chimeric gene designated E2A-PBX1.[102]

The mechanism by which E2A-PBX1 causes leukaemia is still unclear. However, the structure of the fusion protein, in which the majority of PBX1, including the homeodomain, is fused to the transcriptional activation domain of suggests that the oncogenic properties of E2A-PBX1 result from inappropriate regulation of target genes, of which the expression during haematopoiesis is normally regulated by wild-type PBX proteins.[103,104] *In vitro* and *in vivo* data suggest that PBX functions in combination with heterologous homeodomain proteins, including class I HOX proteins. As HOX cofactors, PBC proteins improve HOX specificity due to the increased size of the cooperative binding site and the strength of DNA binding sites by different groups of HOX proteins.[89,93]

In addition, cooperative DNA binding with PBC proteins may act to change the regulatory signal of HOX proteins, from repressors to activators.[78] PBX proteins appear to function as part of large nucleoprotein complexes. The interactions within these complexes are probably decisive factors that allow the DNA binding proteins to discriminate among target regulatory elements. How these complexes are regulated during either early embryonic development or cellular differentiation of somatic cells to control gene expression is still unclear. Abramovich et al. (2000) speculated that the characterization of additional PBX-interacting proteins might shed light on the mechanism of PBX function, and specifically sought to identify novel cofactors or modifiers of PBX1.[105] Although the PBX homeodomain protein is thought to function as a transcription factor, its mechanism of action is still unknown.

1.4.4. The novel haematopoietic PBX-interacting protein (HPIP)

The identification of novel proteins which regulate human stem cells and early progenitor cell fate decisions is one of the major goals for experimental and clinical haematology. Recently, a novel human protein, the haematopoietic PBX-interacting protein (HPIP) that interacts with the homeobox gene and HOX co-factor PBX1 was identified.[105]

The new haematopoietic PBX-interacting protein (HPIP) was identified by a yeast two-hybrid screen of a fetal-liver haematopoietic cDNA library using PBX1 as bait. *HPIP* cDNA encodes a novel protein of 731 amino acid residues containing no homology to any known protein and has a calculated molecular mass of 80 kDa. *HPIP* is predicted to have a coiled-coil domain, suggesting that it interacts with other proteins. *HPIP* can bind to different members of the mammalian PBX family, inhibit the binding of PBX1/Hox complex to DNA and block the transcriptional activity of *E2A-PBX1*. The expression of *PBX1* and *HPIP* was characterized observed by reverse transcription-PCR analysis of RNA obtained from bone marrow: *HPIP* expression was detected in the $CD34^+$ fraction containing the haematopoietic progenitors and at lower levels in the $CD34^-$ matures cell population.

The same pattern was found for *PBX1*, indicating that *HPIP* and *PBX1* are co-expressed in the same haematopoietic compartment. The sub cellular location shows that *HPIP* has a complex sub cellular distribution. It is largely bound to the cytoskeleton, but has the potential ability to shuttle between the nucleus and the cytoplasm by mechanisms involved in nuclear import and export signals.[106] Haematopoietic PBX-interacting protein (HPIP) interacts both with ERalpha and with ERbeta, and increases ERalpha target gene expression through activation of MAPK and AKT and enhanced ERalpha phosphorylation.[107,108]

2. Aims of the study

The aim of the present study was to characterize HPIPs´ function in normal and malignant haematopoietic stem cells. For this purpose we used the constitutive over expression techniques, using a MSCV based retroviral vector system, and all the classic human haematopoietic stem cell assays. The constitutive expression of HPIP was generated in human umbilical cord blood derived $CD34^+$ Lin^- population of stem cells. Complex *in vitro* assays that analyze various stages of differentiation of the haematopoietic lineages (primary and secondary CFC assays for the detection of clonogenic progenitors and analysis of their proliferative capacity, LTC-IC assays for the detection of in vitro equivalents of HSC, MS5 assays for lymphoid development and megakaryocyte assays) as well as an *in vivo* models (NOD/SCID mouse model for the SRC frequency) were used to provide information about the function of HPIP in normal haematopoiesis. To characterize the relevant domains of HPIP, mutant forms of HPIP were generated and tested in the similar functional assays. Alterations in the transcriptome with constitutive expression of WT and mutant forms of HPIP were identified using cDNA micro array techniques. The cDNA micro array technique was used to identify and compare expression profiles of WT vs. mutant HPIP transduced hCB $CD34^+$ Lin^- cells. We also wanted to assess the role of potential collaborating genes with HPIP such as PBX1 and HOXB4, and by using double transduction experiments and then using the vectors stably expressing small hairpin RNA (siRNA) against the respective genes to knockdown their expression in K562 cell lines. The same experiments were performed on the primary CB cells to assess the affect of collaborating partners. In order to determine whether HPIP is differentially expressed in normal versus leukaemic cells or certain subtypes of leukaemia, expression of HPIP was analyzed in different molecularly and cytogenetically defined acute myeloid leukemias (AML) and acute lymphoblastic leukaemia (ALL) samples by using the cDNA micro array techniques.

3. Material and Methods

3.1. Materials

3.1.1. Buffers (All buffers were prepared in deionised water

3.1.1.1. Western Blot

1× TBS (Tris-buffered saline) 1L

4.84 g Tris base	1X TBS
1.60 g NaCl	20 mM Tris-Cl
80 ml Water.	136 mM NaCl

HCl (6N) added till pH = 7.5 (final volume 1L)

1x TBS-T (1L)

100ml 10×TBS	1X TBST
900 ml water	
1 ml Tween 20 (stirring)	0.1% w/v

Transfer buffer

1× Transfer buffer (1L)	1XTB
100 ml 10× Transfer buffer	25 mM Tris
700 ml water	
192 mM Glycine	
200 ml methanol	20% methanol

Ponceau Stain

0.5 g Ponceau Stain	0.5% w/v
1.0 ml glacial acetic acid	
1%v/v	

100 ml water

Blocking Solution

5 g skim milk powder 5% w/v

100 ml 1xTBS

3.1.1.2. Agarose gel electrophoresis

- *TAE buffer*

Tris-HCl (pH 8.2) 40 mM

Acetic Acid 20 mM

EDTA (pH 7.6) 2 mM

3.1.1.3. Stem cell sorting buffers

FACS buffer: (flow cytometry)

Phosphate buffered saline (PBS)

FBS 3%

Propidium Iodide 1 mg/ml

MACS buffer: (Magnetic associated cell sorting)

PBS (pH 7.4)

FBS 3%

EDTA 2 mM

3.1.2. Mediums

3.1.2.1. Serum free medium (SFM)

IMDM (with L-glutamine) supplemented with the following:

• BIT 9500 (BSA, insulin and transferrin) 20%

• 2-Mercaptoethanol 10^{-4} M

3.1.2.2. Haematopoietic cell washing medium

IMDM supplemented with 2% FBS was used for washing of haematopoietic cells. The same buffer was used as a medium for injection of cells in mice.

3.1.2.3. Lymphoid cell culture medium

Lymphoid cell culture medium was prepared with RPMI supplemented with 5% AB human serum and 10% FBS..

3.1.2.4. Maintenance/selection medium for feeder cells

Sl/Sl j-SF-tkneo j-IL-3-hytk cells

- The DMEM medium supplemented with geneticin 418 (0.8mg/ml), hygromycin (0.125mg/ml) and 10% FBS.

M2-10B4 j-GCSF-tkneo j-IL-3-hytk cells

- The RPMI medium supplemented with geneticin 418(0.4mg/ml), hygromycin (0.06mg/ml) and 10%FBS.

3.1.3. Mammalian cell lines

- ***Phoenix Ampho:*** Packaging cell line is used for transient, episomal stable, and library generation for retroviral gene transfer experiments (Stanford University, Medical Centre, and USA).
- ***PG13:*** PG13 is a mouse embryonic fibroblast used as a retroviral packaging cell line. It was purchased from ATCC. Introduction of retroviral vectors into PG13

cells results in the production of retrovirus virions capable of infecting cells from many species excluding mice.

- ***K562:*** An erythroleukemia cell line derived from a chronic myeloid leukaemia patient in blast crisis. K562 cells were purchased from ATCC.
- ***M2-10B4 j-GCSF-tkneo j-IL-3-hytk:*** Murine M2-10B4 fibroblasts engineered to produce high levels of both human granulocyte colony-stimulating factor (G-CSF) and interleukin-3 (IL-3; 190 and 4 ng/mll, respectively), referred henceforth as M2-10B4 G-CSF / IL-3, were provided courteously provided by Connie Eaves (Terry Fox Laboratory, Vancouver, Canada).
- ***Sl/Sl j-SF-tkneo j-IL-3-hytk:*** Sl/Sl fibroblasts engineered to produce high levels of soluble Steel factor (SF), with or without production of the transmembrane form of SF (60 and 4 ng/ ml, respectively), referred henceforth as Sl/Sl SF / IL-3, were provided kindly by Connie Eaves (Terry Fox Laboratory, Vancouver, Canada).
- ***MS-5:*** Mouse stromal cells established by irradiation of the adherent cells in long-term bone marrow cultures derived from C3H/HeNSlc strain mice.

3.1.4. The NOD-SCID Mice

The NOD/LtSz-*scid* strain was generated by crossing the *SCID* mutation from C.B-17- *SCID* mice onto the NOD background. C.B-17-*scid* mice lack functional T & B lymphocytes. The NOD strain mouse is an animal model of spontaneous autoimmune T-cell mediated insulin dependent diabetes mellitus (IDDM); however they have multiple defects in innate immunity. They are deficient in NK cell activity; display defects in myeloid development and function, and cannot generate either the classical or alternative pathways of haemolytic complement activation.

The NOD/LtSz-*scid* lacks an adaptive immune system; due to the absence of T cells, they do not develop autoimmune IDDM and remain insulitis- and diabetes free throughout life. However they carry the innate immune defects present in the parental NOD/Lt stock of mice.[109] NOD/SCID mice were bred from the breeding

pairs originally obtained from Taconic Bomholt, Denmark and maintained in the animal facility located at the GSF-Haematology, Munich. All animals were handled under sterile conditions and maintained under micro isolators. xenografts into immunodeficient NOD-SCID mice are the gold standard readout for human long-term repopulating hematopoietic cells.[110-112]

3.1.5. Mice related reagents and equipment

Sterile Syringes: BD Plastipak 1 ml syringe (BD Biosciences, Palo Alto, CA) for injection of cells in mice and Kendall Monoject 3 ml syringes (Tyco Healthcare, UK) for bone marrow flushing and plating of CFC.

Sterile needles: 0.5 x 25 mm for intra venous injection of cells in mice and 0.55 x 25 mm (BD Microlance, Drogheda, Ireland) for bone marrow aspiration from living mice and flushing of bone marrow from extracted bones. 16 X 1.5 inch needles for dispensing and plating Methocult (CFC) media (Stem Cell Technologies, Vancouver, Canada).

Ammonium Chloride solution: For erythrocyte lysis 0.8% NH4Cl with 0.1 mM EDTA(Stem Cell Technologies, Vancouver, Canada).

Heparinized capillaries: (Microvette CB 300) plastic capillaries for collection of blood, containing 15 units (I.E) Lithium heparin per ml of blood (Sarstedt, Numbrecht, Germany).

3.1.6. Bacterial strain

• E. coli DH5á

3.1.7. Cytokines and antibodies

Cytokine	**Company**
SF Steel factor	**ImmunoTools Friesoythe; Germany**
GM-CSF	**Tebu-bio, Frankfurt, Germany**
G-CSF	**Stem Cell Technologies**

Flt-3-ligand	**PAN Biotech GmbH, Aidenbach, Germany**
IL6	**Tebu-bio, Frankfurt, Germany**
IL3	**ImmunoTools Friesoythe; Germany**

Antibody	**Antigen (BD Pharmingen,Heidelberg, Germany)**
CD45PE	**Leukocyte common antigen**
CD34 PE	**Pluripotential haematopoietic stem cells**
CD38 APC	**Immune cell marker**
CD15PE	**Neutrophils and eosinophil antigen**
CD19 APC	**B-cell antigen**
CD33PE	**Monocytic/myeloid lineage antigen**
CD36 APC	**Platelets, erythrocytes and monocytic antigen**
CD135 PE	**fms-like tyrosine kinase receptor-3 (Flt3) antigen**
CD71APC	**Adult erythropoietic cells**
CD41aAPC	**Platelets, late megakaryocytic antigen**
CD10APC	**Pre-B cells**
CD25APC	**activated B cells, some thymocytes, myeloid precursor antigen**
CD11bPE	**Leukocyte common antigen**
CD34APC	**Pluripotential haematopoietic stem cells**
CD133	**Haematopoietic stem cell antigen**
CD117	**Kit antigen**
Glyco A	**Mature erythroid cells**
Isotype PE/APC	**Negative control**

3.1.8. Commercial kits

- ***MACS CD34^{+} cell Isolation kits*****:** CD34 and CD133 HSC were isolated from UCB using MACS kits from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany.

- ***GenElute™ HP Endotoxin-Free Plasmid Miniprep Kit:*** The kit was used for isolating plasmid DNA from recombinant E. coli cultures (3ml). It was purchased from sigma Sigma-Aldrich, Taufkirchen, Germany.
- ***GenElute™ HP Endotoxin-Free Plasmid Maxiprep Kit:*** The kit was used for isolating plasmid DNA from recombinant E. coli cultures (100ml). It was purchased from sigma Sigma-Aldrich, Taufkirchen, Germany.
- ***Qiaquick gel extraction kit:*** For gel extraction or cleanup of up to 10 ìg DNA (70 bp to 10 kb) from enzymatic reactions. DNA fragments purified with the QIAquick System are ready for direct use in all applications. The kit was purchased from Qiagen GmbH, Hilden, Germany.
- ***QuickChange II XL site directed mutagenesis kit:*** The QuikChange XL system was used to make deletions in *HPIP* cDNA cloned in MSCV vector. The method was performed using PfuTurbo® DNA polymerase and a thermal temperature cycler. The kit was purchased from Agilant technologies, Germany
- ***RNeasy mini kit:*** For purification of up to 100 µg total RNA from cells, tissues, and yeast. The kit was purchased from Qiagen GmbH, Hilden, Germany.
- ***DNeasy mini kit*****:** Genomic DNA extraction kit from small cell numbers purchased from Qiagen GmbH, Hilden, Germany.
- ***ThermoScript^{TM}RT PCR system:*** The ThermoScript RT-PCR System is designed for the sensitive and reproducible detection and analysis of RNA molecules in a two-step. ThermoScript. RT, an avian reverse transcriptase with reduced RNase H activity, is engineered to have higher thermal stability, produce higher yields of cDNA, and produce good amount of full-length cDNA transcripts. The kit was purchased from Invitrogen life technologies, Karlsruhe Germany.
- ***Affymetrix GeneChip probe array:*** mRNA gene expression monitoring was done by using human genome U133 plus 2.0 Array array. The chips were bought from Affymetrix UK Ltd., UK.
- ***Enzymes:*** T4 DNA ligase, *Xho I, Eco RI, SacII* and *Hpa I,* were purchased from New England Biolabs (NEB, Beverly, MA)

- ***DharmaFECT™ Transfection Reagents for siRNA Transfection:*** siRNA transfection in K562 was performed by using DarmaFECT reagents. The kit was purchased from Thermo scientific dharmacon siRNA tech., Bonn, Germany.
- ***TaqMan® Express Plates****:* 96-well format complimenting customized line of individual TaqMan® Gene Expression Assays were used to confirm the microarray mRNA expressions.

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- ### 3.1.9. Umbilical cord blood cells, plasmids and molecular markers

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Biological Materials	**Company**
Peripheral blood (PB) AML patients	**Klinikum Grosshadern MEDIII, Frauen poliklinik**
Cord blood (CB) healthy donors	**Stem cell**
MSCV vector	**BCC, Vancouver, Canada**
Expression arrest TM pSM2 Retroviral shRNA	**Open Biosystems, Germany**
Standards markers, ladders	**Company**
1Kb Plus DNA ladder	**Invitrogen**
100 bp Dann	**NEB, Frankfurt, Germany**
216 Kda Kaleidoscope Prestained protein ladder	**Bio-Rad, Germany**
260 Kda SpectraMulticolor protein ladder	**Fermentas, Germany**

3.1.10. Miscellenous reagents

Reagents	Company
Acetic acid	Sigma-Aldrich, Taufkirchen, Germany
Agar	Sigma-Aldrich, Taufkirchen, Germany
Agarose	Sigma-Aldrich, Taufkirchen, Germany
BIT	Stem Cell Technologies, Vancouver, BC, Canada
Bromphenolblue	Sigma-Aldrich, Taufkirchen, Germany
BSA	New England biotecnologies
Calcium Chloride	Sigma-Aldrich, Taufkirchen, Germany
Ciprobay 400	Bayer
Chloroform	Sigma-Aldrich, Taufkirchen, Germany
DMSO	Sigma-Aldrich, Taufkirchen, Germany
DMEM	PAN Biotech, Aidenbach, Germany
Ethanol	Sigma-Aldrich, Taufkirchen, Germany
Ethidium Bromide	Sigma-Aldrich, Taufkirchen, Germany
Fetal Bovine Serum	PAN Biotech, Aidenbach, Germany
Formaldehyde	Sigma-Aldrich, Taufkirchen, Germany
Geneticin	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
HEPES	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
Hydrocortisone (solucortef)	Stem Cell Technologies, Vancouver, BC, Canada
IMDM	GIBCO, Invitrogen Corporation,Karlsruhe,Germany
Isopropanol	Sigma-Aldrich, Taufkirchen, Germany
LDL	Stem Cell Technologies, Vancouver, BC, Canada
L-Glutamine	Sigma-Aldrich, Taufkirchen, Germany
Methanol	Sigma-Aldrich, Taufkirchen, Germany
Milk powder	Carl Roth Gmbh, Karlsruhe, Germany
Methilcellulose H4434	Stem Cell Technologies, Vancouver, BC, Canada

Pancoll	**PAN Biotech, Aidenbach, Germany**
PBS	**PAN Biotech, Aidenbach, Germany**
Penicillin/Streptomycin	**GIBCO, Invitrogen Corporation, Karlsruhe, Germany**
RPMI	**PAN Biotech, Aidenbach, Germany**
SDS	**Sigma-Aldrich, Taufkirchen, Germany**
Sodium Chloride	**Sigma-Aldrich, Taufkirchen, Germany**
Trypan blue	**GIBCO, Invitrogen Corporation,Karlsruhe,Germany**
Trypsin/EDTA	**GIBCO, Invitrogen Corporation,Karlsruhe,Germany**
Â- Mercaptoethanol	**Sigma-Aldrich, Taufkirchen, Germany**
Cytofix (Cell fixation reagent)	**BD Pharmingen**
Hexadimethrine bromide	**Sigma-Aldrich, Taufkirchen, Germany**

3.1.11. Software and machines

Softwares	**Company**
CellQuest version 3.1f	**Becton Dickinson Immunocytometry Systems**
L calc	**Stem Cell**
Microarray analysis	**Programm R Console (Bioconductor)**
Confocal Microscope	**Ziess**
SigmaPlot 6.0	***SPSS Incorporated, Chicago, USA***
Adobe Illustrator	***Adobe Systems, Unterschleißheim***
Adobe Photoshop	***Adobe Systems, Unterschleißheim***
Cellquest 3.3	***Beckton Dickinson, Heidelberg***
Microsoft Office 2003	***Microsoft, Redmond, WA, USA***

Machines	Company
FACS vantage	**Becton Dickinson FACScan**
FACS vantage	**Becton Dickinson FACSort flow cytometer**
Affymetrix Microarray	**Fuidics Station, Affymetrix** **GeneChip Scanner 3000, Affymetrix**
AMAXA electroporation	**AMAXA GmBH**

3.1.12. Microscopes

Axiovert 25 Inverted light microscope from Carl Zeiss, Germany, was used for normal visualization of cells in culture and for scoring colonies in CFC assays.

Axiostar upright light microscope from Carl Zeiss, Germany, was used for cell counting and visualisation of cytospin preparations.

3.2. Methods

3.2.1. Thawing, passage and freezing of mammalian cells

Umbilical cord blood (CB) was obtained from mothers undergoing cesarean delivery of normal, full-term infants and collected in heparin coated syringes. Approved institutional procedures were followed to obtain informed consent of the mothers. Frozen $CD34^+$ cord blood or bone marrow (BM) cells from healthy donors were buying by (Cell Systems, St. Katharinen, Germany).

Thawing: 10ml of warm culture medium (DMEM or IMDM with 10%FBS) was placed into a sterile 15mL tube. Frozen cells (-135 C or 80 C) cryotubes were thawed quickly in a water bath at 37°C with agitation. 2mL pipette was used to raw up the cell suspention and added very slowly and dropwise to the medium. The cell suspension was centrifuged at 1200rpm for 7 mins. The supernatant was decanted and the cell pellet obtained was resuspended in culture medium and placed into culture dishes or flasks. These were incubated at 37C in a CO_2 incubator. A small aliquot of cells were taken apart for cell counting. For thawing UCB mononuclear

cells, DNase I was used (0.1 mg/ml) to prevent cell loss due to unwanted clumping of dead cells.

Passage: When the cells in dishes or flasks obtained ~100% confluency they were split. Media was removed from the container. The cells were washed with PBS to free FBS traces. 1.5ml of (0.25%) Trypsin-EDTA was added. The container was placed in incubator at 37 c for 5-10 minutes. The adherent cells were checked in between if they were lifted from the surface of the dish. 1mL of culture medium with 10%FBS was added to stop the enzymatic activity of trypsin. About 1/50 cell volume was transferred to a new 10 cm dish/flask ($25cm^2$). The dish/flask was placed at the 37°C incubator with 5% CO_2 in air and ≥95% humidity.

Freezing: confluent cells in 10 cm cell culture dishes were washed with warm PBS and trypsinized. Trypsinized cells were washed using their respective cell culture medium and pelleted down at 1200rpm for 7 mins. The pellet was resuspended in 0.9 ml FBS. DMSO was added drop wise to the cells in FBS to a final concentration of 10%. Aliquots were transferred to cryotubes and stored in freezing containers at -80°C for 24 hours and transferred to liquid nitrogen for long term storage.

3.2.2. MSCV based retroviral vector

HPIP cDNA was cloned in pMSCV-IRES-YFP cassette in the Terry Fox Laboratory, Vancouver, Canada. This plasmid was provided to our lab for analysis of the role of HPIP WT in human stem cells. As a control, the MSCV vector carrying only the IRES yellow fluorescent protein cassette was used.

3.2.3. Mutagenesis

The human gene HPIP was mutated at specific sites for structure-functional analysis of the HPIP WT protein. Mutagenesis was carried out using QuickChange® II XL site-directed mutagenesis kit (Stratagene). Specific primers were designed for desired deletion mutations. Two different regions were chosen for this purpose (1) Microtubule binding region (ΔMBR, 20aa deletion; position

spanning between 189-209) and (2) nuclear receptor and PBX interacting domain (ΔNRPID, deletion of

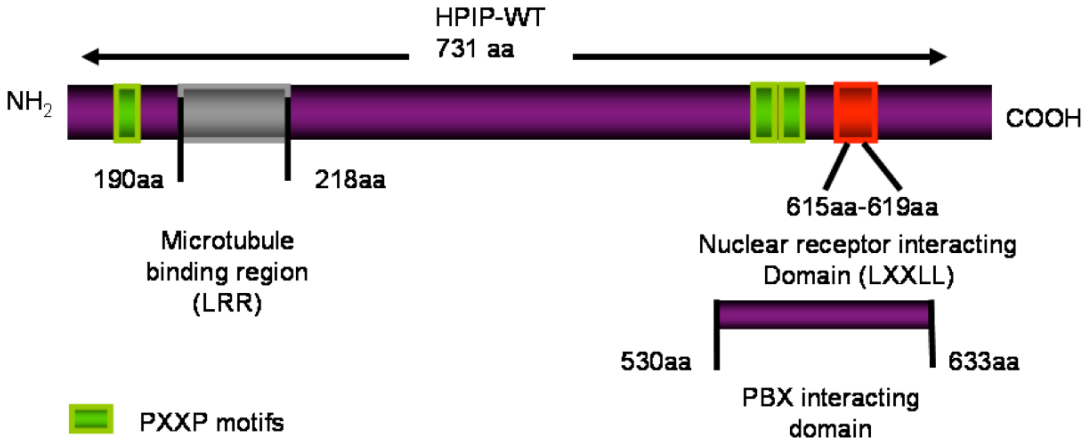


Figure 3.2.3a: Stucture of HPIP protein with potential domains. HPIP is a 731 aa protein with 3 PXXP motifs (shown in green), a microtubule binging region (shown in grey) and a nuclear receptor interacting domain (shown in red).

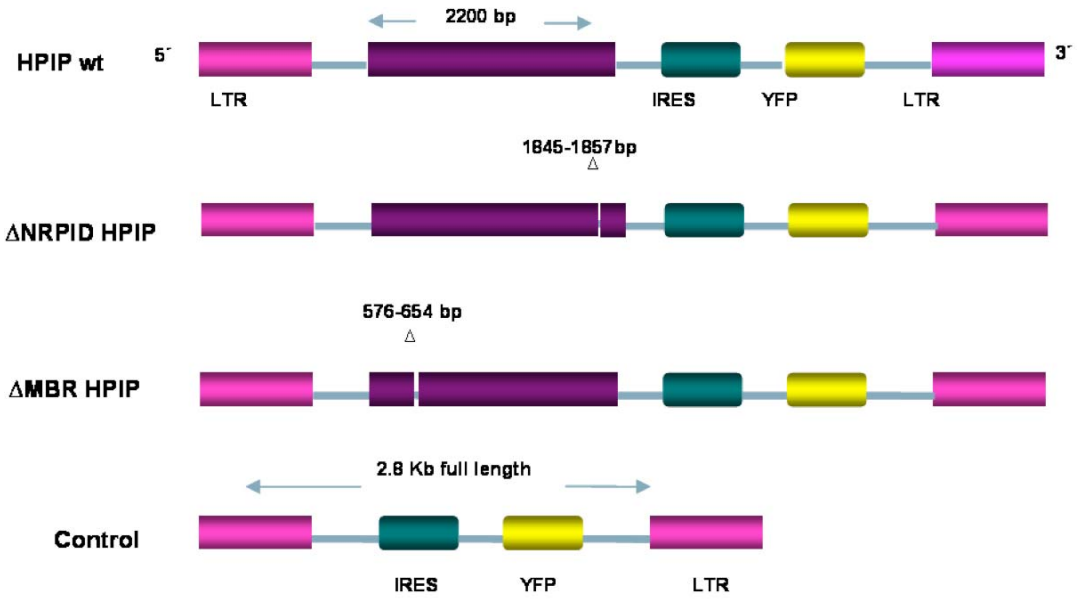


Figure 3.2.3b: Deletion mutants of HPIP: The mutant form ΔNRPID-HPIP-YFP has a set of 3 amino acids artificially deleted between position 615aa-619aa. This region has been implicated as a part of PBX binding domain[105] (PBD position 560aa-633aa Abramovich et al, 2002) and also nuclear receptor interacting domain[108] (N-terminal LXXLL motif position 615aa-619aa; Manavathi et al, 2006). The second mutant ΔMBR-HPIP-YFP has a deletion mutation in microtubule binding sequence (putative leucine repeat rich motif, LRR, position of motif spanning between 190aa-218aa;

LxxxLNxxLLxxLxLLxLxxLL), where a set of 24 amino acids was removed to disrupt the hydrophobic core of the HPIP WT protein [106](Abramovich et al, 2002).

LXXLL motif/ PBX interacting domain, 3aa deletion; position spanning between 615aa-619aa) of HPIP WT. The mutagenesis was performed according to the guidelines provided by the kit manufacturer. Different primers were ordered to specifically perform the in situ mutagenesis. Mutagenesis PCR was carried out with the QuickSolution™ Reagent provided in the kit, and the amplified Plasmid was transformed into XL10- Gold ultracompetent cells (included in the KIT).

Transformed bacteria were plated on agar plates and the plates were incubated at 37°C overnight. The next day early morning the plates were checked for healthy colonies. Selected colonies were picked and grown as mini cultures (minipreps, 3ml) in laura broth (prepared according to the directions on the bottle) overnight. The plasmid was extracted and purified using endotoxin free miniprep kit (Sigma) and sent for sequencing with YFP primers. Only the exact deletion mutant plasmids were chosen after sequence analysis and the remaining bacteria of the mini preps were used to culture a maxi prep (100ml). The final plasmids were purified using endotoxin free maxiprep kit (Sigma), measured and stored at -20°C.

Table 3.2.3: Primers for mutagenesis

HPIP WT mutant forms	Primers for mutagenesis
ΔMBR-HPIP-YFP	**5´-TGCAGGCGGGGAGCTCCTCTTCTCAGG-3´frw** **5´- CCTGAGAAGAGGAGCTCCCCGCCTGCA-3´rev**
ΔNRPID-HPIP-YFP	**5´- GTGCGGCAACAGGAGCTAAGAACATACTTGGC-3´frw** **5´- GCCAAGTATGTTCTTAGCTCCTGTTGCCGCAC-3´ rev**

3.2.4. Generation of shRNA constructs

Small RNA sequences, using online database of cold spring harbour laboratories (www.cshl.org), were searched for generation of shRNA against HPIP mRNA. Five different small 19mer were selected from HPIP nucleotide sequence as small interfering RNA (siRNA) sequences. These sequences together with end

modifications (final nucleotide 22mer, table 2.2.3), and an integrated hairpin (19mer: TGTGAAGCCACAGATGTA) to finish it as small hairpin RNA (shRNA) were ordered at Metabion (Germany) for their manufacture.

Table (3.2.4a): Final 22mer interfering nucleotide sequences against HPIP mRNA (www.cshl.org)

siRNA name	Sequence selected for cloning in mir30MSCV vector
Si-HPIP-1	**5´-AGCGCCCTCAGTCTGGCAGCATTCC-3´ (start position 260)**
Si-HPIP-2	**5´-AGCGGGAGTGGAGTGGAAAGGAAAC-3´ (start position 1469)**
Si-HPIP-3	**5´-AGCGCTTCCACTCCTCTGGAGAAAC-3´ (start position 1700)**
Si-HPIP-4	**5´-AGAAGCCCTGCACTGATGTCACTTC-3´ (start position 2392)**
Si-HPIP-5	**5´-AGCGCCCTCTCTGCTAAGAACATAC-3´(start position 1905)**

The LMP vector was purchased from Biocat technologies (Germany). These MSCV based retroviral (MSCV-mir30-GFP) vectors have been developed in the lab of Scott Lowe at CSHL to enable the efficient expression of shRNAmir constructs from the RNA Polymerase II (Pol II) promoters[113] (Fig.3.2.4a). These vectors produce a highly efficient knockdown of the target protein even when present as a single copy. Other features of the vectors include: efficient and stable single copy knockdown, inducible expression with Tet-responsive promoters, easy cloning of shRNAmir from pSM2 vector into Pol II retroviral vectors and validation for *in vitro* and *in vivo* use.

shRNA design: To be able to generate the new shRNAmir clones, RNAi Central-RNAi oligo retriever (http://katahdin.cshl.org) was used. A typical shRNAmir is a

138 bp PCR product, which has a hairpin loop, the siRNA (22mer) and the flanking blunt

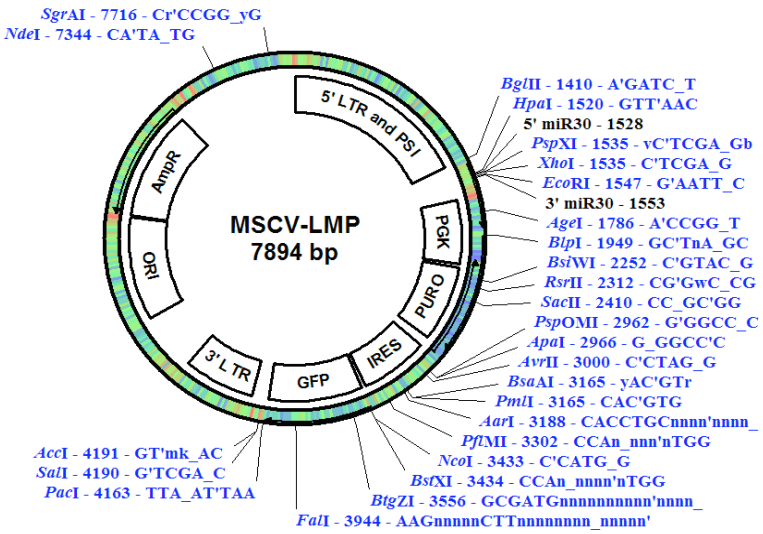
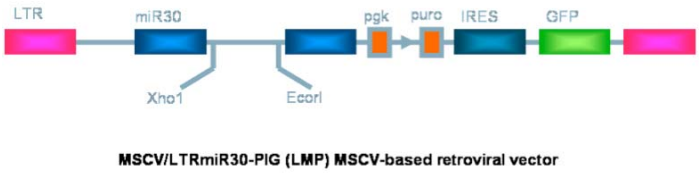
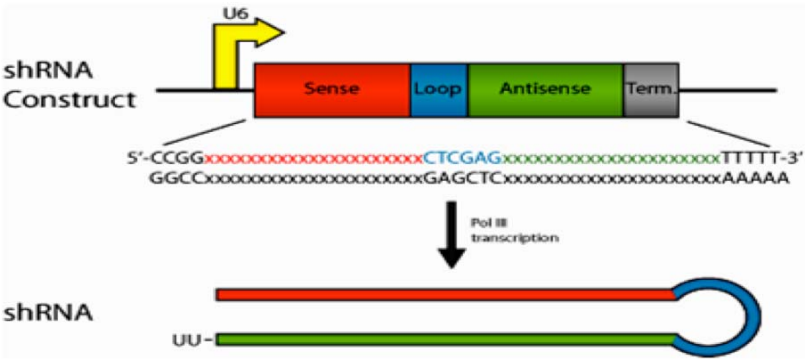


Figure 3.2.4a: LMP microRNA-adapted Vector. The Vector map and sequence file has been compiled from published literature (Open Biosystems). This vector has not been completely sequenced. Unique restriction sites are shown.



(A)



(B)

Figure 3.2.4b: (A) shRNA construct. (B) A simple illustration of shRNA generation in a cell.

ends for ligation into the mir30 backbone of the digested LMP vector. Two different protocols were followed to generate the shRNA-HPIP pertaining to two different conditions to start with shRNA design.

In the first condition we isolated the 22mer commercial sh-HPIP available at Biocat in a pSM2 vector (without GFP marker) and cloned it into LMP vector (with GFP marker)[114] (Paddison et al 2004). In this case we performed a dual restriction digestion of the pSM2 sh-HPIP vector and the empty LMP vector with two enzymes (*Xho*1 and *Ecor*1)

Reaction set up:

- 1µl 10x Buffer (B)
- 6.5µl H_2O
- 2µl DNA (0.3ìg/ìL)
- 0.5µl per Enzyme

b) The entire digest was run on a 1% agarose gel.

c) The 138bp insert from the pSM2 vector and the LMP vector bands was gel isolated and purified using Qiaquick gel extraction kit (Qiagen). The cut insert and plasmid were eluted in 50ìl nuclease free water and quantified.

d) To retrieve back the flanking ends of the sh-HPIP commercial hairpin, we performed a PCR using 5' and 3' miR30 primers (Table 2.2.3b), namely mir30_Xho1 frw and mir30-Ecor1 rev. the PCR amplified product (138 bp) was run on a 1% gel and isolated.

d) Ligation was performed by using the sh-HPIPmir30 inserts and the LMP vector cut with EcoRI and XhoI (quantity taken in a ratio 1:3 respectively). 2µl of 10x ligase buffer and ligase enzyme (0.5µl) was used making a final reaction volume of 20µl. The ligation was performed for 3 hrs at room temperature.

e) 2ìl of the diluted ligation mix was transformed into competent *E.coli*. The transformed cells were plated onto agar plates containing 100ìg/ml ampicillin.

Plates were incubated at 30°C overnight.

In the second condition we ordered the complete shRNAmir30 (97 bp) constructs (Metabion, Germany). After a simple PCR we could insert the Xho1 and Ecor1 flanking sites to the oligo. The final shRNAmir30 oligos contained the respective sh-HPIP (1, 2, 3, 4, 5; table 2.2.3a), a hairpin backbone and the flanking *Xho*I and *Ecor*I blunt ends. We then continued with the restriction digestion step of the LMP vector and ligation of all the shRNAmir30 constructs as mentioned above.

Table 3.2.4b: Common 5' and 3' miR30 primers ordered were as follows:

Primer name	Oligo sequences
mir30_Xho1 frw	5-CAGAAGGCTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3´
mir30-Ecor1 rev	5´-CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA-3´
LMP_check	5´-CCCTTGAACCTCCTCGTTCGACC-3´

Figure 3.2.4a

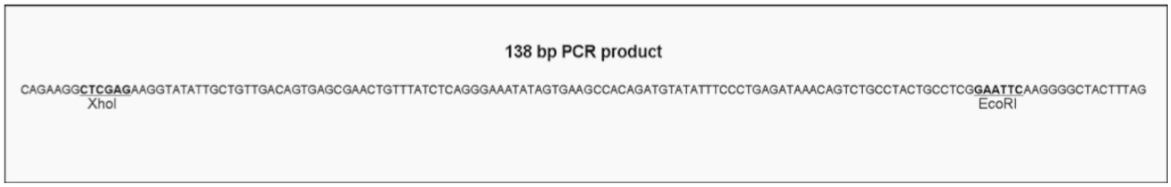


Figure 2.2.3a: A typical 138bp shRNAmir30 nucleotide construct. A typical shRNAmir is a 138 bp PCR product, which has a hairpin loop, the siRNA (22mer) and the flanking blunt ends with Xho1 and Ecor1 digestion sites.

The LMP_check primer was also ordered to check the sequence (Sequiserve) of the final plasmids obtained. The knockdown of endogenous or overexpressed HPIP WT-YFP was tested on K562 cells at mRNA level using RT-PCR and at the protein level with the help of Western Blot.

3.2.4. Establishment of stable PG13 packaging cell lines

PG13 packaging cell lines were generated for different plasmids established for the current study as described before. These cell lines were used to perform infections on human umbilical cord blood $CD34^+$ cells. PG13 cells are derived from TK-NIH/3T3 (mouse fibroblast) cells. The hybrid cell line is expressing a GALV envelope and the MoMLV virus core. The viruses produced have a wide host range (rat, hamster, cat, dog, monkey, bovine, human cells). Phoenix amphotropic cells were used for the transient transduction of PG13 with various viruses. Both these cell lines were cultured in DMEM with 10% fetal bovine serum and plated on corning dishes for transfections and transient transduction (2.5×10^6 cells per 10 cm plate) a day prior to the experiment.

Transfection of Phoenix Ampho: Medium from Phoenix Ampho was changed 4 hours prior to transfection. In a 5 ml tube 20 µg of plasmid DNA, 62.5 ml of 2M $CaCl_2$ and dH_2O to make up to 500 ml were mixed together. 500 ml of 2x HBS (ph=7) was added drop wise to form a precipitate. This mixture was added drop wise over the phoenix cells. After 12 hours of transfection, the medium was changed again over the Phoenix Ampho cells. The plates were left untouched for over 24 hrs for plasmid expression and virus collection in the medium.

Infection of PG13 cells: The virus containing medium (VCM) was collected after 24 hours, filtered through 0.45 µm filter, supplemented with hexadimethrine bromide (polybrene, 5µg/ml) to give a final concentration of 0.5ng/ml. This VCM was added on PG13 packaging cells for viral infection. After repeated infections (3-5 times), PG13 cells were left untouched for 48 hrs to allow the expression of YFP marker enclosed in the plasmid vector. The YFP expressing cells were sorted out using FACS and cultured for up to 2 weeks and resorted for the second time to acquire the most stably transduced PG13 cells. From these PG13 YFP^+ cells, single cells were sorted into 96 well plates, expanded and viral production was titered on K562 cells. Individual PG13 YFP^+ clones that produced the highest viral titre were identified and used for infecting umbilical cord blood $CD34^+$ cells.

The various plasmids used for transient transductions were: HPIP WT-YFP, ΔNRPID-HPIP-YFP, ΔMBR-HPIP-YFP, sh-HPIP-GFP (1, 2, 3, 4, 5), YFP and GFP.

3.2.6. Purification of umbilical cord blood CD34$^+$ cells (CB CD34$^+$) from mononuclear umbilical cord blood (UCB).

Umbilical cord blood was collected in heparinised syringes according to institutional guidelines following normal full-term deliveries. Informed consent was obtained in all cases. Mononuclear cells (MNC) were separated using density gradient centrifugation. Fresh umbilical cord blood, not older than 12 hours, was diluted with 2 volumes of PBS and layered over Pancoll. Usually 35 ml of diluted blood was layered over 15 ml Pancoll in a 50 ml conical tube. This was centrifuged at 400x g for 30 minutes at 20°C in a swinging-bucket rotor without brakes. The upper layer was aspirated and discarded, leaving the interphase undisturbed. The interphase containing MNC such as lymphocytes, monocytes and thrombocytes was then transferred to a new 50 ml tube, washed twice with large volumes of PBS, and then counted before labelling with magnetic bead or fluorochrome conjugated antibodies.

hCB CD34$^+$ cell purification was conducted using MACS CD34$^+$ Cell Isolation Kit that uses positive selection method. Cells were resuspended in a volume of 300 ml per 1x10^8 MNCs These were blocked with 100 ml of FcR Blocking Reagent and labelled with 100 ml of CD34 Microbeads. When working with higher cell number, all the reagent volumes & the total volume was scaled up accordingly. This was followed by incubation for 30 minutes at 4-8°C. Cells were then washed twice by adding 10x the labelling volume of buffer and centrifuged at 300 x g for 15 minutes. The resultant cell pellet was then resuspended in 500 ml of MACS buffer and loaded into MS Column mounted on magnetic separator. The negative cells were allowed to pass through and the column was washed at least three times with 2 ml buffer.

The column was then removed from the separator, placed on a collection tube, loaded with fresh buffer, and the magnetically labelled cells flushed out using the

plunger. The magnetic separation was usually repeated to get a purity of more than 95%. Purified cells were then frozen in FBS with 10% DMSO and thawed when needed for pre-stimulation and transduction. hCB $CD34^+$ cell enrichment was done by FACS. For separation by FACS, MNCs were thawed from frozen stocks or prepared freshly from UCB and labelled using anti CD34-PE antibody (100 ml per 10^8 cells), for 30 minutes on ice. Labelled cells were then washed twice with PBS, resuspended in FACS buffer and sorted. The sorted cells with purity above 95 % were used for 48 hour pre-stimulation followed by transduction.

3.2.7. Feeders and Co-cultures

For the general feeder dependent long term culture initiating cell (LTC-ICs) assays, a mixture of M2-10B4-J-GCSF-tkneo-J-IL3-hytk (M2-10B4) fibroblasts and SL/SL-J-SF-tkneo-J-IL3-hytk (SL/SL) fibroblasts were used. M2-10B4 cells are a cloned line of mouse bone marrow originally engineered to produce G-CSF and IL-3 (190 and 4 ng /ml respectively) and the SL/SL fibroblasts are a cell line originally established from SL/SL mouse embryos engineered to produce high levels of soluble Steel factor with or without production of the transmembrane form of SF (60 and 4 ng / ml respectively). The M2-10B and the SL/ SLcells were maintained by plating at a concentration of 1-2 $x10^5$ cells per 10 cm tissue culture dish (Corning) in RPMI, 10 % FCS, 0,4 mg/ml G418 and 0,06 mg/ml hygromicin and DMEM, 15 % FCS, 0,8 mg/ml G418 and 0,125 mg/ml hygromicin respectively.

Both murine feeders were irradiated with 8000 Rads (80 Gy) before being cocultured with CB $CD34^+$ cells. Corning dishes (35 mm tissue culture) were pre-coated with collagen solution (StemCell Technologies) to form a film that helps in the adherence of fibroblast cell lines. Both cell lines were mixed in a 1:1 ratio before plating on the dishes for LTC-IC assays.

3.2.8. Transduction of CB $CD34^+$ stem cell population

Specific PG13 cell lines were split into a couple of dishes to generate maximum possible VCM for the entire experiment. Enriched CB $CD34^+$ cells were

thawed and counted. 1.5-2 $\times 10^6$/ml cells were pre-stimulated for 48 hours in Iscoves modified Dulbecco medium (IMDM) containing a serum substitute BIT.(Stem Cell Technologies), 10^{-4}M mercaptoethanol (Sigma), and supplemented with the following recombinant human cytokines: 100 ng/ml Flt-3 ligand, 100 ng/ml Steel factor, 20 ng/ml interleukin-3, 20 ng/ml IL-6, and 20 ng/ml granulocyte colony-stimulating factor (G-CSF). Corning dishes were coated with the 2ml of filtered VCM harvested from PG13 cells (the step was repeated at a difference of one hour

After 48 hours in culture, hCB $CD34^+$ cells were harvested and resuspended in 8ml of filtered virus-containing medium (VCM) supplemented with the same cytokines combination as mentioned above and hexadimethrine bromide (polybrene, 5µg/ml). This cell suspension was dispensed in the coated corning dishes that were prepared before. This procedure was repeated on the next two consecutive days for a total of 3 infections. At the end of third day, cells were harvested and transfered into fresh suspension dishes with serum-free medium supplemented with the same cytokine cocktail as above. These were incubated for an additional 48 hours prior to establishment of any in vitro assays.

For in vitro assays the cells were harvested and stained with PE-labelled anti-CD34 antibody (Becton Dickinson, San Jose, CA) and isolation of the YFP^+/GFP^+/ $CD34^+$ cells on a FACS Vantage (Becton Dickinson) sorter was performed. However, for in vivo studies, transduced cells were injected into non obese diabetic/severe combined immunodeficiency (NOD/SCID) mice directly after the third day of transduction (< 6 hours after the last exposure to fresh VCM) without any pre-selection of YFP expressing cells. In certain experiments some extra irradiated CB mononuclear cells were also used as carrier cells for injections. An aliquot was kept in vitro for further for the determination of the proportion of transduced cells to be injected into mice while performing limiting dilution experiments in mice.

In another setup of experiments we performed the double transductions on CB $CD34^+$ cells. These were performed by collecting the VCM from two different PG13 packaging cell lines, producing viruses' containing 2 different plasmids. In this case

it was HPIP WT-YFP and HOXB4-GFP. The VCMs were mixed in a ratio of 1:1 before coating it on the corning dishes. The cells were centrifuged at 600-700rpm for 20 minutes to facilitate the co-localization of different viruses on CB $CD34^+$ cells. The cells were resuspended in dual VCM with polybrene for transduction to take place.

3.2.9. Detection of gene and protein expression

For amplification of HPIP WT cDNA (PCR) following primers were used: forward- 5´- CAGAGTCTGAGACTGGGCC -3´, reverse-5´-GCTGCAGCTCTGACTCCA-3´ and a human beta-actin mRNA was used as control, forward: 5´-CTTCAACACCCCCAGCCAT and reverse: 5´-TAATGTCACGCACGATTTCC-3´. These primers were used also to detect the presence of cDNA expression of all the mutant forms of HPIP WT in the PG13 cells. The two step RT-PCR was performed to check the cDNA expression.

The ThermoScript RT-PCR system (Invitrogen) was used for the same. Protein expression of the HPIP WT and the mutant forms was documented using standard Western blotting. All the HPIP constructs are Flag tagged. Furthermore, two different monoclonal HPIP antibody specific for HPIP protein were designed and raised at the monoclonal peptide core facility (Dr E Kremmer), Hematologikum together with the Peptide Speciality Laboratories GmbH, Heidelberg. These antibodies were used to test endogenous and retrovirally driven HPIP WT protein levels.

Total cellular protein was extracted from PG13-HPIP WT-YFP, PG13-ÄNRPID-HPIP-YFP and PG13-ΔMBR-HPIP-YFP packaging cell lines by using RIPA lysis buffer. Whole cell lysates were run and separated on 10 % SDS-page gel and transferred to nitrocellulose membrane. Membranes were probed with an anti-FLAG (Sigma) and anti-HPIP monoclonal antibody (Dr. Kremmer). The membrane was re-stained with secondary goat anti-mouse immunoglobulin for Flag tagged HPIP WT and with anti-goat secondary antibody for detection of endogenous expression of HPIP. Proteins were visualized using an ECL plus kit, according to manufacturer's recommendations.

The K562 cell line was transduced with different shRNAs generated against HPIP mRNA. These cells were double transduced with the HPIP-YFP virus and the

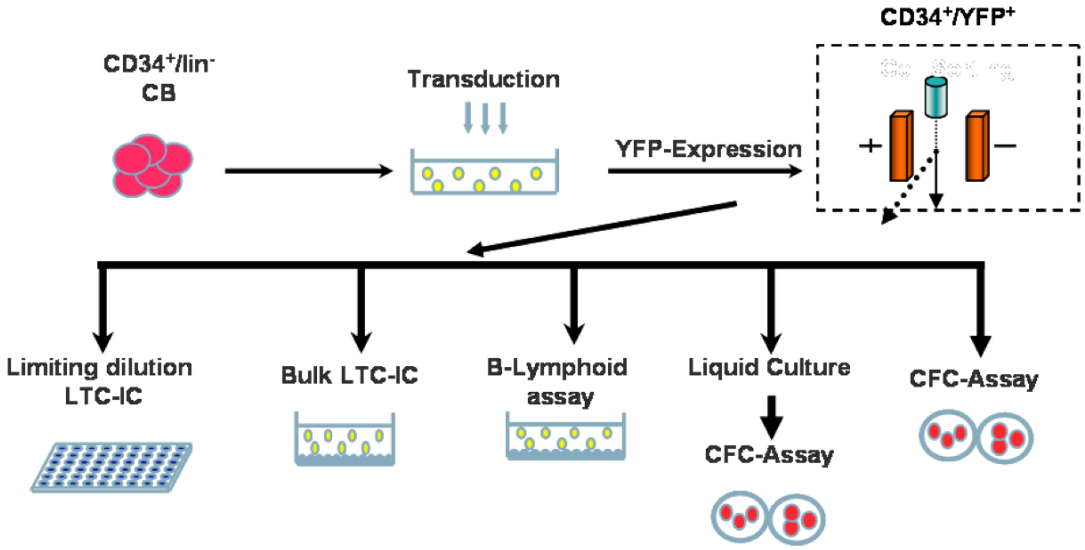


Figure 3.2.8: A schematic representation of transduction experiment and all the following functional assays that are performed on primary human stem cells.

shRNA virus to use this model for testing the efficacy of the different shRNAs. The protein was extracted and detected as mentioned above to estimate the downregulation of the HPIP protein and choose the best working shRNA against HPIP WT.

3.2.10. Sub cellular localization of constitutively expressed HPPWT and its mutant forms.

For sub cellular localization studies, NIH3T3 and PG13 mouse fibroblasts were grown on coverslips and transfected with Flag tagged HPIP WT-YFP, ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP. After 24 hrs, cells were fixed with phosphate-buffered saline (PBS), 2% paraformaldehyde for 10 min, permeabilized with PBS 0.1% Triton X for 10 min and blocked with PBS 10% fetal calf serum (FCS) for 1 hr. Coverslips were incubated with monoclonal mouse Flag antibody (Sigma). Following extensive washing with PBS 0.1% Tween, Alexa 555 and Alexa 488-conjugated secondary antibodies were added for 1 hr. After further washing steps, cells were stained with DAPI and

mounted using Cytomat medium (DAKO, Glostrup, Denmark). Finally, immunostained species were analysed in a confocal fluorescence laser scanning system (TCS-SP2 scanning system and DM IRB inverted microscope, Leica, Solms, Germany).

3.2.11. Colony forming cell assay

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Haematopoietic colony-forming cells (CFC) were assayed using methylcellulose-based medium (MethoCult H 4434) (fig. 3.2.11). Required number of pre-aliquoted tubes of MethoCult medium was thawed overnight under refrigeration (2-8°C) or at room temperature. The cells were diluted with IMDM + 2% FBS to 10X the final concentration(s) required for plating. For a duplicate assay 0.3 ml of diluted cells was added to 3 ml MethoCult tube and the contents vortexed vigorously. After about 5 minutes, 1.1 ml of cell: methylcellulose mixture was dispensed into 35 mm culture dishes using sterile a 3 ml syringe and16-gauge bunt-end needle. The 35 mm culture dishes were placed into 10 cm petri dish along with an extra 35 mm dish containing sterile water to maintain humidity and placed in a CO2 incubator at 37°C and >95% humidity.

CFC numbers were evaluated after an incubation period of 12-14 days and distinguished into following classes: Colony-forming unit-erythroid (CFU-E): Produces 1-2 cell clusters containing a total of 8-200 erythroblasts. A CFU-E consists of mature erythroid progenitors that require erythropoietin (EPO) for differentiation. Burst-forming unit-erythroid (BFU-E): Produces a colony containing >200 erythroblasts in a single or multiple clusters. A BFU-E consists of more immature progenitors than CFU-E and requires EPO and cytokines with burst-promoting activity such as Interleukin-3 (IL-3) and Stem Cell Factor (SCF) for optimal colony growth.

Colony-forming unit-granulocyte, macrophage (CFU-GM): Produces a colony containing at least 20 granulocyte cells (CFU-G), macrophages (CFU-M) or cells of both lineages (CFU-GM). CFU-GM colonies arising from primitive progenitors may contain thousands of cells in single or multiple clusters. Colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM): A multi-

potential progenitor that produces a colony containing erythroblasts and cells of at least two other recognizable lineages. Due to their primitive nature, CFU-GEMM tend to produce large colonies of >500 cells.

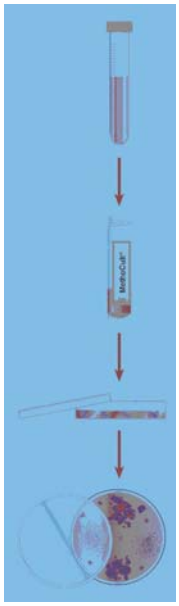

Figure 3.2.11: Human haematopoietic CFC assay in methylcellulose media detects haematopoietic progenitors (Figure adapted from the Stem Cell Technologies Catalogue).

3.2.12. Liquid expansion culture

For in vitro liquid expansion assays, transduced cord blood $CD34^{+}$HPIP WT-YFP^{+} ,$CD34^{+}$ ΔNRPID-HPIP-YFP^{+} and $CD34^{+}$ ΔMBR-HPIP-YFP^{+} cells were placed in cytokine-supplemented serum-free medium containing 10^{-4} M ß-Mercaptoethanol, (Gibco) 20 % BIT (StemCell). For cord blood transduced cells 100 ng/ml each of Flt-3 ligand and SF and 20ng/ml each of IL-3, IL-6 and G-CSF cytokines was added in the culture medium. Half-media change was performed every week and morphology of cells present in cultures at various time points was determined by performing cytospins. For cytospins $1x10^{5}$ cells were fixed on a slide and stained with Wright-Giemsa. Every week $1x10^{4}$ cells were plated in CFC assay after half-media change. In addition, separate aliquots were taken and incubated for 30 minutes on ice with a mouse isotype-matched control antibody (Becton

Dickinson) and large antibody panel against human antigens and analyzed by FACS for the expression of different lineages.

3.2.13. B-lymphoid progenitor cell assay

To test the influence of HPIP on B-cell development, two culture conditions were chosen (L. Coulombel, personal communication). Cells were cultured on murine MS-5 stromal cells in RPMI 1640 with 10% FCS and 5% human AB serum with either a combination of 3 growth factors as SCF (50ng/mL), IL-2 (10ng/mL) and IL-15 (10ng/mL), to allow the development of predominantly B-cells, or a combination of 6 growth factors (SCF (50ng/mL), IL-2 (10ng/mL) and IL-15 (10ng/mL), IL-7 (10ng/mL), Flt-3 L (100ng/mL) and TPO (50ng/mL) to allow the development of B-, NK cells and granulocytes / macrophages. After 4 weeks adherent and non-adherent cells were collected and analysed by FACS for the expression of CD34, CD38, myeloid (CD15, CD33), lymphoid (CD19, CD10), erythroid (Glyco A, CD71) and megakaryocytic (CD41) cell surface markers.

3.2.14. Human bulk long term culture initiating cell assay (Bulk LTC-IC).

M2-10B4 G-CSF / IL-3 and Sl/Sl SF / IL-3 cells (1:1 mixture) were established in 24-well flat-bottom culture plates at a density of 2 $x10^5$ cells per dish. On the day of assay, medium was removed from the wells. The test cells were added to the wells in 2ml of medium with cortisol (solucortef). The number of cells seeded per well were 5000. LTC-IC cultures were incubated at 37°C in humidified incubator (>95%) with 5 % CO_2 in air for six weeks. For weekly half media changes, one half of the medium was removed and replaced with fresh LTC-IC medium each week for five weeks. To harvest the LTC-IC, all the adherent and non-adherent cells were removed from wells and place into individual 12x75 mm sterile tubes using a pipette and sterile tips. Single wells were harvested at a time to avoid cross contamination of samples. Wells were rinsed once with 1 ml PBS and added to tube. 1 ml Trypsin-EDTA was added to each well and incubated for 3 to 5 minutes

and examined for detached cells. Once the adherent cells are detached, the wells are washed with more PBS and the medium collected in the appropriated tube.

The wells are finally washed with 1 ml IMDM containing 2 % FBS and transferred to the specific tube. The tubes were centrifuged at 1200 rpm for 10 minutes and the supernatant was removed without disturbing the cell pellet. Approximately 200ìL of medium was left along with the cell pellet and vortexed. To this 3ml of Methocult (H4434) methylcellulose medium was added and vortexed again. Each tube (contents of one well) was plated individually into 2 different (1.5 ml/dish) 35 mm petri dish with 1 ml syringe (without needles attached). Different dishes (6-8) were placed in a 15 cm petri-dish along with an additional 60 mm open dish containing 5 ml sterile water to maintain humidity. The dishes are incubated at 37°C in humidified incubator (>95 %) with 5% CO_2 in air for 16-20 days. Colonies were scored as positive if one or more BFU-E, CFU-GM or CFU-GEMM were detected or scored as negative if no colonies were present.

The number of LTC-IC for the test cell population was calculated by dividing the total number of CFC detected in the culture by the average number of clonogenic progenitors per LTC-IC for the standard conditions used. Alternatively the values were expressed as LTC-IC derived CFC per number of test cells.[115]

3.2.15. Fluorescence associated cell sorting

To determine the gene transfer efficiencies of cultured $CD34^+$ cord blood cells after transduction, aliquots of cells were stained with anti-human CD34-PE antibody, washed twice with PBS and stained with propidium iodide 2µg/mL to exclude non-viable cells. Cells were analyzed using a FACS Calibure (Becton Dickinson) with Cellquest software (Macintosh, Cupertino,CA). 20.000 events were acquired to determine the proportion of positive cells present; positive cells were defined as those exhibiting a level of fluorescence exceeding 99.98% of that obtained with isotype-control antibodies labelled with the same fluorochromes. Green fluorescent protein (GFP) positive cells were detected by their increased fluorescence intensity within the fluorescence 1 channel. The percent gene transfer

efficiencies were calculated by the number of YFP^+/GFP^+CD34^+ cells relative to the total number of $CD34^+$ cells.

3.2.16. Xenotransplantation models

NOD/LtSz-scid/scid (NOD/SCID) mice were bred and maintained in the animal facility of the Helmholtz- zentrum (Haematologikum) in microisolator cages containing autoclaved food and water. For competitive repopulating unit assay (CRU) in NOD/SCID mice, the mice were ordered from Taconic Farms, Bomholt, Denmark. Eight to nine weeks old NOD/SCID mice were sublethally irradiated with 250 cGy from a 137Cs source an evening prior to injection. For transplantation, transduced cells were washed, counted, resuspended in PBS and injected into the lateral tail vein of irradiated mice (300-350µL/mouse). After 6-8 weeks mice were sacrificed by CO_2 inhalation (Fig 3.2.16). The cells from both tibiae and femurs of each mouse were collected for additional analyses.

The absolute number of cells in the marrow of each mouse was calculated assuming that the contents of both femurs and both tibiae represent 25% of the total marrowFor the bulk cell experiments, a certain fixed amount of cells were used to inject in mice and engraftment level, differentiation and proliferation levels in vivo were estimated 6-8 weeks post transplantations. However, in limiting dilution assays carried out in mice, different numbers of cell dilutions were generated and cohorts of mice were injected with different number of test cells. After BM harvest the frequency of competitive repopulating unit (CRU) or Scid repopulating cells (SRC) was assessed using L calc software (stem cells)

3.2.16. Analysis of the BM of sacrificed mice.

Cells harvested from bone marrow of NOD/SCID mice were resuspended in 7 % ammonium chloride (Stem Cell Technology, Vancouver, Canada) and placed on ice for 20-30 minutes for lyses of red blood cells. After this, cells were washed and re-suspended in Hanks balanced salt solution (Stem Cell Technologies,

Vancouver, Canada). Cells were washed and stained with anti-human CD45-PE (Becton Dickinson).

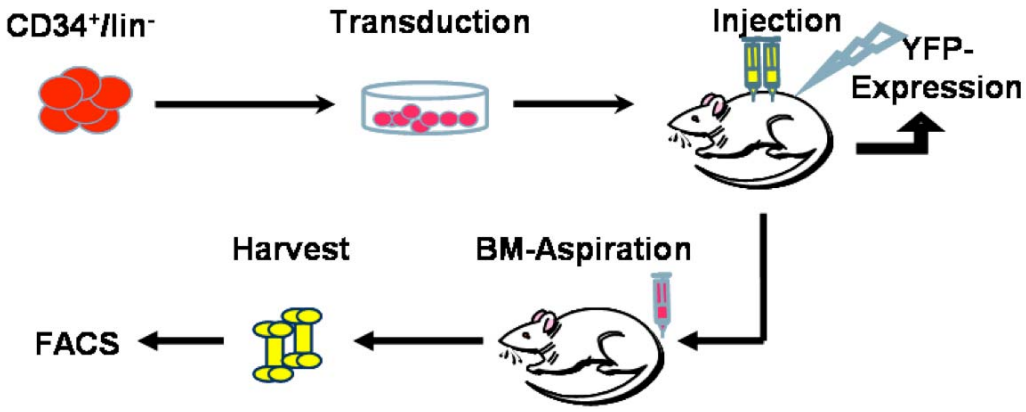

Figure 3.2.16: A schematic presentation of *in vivo* experiment set in laboratory. The transduced cells are injected in the tail of mice without any YFP expression pre-selection. After 6-8 weeks of transplantation the surviving mice are sacrificed and the BM is harvested for engraftment of human CB cells and levels of YFP^+ expression in these mice.

A proportion of the cells were incubated 30 minutes on ice with a mouse IgG1 isotype control (Becton Dickinson Immunocytometry Systems, San Jose, CA) to evaluate non-specific immunofluorescence. The remaining cells were incubated with fluoresceinated anti-CD45 a human-specific pan-leukocyte marker to detect human cells. The percentage of human $CBCD45^+$ cells was determined after excluding 99.9 % of nonviable (propidium iodide) cells and at least 99.9 % of cells labelled with isotype control antibodies. To determine lineage differentiation and multilineage engraftment, cells were stained for 30 minutes at 4°C, with the antihuman CD45-PE phycoerythrin (PE;Becton Dickinson) and antihuman CD71-APC antibodies (OKT9) to quantify the total number of human cells present ($CD45^+/71^+$), with antihuman CD34 8G12-Cy5 and antihuman CD19-PE to quantify the number of human B cells present, and with antihuman CD15-PE (Becton Dickinson) to quantify the number of human myeloid cells present.

Additional antibodies used for the detection of erythroid positive cells fraction were the antihuman GlyA-PE and antihuman CD36-APC (Becton Dickinson), and antihuman CD38- PE (Becton Dickinson). Expression of basophiles was detected

using CD25-APC (IL-2Rá), antihuman CD40-APC and Cd11b-PE and antihuman CD41a-PE (Becton Dickinson). Multilineage engraftment was considered if there were >5 $CD34^+CD19^+$ human cells and > 5 $CD45^+CD15^+$ human cells per $2x10^4$ viable cells analyzed.[116]

3.2.17. Generation of monoclonal antibody against HPIP WT

In cooperation with Dr. E Kremmer (GSF, München) and Dr. HR Rackwitz (PSL GmbH, Heidelberg) two HPIP peptides, namely HP1 (PQSGSILTEETV) and HP2 (KQKQPRWREGTKD) were synthesized and used to immunize rats. Antibody-secreting hybridoma cells were produced by fusing the spleen cells from the immunized rats with myeloma cells. Successfully fused cells were propagated in selective medium and screened for antibody production. Using ELISA and western blot productive hybridoma clones were identified to specifically detect the protein. Productive hybridoma clones were expanded and cloned to establish monoclonal hybridoma cell lines. Supernatants of these cell lines were used for western blots and immunoprecipitations.

3.2.18. Affymetrix Genechip expression analysis

Fresh RNA was prepared using RNA easy micro kit using ≤ $2X10^5$ retrovirally transduced Cord blood $CD34^+$ cells from each arm. Biotinylated target from these purified RNA samples suitable for hybridization to gene chip expression probe arrays was performed using a Two-Cycle target labelling and control KIT.

3.3. Analysis

3.3.1. Statistics

Data were statistically tested using Student's t-test (Microsoft Excel). Differences with p-values ≤ 0.05 were considered statistically significant. Statistical analysis for calculating CRU frequency was performed using L-Calc™ software for limiting dilution assays.

3.3.2. Gene expression profile

Differential expressions of RNA in this over expression model were performed using R program, Tinn R program.

3.3.3 Confocal Microscope

Expression of proteins in cells was examined using an anti-flag antibody. The photos from different channels were acquired using Leica Confocal Software.

4. Results

4.1. Transduction efficiency measured on the CD34$^+$ progenitor pool of umbilical cord blood cells (hUCB derived CD34$^+$Lin$^-$ cells).

Human primary umbilical cord blood cells (hUCB derived CD34$^+$Lin$^-$) were purchased from Stem Cell Technologies. The hCB CD34$^+$ stem cell pool was retroviral transfected with bi-cistronic vectors containing either WT or HPIP mutant cDNA or a yellow fluorescent protein (YFP) as a reporter gene (see methods, p.48). The analogous vector containing YFP only was used as a control. The gene delivery efficiencies were determined by measuring the proportion of cells expressing the YFP, with the help of the FACS Calibur. The mean transduction efficiency of the double positive cells (CD34$^+$YFP$^+$) was 9.55% (±5.92%) for HPIP WT-YFP$^+$ and 11.14% (±6.23%) for YFP$^+$ cells respectively, in hCB CD34$^+$ cells relative to the total number of the input hCB CD34$^+$ cell population (n=13).

The mean transduction efficiency was 9.11% (±2.02%) for CD34$^+$ΔNRPID-HPIP-YFP$^+$ cells (n=6) and 7.1% (±0.15%) for CD34$^+$ΔMBR-HPIP-YFP$^+$ cells (n=6, Fig. 4.1), relative to the total input hCB CD34$^+$ cell population. There were no significant differences observed between the mean transduction efficiencies of all the vectors in human hCB CD34$^+$ cells.

4.2. RNA and protein expression analysis

The complete cDNA of HPIP WT was cloned into the bi-cistronic vector enclosing an IRES-YFP cassette based on the murine stem cell virus (MSCV) backbone (HPIP virus). Mutagenesis of MSCV-HPIP WT-IRES-YFP was performed to create the two mutants of HPIP WT namely ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP (see methods, p-39). In parallel MSCV–IRES–YFP vector was used as a control. High titre virus producing cell lines were generated from the GALV pseudo-typed PG13 packaging cell line in case of all the four constructs. K562 cell lines were retro-virally transduced and the full-length provirus integration and expression

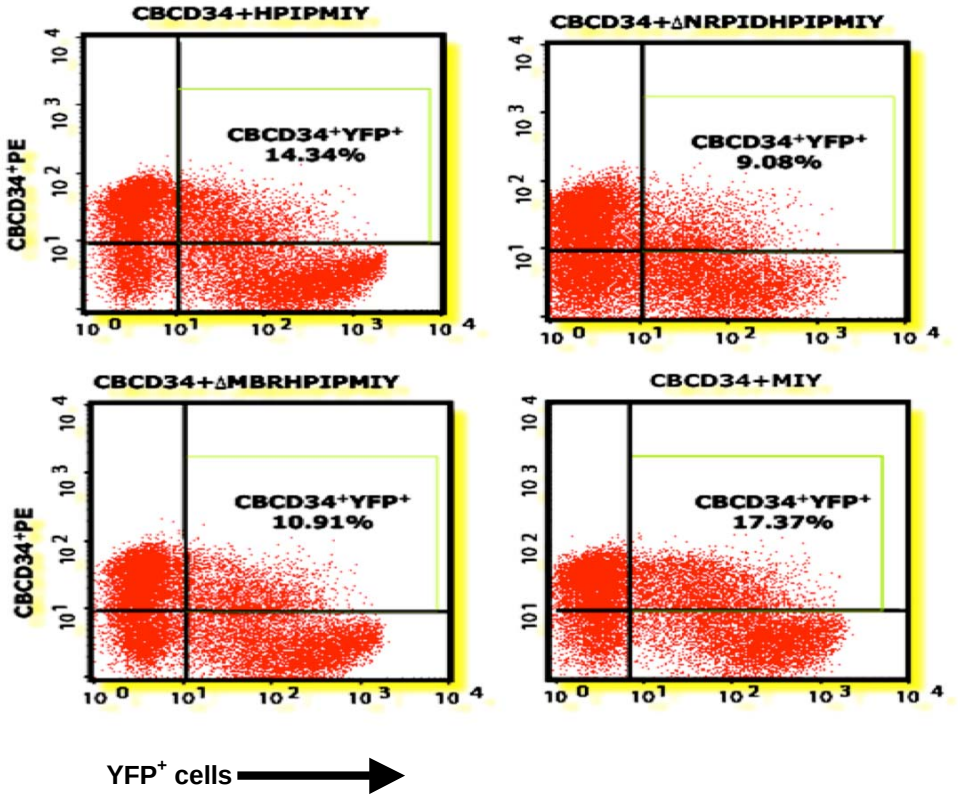

Figure 4.1: Acquisition plots representing retroviral transduction efficiency in hUCB CD34$^+$ Lin$^-$ cells. hCB CD34$^+$ cells were retroviral transduced with HPIP WT (dot plot above left), ΔNRPID-HPIP-YFP (dot plot above right), ΔMBR-HPIP-YFP (dot plot below left) and YFP (dot plot below right). The cells were stained with CD34 PE cell surface marker, washed with PBS and treated with medium containing Propidium Iodide (FACS buffer, see methods, p-30) and analysed with the help of the FACS Calibur. Each dot plot represents four different compartments of the cells. These are four different kinds of enriched populations shown in four different sections in each plot; (please refer to a single plot presented above) hCD34$^+$ only (upper left), hCD34$^+$YFP$^+$ (upper right), hCD34$^-$ YFP$^+$ (lower right) and hCD34$^-$YFP$^-$ (lower right) The population that was sorted further for the experiment in each case is marked with the green box (hCD34$^+$YFP$^+$), showing the % positive CD34$^+$YFP$^+$ cells. These double positive cells were sorted and used further for *in vitro* and *in vivo* assays.

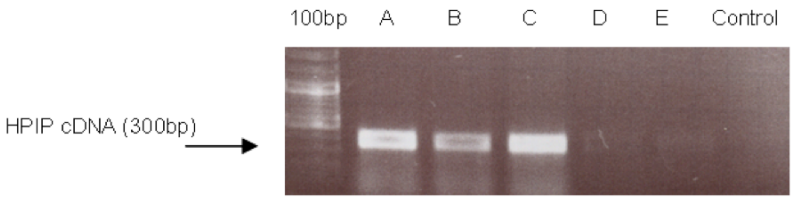

Figure 4.2a: cDNA expression analysis using RT-PCR in K562 cell line. K562 cells were transduced with WT and HPIP mutant forms and YFP control viruses. RNA was extracted from

stable K562 cell lines and the cDNA was generated by performing RT-PCR, using primers specific for HPIP WT, frw: 5´-CAGAGTCTGAGACTGGGCC-3´ and reverse- 5´-GCTGCAGCTCTGACTCCA-3´ primers were able to amplify all the constitutively expressed WT and mutant HPIP cDNA (see methods, p-50). (A) Constitutively expressed HPIP WT-YFP, (B) constitutively expressed ΔNRPID-HPIP-YFP, (C) constitutively expressed ΔMBR-HPIP-YFP, (D) endogenous HPIP expressed in K562-YFP cell line, (E) endogenous HPIP in K562 cell line.

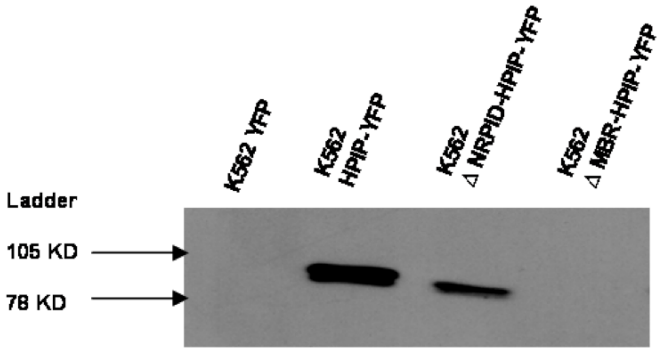

Figure 4.2b: Western blot of flag tagged proteins in stably transduced K562 cell lines. The above blots depict the presence of the WT and HPIP mutant form proteins in cell lysates from K562 stably transduced cell lines. The lysates from PG13 and K562 were prepared using RIPA cell lysis buffer 2. The samples were run on a 7.5% SDS gel. In both above cases the HPIP-WT-YFP (105 KD) and ΔNRPID-HPIP-YFP (a little lower < 105 KD) expressing cells show strong protein expression. However the expression of ΔMBR-HPIP-YFP was unpredictable.

was confirmed by RT–PCR (Fig. 4.2a) and immunoprecipitation on western blot (Fig. 4.2b).

An approximate equal expression of all the constitutively overexpressed cDNAs could be verified with the help of RT-PCR. On the western blot, the flag tagged protein expression could be predicted for overexpressed/flag tagged HPIP WT and ΔNRPID-HPIP-YFP proteins (the same has been demonstrated with an immunoprecipitation blot in figure 4.2b). Expressions could also be depicted with a monoclonal antibody generated specifically for the detection of endogenous HPIP WT protein for both the above mentioned HPIP forms. However, the protein expression of ΔMBR-HPIP-YFP was low to unpredictable on western blot.

4.3. Subcellular localization of the constitutively expressed protein

Subcellular localization of WT and HPIP mutant forms was investigated with immunofluorescence staining at the single cell level. Protein expression at the cell level and protein localization was predicted using immunoassaying using the anti-FLAG antibody staining. For microscopy, the cells were grown on 18x18 mm cover slips. After overnight staining with anti-flag, cells were washed with PBS and fixed for 10 min in 3.7% formaldehyde/PBS.

All the photos were taken on an automated Axiovert 200M microscope equipped with single band pass filter sets for visualization of DAPI (blue fluorescence), CFP (Red fluorescence) and YFP (Yellow fluorescence). Images were recorded and processed using open *lab* 3.08. Clearly, expression of proteins at the cell level and their cytoplasmic localization were detectable (Fig. 4.3) with the help of confocal microscopy. There were no visible differences observed in the fluorescence intensity of CFP (anti-FLAG) and YFP, suggesting the positive expression correlation of the test proteins and the control YFP. The figure 4.3 shows the presence of HPIP- WT-YFP, ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP proteins in cytoplasm. There was no CFP signal detectable in the nucleus of the cells.

This confirms that WT and mutant forms are primarily located in cytoplasm of the cells. The deletion in both of these domains of HPIP could not render HPIP delocalized inside the cells. We could clearly see a signal of CFP tagged FLAG-ΔMBR-HPIP (Fig 4.3-III), protein in this experiment, which was previously not detectable in the western blots.

4.4. In vitro *proliferation assay of the human haematopoietic stem cell.*

4.4.1. *In vitro* commitment and differentiation of transduced cells.

Liquid expansion culture system for human haematopoietic progenitor cells has been developed to monitor the commitment and differentiation of hCD34^{+} cells to

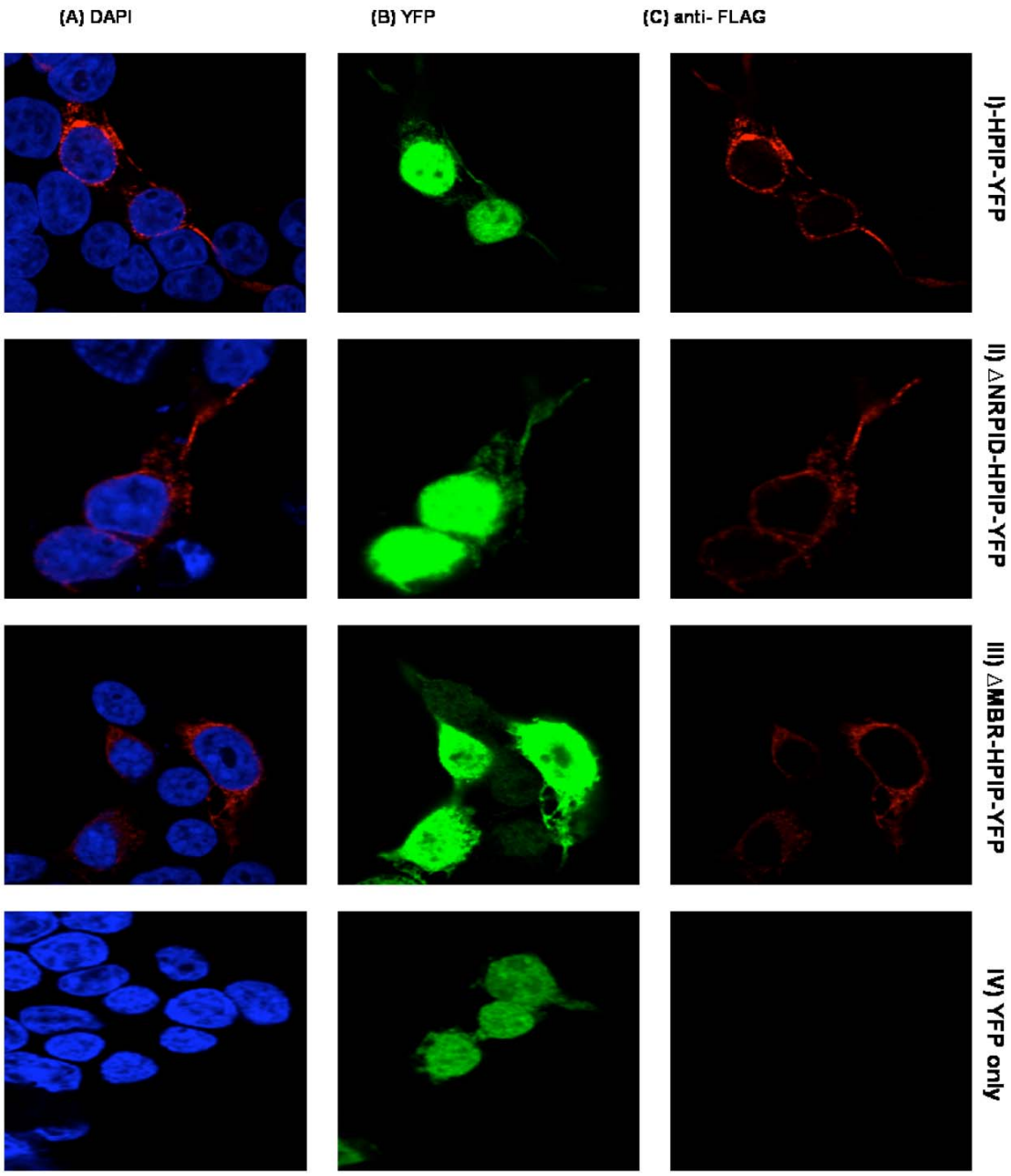

Figure 4.3: Immunostaining and confocal laser scanning of flag tagged WT and HPIP mutant form of proteins. HEK 293T cells were grown on cover slips. HPIP-WT and its mutant forms (ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP) were transfected and expressed in HEK 293T cell lines. As a control MSCV-YFP construct was used. The cells were fixed with PSA and stained with DAPI (Blue in colour), YFP (Yellow in colour) and anti-FLAG (Red in colour). Pictures were captured using Axiovert 200M/confocal microscope. In the figures above you can observe (third photo on the right

hand side) the staining with anti-Flag alone that revealed the presence of : (I) HPIP WT-YFP expression, primarily in cytoplasm (Red) (II) ΔNRPID-HPIP-YFP expression primarily in cytoplasm (Red) (III) ΔMBR-HPIP-YFP expression primarily in cytoplasm (Red) and (IV) YFP only with negative results for anti-Flag staining. Confocal laser scanning demonstrates that HPIP-WT and its mutant form proteins are primarily localized in the cytoplasm.

specific lineages and the maintenance and expansion of $hCD34^+$ cells *in vitro*. As an initial test to determine whether HPIP-WT or its mutant forms would affect the commitment and differentiation capacity of human haematopoietic progenitor cells, a total of $1x10^4$ highly purified (FACS) $hCD34^+$HPIP WT-YFP^+, $hCD34^+$ΔNRPID-HPIP-YFP^+, $hCD34^+$ΔMBR-HPIP-YFP^+ and $hCD34^+YFP^+$ cells were cultured in serum free medium supplemented with cytokines for 5 weeks (see methods, p-53).

Constitutive expression of HPIP-WT and its mutant forms decreased the cell proliferation *in vitro* at every time point measured between week 2 to 4: at week two, *HPIP* transduced hCB $CD34^+$ cells expanded from 10,000 cells plated at the beginning of the culture to $3.1x10^6$ cells, for Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells expanded to $8.7x10^5$ cells and for Δ*MBR-HPIP* transduced hCB $CD34^+$ cells expanded to $1.27x10^6$ cells as compared to $5.1x10^6$ cells in control YFP. At week three *HPIP* transduced hCB $CD34^+$ cells expanded to $4.19x10^6$, Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells expanded to $1.4x10^6$ and Δ*MBR-HPIP* transduced hCB $CD34^+$ cells expanded to $1.6x10^6$ as compared to $6.03x10^6$ cells in the control YFP.

The average cell expansion decreased between week 2 and week 4 for *HPIP* transduced hCB $CD34^+$ cells by 39% and 30.5% respectively, for Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells by 82% and 77.5% respectively ($p \leq 0.01$) and for Δ*MBR-HPIP* transduced hCB $CD34^+$ cells by 75% and 73.5 % ($p \leq 0.01$) respectively, relative to the control YFP (n=3). The average fold change in the number of cells measured between week 2 and week 4 was 1.5 fold decrease in cell number ($p \leq 0.05$) for cells expressing HPIP-WT, 4.1 fold decrease in cell number ($p \leq 0.01$) for cells expressing ΔNRPID-HPIP, and 3.5 fold decrease in cell number ($p \leq 0.01$) for cells expressing ΔMBR-HPIP as compared to control YFP expressing cells.

In addition the frequency and absolute number of clonogenic progenitors was assessed on weekly intervals for a time period of 5 weeks in liquid expansion culture. During the half medium change of the liquid expansion culture one third of the total harvested cell number was plated in methylcellulose (Methocult-H4434) to

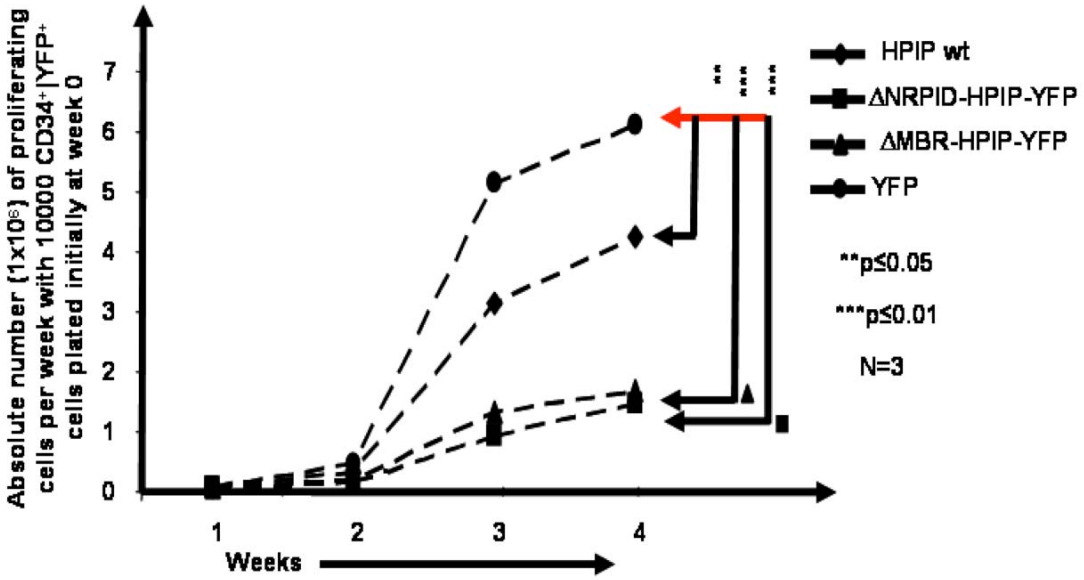

Figure 4.4a: Proliferation of transduced cells *in vitro*. A total number of 10, 000 hCD34$^+$ YFP$^+$ cells were sorted after the retroviral transduction of WT and HPIP mutant forms in hCD34$^+$ cells. These cells were suspended in cytokine-supplemented (Flt-3 ligand, SF, IL-3, IL-6 and G-CSF) serum-free medium (see methods, p-53). As demonstrated above, the expression of the mutant forms of HPIP showed a significant decrease in the number cell proliferation in suspension liquid culture between 3-4 weeks. There was a significant decrease of 3.5 fold in the proliferating cells in the case of ΔMBR-HPIP-YFP (***p≤0.01) and 4.1 in the case of ΔNRPID-HPIP-YFP (***p≤0.01, n=3) as compared to the control YFP (indicated with red line).

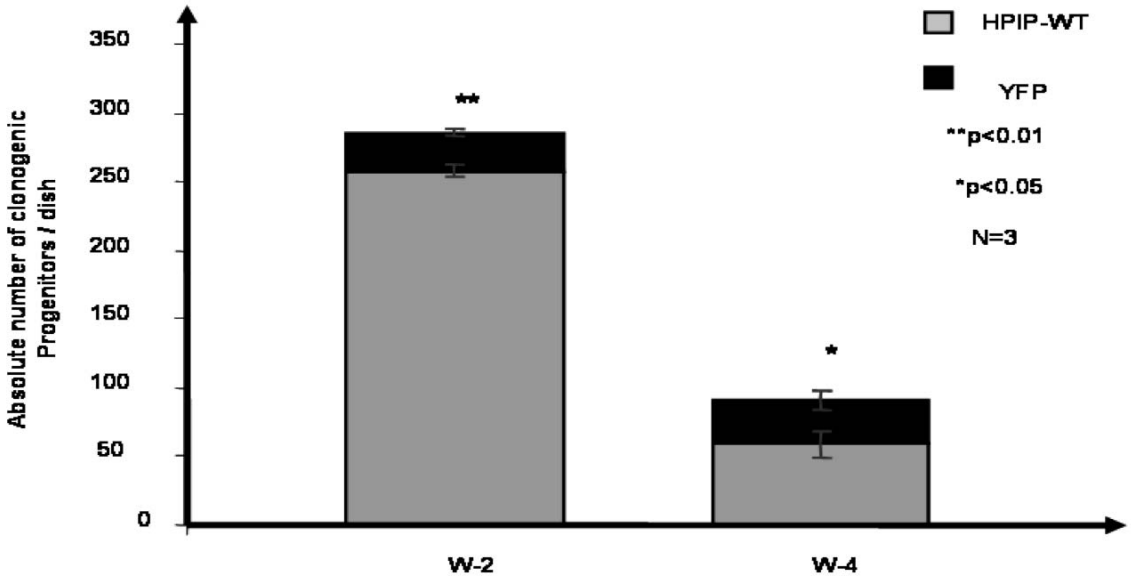

Figure 4.4b: Absolute number of clonogenic progenitors per dish in liquid expansion assay. The graph illustrates the absolute number of clonogenic progenitors per experimental group at the week 2 and week 4. Expression of HPIP-WT ($p<0.01$, n=3) was able to enhance significantly, the number of absolute progenitors per dish at week 2 relative to control YFP. At week 4 we could still see a similar significant difference ($p<0.05$) in the number of absolute clonogenic progenitors, with an increase in the number of progenitors in the case of HPIP relative to the YFP control.

evaluate the colony forming potential of the proliferating cells in the long term expansion culture *in vitro.* Due to very less number of proliferating cells in both the mutant forms of HPIP, we could not perform CFC assays from the liquid expansion cultures.

At week two, the average number of colonies for *HPIP* transduced hCB $CD34^+$ cells was equal to 43.63 (±4.58) per $5x10^5$ input cells ($p\leq0.05$, n=3)(harvested from the liquid expansion culture) as compared to control YFP with an average number of colonies equal to 26.8 (±2.6) per $5x10^5$ cells (n=3). A 1.6 fold increase in the number of colonies generated by *HPIP* transduced hCB $CD34^+$ cells ($p\leq0.05$, n=3) was observed at week 2 as compared to the colonies generated by the control *YFP* transduced hCB $CD34^+$ cells. At week four, the average number of colonies for *HPIP* transduced hCB $CD34^+$ cells was equal to 28.03 (±9.4) per $1.5x10^6$ input cells ($p\leq0.05$) as compared to the control YFP with an average number of colonies equal to 11.2 (±6.7) per $1.5x10^6$ input cells (n=3). A 2.5 fold increase in the number

of colonies generated by *HPIP* transduced hCB $CD34^+$ cells ($p \leq 0.05$, n=3) was observed at week 4 as compared to the colonies generated by the control *YFP* transduced hCB $CD34^+$ cells.

Furthermore the absolute number of progenitors was calculated considering the number of colonies generated, for each experimental arm from the total number of cells at week 2 and 4. At week 2, the expression of HPIP-WT in hCB CD34+ cells produced 258.2 (±5) absolute numbers of progenitors, with a significant increase relative to the 27.28 (±3) numbers of absolute progenitors generated by control YFP. At week 4, HPIP-WT expression could induce 58.98 (±9) number of absolute progenitors over 32.3 (±11) numbers of progenitor cells generated in the control YFP.

4.5. Colony forming cell (CFC) assays

4.5.1. Quantification of committed haematopoietic progenitor cells in vitro

Colony-forming cell (CFC) assay, also referred to as the methylcellulose assay, is the commonly employed *in vitro* assay for the quantification of committed haematopoietic progenitors. Clonogenic haematopoietic assays detect progenitor cells committed to a specific lineage by seeding candidate populations into semi-solid methylcellulose media. Progenitors identified by this assay are retrospectively classified as colony forming cells (CFC) and can be quantitatively subdivided into lineage restricted subtypes by examining the composition of the resulting progeny (methods, p-51)(Fig. 4.5.1a).

In order to test the impact of HPIP and its mutants on the growth of clonogenic progenitors, a total number of 3,300 hCB $CD34^+YFP^+$ cells transduced with WT and mutant forms (*HPIP-WT-YFP,* ∆*NRPID-HPIP-YFP,* ∆*MBR-HPIP-YFP)* and *YFP* control, were suspended in methylcellulose-H4434 (Methocult H4434) and plated into 2 methylcellulose dishes with an approximate number of 1000 $CD34^+YFP^+$cells plated per dish. The colony formation was evaluated after 14 days of incubation at 37ºC. The *HPIP* transduced hCB $CD34^+$ cells formed an average number of 112 (±9.2) colonies per 1000 hCB $CD34^+YFP^+$ initially plated cells, the ∆*NRPID-HPIP-YFP* transduced hCB $CD34^+$ formed 97 (±9.3) colonies per 1000 hCB $CD34^+YFP^+$ initially plated cells ($p \leq 0.05$) and the ∆MBR-*HPIP-YFP* transduced hCB $CD34^+$ formed 102 (±4.2) colonies per 1000 hCB $CD34^+YFP^+$ initially plated cells ($p \leq 0.05$) relative to 80 (+11.2) colonies formed by the control YFP transduced cells (n=6; Fig 4.5.1b).

An increase in the number of erythroid colonies was observed in the case of *HPIP* transduced hCB $CD34^+$ cells with an average number of 46.35 (±6.9) colonies per 1000 hCB $CD34^+YFP^+$ initially plated cells ($p \leq 0.01$, n=6) and ∆*MBR-HPIP-YFP* transduced hCB $CD34^+$ cells with an average number of 40.8 (±5.9) colonies generated per 1000 hCB $CD34^+YFP^+$ initially plated cells ($p \leq 0.01$, n=6), as

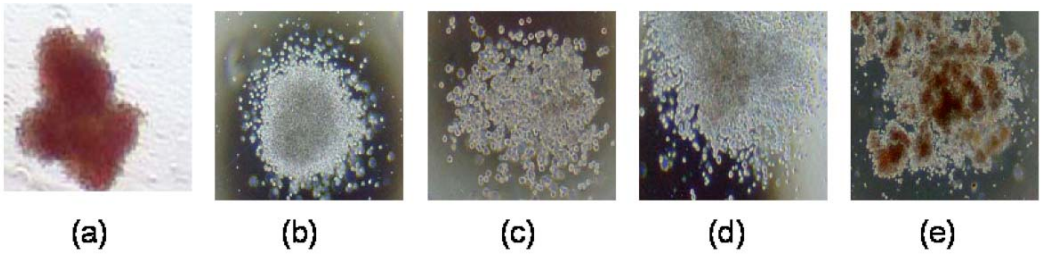

Figure 4.5.1a: Images of colonies obtained from hCB CD34+ cells grown on Methocult H4434. An illustration of (a) Erythroid E ; (b) Granulocytic G; (c) Megakaryocytic M; (d) Mixed GM and (e) Mixed GEMM colonies formed after plating human hCB $CD34^+$ cells in Methocult H4434 and scored after 14 days of culture . The colonies are thoroughly analysed for their morphology and scored accordingly. The above pictures represent the colonies formed by normal human haematopoietic $CD34^+$ cells

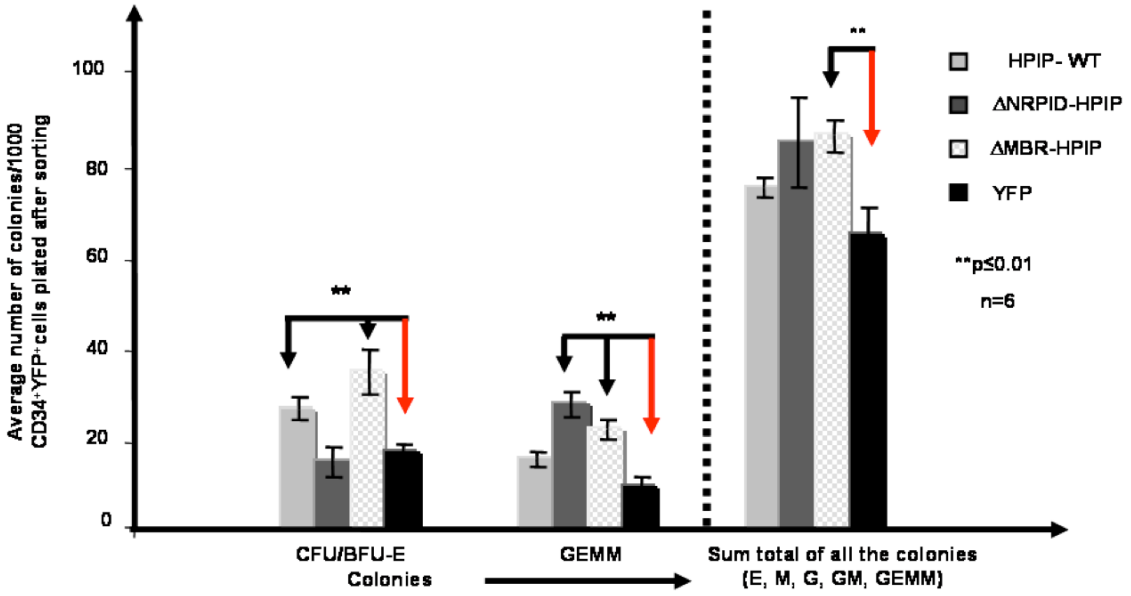

Figure 4.5.1b: The Colony Forming Cell (CFC) Assay. The graph illustrates the number of colonies obtained for $hCD34^+YFP^+$ cells expressing WT and HPIP mutant forms per 1000 cells plated initially per dish in Methocult H4434. The dishes were incubated for 14 days and thereafter colonies were scored and morphology was assessed. HPIP-WT increased the number of erythroid colonies ($p \leq 0.01$, $n = 6$) and mutation in the Nuclear receptor and PBX1 binding domain (NRPID) nulls the effect. The microtubule binding region mutant (MBR) increases the formation of erythroid colonies ($p \leq 0.01$, $n = 6$), similar to the effect produced by HPIP-WT as compared to control YFP (indicated by red lines). Both the mutant forms of HPIP increased the number of GEMM colonies ($p \leq 0.01$) as compared to the control YFP.

compared to an average of 25.4 (±3.5) colonies generated per 1000 hCB $CD34^+YFP^+$ initially plated cells, by control YFP transduced cells (n=6). Δ*NRPID-HPIP-YFP* transduced hCB $CD34^+$ cells generated 25.3 (±7.1) colonies per 1000 hCB $CD34^+YFP^+$ initially plated cells with no significant change observed as compared to the control YFP transduced cells (p=ns, n=6).

There was a slight increase observed in the primitive colony-forming unit-granulocyte, erythroid, macrophage, megakaryocytic (GEMM colonies), in the case of *HPIP* transduced hCB $CD34^+$ cells with an average of 18.1 (±2) colonies per 1000 hCB $CD34^+YFP^+$ initially plated cells (p=ns, n=6). A significant increase was observed in the number of GEMM colonies in the case of Δ*NRPID-HPIP*-YFP transduced hCB $CD34^+$ cells with and average of 32.5 (±3.2) colonies generated per 1000 hCB $CD34^+YFP^+$ initially plated cells (p≤0.0005, n=6) and Δ*MBR-HPIP*-YFP transduced hCB $CD34^+$ cells with an average of 25.8 (±2.7) colonies generated per 1000 hCB $CD34^+YFP^+$ initially plated cells as compared with 10.9 (±2.8) (p≤0.003, n=6) colonies generated by control YFP transduced cells (p≤0.01, n=6).

The absolute number of cells harvested *per CFC assay* did not show a significant change in the cell number generated by *HPIP* transduced hCB $CD34^+$ cells ($3.4x10^6$ ± $5.2x10^5$ cells, p=ns), for Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells ($2.5x10^6$ ± $4.6x10^5$, p=ns) relative to the control YFP per 1000 hCB $CD34^+YFP^+$ initially plated cells (n=6). A significant decrease was observed in the absolute number of cells harvested per CFC assay (all the cells from one complete CFC dish) for Δ*MBR-HPIP-YFP* transduced hCB $CD34^+$ cells ($1.6x10^6$ ± $5.2x10^5$) (p≤0.06, n=6) relative to the absolute number of cells in the case of control YFP per 1000 hCB $CD34^+YFP^+$ initially plated cells.

Further we calculated the absolute number of cells harvested *per colony* from each CFC assay. The number of cells per colony did not show a significant change in the case of *HPIP* transduced hCB $CD34^+$ cells ($3.8x10^4$ ± 5915 cells/ colony, p=ns), and for Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells ($2.6x10^4$ ± 5615 cells/ colony, p=ns) relative to the control YFP transduced hCB $CD34^+$ cells ($4.2x10^4$± $1x10^4$ cells/colony)(p=ns, n=6). In the case of Δ*MBR-HPIP* transduced hCB $CD34^+$

cells, the absolute number of cells per colony showed a significant decrease equivalent to $1.5x10^4$ ± 4510 cells/colony relative to control YFP ($p \leq 0.03$, n=6).

Morphologically, Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells generated smaller M and GEMM colonies as compared to *HPIP* transduced hCB $CD34^+$ cells. Also Δ*MBR-HPIP* transduced hCB $CD34^+$ cells generated larger GEMM colonies as compared to *HPIP* transduced hCB $CD34^+$ cells and YFP transduced hCB $CD34^+$ cells. The figure 4.5.1a illustrates the morphology of colonies that are obtained in CFC assay performed with $hCD34^+$ cells. The figure 4.5.1b and figure 4.5.1c illustrates graphically the primary CFC data from the assays.

4.5.3. CFC replating Assays

In order to quantify the self-renewal potential of clonogenic progenitors, secondary CFC assays were performed. The methylcellulose dishes containing primary colonies were harvested, and replated (1/4 cells generated per primary CFC assay plated per secondary CFC assay) into fresh methylcellulose dishes and scored after 14 days. The constitutive HPIP-WT expression in *HPIP* transduced hCB $CD34^+$ cells decreased the number of secondary colonies with the generation of 27.75 (± 2.79) average number of secondary colonies per $1x10^6$ cells harvested from the primary CFC assay, as compared to the control YFP transduced hCB $CD34^+$ cells, with 34.9 (±7.9) generated colonies per $1x10^6$ cells harvested from the primary CFC assay.

The constitutive expression of HPIP mutant forms Δ*NRPID-HPIP* in hCB $CD34^+$ cells generated a total number of (sum total of E, G, M, GM, GEMM) 30.8 (±2.79) colonies per $1x10^6$ cells harvested from the primary CFC assay and Δ*MBR-HPIP* in hCB $CD34^+$ cells generated 36 (±6.1) colonies per $1X10^6$ cells harvested from the primary CFC assay with no significant changes as compared to control YFP transduced hCB $CD34^+$ cells (34.9 ±7.9) (p=ns, n=6)(Fig. 4.5.2a). replating of CFCs formed by Δ*NRPID-HPIP*-YFP transduced hCB $CD34^+$ cells showed a significant increase in the formation of granulocytic colonies with an absolute

number of 14 (±3.41) colonies per 1X10^6 cells harvested from the primary CFC assay (p≤0.05,

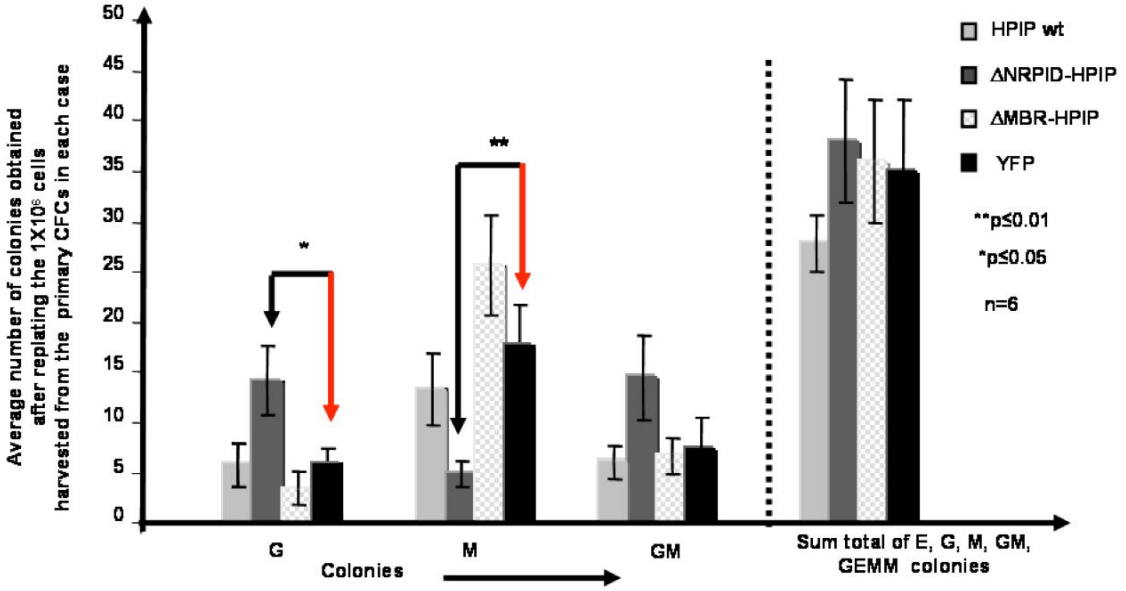

Fig 4.5.2a: Replating (Secondary) of colony forming cells: The graph illustrates the number of colonies obtained by replating the 1X10^6 cells harvested form the primary colony forming cell assay (section 4.5.1). The ΔNRPID-HPIP-YFP transduced cells showed a significant increase in granulocytic colonies (p ≤ 0.05, n = 6) and a decrease in macrophage colonies (p ≤ 0.01, n = 6) as compared to control YFP (indicated with red lines). An increase in more primitive GM colonies was observed in ΔNRPID-HPIP- YFP transduced cells compared to HPIP-WT and control YFP, but was non-significant.

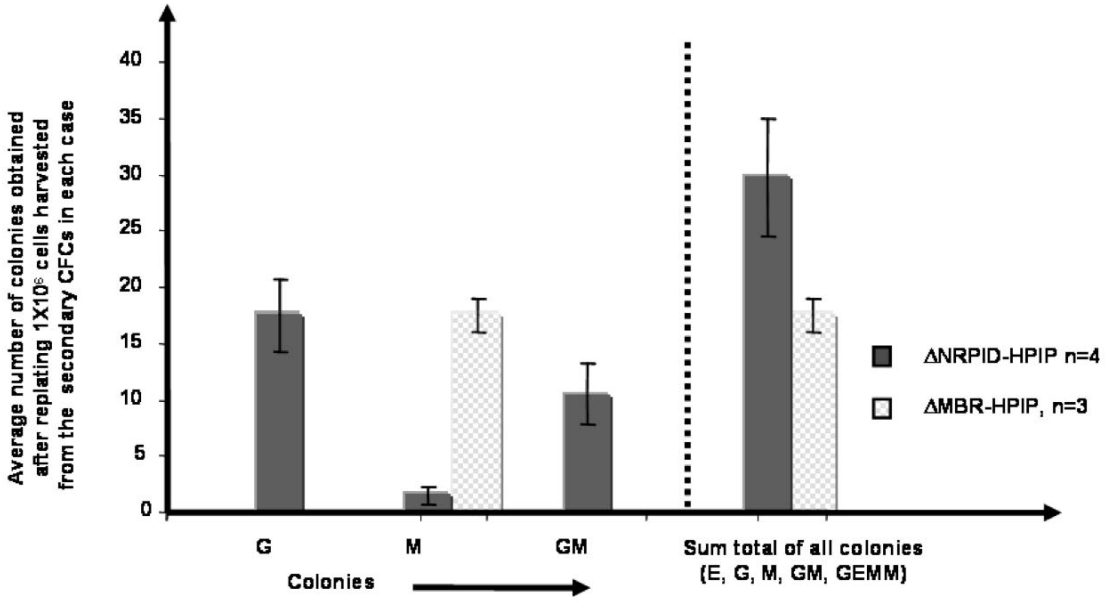

Fig 4.5.2b: Replating (Tertiary) of colony forming cells: The graph illustrates the number of colonies obtained by replating the $1X10^6$ cells harvested form the secondary colony forming cell assay (fig 4.5.2a). The values shown in above figure are normalised to a total number of $1X10^6$ input cell number (obtained from a secondary CFC) per experimental group. The ΔNRPID-HPIP-YFP was able to generate again G, M and GM colonies. In contrast the HPIP-WT and control YFP cells could only generate small clusters of megakaryocytes.

n=6) as compared to control YFP with 6 (±1.6) colonies per $1X10^6$ cells harvested from the primary CFC assay and HPIP-WT that showed 5 (±2) colonies per $1X10^6$ cells harvested from the primary CFC assay.

In addition, the constitutive expression of Δ*NRPID-HPIP* in hCB $CD34^+$ cells induced a decrease in the number of megakaryocytic colonies (5±1.3 colonies per $1X10^6$ cells harvested from the primary CFC assay) ($p \leq 0.01$, n=6) as compared to the colonies generated by expression of the control YFP (17.55±4.22 colonies per $1X10^6$ cells harvested from the primary CFC assay). There was an average increment of 60% of granulocytic colonies and a decrease of 67% in number of megakaryocytic colony formation by the expression of Δ*NRPID-HPIP* in hCB $CD34^+$ cells in replating assay as compared to the colonies generated by expression of *HPIP* and control YFP in hCB $CD34^+$ cells.

At the end of the secondary CFC assays, half of the harvested cells were replated into 2 methylcellulose dishes (1/4 number of cells from secondary CFC assay per tertiary CFC assay) and the average number of colonies generated were scored after 14 days of incubation (Fig. 4.5.2b). The expression of ΔNRPID-HPIP in transduced hCB $CD34^+$ cells formed on an average of 27.45 (± 3.41) total colonies per $1X10^6$ cells harvested from the secondary CFC assay in the tertiary replating while there were no colonies observed in control YFP. The former cells could sustain the formation of granulocytic colonies (17.5 ± 3.2), megakaryocytic colonies (1.5 ± 0.78) and mixed GM colonies (10.5 ± 2.6) per $1X10^6$ cells harvested from the secondary CFC assay. The expression of ÄMBR-HPIP in transduced hCB $CD34^+$ cells generated on an average 17.5 (±1.5) total colonies per $1X10^6$ cells harvested from the secondary CFC assay and all of them were megakaryocytic

colonies. There were small clusters of cells to no colonies observed in the case of HPIP-WT and YFP.

4.6. B-Lymphoid progenitor in vitro *assay*

In an *in vitro* B-lymphoid progenitor assays, a total number of $1x10^4$ hCB $CD34^+YFP^+$ cells were sorted for each experimental group (hCB $CD34^+$ cells transduced with HPIP-WT, ΔNRPID-HPIP-YFP, ΔMBR-HPIP-YFP and YFP) and co-cultured with MS-5 feeder with two different cytokine cocktails: in the first set of co-culture the cells and feeder was supplemented with a cocktail of three cytokines (SCF, IL-2, IL-15) for a period of 4 weeks. Every week a half medium change was performed without disturbing the experimental cells that were sticking to the MS-5 feeder layer. The total number of harvested cells, from the 10,000 initially plated cells was calculated at the end of the four weeks in culture.

The *HPIP* transduced hCB $CD34^+$ cells increased to $2.9x10^5 \pm 2x10^5$ (with >20 fold increase in cell number as compared to initially plated 10,000 cells, n=4, p= ns), the Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells decreased to 8983 ±230 cells (with <1 fold decrease in cells number as compared to initially plated 10,000 cells, n=4, $p \leq 0.05$)), the Δ*MBR-HPIP* transduced hCB $CD34^+$ cells increased to $2.4x10^4 \pm 1.5x10^4$ cells (with >2 fold increase in cell number as compared to initially plated 10,000 cells, n=4, p=ns) and the YFP transduced hCB $CD34^+$ cells increased to $3x10^4 \pm 4300$ cells (with >3 fold increase in cell number as compared to initially plated 10,000 cells, n=4)(Table 4.6a).

Further we tested the fractions of cells with various lymphoid and myeloid surface markers, in the bulk harvested cells, from each experimental arm. The *HPIP* transduced hCB $CD34^+$ cells generated an increase observed in the absolute number of $CD34^+$ $CD38^{low/-}$cells (960 ± 615 cells, p=ns), a significant increase was observed in the proportion of the $CD10^+$ cells (2328 ± 1292 cells, $p \leq 0.05$), and $CD19^+$ cells (4179 ± 2558 cells, $p \leq 0.05$) as compared to the absolute number of cells generated by control YFP transduced hCB $CD34^+$ cells (Table 4.6a).

In the second setting, the feeder layer was supplemented with six cytokines (SCF, IL-2, IL-15, IL-7, FLT3-L, and TPO), and the cells were cultured for a period of four weeks. For establishing this biphasic culture a total number of $1x10^4$ CB $CD34^+YFP^+$ cells were sorted for each experimental group (hCB $CD34^+$ cells transduced with HPIP-WT, ΔNRPID-HPIP-YFP, ΔMBR-HPIP-YFP and YFP). Every week a half medium change was performed without disturbing the experimental cells that were bonded to the MS-5 feeder layer. The total number of harvested cells (from 10,000 initially plated cells), were calculated at the end of four weeks for each culture.

The *HPIP* transduced hCB $CD34^+$ cells increased to $3.8x10^4$ ± 7461 cells in number (with ≥3.8 fold increase in cell number as compared to initially plated 10,000 cells, n=5), the Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells increased to $3x10^4$ ± 9129 cells in number (with ≥3 fold increase in cells number as compared to initially plated 10000 cells, n=4), the Δ*MBR-HPIP* transduced hCB $CD34^+$ cells increased to $5.2x10^4$ ± 8052 cells in number (with ≥5.2 fold increase in cell number as compared to initially plated 10,000 cells, n=4) and the YFP transduced hCB $CD34^+$ cells increased to $4.9x10^4$ ± 6461 cells in number (with >4.9 fold increase in cell number as compared to initially plated 10,000 cells, n=4).

The constitutive expression of HPIP- WT showed a significantly increased absolute number of the $CD10^+$ ($p \leq 0.01$, n=5), $CD15^+$ ($p \leq 0.05$, n=5) cells as compared to the absolute number of cells in the case of control YFP transduced hCB $CD34^+$ cells (n=5)(Table 4.6b). The Δ*NRPID-HPIP* transduced hCB $CD34^+$ cells did not show any significant changes in the number of $CD33^+$ (p=ns, n=4) but could significantly increased the absolute number of $CD15^+$ ($p \leq 0.05$, n=4) cells as compared to the proliferation in the case of control YFP transduced hCB $CD34^+$ cells (n=5)(Table 4.6b). The Δ*MBR-HPIP* transduced hCB $CD34^+$ cells also did not show any significant changes in the absolute number of cells with different surface markers expressing cell fractions, as compared to the cell proliferation in the case of control *YFP* transduced hCB $CD34^+$ cells.

Table 4.6a: The table illustrates the development of hCB CD34$^+$ cells expressing HPIP-WT, ΔNRPID-HPIP-YFP, ΔMBR-HPIP-YFP, and YFP in a 3 cytokine supplemented setup of the B-lymphoid assay.

hCB CD34$^+$ cells transduced with	*Average Number of hCD45$^+$ cells generated (no. of cells ± SEM)* (% proportion of YFP+ cells)	*Average Number of hCD34$^+$ CB38$^{low/-}$ cells (no. of cells ± SEM)* (% proportion of YFP+ cells)	*Average Number of hCD10$^+$ cells (no. of cells ± SEM)* (% proportion of YFP+ cells)	*Average Number of hCD19$^+$ cells (no. of cells ± SEM)* (% proportion of YFP+ cells)	*Average Number of hCD15$^+$ cells (no. of cells ± SEM)* (% proportion of YFP+ cells)	*Average Number of hCD33$^+$ cells (no. of cells ± SEM)* (% proportion of YFP+ cells)
HPIP-WT n=4	2.9x10^5± 2x10^5 (96.36%) •p=ns	960 ± 615 (0.97%) p=ns	2328 ± 1292 (2%) p≤0.05	4179 ± 2558 (1.6%) p≤0.05	712± 477 (0.7%) p = ns	2.6x10^5± x10^5 (83.5%) p=ns
ΔNRPID-HPIP n=4	8983 ±230 (90%) p≤0.05	306 ± 121 (3%) p=ns	74 ±47 (0.7%) p=ns	13± 9 (0.5%) p=ns	387.5±172.6 (2.87%) p=ns	8432± 418.3 (84%) p=ns
ΔMBR-HPIP n=4	2.4x10^4 ± 1x10^4 (96.8%) p=ns	196 ± 20.7 (1.7%) p=ns	170 ± 88 (1.2%) p=ns	9.9 ± 8 (0.5%) p=ns	40.5±24.4 (3.4%) p≤0.05	27293 ±17523 (95.7%) p=ns
YFP n=4	6x10^4 ± 4298 (95.18%)	246 ±31 (0.4%)	218 ± 55 (0.2%)	15 ± 2 (0.3%)	201±48 (0.9%)	5.2x10^4 ± 3547 (85.2%)

• P values refer to the significant differences between the absolute numbers of cells with respect to control YFP.

Table 4.6b: The table illustrates the development of hCB CD34$^+$ cells expressing HPIP-WT, ΔNRPID-HPIP-YFP, ΔMBR-HPIP-YFP, and YFP in a 6 cytokine supplement setup of B-Lymphoid assay.

hCB CD34$^+$ cells expressing	*Average Number of hCD45$^+$ cells generated (no. of cells ± SEM)*	*Average Number of hCD34$^+$ CD38$^{Low/-}$ cells (no. of cells ± SEM)*	*Average Number of hCD10$^+$ cells (no. of cells ± SEM)*	*Average Number of hCD19$^+$ cells (no. of cells ± SEM)*	*Average Number of hCD15$^+$ cells (no. of cells ± SEM)*	*Average Number of hCD33$^+$ cells (no. of cells ± SEM)*
HPIP WT N=5	3.8x10^4 ± 7461 (99%) •p=ns	977 ± 352 (3.5%) p=ns	32166 ± 0830 (1.2%) p≤0.01	301 ± 113 (2.3%) p=ns	294 ± 109 (3.8%) p≤0.05	36688 ± 7723 (95%) p=ns
ΔNRPID-HPIP N=4	3x10^4 ± 9129 (95.32%) p=ns	1634 ± 943 (2.5%) p=ns	172 ± 172 (0.8%) p=ns	517 ± 517 (0.2%) p=ns	2242 ± 980 (7.8%) p≤0.05	26629 ± 7791 (90.7%) p=ns
ΔMBR-HPIP N=4	5.2x10^4 ± 8052 (97.37%) p=ns	3208 ± 2856 (1.1%) p=ns	938 ± 487 (1.6%) p=ns	2384± 2384 (3.17%) p=ns	1355 ± 1028 (3.3%) p=ns	49762 ± 751 (95.90%) p=ns
YFP N=5	4.9x10^4 ± 6461 (97.37%)	2560 ± 118 (2%)	210 ± 5 (0.4%)	1263 ± 61 (3.8%)	30 ± 2 (0.1%)	47828 ± 522 (94%)

• P values refer to the significant differences between the absolute numbers of cells w.r.t control YFP.

4.7. Long term culture initiating cell assay (LTC-IC) in vitro.

In an attempt to test the influence of HPIP-WT expression on the maintenance of primitive human progenitors in long-term cultures, we performed the LTC-IC assay: highly purified 1x10^4 CD34$^+$ YFP$^+$, CD34$^+$ HPIP-YFP$^+$, CD34$^+$ ΔNRPID-HPIP-YFP$^+$ and CD34$^+$ ΔMBR-HPIP-YFP$^+$ cells were seeded on a mixture of M2-10B4-J-GCSF-tkneo-J-IL3-hytk fibroblast and SL/SL-J-SF-tkneo-J-IL3-hytk fibroblast feeder cells engineered to produce human SF, IL-3, and G-CSF under LTC-IC conditions. The bulk LTC-IC cultures were carried out for a period of 6 weeks with a weekly half medium change. At the end of week 6 all the cells were harvested from the dishes and the number of cells obtained was registered. These cells were resuspended in Methocult-H4434 and plated in methylcellulose dishes (one half of the total number of cells obtained in each dish). Colonies generated after 16-20 days were scored. The average number of CFC per 1x10^6 initially plated cells was calculated (see methods, p-53&54). The expression of HPIP-WT in hCB CD34+ cells increased the CFC output by 10 fold with an average value of 14315 ± 87 colonies per 1X10^6 cells initially plated versus the colonies obtained by the expression of YFP control alone, with an average number of 1371.4 ± 672 colonies per 1x10^6 cells initially plated (n=8, p≤0.05)Table 4.7)

The cells expressing ΔMBR-HPIP-YFP mutant form did not show an increase in the number of colonies generated equivalent to 1337 (±493) colonies per 1X10^6 cells as compared to the control YFP (n=3, p=ns). The cells expressing ΔNRPID-HPIP-YFP mutant form showed a decrease in the number of colonies generated with an average number equivalent to 424 (± 64) (p=ns) colonies per 1X10^6 cells as compared to the control YFP.

The total yield of LTC-IC was calculated relative to the number of CFC obtained per 1x10^6 initially plated cells. The average CFC output per LTC-IC[115] has been standardized to a calculated factor of 8. The CFC output per million cells was divided by 8 to obtain the absolute number of LTC-IC generated at the end of the co-cultures, after 6 weeks. The number of LTC-IC generated per million cells for the cells expressing HPIP-WT was calculated. The expression of HPIP-WT in

Table 4.7 shows the average number of LTC-IC and CFC generated per million of initially plated cells in bulk LTC-IC assay *in vitro*.

The experimental form retrovirally transduced in hCB CD34+ cells	*Average number of CFC per 1x10^6 initially plated cells (± SEM)*	*Average number of LTC-IC generated per million cells (± SEM)*
HPIP-WT-YFP, (n=8)	**14315 ± 87**	***545 ± 169**
ΔNRPID-HPIP-YFP (n=3)	**424 ± 64**	**53 ± 8**
ΔMBR-HPIP-YFP (n=3)	**1338 ± 493**	**180.4 ± 58**
YFP (n=8)	**1371.4 ± 672**	**165.8 ± 67**

*Significance calculated with respect to control YFP (p≤0.05)

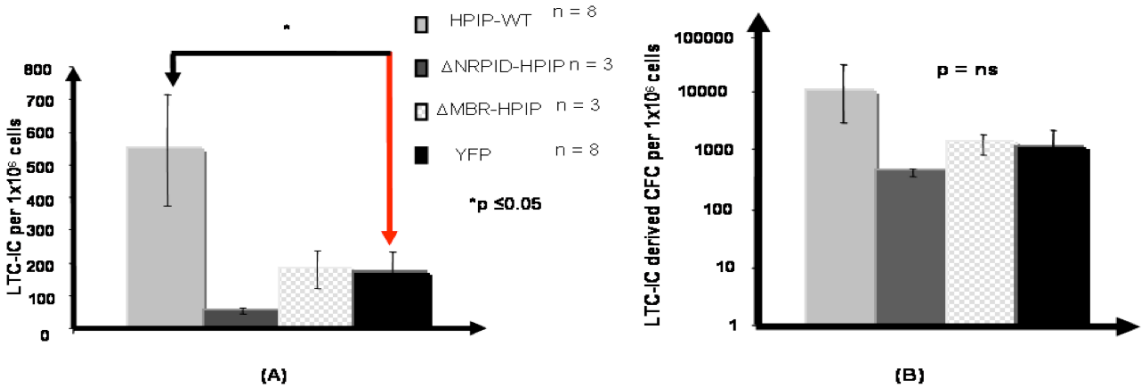

Figure 4.7: Long term culture initiating cell assay *in vitro*. The graphs show the long term culture initiating cells (LTC-ICs) obtained with the constitutive expression of WT and mutant forms of HPIP in hCB CD34$^+$ cells. The WT and mutant HPIP forms were seeded (10,000 cells per experimental group) on a mixture of M2-10B4-J-GCSF-tkneo-J-IL3-hytk fibroblasts and SL/SL-J-SF-tkneo-J-IL3-hytk fibroblasts feeder cells and co-cultured using LTC-IC medium and (see methods, 53&54), for 6 weeks. Every week a half medium change was performed not disturbing the cells sticking to the feeder layers. At the end of 6 weeks al the cells were harvested, counted and suspended in Methocult-H4434, and plated into methylcellulose dishes. After 19-20 days in methylcellulose culture, colonies were scored and LTC-IC bulk frequency was calculated. hCB CD34$^+$ cells transduced with HPIP-WT showed a significant increase of LTC-IC per 1x10^6 cells (A) (p≤0.05, n=8) as compared to YFP control (indicated with red line).

hCB CD34+ cells increased the number of LTC-IC by 2.5 fold, with an average number of 545 (±169) bulk LTC-IC per million cells initially seeded as compared to the control YFP with an average number of 102 (±33) bulk LTC-IC per million cells (n=8, p≤0.05). The mutant forms of HPIP-WT did not show any significant changes

in the calculated absolute number of LTC-IC per million cells as compared to the control YFP.

4.8. Double transductions with HPIP-WT-YFP and HOXB4-WT-GFP construct.

To evaluate whether genes such as *HOXB4* are able to collaborate with *HPIP,* double transductions were performed in primary cord blood progenitors. However, there were no significant differences found in *in vitro* Colony forming cell (CFC) assays or Long term culture initiating cell (LTC-IC) assays for CBCD34^{+}HPIP WT-YFP^{+}/HOXB4-GFP^{+} and the control vector YFP^{+}/GFP^{+} (Figure 4.8). The double transduction experiment demonstrated that there was no impact of HOXB4-WT constitutive expression on HPIP-WT function in this functional assay setup of CFC and LTC-IC.

In order to test the impact of constitutive expression of HPIP-WT and HOXB4-WT together on the growth of clonogenic progenitors 3300 cells triple positive for HPIP- WT-YFP, HOXB4-WT- GFP and CD34 expression were suspended in methylcellulose-H4434 (Methocult-H4434) and plated into 2 methylcellulose dishes (1000 CD34^{+}YFP^{+}GFP^{+}cells/ dish). In parallel separate CFC assays were carried out with HPIP-WT-YFP only, HOXB4-WT-GFP only expressing cells and YFP only or YFP/GFP expressing cells as control. The methylcellulose dishes were scored on the 15th day for colonies. The outcome of colony formation is illustrated in the figure 4.8b. There were no significant synergistic effects observed on the total number of colonies and the types of colonies generated in double transduced cells as compared to the control YFP.

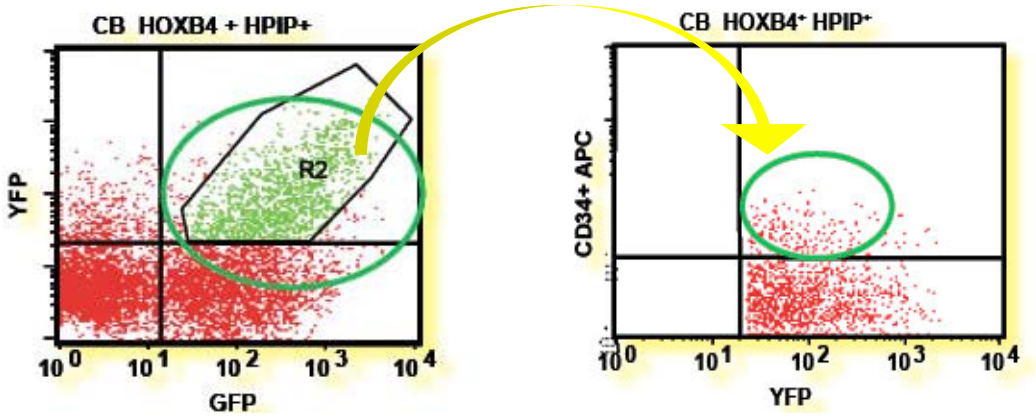

Figure 4.8: Double transductions with HPIP-WT and HOXB4 construct. The above acquisition plots illustrate a simple setup of sorting double transduced cells expressing HPIP-WT-YFP and HOXB4-GFP in primary cord blood cells. Only YFP and GFP double positive cells were gated (gate R2). Gated cells of R2 were further gated to define $CD34^{+}APC^{+}$ cells. These triple positive cells $CD34^{+}YFP^{+}GFP^{+}$ were sorted for different *in vitro* assays such as CFC and LTC-IC.

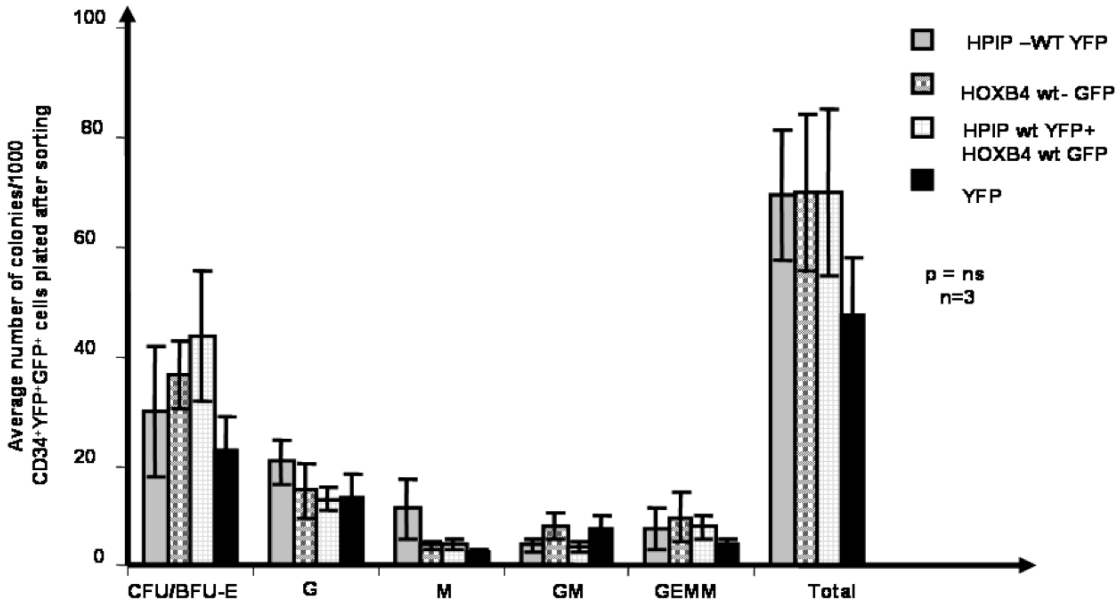

Figure 4.8b: CFC assays with double transduced hCB $CD34^{+}$ cells. The graph illustrates the number of colonies obtained by the hCB CD34+ cells transduced with HPIP-WT, HOXB4-WT, HPIP- WT+ HOXB4-WT and control YFP vectors. There were no significant changes observed in the colony numbers of E, G, M, GM and GEMM or total colony numbers in the CFC assay done with double transductions (n=3, p=ns).

4.9. Analysis of WT and HPIP mutant expression functions in the xenotransplant NOD/SCID model.

To evaluate the effects of constitutive expression of HPIP-WT and its mutant forms, on the developmental pathways of human haematopoietic progenitor cells *in vivo*, xeno-transplantations of hCB $CD34^+$ transduced with HPIP-WT, ΔNRPID-HPIP-YFP, ΔMBR-HPIP-YFP and YFP, were performed in NOD/SCID mice. The hCB $CD34^-YFP^+$ and hCB $CD34^-YFP^-$ cells were co-injected with the $CD34^+$ transduced cells to support their engraftment did you irradiate them?. The initial number of cells taken for injections (double positive for hCB CD34 and YFP) for bulk *in vivo* assays was $2.0x10^4$ hCB $CD34^+YFP^+$ cells for all the irradiated cohorts of mice. At the end of 6-8 weeks, mice were sacrificed and the engraftment of transduced human hCB cells (YFP+) and non-transduced human hCB (YFP-) cells was assessed by FACS. In order to characterize long-term engrafts in mice bone marrow, the multilineage differentiation of the engrafted hCB CD34+YFP+ cells (engrafts) was assessed. Figure 4.9 illustrates how the analysis was performed.

4.9.1. Engraftment of transduced hCB $CD34^+$ cells in the bone marrow of NOD/SCID mice.

Lympho-myeloid engraftment was defined by the presence of at least 5 human transduced $CD19^+$ B cells plus at least 5 human transduced myeloid $CD15^+$ cells per $2×10^4$ $CD45^+$ human cells analyzed from harvested mice bone marrow. The harvested BM cells from all the cohorts of xenotransplanted mice, that were engrafted, were analyzed for expression of a panel of surface markers (CD45, CD34, CD38, CD15, CD19, CD33, CD36, CD41a, CD71, CD10, CD133, CD117 and Glycophorin A) to distinguish the different myeloid, lymphoid or erythroid fractions of human haematopoietic cell lineages.

The engraftment levels of YFP^+ human haematopoietic cells, at week 6, ranged from 0.36 to 8.6 % of $CD45^+$ YFP^+ for the HPIP-WT cohort (n=6) and from 0.8-13.07 % for the YFP control group (n=8). Both groups showed lymphoid

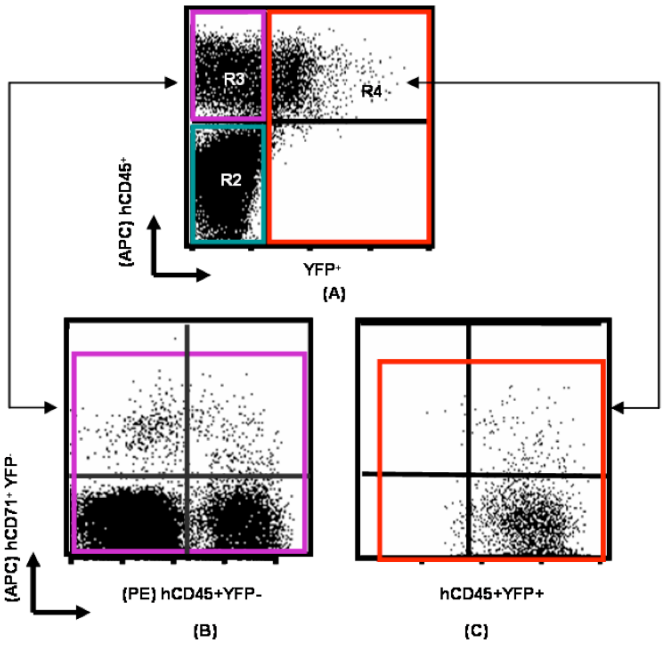

Figure 4.9.: Representative acquisition dot plots from the cells harvested from the BM of the NOD/SCID mice. The dot graphs above are a simple illustration of the criteria that was used to assess the presence and repopulation of engrafted human haematopoietic cells in NOD/SCID mice. After the harvest of NOD/SCID bone marrow, the cells were stained for hCD45 (PE) cell surface marker. In the dot graph a mix of mouse and human cells that were gated as R1(shown in green box); (A) all the cells gated in R1 were further separated into three different gates R2 (shown in turquoise box), R3 (shown in pink box) and R4 (shown in red box); (C) the cell population in R2 region presents murine bone marrow cells; (B) The cell population in R3 region is the human $CD45^+$ YFP^- cell population and (C) the cell population gated in R4 region is the $hCD45^+YFP^+$ cells. Similarly all the other staining were analysed in engrafted mice using only the $hCD45^+YFP^+$ human compartment of cells. As an internal control for comparison with the transduced compartment the $hCD45^+YFP^-$ compartment was used. In this experimental set up different cell surface markers were used to determine the differentiation stage of cells such as e.g. CD34 (PE)/CD38 (APC).

predominance of engrafted human cells with lymphoid/myeloid ratios (CD19/CD15) ratio ranging between 3 to10. Similarly, engraftment levels were measured in the cohorts of mice transplanted with ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP expressing cells At week 8, the engraftment levels of YFP^+ human cells ranged from 0.54 to 2.41% for ΔNRPID-HPIP-YFP (n=4) and were 2.4% for ΔMBR-HPIP-YFP(n=1). These results demonstrate that a long-term lymho-myeloid engraftment in all experimental groups was achieved, indicating efficient gene transfer into long-term repopulating stem cells (Table 4.9.1).

Table 4.9.1: Multilineage engraftment generated by the hUCB CD34$^+$ Lin$^-$ cells transduced with WT and mutant forms of HPIP.

Cohort	*Lymphoid engraftment in NOD/SCID mice*		*Myeloid engraftment in NOD/SCID mice*			
	% of CD45$^+$CD19$^+$ cells		% of CD45$^+$CD15$^+$ cells		% of CD45$^+$CD33$^+$ cells	
	°° % of all YFP$^+$ and YFP$^-$ cells (±± SEM)	°°° % of YFP$^+$ only (±± SEM)	°° % of all YFP$^+$ and YFP$^-$ cells ±± SEM	°°° % of YFP$^+$ only ±± SEM	°° % of all YFP$^+$ and YFP$^-$ cells ±± SEM	°°° % of YFP$^+$ only ±± SEM
HPIP WT-YFP (n=8)	53.24±±1.17	82.69±±4.29	8.45±±4.5	36.26±±3.79	34.88±±8	69.16±±7.18
∆NRPID-HPIP-YFP (n=4)	28.02±±2.24	20.34±±5.77	10.04±±0.7	7.35±±1.5	15.0±±4.3	53.36±±9.89
∆MBR-HPIP-YFP (n=1)	33.51	16.57	2.41	18.67	8.01	61.08
YFP (n=6)	59.89±±5.88	65.00±±12	11.21±±5.0	14.50±±4.8	27.99±±9.0	70.68±±7.37

° Representative of gates R3and R4, as shown in figure 4.9 above.

°° Representative of gate R4 only, as shown in figure 4.9 above.

∆NRPID-HPIP–YFP expression decreased the human cell engraftment into NOD/SCID mice. Of 31 mice that received a transplant, only 9 mice (29%) exhibited human cell engraftment after 6-8 weeks and the engraftment levels were low (medien=1.9%, range=0.58%-3.42%) relative to the YFP control (medien=7.71, range=0.35%-13.8%).

4.9.2. The assessment of the lymphoid/ myeloid ratio in vivo.

Balanced HSCs repopulate peripheral white blood cells in the same ratio of myeloid to lymphoid cells as seen in unmanipulated mice (on average about 15% myeloid and 85% lymphoid cells, or 3≤ñ≤10). In the recipients of transduced HPIP-WT-YFP, the number of $CD15^+$ Myeloid cells generated was found to be 2.5 fold higher thanthe $CD15^+$ YFP^+ cells in the BM of the control YFP mice. The lymphoid/myeloid ratio was 1.8, in the case of HPIP-WT as compared to 4.4 in the control YFP mice (n=6, p≤0.05) (Fig 4.9.2). The expression of the ΔMBR-HPIP mutant form of HPIP resulted even in an inversion of the lymphoid/myeloid ratio of 0.8. The expression of the ΔNRPID mutant form of HPIP did not show any significant inversion of CD19/CD15 ratio as compared to the control YFP or HPIP-WT (slightly lower value of 2.7 as compared to YFP control (n=4, p=ns). Thus, constitutive expression of the WT HPIP significantly reduced the lymphoid/myeloid ratio of human engrafted cells depending on its ΔNRPID domain.

4.9.3. Short term (hSTRCs) and long term (hLTRCs) repopulating populations in NOD/SCID bone marrow.

The harvested bone marrow from all the cohorts of NOD/SCID mice was analysed in details by staining the $CD45^+$ cell population with various hSTRCs and hLTSCs classifying surface markers. At least $5x10^5$ cells were chosen to be stained with antibodies specific for surface antigens of CD34, CD38, cKIT (CD117) and CD133 expression. The proportion of $CD34^+$ cells in the engrafts transduced with HPIP- WT-YFP showed no significant changes with a mean value of 43.7% ($CD34^+YFP^+$ cells, p≤0.05, n=6) as compared to 42.7% in the control mice (n=6).

The proportion of $CD34^+$ cells in the engrafts transduced with ΔNRPID-HPIP-YFP was significantly decreased with a mean value of 14.45% ($CD34^+YFP^+$ cells; p≤0.05, n=4) as compared to the YFP control. The proportion of $CD34^+$ cells in the engrafts transduced with ΔMBR-HPIP-YFP also did not show any significant changes with a mean value of 34.85% ($CD34^+YFP^+$ cells, p=ns, n=1), as compared to the proportion of $CD34^+$ cells in the engrafts transduced with YFP with a value of

42.7% (n=6)(Fig 4.9.3a). The hCD34^{+} cells were checked for the presence of the early differentiation marker CD38. The proportion of cells, expressing CD34^{+} and

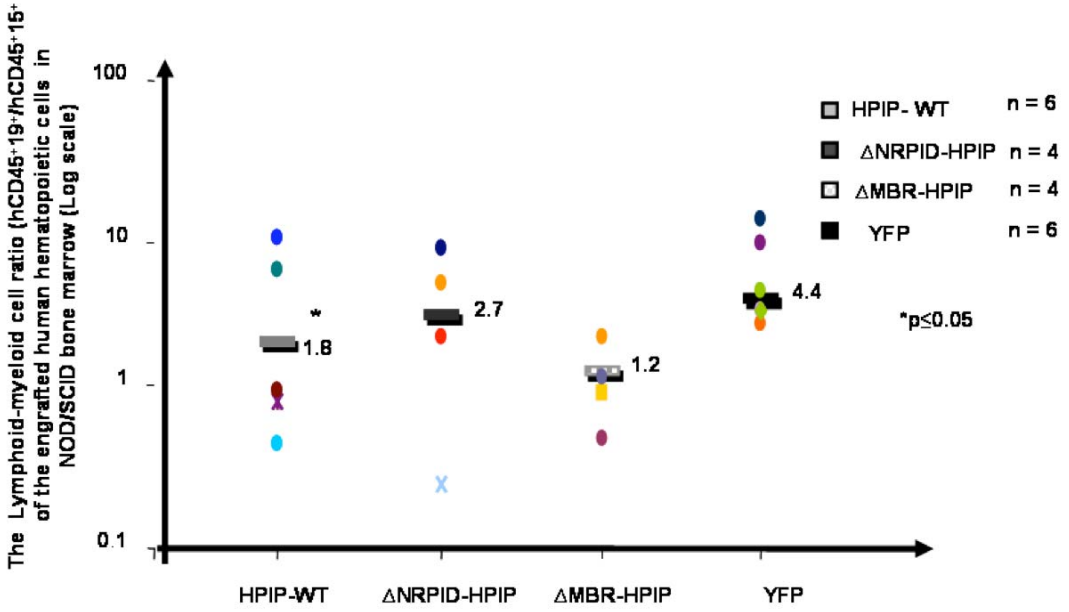

Figure 4.9.2: The lymphoid / myeloid cell ratio assessment. The figure demonstrates the calculated lymphoid to myeloid cell ratio (CD19^{+}/CD15^{+}), that was assessed in the bone marrow of engrafted NOD/SCID mice in each experimental group. (A)After 8 weeks of transplantation with hCB CD34^{+}YFP^{+} cells, the bone marrow of NOD/SCID mice was harvested and checked for engraftment. The CD45^{+} YFP^{+} cell proportions from each of the engrafted mice were analysed for the presence of CD19^{+} and CD15^{+} surface markers. Engrafted mice transplanted with CB CD34^{+} HPIP-WT-YFP^{+} cells showed a significant change in the ratio between lymphoid and myeloid cells of 1.8 (n=8, **p≤0.05) as compared to the YFP control with 4.4 (n = 6), indicating a generation of a more myeloid biased population by HPIP-WT.

CD38^{+} [31](hCD34^{+}CD38^{+}, hSTRCs) was found to be significantly increased in engrafts transduced with HPIP-WT with a mean value of 43.16% (p≤0.05, n=6) as compared to the YFP control with a mean value of 22.39% (n=6)(Fig 4.9.3a). This effect of HPIP was depending on the ΔNRPID domain as in ΔNRPID-HPIP mice there was a significant decrease in hSTRCs with a mean value of 7.69% (p≤0.01, n=4) compared to HPIP- WT and the YFP control. The results showed that expression of HPIP-WT or its mutant forms can significantly change the proportions of the hSTRCs *in vivo.*

The second analysis focused on the proportions of hLTRCs in the xenotransplated NOD/SCID mice with WT and mutant HPIP transduced hCB $CD34^+$ cells (Fig. 4.9.3b). The percentage of $CD45^+YFP^+$ cells in all the engrafts was checked for the presence of primary haematopoietic progenitors (HPCs), expressing the surface marker $hCD34^+CD38^-Lin^-$. The proportion of cells with $CD34^+CD38^-$ phenotype was calculated. The proportion of $hCD34^+CD38^-$ cells was found significantly decreased in the engrafts transduced with ΔNRPID-HPIP-YFP cells with a mean value of 0.21% ($p \leq 0.05, n=6$) cells, as compared to the control YFP with a mean value of 2.7% cells expressing $hCD34^+CD38^-$. The expression of HPIP-WT in the engrafts did not show any significant changes as compared to the control YFP expressing engrafts (fig 4.9.3b).

The percentage of $hCD45^+YFP^+$ cells in all the engrafts was checked for the presence of haematopoietic progenitors (HPCs), expressing the surface marker $cKIT^+$ ($hCD117^+$). The proportion of $hCD45^+CD117^+YFP^+$ cells was found significantly increased in the engrafts transduced with HPIP-WT-YFP cells with a mean value of 26.85% ($p \leq 0.05, n=6$) cells, as compared to the control YFP with a mean value of 7.72% cells expressing cKIT. The expression of HPIP-WT in the engrafts increased significantly the number of hLTRCs by 3.4 fold as compared to the YFP control. No significant difference was observed in the proportion of most primitive haematopoietic cells[31] ($hCD34^+CD38^-$, STRC-ML) in all the engrafts analysed above. Interestingly, there was an increase in the proportions of CD133 expressing cells in the engrafts of ΔNRPID-HPIP-YFP transduced hCB cells with a mean value of 14.9% cells ($p \leq 0.01$, $n=4$) as compared the control YFP with a value of 0.4% cells ($n=6$), however there were no significant differences observed in the case of HPIP-WT as compared to the control YFP.

The expression of ΔNRPID-HPIP-YFP ($p \leq 0.05$, $n=4$) significantly decreased the number of hLTRCs by 3.4 fold as compared to the control YFP ($n=6$). The above results demonstrated a crucial role of the NRPID domain in making the stem cell fate decision at the haematopoietic multiprogenitor cell level. The deletion of this domain strongly affected the proportion of human long term repopulating cells ($hCD34^+$ and $hCD117^+$ cells) *in vivo,* generating a lower number of hLTRCs. Also

this deletion induced a decrease in human short term repopulating cells (hCD34^{+}38^{+} cells) *in vivo*,. The results implicate that HPIP-WT is a putative regulator of short

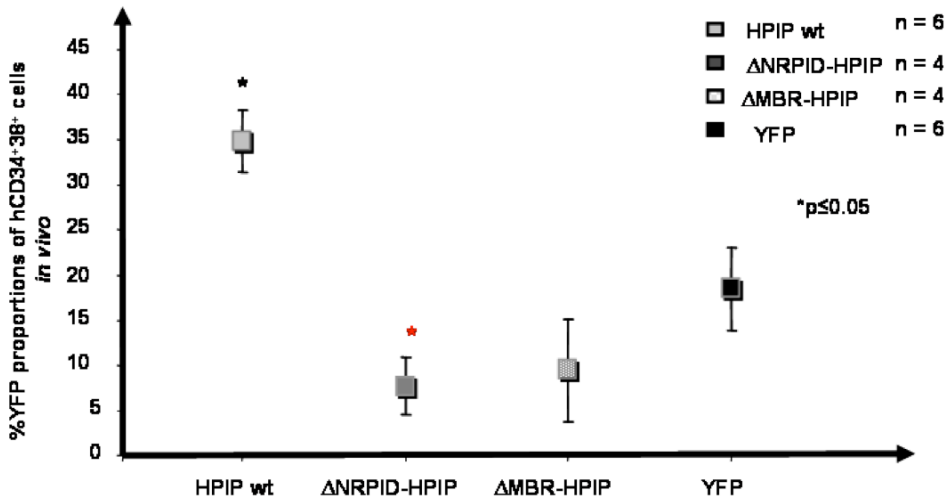

Figure 4.9.3a: Short term repopulating cells *in vivo*. The above graph illustrates of the proportion of short term repopulating cells in the BM of engrafted NOD/SCID mice. HPIP expression increased significantly the proportion of short term repopulating cells (CD34^{+}CD38^{+} cells) *in vivo* (**p≤0.05). The mutant form of HPIP, ΔNRPID-HPIP significantly decreased the proportion of hSTRCs in the BM of engrafted NOD/SCID mice as compared to the YFP control and HPIP-WT (**p≤0.05).

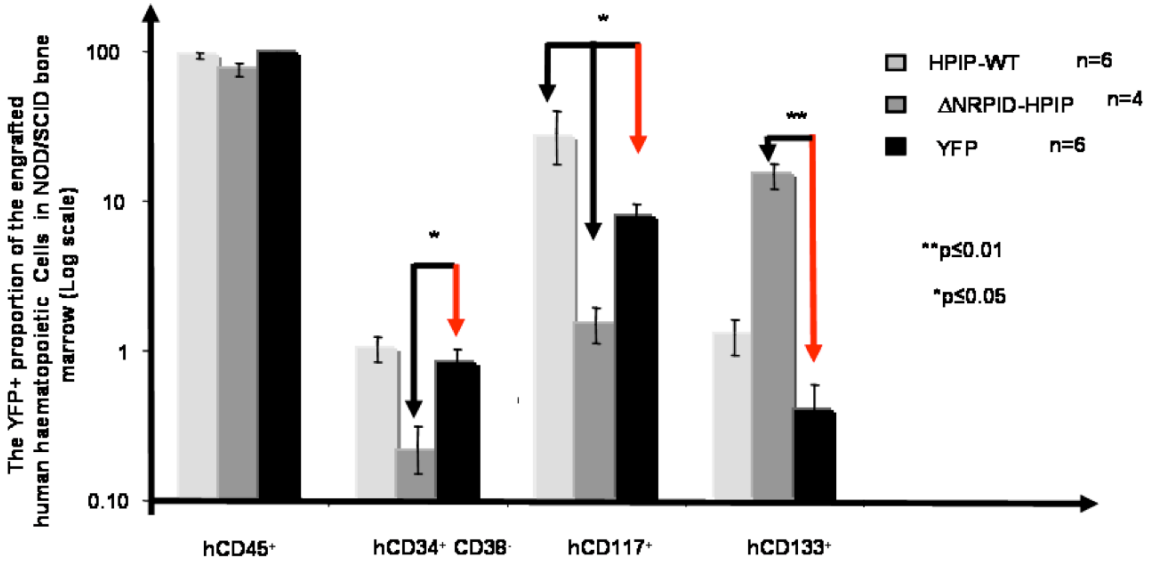

Figure 4.9.3b: Engraftment of human progenitor cells in NOD/SCID mice. The graph illustrates the proportions of the human CB CD45^{+}, CD34^{+}, CD117^{+}, and CD133^{+} transduced cells engrafted in the bone marrow of NOD/SCID mice

term repopulating cells and progenitor cells *in vivo* and that for this effect the ΔNRPID domain is crucial.

4.9.4. The effect of constitutively expressed WT and mutated HPIP on the multilineage repopulation of human haematopoietic cells in NOD/SCID engraft model.

In an attempt to characterize the function of WT, or mutated HPIP on human haematopoietic differentiation, the harvested BM cells were evaluated for expression of a panel of surface markers (CD45, CD34, CD38, CD15, CD19, CD33, CD36, CD41a, CD71, CD10, CD133, CD117 and Glycophorin A) to quantify the myeloid, lymphoid or erythroid human subpopulations.

The proportion of $hCD45^+YFP^+$ cells expressing CD19 did not differ between HPIP- WT mice with a mean value of 68.55% cells (p=ns, n=6), as compared to the YFP control mice with a mean value of 65.06%. A very significant decrease in the proportion of $hCD45^+CD19^+YFP^+$ cells was observed for the ΔNRPID-HPIP cohort with a mean value of 20.34% cells (p≤0.01, n=4), as compared to the HPIP-WT and control YFP(fig 4.9.4). Also ΔMBR-HPIP mice showed a significant decrease of $hCD45^+$ $CD19^+$ YFP^+ cells with a mean value of 19.59% cells (n=1), as compared to the HPIP-WT and control YFP. The proportion of $hCD45^+CD71^+YFP^+$ cells did not show any significant difference between HPIP-WT, with a mean value of 41.8% $hCD45^+$ $CD71^+YFP^+$ cells (p=ns, n=6), compared to the control with a mean value of 32.4% (n=6).

However a significant differences were observed in the proportion of the $hCD45^+CD71^+YFP^+$ cells in ΔNRPID-HPIP-YFP mice with a mean value of 2.64% $hCD45^+CD71^+YFP^+$ cells (p≤0.01, n=4), and in ΔMBR-HPIP mice with a mean value of 3.39% $hCD45^+$ $CD71^+YFP^+$ cells (n=1), as compared to the HPIP-WT and control YFP. There was a 4 fold decrease (p≤0.01) in the proportion of human erythroid cells in NOD/SCID mice transplanted with CB expressing both the mutant forms of HPIP as compared to the WT HPIP and control YFP. However we did not observe any significant differences between the case of HPIP-WT and YFP control.

The proportion of hCD45$^+$YFP$^+$ cells expressing CD15 were significantly increased by HPIP-WT expression with a mean value of 37.26% of hCD45$^+$CD15$^+$YFP$^+$ cells (p≤0.05,n=6), as compared to the YFP control with a

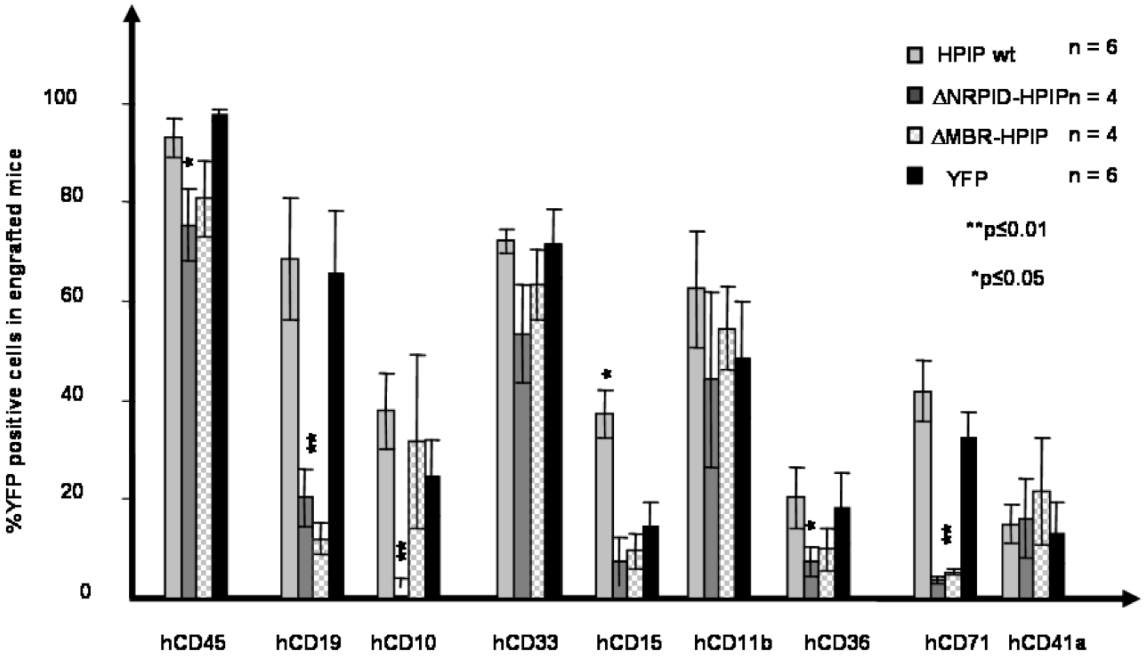

Figure 4.9.4: Multilineage repopulation of human haematopoietic cells in bone marrow of NOD/SCID mice. The above graph illustrates the percentage of transduced YFP$^+$ human cells of the different lineages engrafted in NOD/SCID bone marrow: lymphoid lineage (hCD19$^+$, hCD10$^+$), myeloid lineage (hCD15$^+$, hCD33$^+$, and hCD36$^+$), and erythroid lineage (hCD71$^+$). The bone marrow from engrafted mice was harvested and stained for various cell surface markers to quantify the proportion of different cell lineages in the YFP$^+$ compartment of the whole bone marrow. A significant decrease in the number of cells expressing CD19 and hCD10 was observed in the case of human haematopoietic cells transduced with ΔNRPID-HPIP-YFP (n=4) and ΔMBR-HPIP-YFP (n=1) mutant forms of HPIP as compared to HPIP-WT (n=6) and control YFP (n=6, *p≤0.05). There was a significant decrease observed in the number of cells expressing erythroid cell specific marker hCD71, on all the human haematopoietic cells transduced with mutant forms of HPIP (fold decrease = 4, **p<0.01).

mean value of 15.59% of hCD45$^+$CD15$^+$YFP$^+$ cells (n=6). A significant decrease in the proportions of hCD45$^+$CD15$^+$YFP$^+$ cells was seen in the case of ΔNRPID-HPIP-YFP transplanted mice with a mean value of 7.38% of CD45$^+$CD15$^+$YFP$^+$ cells (p≤0.01, n=4), as compared to the HPIP-WT and the control.

There were no significant changes observed in the proportions of $hCD45^+CD33^+YFP^+$ or $hCD45^+CD36^+YFP^+$ cells between ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP as compared to the HPIP-WT and YFP control. Also we did not observe significant differences between the proportions of $hCD45^+CD33^+YFP^+$ or $hCD45^+CD36^+YFP^+$ cells in the case of HPIP-WT and YFP control. The results indicate that WT and mutant HPIP protein can deregulate the developmental pathways of human haematopoiesis.

4.9.5. Limiting Dilution Assay (LDA) in NOD/SCID mice: Quantification of SCID Repopulating (SRC) cell frequency.

To determine if WT or mutant HPIP expression affects the frequency of SCID repopulating cells (SRC) in the NOD/SCID xenotransplantation model, limiting dilution assays with the CB CD34+ cells transduced with the HPIP-WT and control YFP, were carried out. For this hCB $CD34^+YFP^+$ cells, that were retroviral transduced with HPIP-WT or the empty control vector, were injected intravenously in cohorts of mice with different dilution of these cells. (Table 4.5.9a and Table 4.5.9b).

The mice were sacrificed after 6-8 weeks and the bone marrow was analysed. Based on the number of engrafted mice per cohort the frequencies were calculated by Poisson statistics using the L-CALC® software (StemCell Technologies Inc.). Four independent experiments were performed and the SRC frequency was calculated by pooling all the experiments together. The SRC frequencies was 1 in 31866 for HPIP- WT transduced $CD34^+$ cells and 1 in 19098 YFP transduced $CD34^+$ cells (n=4, p=ns, Table 4.9.5a).

To determine if the deletion mutants form of HPIP, ΔNRPID-HPIP-YFP affects the frequency of SCID repopulating cell (SRC), limiting dilution analysis in NOD/SCID mice were performed on a similar pattern as mentioned above. The hCB $CD34^+$ cells were transduced with HPIP-WT-YFP, ΔNRPID-HPIP-YFP and a control YFP in a single experiment. The mice were sacrificed after a period of 8 weeks and the BM was analysed for multilineage engraftment. Based on these

limiting dilution results, the frequencies of SRC in the transduced hCB cells were calculated. The frequency of engraftment was 1 in 11348 for HPIP-WT, 1 in 36814 for ΔNRPID-HPIP-YFP and 1 in 32729 YFP only transduced $CD34^+$ cells (p=n.s.).

Table 4.9.5: SRC frequencies of CB $CD34^+YFP^+$ were calculated by limiting dilution transplantation for HPIP-WT, ΔNRPID-HPIP-YFP and the control YFP.

		Analyzed mice/Engrafted mice (n=1)		
Serial no	**Dilution/ Cell dose $CB34^+YFP^+$ cells**	***YFP control* (Number of analysed mice/engrafted mice)**	***HPIP- WT* (Number of analysed mice/engrafted mice)**	***ΔNRPID-HPIP* (Number of analysed mice/engrafted mice)**
1	**50000**	**1/1**	**3/3**	**5/3**
2	**10000**	**3/1**	**3/2**	**3/2**
3	**3000**	**2/0**	**2/0**	**2/0**
Frequency of LTC-IC in Bone marrow of NOD/SCID mice		**1 in 32729**	**1 in 13248**	**1 in 36814**
Proportion (95% confidence interval)		**1/5656-1/99549**	**1/3539-1/36392**	**1/13914-1/97402**

4.10. Affymetrix differential gene expression profile

4.10.1. Comparative analysis of gene expression: HPIP-WT-YFP, ΔNRPID-HPIP-YFP and ΔMBR-HPIP-YFP relative to control YFP transduced human cord blood CD34$^+$ cells.

In an attempt to provide a molecular basis for the observed functional differences, we analyzed the gene expression of CD34$^+$ cells transduced with HPIP- reported here are based on four micro arrays, each profiling gene expression of hCB CD34$^+$ cells transduced with HPIP-WT, ΔNPRID-HPIP-YFP, ΔMBR-HPIP-YFP or YFP cells, 48 hours after the end of transduction. At that time point the cells were harvested and hCD34$^+$YFP+ cells were sorted out by FACS for each experimental group. RNA was prepared from each set of CD34$^+$YFP$^+$ cells. We analyzed the data by defining gene expression levels of hCB CD34$^+$ cells transduced with YFP$^+$ only as a baseline for comparison, and the relative differences in gene-expression levels (see methods, p-57)(Fig 4.10.1).

Further, a numerical threshold of 1.5-fold was set to identify target genes, which were differentially expressed. This threshold was chosen, as a 1.5-fold difference can be validated by other assays such as Low Density Array and Q-RT-PCR. Based on these criteria, a comparative analysis showed that 28 genes were differentially expressed between HPIP-WT compared with hCD34$^+$YFP$^+$ control cells. Ten genes showed significantly decreased expression in CD34$^+$ HPIP-WT-YFP cells, whereas the expression of 18 genes was increased as compared to the YFP control (Fig 4.10.1).

Among the differentially expressed genes specific groups of genes involved in different cellular pathways were found. Seven of the deregulated genes are involved in MAPK pathway namely: *CD14, FGFR3, FLNA, FOS, HRAS, RAPIA* and *SOS1*. Along with this many genes involved in cytokine-cytokine interactions were also differentially expressed, namely: *CCL3, CXCL2, CXCL5, CXCR4, HGF, IL10RB, PF4* and *TNFRSF19.* Genes involved in CXCR/SDF1 axis/Pathway

namely: *DPP4, HRAS, SOS1, and CXCR4*. The majority of the gene expressions mentioned in

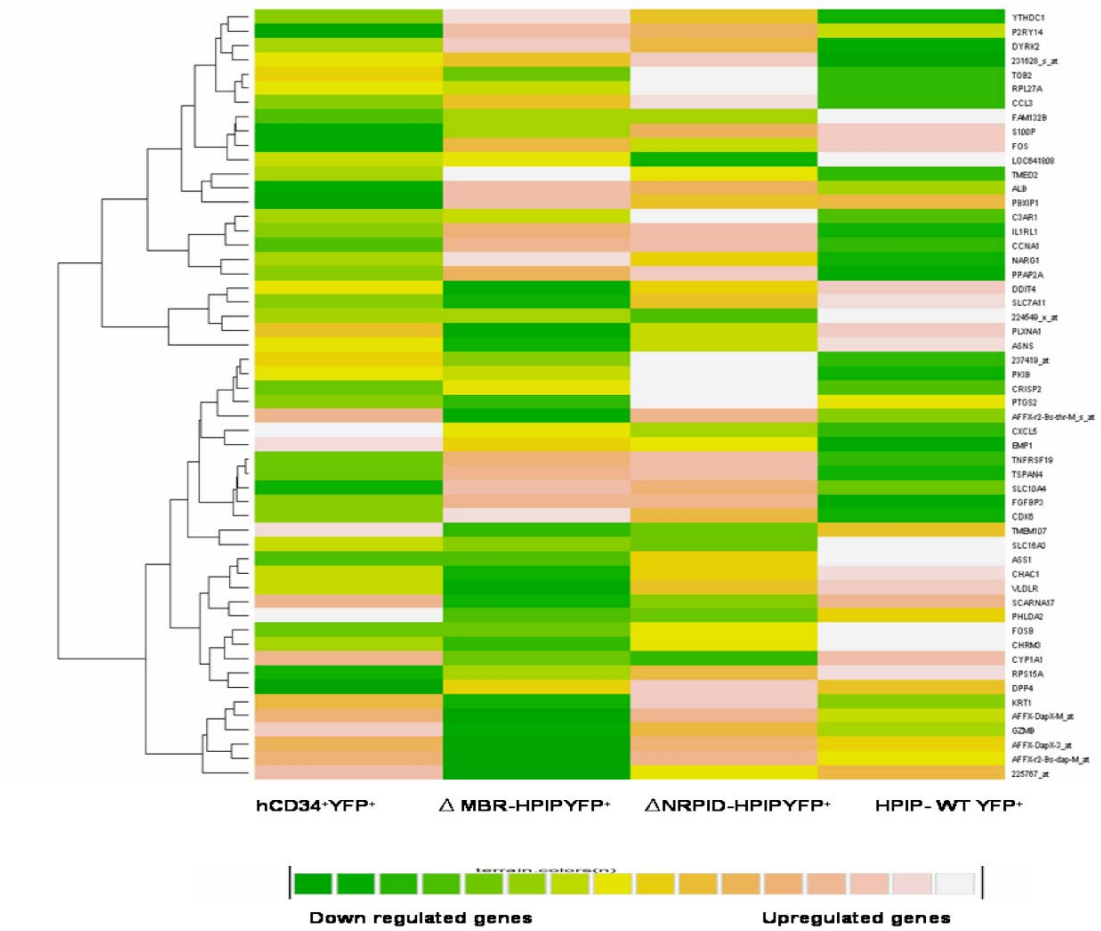

Figure 4.10.1: Heat map representative of Affymetrix differential gene expression profile. The heat map above illustrates the differential expression of genes in hCB CD34$^+$ cells transduced with YFP only as a control, ΔMBR-HPIP, ΔNRPID-HPIP, and HPIP-WT. A differentially expression of 1.5 fold (up or down regulated genes) compared to the YFP control was defined as threshold. The comparative expression profile shown here is an expression calculation with respect to the control YFP expression values. Downreglation or upregulation is shown by different colours as indicated.

these pathways could be confirmed at Q-RT-PCR level. A few more genes with expression differences of > 2 fold e.g. *FOSB, GZMB, SCARNA17*, *CHRM3, CNN3* and *ASS1* were confirmed by performing Q-RT-PCR in triplets using inventory express plates from Applied Biosystems (Table 4.10.1)

Besides the above mentioned genes, there were significant differences observed in the gene expressions between human hCB CD34$^+$ cells transduced with HPIP-WT

and the ΔNRPID-HPIP mutant form. A comparative analysis carried out between expression arrays of HPIP-WT and its mutant form ΔNRPID-HPIP-YFP showed, in total 186 genes that were differentially expressed. Out of these 186 genes there were 129 genes that were upregulated in the cells expressing ΔNRPID-HPIP-YFP mutant form as compared to expression profile of the HPIP-WT expressing cells.

In total, 14 genes showed a 1.5 to 2 fold higher expression in hCB $CD34^{+}YFP^{+}$ cells transduced with ΔNRPID-HPIP-YFP, as compared to the cells transduced with HPIP- WT-YFP: these genes were namely: *KRT1, PKIB, PPAP2A, PTX3, C3AR1, NARG1, FGFBP3, CCL3, C1orf43, CRISP2, ZNF618, GZMB, IL1RL1,* and *CCNA1*. Another set of 10 genes showed a 1.5 to 2 fold lower gene expression in the Affymetrix expression analysis carried out for the hCB $CD34^{+}YFP^{+}$ cells transduced with ΔNRPID-HPIP-YFP mutant form as compared to the HPIP-WT transduced cells. These genes were namely: *TGFB1I1, CHRM3, SLC16A3, ASS1, TNS1, WSB1, SCARNA17, ASNS, CYP1A1, and PLXNA1*. In the tables 4.10.1a and 4.10.1b, all the genes that were differentially expressed between HPIP-WT and the mutant ΔNRPID-HPIP compared to the genes with respect to the expression of genes in the case of control YFP only. Functions of some of the genes are explained in table 4.10.1b.

Table 4.10.1: Differential gene expression in hCB CD34$^+$ cells transduced with WT and ΔNRPID-HPIP mutant form of HPIP as compared to the YFP control.

	Fold change Compared to the YFP control	Genes deregulated due to expression of HPIP-WT in hCB CD34$^+$ cells relative to YFP	Genes deregulated due to expression of ΔNRPID-HPIP YFP in hCB CD34$^+$ cells relative to YFP
Down regulated gene expressions	2 to 3 fold	*CXCL5, GZMB*	*CXCL5*
	1.5 to 2 fold	*EMP1*	*PHLDA2, SCARNA17, CYP1A1 TMEM107*
	1 to 1.5 fold	*CD9, PNA3, PLTP, CARHSP1, G6PD, HEY1, NARG1, PECAM1, EXOSC4, HIST1H2BG, ELA2, HEATR2, CYB561, ACBD3, PRSS1, CTSL1, SPINK2, 17orf81, TMEM107, SGOL2, SCD, TGFBI, RTKN, SLC38A5 SCD5*	*NR5A2, CD163, 229815_at, HBZ, KDELR3, SOS1, IGHM, UGT1A1, HIST1H2BG, CTHRC1, 28919_at, SLC22A18, RAP1A, CNN3, RBM6, HIST1H1C, PF4, BIN1, FGD5, CMTM5, SLC16A14, UGT1A6, 214349_at, TGFB1I1, CD14, hCG_2045751, TNS1,*
Upregulated gene expression	2 to 3 fold	*FOS, ASS1, PBXIP1*	*PBXIP1*
	1.5 to 2 fold	*PTPRO, CHAC1, GLT25D2, SLC7A11, FLJ37034, DDIT4, CHRM3, FOSB, RPS15A, VLDLR*	*DPP4*
	1 to 1.5 fold	*CCPG1, S100P, LOC641808, FLNA, RKHD2, TRIP12, CHD2, LOC643641, CXCL2, OGT, MAD1L1, MAP7D2, WARS, NFKBIZ, SFRS3, SLC16A3, ATF3, CTH, ST6GALNAC3, SFRS1, CD69, CNOT7, ANXA3, PLXNA1, PSAT1, HNRPA1, CTH, ASNS.*	*SLC10A4, P2RY14, TNFRSF19, S100P, C3AR1, TSPAN4, CXCR4, CCL3, CCNA1, ENPP2, SGK, DLC1, IL1RL1, NETO2, TRIB2, PPAP2A, RDH10, C5orf20, PKIB, SIGLEC6, KCTD12, 31628_s_at, MGC10850, RGS2, RPS15A, IL10RB, PTGS2, DLC1, HPGD, PTX3, EPC1, GLT25D2, FGFBP3, TRIM59, LMO4, C5orf5, HS2ST1, RNASE2*

Table 4.10.1b. The table demonstrates some important differentially expressed genes within different pathways.

Gene Name	Function in brief
FGFR3	Receptor for acidic and basic fibroblast growth factors. Preferentially binds FGF1
FLNA	Anchors various transmembrane proteins to the actin cytoskeleton and serves as a scaffold for a wide range of cytoplasmic signalling proteins
HRAS	Ras proteins bind GDP/GTP and possess intrinsic GTPase activity
SOS1	Promotes the exchange of Ras-bound GDP by GTP
CXCR4	Receptor for the C-X-C chemokine CXCL12/SDF-1
TNFRSF19	Can mediate activation of JNK and NF-kappa-B. May promote caspase-independent cell death.
CCL3	Monokine with inflammatory and chemokinetic properties. Binds to CCR1, CCR4 and CCR5.
CXCL2	Hematoregulatory chemokine, which, *in vitro*, suppresses haematopoietic progenitor cell proliferation
CXCL5	Involved in neutrophil activation
PF4	Chemotactic for neutrophils and monocytes
HGF	Hepatocyte growth factor (HGF) regulates cell growth, cell motility, and morphogenesis by activating a tyrosine kinase signaling cascade after binding to the proto-oncogenic c-Met receptor
IL10RB	Receptor for IL10 and IL22. Serves as an accessory chain essential for the active IL10 receptor complex and to initiate IL10-induced signal transduction events
WNT5B	implicated in oncogenesis and in several developmental processes, including regulation of cell fate and patterning during embryogenesis. This gene is a member of the WNT gene family.
ASS1	The protein encoded by this gene catalyzes the penultimate step of the arginine biosynthetic pathway.
GZMB	linked to an activation cascade of caspases (aspartate-specific cysteine proteases) responsible for apoptosis execution
MLL	Histone methyltransferase. Methylates 'Lys-4' of histone H3. H3 'Lys-4' methylation represents a specific tag for epigenetic transcriptional activation. Promotes

	PPP1R15A-induced apoptosis.
SCARNA17	**small Cajal body-specific RNA 17**
DDIT4	**DNA damage inducible transcript 4, inhibits cell growth by regulating the FRAP1 pathway upstream of the TSC1-TSC2 complex**
DPP4	**Plays a role in T-cell activation and human stem cell homing pathways.**
FOSB	**FosB interacts with Jun proteins enhancing their DNA binding activity**

4.11. Gene expression profiling in leukaemia patient samples

4.11.1. cDNA microarray analysis from AML samples and normal bone marrow from healthy donors.

To extend the analysis to primary AML samples bone marrow samples from 147 thoroughly characterized patients with newly diagnosed and untreated AML as defined by the World Health Organization classification were used and processed immediately as described[117,118] and compared to fresh bone marrow samples from healthy volunteers. Each AML subgroup comprises approximately 10 samples.

Samples were analyzed by cytomorphology, cytochemistry, immunophenotyping, cytogenetic, and molecular genetics in all cases and were characterized by specific chromosomal aberrations or molecular genetic alterations from the leukaemia diagnostics laboratory, Klinikum Großhadern, Munich. The studies were done in accordance with the rules of the local internal review board and the tenets of the revised Helsinki protocol. First cDNA micro arrays documented high expression of HPIP in AML patients with specific cytogenetic aberrations such as CALM/AF10 translocation, FLT3 length mutation or translocation t(15;17) compared to bone marrow from healthy donors as well as compared to other cytogenetic subtypes such as MLL-PTD, complex, AMLM2 andM4Eo (Fig 4.11).

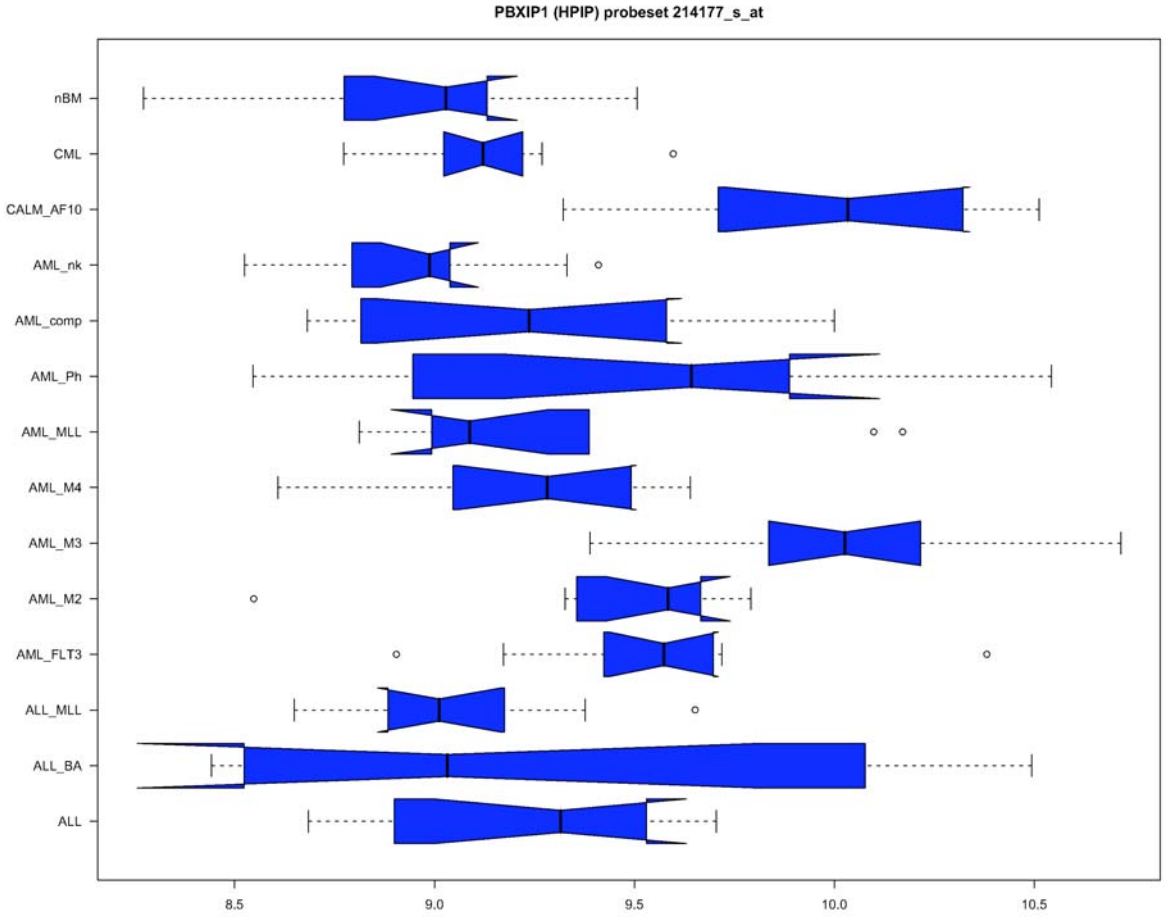

Figure 4.11: Expression levels of *HPIP* in cytogenetically different subgroups of patients with AML. Bone marrow samples from patients with newly diagnosed and untreated AML and fresh bone marrow samples from healthy volunteers were analyzed. Expression of *HPIP* was found to be significantly elevated in AML samples from patient carrying different cytogenetic aberrations especially CALM-AF10, AML-M3, AML-M2, AML-FLT3 (N=10, ***p≤0.01) compared to healthy controls. Significance was determined by Student's t-test and is indicated above in the box plots. The indicated statistically significant differences are in comparison to the normal bone marrow (indicated with red line), used as control.

5. Discussion

In the present study, the human PBX interacting protein (HPIP-WT) was characterized as a novel regulatory protein of human haematopoiesis. It was furthermore possible to demonstrate that the C´ terminal nuclear receptor/PBX interacting domain (NRPID), is a critical functional domain of HPIP-WT. All the data were obtained using the human haematopoietic stem cell functional assays.

HPIP-WT is able to enhance the clonogenic progenitors in the liquid suspension cultures and colony forming assays

HPIP-WT has been reported previously as a novel PBX1 interacting protein. It can bind to the different members of the mammalian PBX family, inhibit the binding of PBX1/HOX complex to DNA and block the transcriptional activity of E2A-PBX.[105] In the liquid suspension cultures, the expression of HPIP-WT on huCB derived $CD34^+$ cells could significantly decrease the cell proliferation when compared to the control. One of the most striking effects in vitro was the significant increase in the number of BFU-E/CFU-E colonies, when compared to the control. The multi-potential progenitors (e.g. CFU-GEMM) and the lineage-committed progenitors (e.g. CFU-E, CFU-GM), representing various stages along the differentiation pathway, can be identified by *in vitro* assays and by the expression of known surface antigens.[119,120]

A selective increase in erythroid colonies compared with the control has been previously reported with the expression of HOXB4-WT in hCB $CD34^+$ cells in the replating colony assays.[121]. As an interactor of the HOX co-factor PBX1 a similar effect was now seen for HPIP. It was previously shown that HOXB4 binds to PBX1, thus both proteins form complexes with PBX1. It remains open whether this impact on erythroid colony formation is linked to PBX1 protein complexes.

HPIP-WT is able to enhance the frequency and yield of the long term culture initiating cells *in vitro,* but does not increase the SCID Repopulating Cell (SRC) frequency *in vivo*.

A potential role of HPIP-WT in the regulation of most primitive haematopoietic cells was tested using long term culture initiating cell (LTC-IC) assay *in vitro* and the competitive repopulating unit (CRU) assay in *vivo*. The constitutive expression of HPIP- WT elevated the number of LTC-IC per million cells *in vitro* relative to the control YFP. The result states that constitutive expression of HPIP-WT influences the growth kinetics of primitive progenitor cells on a larger scale, for example, by shortening the doubling time, or enhanced self-renewal of HSCs, as it has been implicated in the over expression model of *HOXB4* and *PBX1.*[79,121-123] Also in the competitive repopulating assays carried out in NOD/SCID mice using different limiting dilutions, we could observe a trend towards an increased frequency of SRC in the case of HPIP-WT as compared to the control, but without a statistical significance. Thus, again similar to HOXB4, HPIP can amplify primitive human progenitors at least in vitro, again suggesting that both proteins might have overlapping functions.

HPIP-WT expression is able to increase the short term SCID repopulating cells (huST-SRCs) *in vivo.*

We further tested the effects of HPIP-WT expression on the huST-SRCs in the NOD/SCID xenotransplantation model. With the expression of HPIP-WT-YFP, the proportion of human cells expressing the $hCD34^+$ and the early differentiation marker hCD38 ($hCD34^+38^+$ cells) was found to be significantly increased in the BM of the engrafted mice, relative to the control. This indicates that the HPIP-WT affects the human haematopoietic cells with short–term *in vivo* repopulating activity that has been defined before as the hSTRC population *in vivo*. These phenotypic separable populations of haematopoietic stem cells are pluripotent and capable of self renewal, and have been clearly identified in the engrafted NOD/SCID mice.[31] This analysis elucidated a regulatory effect of HPIP-WT expression in the hST-SRCs in repopulating this particular HSC population.

HPIP-WT could alter the lympho/myeloid cell ratio *in vivo*.

The HPIP-WT expression also induced an alteration in the lympho/myeloid cell ratio with a shift towards the myeloid compartment that is characterized by expression of CD15 marker. This increase in the myeloid lineage cells did not come on expense of the lymphoid cells. The expression of HPIP-WT did not produce any changes in the lymphoid cell population ($hCD19^+$) compartment, when compared to the control. This observation revealed that the expression of HPIP-WT is able to increase generation of myeloid cells *in vivo* without affecting the lymphoid cell compartment. Strikingly, it suggests that HPIP-WT expression does not interfere with the lineage determination of the early progenitor cells *in vivo*. This stands in contrast to HOXB4, which in the same setting does not influence lineage differentiation.

HPIP-WT expression affects the expression of genes involved in the cell signalling and growth pathways.

In an affymetrix differential cDNA expression analysis, HPIP-WT expression in huCB $CD34^+$ cells was able to up-regulate the expression of genes such as *DDITP4, FLNA, FOSB* and *CXCL2* genes, relative to the control. The *DDIT4* (DNA damage inducible transcript 4) expression can inhibit cell growth by regulating the FRAP1 pathway.[124,125] (CXCL2 is secreted by haematopoietic stem cells and it mobilizes cells by interaction with a cell surface chemokine receptor called CXCR2.[126] CXCL2 has been implicated as a hematoregulatory chemokine that suppresses haematopoietic progenitor cell proliferation, *in vitro.*[127] *DDIT4* and *CXCL2* up-regulation might contribute to the decreased proliferation of HPIP-WT-YFP^+ cells in the liquid suspension cultures. However, it remains open why HPIP-WT expression increased the absolute number of clonogenic progenitors relative to the YFP control in liquid expansion cultures parallel to decreasing the proliferation of the bulk population in the suspension.

Previously in the literature HPIP-WT has been described as a scaffold protein that is able to tether the estrogen receptor-alpha (ESR1) to the microtubules and allows them to influence estrogen receptors-alpha signalling.[107,108] HPIP induced

increased expression of FLNA (Flamin A), a protein which anchors various transmembrane proteins to the actin cytoskeleton and serves as a scaffold for a wide range of cytoplasmic signalling proteins.[128,129] Upregulation of *FLNA* could contribute to the changes in growth kinetics of the cells in the liquid suspension cultures.

The expression of HPIP-WT in primary cells, down regulated *CXCL5, GZMB, TGFB1, and HEY1* genes in differential cDNA expression analysis relative to the control. TGFB1 is a multifunctional peptide that controls proliferation, differentiation and other functions in many cell types. It regulated the actions of many other growth factors and determines a positive or negative direction of their effects[130]. *HEY1* gene encodes a nuclear protein belonging to the hairy and enhancer of split-related (HESR) family of basic helix-loop-helix (bHLH) type transcriptional repressors. Expression of this gene is induced by Notch and c-Jun signal transduction pathways.[131-133] HPIP WT has also been implicated as an estrogen receptor-Interacting protein that not only activates MAPK and AKT pathways but also potentates' estrogen-responsive gene expression in breast cancer cell lines.[107,108] The genes that we have observed to be deregulated above are belonging to cytokine-cytokine interaction pathways (*CXCL2, CXCL5*) and MAPK (*FLNA*) pathways. This further indicates that HPIP-WT expression is able to affect important cell signalling pathways.

HPIP-WT when expressed together with HOXB4-WT does not produce the synergistic effects on haematopoietic progenitors.

HPIP-WT is strongly expressed in the hCB $CD34^+$ fraction containing the haematopoietic progenitors and at lower levels in the hCB $CD34^-$ fraction comprising the mature cell population. The same pattern of expression was found for *PBX1*, indicating that HPIP and PBX1 are co-expressed in the same human haematopoietic cell compartment. This expression level decreases with differentiation of the HSCs. The pattern of HPIP-WT expression is similar to the expression of other homeodomain genes such as *HOXB4.*[105] or like *ABCG* transporters expression.[134] Among the best characterized co-factors of HOX genes are the mammalian pre-B cell leukaemia homeobox (Pbx)[89] and the myeloid ecotropic viral insertion site (Meis)[91] family members.

It is predicted that Hox and Pbx proteins interact within multiprotein complexes that have the highest level of specificity in terms of DNA binding and co-ordination of activation or repression events.[82] In another attempt we tried to express both HPIP-WT and HOXB4-WT proteins by making double transductions on hCB $CD34^+$ using YFP and GFP reporters respectively. The hCB $CD34^+YFP^+GFP^+$ cells were sorted out and *in vitro* CFC and LTC-IC assays were carried out. We did not observe any synergistic effects produced by both the proteins together. We did see a trend towards the increased CFC numbers in this case but is was statistically non significant relative to control. Sustained *in vitro* trigger of self-renewal divisions in Hoxb4hiPbx1 haematopoietic stem cells has been already been implicated.[135] Also, genetic interactions between two well-recognized regulators of this process: Bmi1 and Hoxb4, using complementation and over expression strategies in mouse HSCs, are well documented.[136]

Our results do not formally exclude functional collaboration between HPIP-WT and HOXB4-WT: it might be that endogenous HOXB4-WT is sufficient for an optimal effect of HPIP-WT and that over expression of HOXB4 is not able to further augment the impact of HPIP-WT on early progenitor cells. Another hypothesis could be that the role of HPIP-WT protein and its collaboration is not extended to HOXB4; however we do not have enough evidence to show that at present.

Importance of the functional domains of HPIP-WT

Next we were interested in the functional domains of HPIP. The mutant form ΔNRPID-HPIP-YFP is unique in a way that a set of 3 amino acids between position 615aa-619aa (deletion between 1845 bp-1857 bp on cDNA) was deleted artificially. This region has been implicated as a partial part of the 'PBX binding Domain' (PBD position 560aa-633aa; Abramovich *et al,* 2002) and also as a nuclear receptor interacting domain (C-terminal LXXLL motif position 615aa-619aa). The LXXLL-type motifs have been shown to mediate the receptor interactions of co activators and corepressors.[137,138]

This mutant was generated to understand if a deletion in nuclear receptor/PBX binding domain could affect the expression or normal function of HOX or Para HOX

genes. Since we understand that this particular region is interacting with PBX and the protein is a regulator of pre-B-cell leukaemia transcription factors (PBX) function. It has been shown to inhibit the binding of PBX1-HOX complex to DNA and to block the transcriptional activity of E2A-PBX1.[105]. We have hypothyzed that a deletion in such a region could probably affect the stem cell fate. Previously similar cases have been shown for Hox genes like preferential targeting of a small region in the Hoxa gene locus in E2A-PBX1-induced lymphoid leukaemia resulting in the over expression of several Hoxa genes.[139]

The second mutant ΔMBR-HPIP-YFP has a deletion mutation in the microtubule binding sequence (putative leucine repeat rich motif, LRR, position of motif spanning between 576 bp-654 bp (190aa-218aa) in the cDNA; LxxxLNxxLLxxLxLLxLxxLL), where a set of 20 amino acids was removed to disrupt the hydrophobic core of the HPIP-WT protein. This region has been implicated in association of HPIP to cytoskeletal fibers. Leucine-rich repeats (LRRs) are 20-29aa residue sequence motifs, present in a number of proteins with diverse functions. The primary function of these motifs is to provide a versatile structural framework for the formation of protein-protein interactions.[140]

A number of human diseases have been shown to be associated with mutation in the genes encoding leucine-rich-repeat (LRR)-containing proteins.[141] Protein localization at the sub cellular level was investigated using flag antibody and the images were captured using confocal microscope. Visibly there was no change in localization of WT and mutant form of HPIP proteins at the sub cellular level, stating that the deletions did not effect the distribution of the protein at the sub cellular level.

The NRPID mutant abrogates the effects of wild type HPIP and induces a replating capacity in the colony forming assays.

In the colony forming assays, the deletion of NRPID in HPIP-WT led to the increase in the number of colonies consisting of multilineage committed progenitors (CFU-GEMM/CFU-GM). The expression of the NRPID mutant did not show increase in the number of erythroid (BFU/CFU-E) colonies relative to the YFP control; this is in complete contrast to the results obtained with HPIP-WT relative to the YFP control.

This indicates an apparent role of HPIP-WT at committed human haematopoietic progenitor level. The mutation of LXXLL motifs can prevent essential activated complex formations and eliminate activation of pathways such as in the case of Estrogen Receptor-Modulator of Nongenomic Actions of Estrogen Receptor (ER-MNAR) complex formation and its effects on the Src/ Mitogen Activated Protein Kinases (Src/MAPK) pathway.[142] Furthermore the role of Ras and MAP kinases (MAPKs) in the regulation of erythroid differentiation has been demonstrated.[143-145]

In addition a serially replatable potential was observed in the colony forming cells transduced with ΔNRPID-HPIP-YFP, in contrast to the CFCs transduced with HPIP-WT or ΔMBR-HPIP-YFP. This suggests that the domain deleted in the ΔNRPID-HPIP mutant might be important for the self – renewal program of early human progenitors. In the differential gene expression analysis, the expression of ΔNRPID-HPIP-YFP up regulates expression of *WNT5B* gene, relative to HPIP-WT and also the control YFP. The Wnt family genes are implicated as the genes involved in the regulation of self renewal of stem cells (Willert K, 2003, Reya T, 2003). The up regulation of WNT5B in ΔNRPID-HPIP-YFP$^+$ cells explains the enhanced replating capacity of the clonogenic progenitors in this case, relative to the control. A number of other transcription factors have also been suggested to be involved in HSC self-renewal, including the over expression of *HOXB4*.[79], activation of *NOTCH.*[146] and knockout of *BMI1* [147,148]

NRPID mutant does not affect the human short term SCID repopulating cells (hST-SRCs) in the NOD/SCID model

A sharp decrease in the proportion of hCD34$^+$38$^+$ cells was observed in the BM of the mice transplanted with ΔNRPID-HPIP-YFP$^+$ cells, when compared to the HPIP WT$^+$ cells. However, no significant changes were observed relative to the control YFP. The hCB CD34$^+$38$^+$ cell population in the BM of NOD/SCID mice have been characterized as a set of short term repopulation cells (hSTRCs), with higher erythroid and megakaryocytic lineage growth properties (hSTRC-M). We also looked for the changes in the proportion of hCD34$^+$38$^-$ cells, i.e. hSRTC-LM population with broader lympho-myeloid differentiation potential[149], with the expression of ΔNRPID-HPIP-YFP. The hST-SRCs were significantly lower in number with NRPID mutant

expression as compared to HPIP-WT and control YFP. This reveals that the deletion of nuclear receptor interacting domain from the HPIP-WT protein radically affects the hSTRC-LM.

NRPID mutant decreases the proportions of lymphoid and myeloid cell compartments *in vivo*

The hCBC45^{+}ΔNRPID-HPIP-YFP^{+}-SRCs showed a significant decrease in the myeloid (hCD15) and lymphoid (hCD19) cell compartments, relative to HPIP-WT and control. The result directly indicates that the nuclear receptor/PBX binding domain of HPIP-WT is functionally essential for normal function of HPIP-WT in haematopoietic progenitor fate. The role of nuclear receptor/PBX interacting domain of HPIP could play a role in the normal functions of PBX in HSCs. Studies of Pbx1-/- mutant mice have revealed that Pbx1 is also required for the generation of common lymphoid precursors of B, NK (natural killer) and T cells.[150].

In the affymetrix gene expression data, gene groups showing preferential expression in quiescent and normal dividing cells could be observed as deregulated in ΔNRPID-HPIP^{+} cells relative to the control YFP. The genes involved in cytokine-cytokine interactions were found to be differentially expressed: *CCL3, CXCL2, CXCL5, CXCR4, HGF, IL10RB, PF4* and *TNFRSF19*. The result also points towards a possible role played by the deregulated cytokine-cytokine interactions pathway in the decreased cell proliferation of the ΔNRPID-HPIP^{+} cells relative to the control YFP.[151,152]

NRPID mutant expression up regulates the expression of *CXCR4* gene and reduces the engraftment levels of the HSCs in NOD/SCID mice.

The NRPID mutant expression decreased the human cell engraftment into NOD/SCID mice. Of 31 mice that received a transplant, only 9 mice (29%) exhibited human cell engraftment after 6-8 weeks and the engraftment levels were low (medien=1.9% CD45^{+} cells, $p<0.05$) relative to the HPIP-WT, with 31 mice engrafted out of 65 mice (47%) and YFP control with 32 mice engrafted out of 53 (60). This low engraftment of ΔNRPID-HPIP–YFP^{+} cells could result from an impaired migration or

their reduced capacity to establish human haematopoiesis (requires proliferation and differentiation in the bone marrow environment) [153,154]

The Affymetrix differential gene analysis data in the present study revealed the upregulated expression of *CXCR4* and *DPP4* in hCB $CD34^+$ cells transduced with ΔNRPID-HPIP–YFP cells *in vitro*, 1.5 fold increase in expression as compared to control YFP. The chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 are stated to be critical for murine bone marrow engraftment by human severe combined immunodeficient (SCID) repopulating stem cells. The CXCR4 ligand SDF-1á is a locally secreted small cytokine and is expressed constitutively in some tissues, including bone marrow and lung, which are major homing organs for metastatic breast cancer.[155] CXCR4 is required for invasion, migration, and adhesion activities under SDF-1á-stimulated conditions, suggesting that CXCR4 may play a role in these steps toward malignant metastases.[156]

Treatment of human cells with antibodies to CXCR4 has been shown to prevent engraftment.[157-159] Despite of the fact that CXCR4 is up regulated in the ΔNRPID-HPIP–YFP^+ cells, the engraftment capacity did not increase. This affect can be explained with a decreased proliferation and differentiation for ΔNRPID-HPIP–YFP^+ cells in bone marrow niches. This is a possible reason for the fact that ΔNRPID-HPIP–YFP^+ cells could not reconstitute an efficient human haematopoietic system in NOD/SCID mice.

The expression of NRPID mutant deregulates important genes relative to HPIP WT.

While analysing the differentially expressed genes between the expression profiles of the CB transduced with HPIP-WT and NRPID-HPIP mutant form, we could observe many important genes differentially expressed. Amongst them are *BLNK* (B-cell adapter containing a SH2 domain protein), *HEY1* (Tumour suppressor gene), *PPAP2A* (Major enzyme responsible of dephosphorylating LPA in platelets), *CCL3* (Chemokine), *ZNF618, GZMB, IL1RL1,* and *CCNA1* (cell cycle regulatory gene) that are up-regulated in the case of ΔNRPID-HPIP compared with HPIP-WT. The BLNK (SLP-65) protein has a function in the pre-BCR-signalling pathway, which constitutes

a critical developmental checkpoint in the B cell differentiation.[160] Up-regulated genes such as *BLNK* can explain the differences observed with the reduced expansion of the lymphoid compartment with expression of NRPID mutant relative to the HPIP-WT, *in vivo*.

Another set of genes down-regulated in the case of NRPID-HPIP compared with HPIP-WT were: *TGFB1I1, CHRM3, TNS1* (Src homology 2 domain protein, that is often found in molecules involved in signal transduction), *SCARNA17* (small Cajal body-specific RNA 17)*, ASNS* (Cell cycle control protein TS11)*,* and *CYP1A1* (cytochrome P450 superfamily of enzymes)*,* Amongst the down regulated genes in the above case, *TNS1* gene is an actin binding protein and may be involved in linking signal transduction pathways to the cytoskeleton.[161] Affymetrix differential expression analysis also demonstrated deregulated expression of three MAPKs pathway genes in the case of NRPID mutant, namely: *CD14, FGFR3,* and *RAPIA*. The result indicates that loss of the functional nuclear receptor/PBX interacting domain (LXXLL) of HPIP WT is associated with altered expression of MAPKs. A direct role of HPIP in signal transduction pathways can be suspected with this data.

Expression of HPIP is elevated in CALM/AF10 and FLT3 length mutation specific cytogenetic subtypes of AML.

The Affymetrix expression data from AML patient samples indicates that HPIP WT is highly expressed in selected subtypes of AML. AML cases with specific cytogenetic aberrations such as the CALM/AF10 translocation, FLT3 length mutation or translocation t(15;17) had higher levels of *HPIP* expression as compared to the bone marrow from healthy donors as well as compared to other cytogenetic subtypes such as MLL-PTD, complex karyotype, AMLM2 and M4Eo. It has been shown that the MLL protein binds to the promoter of *Hox* genes to maintain their expression. MLL fusion proteins have also been reported to directly up regulate the *Hox* genes along with expression of co-factor *Meis1*. We analysed the expression of HPIP in a group of ALL-MLL patients, however there was no differences of HPIP expression compared to the normal bone marrow control.

Our data has clearly pigeonholes HPIP-WT as a novel regulator/mediator of

human haematopoietic stem cells with an important C´terminal nuclear receptor/PBX interacting domain. Expression of HPIP-WT promotes the growth and repopulation of clonogenic progenitors and long term culture initiating cells *in vitro*. It is also able to promote the expansion of hST-SRCs *in vivo.* It was able to effect the expression of important genes involved in cell signalling, cell cycle, proliferation and differentiation. We could demonstrate the importance of HPIPs´ nuclear receptor/PBX interacting domain in self renewal, hST-SRCs-LM cell compartment, lymphoid/myeloid and erythroid cell differentiation and its effects on the expression of genes involved in cell signalling, self renewal and engraftment/migration in NOD/SCID mice. However, there is still a need to further describe the molecular mechanisms which are underlying the regulatory role of HPIP in early human haematopoiesis.

6. Summary

The characterization of new genes involved in the regulation of normal/leukaemia haematopoiesis and the development of therapies against deregulated processes in haematopoiesis are the major goals in experimental and clinical haematology. The focus of this thesis was the characterization of haematopoietic PBX-interacting protein (HPIP), a novel putative regulatory protein of early human haematopoiesis. Using complex *in vitro* and *in vivo* assays we analyzed the impact of constitutive expression of HPIP- WT and its functional domains (nuclear receptor/PBX interacting domain and microtubule interacting domain) on the stem cell and early human haematopoietic development. To detect the affect of WT and mutated HPIP on the clonal progenitor cells, primary and secondary colony-forming-unit (CFC) assays were performed. Additionally the *in vitro* equivalent of HSC long-term culture initiating cells was detected with the (LTC-IC) assay.

We were able to show that the constitutive expression of HPIP-WT increases the number of cells detected on the level of committed clonogenic progenitors and long term culture initiating cells in the LTC-IC assays. In addition, HPIP-WT^+ cells show enhanced number of CFCs generated per LTC-IC as compared to the control. Besides its effect on maintenance of primitive haematopoietic progenitor cells, constitutive expression of HPIP-WT could not block terminal haematopoietic differentiation. It also increases the proportions of the short term SCID repopulating cells (ST-SRCs, $hCD34^+38^+$) *in vivo*, when compared with the empty vector control. Additionally we could show that the constitutive expression of HPIP leads to an increase of myeloid cells in transplanted in the NOD/SCID mice. These data characterizes HPIP-WT as a novel regulator of the early human haematopoietic stem cells, whose constitutive expression has a notable impact on the short term committed progenitors and myeloid cell repopulation of human haematopoietic system.

We could further characterize the functional domains of HPIP-WT. We could demonstrate the importance of HPIPs´ nuclear receptor/PBX interacting domain in self renewal, hST-SRCs-LM cell compartment (ST-SRCs, $hCD34^+38^+$) *in vivo*,

lymphoid/myeloid and erythroid cell differentiation and its effects on the expression of genes involved in cell signalling, self renewal and engraftment/migration in NOD/SCID mice. Overall HPIP is a very important interactor of para HOX gene PBX. In the present study we could clearly prove it for the first time that HPIP´s expression in hu $CD34^+$ cells holds an important role in the regulation of human haematopoiesis.

7. Zusammenfassung

Die Charakterisierung neuer Gene, die in die Regulation der normalen/leukämischen Hämatopoese involviert sind, und die Entwicklung von Therapien gegen deregularisierte Prozesse in der Hämatopoese sind die Hauptziele der experimenteller und klinischer Hämatologie. Der Schwerpunkt dieser Promotionsarbeit war die Charakterisierung des hämatopoetischen PBX-interacting Protein (HPIP), einem neuartigen mutmaßlichen regulatorischen Protein der frühen menschlichen Hämatopoese. Indem komplexe *in vitro* und *in vivo* Assays genutzt wurden, analysierten wir die Auswirkungen der konstitutiven Expression von HPIP-WT und seiner funktionalen Domänen (Kernrezeptor/PBX-interagierende Domäne und Mikrotubuli interagierende Domäne) auf die Stammzelle und frühe menschliche hämatopoetische Entwicklung. Um die Auswirkung von WT und mutierter HPIP auf klonale Vorläuferzellen zu erkennen, wurden primäre und sekundäre koloniebildende Einheiten (CFC) Assays durchgeführt. Zusätzlich wurde das *in vitro* Äquivalent von HSC, die Langzeitkultur initiierenden Zellen, nachgewieser mit dem sog. LTC-IC Assay.

Wir waren in der Lage zu zeigen, dass der konstitutive Ausdruck von HPIP-WT die Anzahl der Zellen erhöht, die auf der Ebene der beteiligten klonalen Vorläuferzellen und Langzeitkultur initiierenden Zellen in den LTC-IC Assays erhöht. Weiterhin zeigen HPIP-WT*-Zelllen eine vergrößerte Anzahl von CFC, die per LTC-IC generiert wurden, als die Kontrolleinheit. Abgesehen von seiner Auswirkung auf die Aufrechterhaltung primitiver hämatopoetischer Vorläuferzellen, konnte die konstitutive Expression von HPIP-WT die abschließende hämatopoetische Differenzierung nicht blockieren. Sie erhöht außerdem die Proportionen von kurzfristig SCID wiedervermehrten Zellen (ST-SRCs, hCD34*38*) *in vivo* im Vergleich zur leeren Vektorkontrolleinheit. Zusätzlich konnten wir zeigen, dass die konstitutive Expression von HPIP zu einer Erhöhung der myeloiden Zellen führt, wenn sie NOD/SCID-Mäusen transplantiert werden. Diese Daten charakterisieren HPIP-WT als einen neuen Regulator der frühen menschlichen hämatopoetischen Stammzelle, dessen konstitutive Expression einen beträchtlichen Effekt auf die kurzzeitig agierenden Vorläuferzellen und die

Repopulation der myeloiden Zellen des menschlichen hämatopoetischen Systems hat.

Darüber hinaus konnten wir die funktionalen Domänen des HPIP-WT charakterisieren. Wir konnten die Bedeutung des Kernrezeptors von HPIP/PBX-interagierender Dömane in Hinsicht auf die folgenden Bereiche zeigen: Selbsterneuerung, hST-SRCs-LM Zellabschnitt (ST-SRCs, $hCD34^{+}38^{+}$) *in vivo*; lymphoider/myeloider and erytroider Zelldifferentiation und seiner Auswirkungen auf die Expression von Genen, die bei der Zellsignalgebung, Selbsterneuerung und Anwachsen/Migration bei NOD/SCID-Mäusen beteiligt sind. Das umfassende HPIP ist ein sehr wichtiger interagierendes Element des para HOX-Gens PBX. In der vorliegenden Studie konnten wir erstmalig eindeutig beweisen, dass die Expression von HPIP in humanen CD34*-Zellen eine bedeutende Rolle in der Regulation der menschlichen Hämatopoese spielt.

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9. Abbreviations

- µl Microliter
- µM Micromola
- ALL Acute lymphoid leukemia
- AML Acute myeloid leukemia
- BFU-E Burst forming unit- Erythroid
- BM Bone marrow
- CB Cord blood
- CD Cluster of Differentiation
- CFC Colony forming cell
- CSF Colony stimulating factor
- DMSO Dimethylsulfoxid
- DNA Desoxyribonucleic acid
- EPO Erytropoietin
- FAB French American British
- FACS Fluoresence Activated Cell Sorting
- FISH Flurecence in situ hybridisation
- FLT3 FMS-like tyrosine kinase 3
- G-CSF Granulocite-Colony stimulating factor
- GM-CSF Gramulocite-Macrophage-Colony stimulating factor
- HB Homeobox gene
- HD Homeodomain
- IL interlulukin
- ITD Internal tandem duplication
- LDA-LTC-IC Limiting dilution assay LTC-IC
- LM Lenght mutation
- LTC-IC Long-term culture-initiating cell
- LTR Long terminal repeat
- M-CSF Macrophage-Colony stimulating factor
- MIG MSCV-IRES-EGFP
- MNC Mononuclear cells
- MSCV Murine Stem Cell Virus
- NBM Normal bone marrow
- NOD/SCID Deficent Non Obese Deficent-Sever Compromise Immuno

- PB Peripheral blood
- PCR Polymerase Chain Reaction
- PI Propidium iodide
- PTK Phospho tyrosine kinase
- PTK Protein Tyrosine Kinase
- PTKI Protein Tyrosine Kinase Inhibitor
- RNA Ribonucleic acid
- S-CFU-S Spleen-Colony forming unit
- SC-IC Suspension culture- initiating cell
- SEM Standard error of the mean
- t translocation
- TDK Tyrosine kinase domain
- TM Transmembrane domain
- VCM Virus containing medium
- WBC White blood cell
- WT Wild type
- YFP Yellow Fluorescent Protein

10. Acknowledgment

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CURRENT STATUS/ EDUCATION/QUALIFICATION

Technical University Muenchen
Stem cell Physiology Laboratory, AG Oostendorp/Götze (current employer)
Department of haematology, Klinikum recht der Isar Munich, Germany
Post Doctoral position
09/2009-12/2011

- **Research Topic: Characteristation of microenvironment in normal and AML-ITD positive Leukemia haematopoiesis**

Helmholtz Zentrum
Clinical Cooperative Group ''Leukemia'',
Ludwig-Maximilians University, AG Feuring Buske
Department of Medicine III, Klinikum Grosshadern Hospital Munich Germany
Ph.D position (magna cumlaude) **09/2005-09/2009**

- **Research topic**: **Functional characterization of a novel ´Hematopoietic PBX1 interacting protein, (HPIP), and its role in normal and malignant human haematopoiesis.**

Guru Nanak Dev University
M.Sc. in Human Genetics (Honors) Guru Nanak Dev University,
Amritsar (GNDU), India (Aggregate Marks- 62.2%, First Division) Amritsar, India
Master Thesis
2003-2004

- **Research topic: Lymphocytic chromosomal instability in gastrointestinal tract cancer patients and their first-degree relatives.**

SKILLS

Cell Culture

- Protein arrays (on membrane), Retroviral transduction on Umbilical cord blood cells ($CD34^+$ Human Hematopoietic stem cells) single and double/mixed virus transductions/ transfections, Liquid expansion cultures, Long term culture initiating cell, assays (LTC-IC), Colony forming unit assay, B-Lymphoid assay, RNA interference using shRNA cloned in MSCV vector, Gene expression profiling, DNA/RNA/protein isolation,.

Mouse techniques

- Competitive repopulating cell assay in NOD/SCID mouse model, Intravenous injections in the tail of mice, Intra-peritonial injections, Intra hepatic injections, mouse bone marrow harvest.

Molecular Techniques

- Flow cytometry, PCR, Real time quantitative, siRNA, shRNA development, Electroporation, Preparation and cloning of shRNA in MSCV based vector, Western blot, cloning.

Cytogenetics

- Human lymphoid cell cultures, Metaphase screening, Chromosome banding and Karyotyping

Softwares

- L- calc (SRC frequency analysis), Microsoft (Excel, Word, Powerpoint), BD quest, Flowjo (Flow cytometry analysis), Image J.

COURSES ATTENDED

Wellcome Trust Scientific Workshop Cambridge, **UK**
Perspectives in stem cell proteomics training workshop 2009

MDC/DKFZ Summer School 2007 Berlin, **Germany**
Signal transduction and transcriptional regulation in cancer 2007

European School of Genetic medicine in Bertinoro di Romagna, Bertinoro, **Italy**
7th course in Bioinformatics for Molecular Biologists 2007

EUCOMM/GSF training event: Munich, **Germany**
Protection and Commercialization of Intellectual Property 2006

TRAINING

All India Institute of Medical Sciences (AIIMS)
Delhi**, India** Institute Rotary Cancer Research Hospital, Department of Hematology, Leukemia Research Lab, October, 2004- February, '05

- **RFLP** [To Characterize MTHFR gene polymorphism in ALL patients (<A1298C> and <C677T>)].
- **RT- PCR** (Molecular detection of BCR-ABL transcript in CML patients).

AWARDS

- Recipient of travel award in EHA conference, 2009 held in Berlin, Germany
- Recipient of travel award in ISEH conference, 2007 held in Hamburg, Germany

- Recipient of National scholarship by Government of India to follow Masters study, 2004, India

MEETINGS ATTENDED

- Perspectives in stem cell proteomics, Welcome trust conference, 2009, Cambridge, UK
- American Society of Hematology (ASH), 2008, San Francisco, US.
- International Society of Experimental Hematology ISEH, 2008 Boston Massachusetts, US)
- American society of Human Genetics, (ASGH), 2007, San diego, US.
- International Society of Experimental Hematology (ISEH), 2007 Hamburg, Germany
- International Symposium on RNAi (SMPRNAi/NGFN) 2006, Neuherberg, Munich
- Indian Society of Human Genetics (ISHG) 2005, CCMB, Hyderabad, India.
- Indian Society of Human Genetics (ISHG) 2004, NIMHANS, Banglore, India.
- Indian Society of Human Genetics (ISHG) 2002, Kerela, Rajiv Gandhi institute of –Biotechnology, India.

PUBLICATIONS (ABSTRACTS/POSTERS/PAPERS)

Abstract publications

- **Kaur P**, Stadler C, Humpheries KR, Hiddemann W, Buske C and Feuring-Buske M. Functional characterization of a novel ´Hematopoietic PBX1 interacting protein (HPIP) and its role in normal and malignant haematopoiesis. **Abstract published in November online supplement of Blood, 2008**.

Poster abstract

Kaur P, Stadler C, Ahmed F, Humpheries KR, Hiddemann W, Buske C and Feuring-Buske M: *Functional characterization of a novel ´Hematopoietic PBX1 interacting protein (HPIP) and its role in normal and malignant human hematopoiesis* ***ISEH, 2008 San Francisco, California, US.***

Kaur P, Stadler C, Ahmed F, Humpheries KR, Hiddemann W, Buske C and Feuring-Buske M: *Functional characterization of a novel ´Hematopoietic PBX1 interacting protein (HPIP) and its role in normal and malignant human hematopoiesis* ***ISEH, 2008 Boston, Massachusetts, US.***

Kaur P, Stadler C, Ahmed F, Humpheries KR, Hiddemann W, Buske C and Feuring-Buske M: *Functional characterization of a novel ´Hematopoietic PBX1 interacting protein*

(HPIP) and its role in normal and malignant human hematopoiesis ***ASHG, 2007, San Diego, US.***

Kaur P, Arseni N, Stadler C, Ahmed F, Humpheries KR, Hiddemann W, Buske C and Feuring-Buske M: *Functional characterization of a novel´Hematopoietic PBX1 interacting protein (HPIP) and its role in normal and malignant human hematopoiesis* ***ISEH, 2007 Hamburg, Germany.***

Kaur P, Arseni N, Ahmed F, Humpheries KR, Hiddemann W, Buske C and Feuring-Buske M: *Functional characterization of a novel ´Hematopoietic PBX1 interacting protein (HPIP) and its role in normal and malignant hematopoiesis International Symposium on RNAi (SMPRNAi/NGFN)* ***2006, Neuherberg, Munich***

Pawandeep Kaur and Vasudha Sambyal: *Lymphocytic Chromosomal Instability in Sporadic Gastrointestinal Tract (GIT) Cancer Patients and their First-Degree Relatives.* ***ISHG, 2005 CCMB, Hyderabad.***

Paper publications/ in preparation

- **Pawandeep Kaur** and Vasudha Sambyal; Lymphocytic Chromosomal Instability in Sporadic Gastrointestinal Tract (GIT) Cancer Patients and their First-Degree Relatives. Kamla-Raj. **Int J Hum Genet, 8(4): 335-342 (2008)**
- **Pawandeep Kaur**, Christiane Stadler, Vindi Jurinovic, R Keith Humphries, Wolfgang Hiddemann, Stefan Bohlander, Christian Buske and Michaela Feuring-Buske. The Human PBX interacting Protein (HPIP) is a novel regulatory protein of early human hematopoietic development .(Manuscript in preparation)
- **Pawandeep Kaur** and Robert A J Oostendorp. Role of soluble factors in regulation of HSCs in bone marrow microenvironment. (review for the journal Frontiers of Biosciences, in press)

LANGUAGE SKILLS

English (excellent level), German (basic level), French (beginner)
Native languages: Hindi and Punjabi (excellent levels).

ADDITIONAL ACTIVITIES AND SKILLS

Teaching experience
Part-time lecturer: Uppal College of Paramedical Sciences
(July 2004- Oct 2004) PunjabTechnical University.
Subjects taught: Molecular Biology, Genetic Engineering and Biophysics.

Youth programs on all India radio
To my credit, I have presented programs on Human Genetics for the youth programs

on Radio (part-time) under an unofficial name Pawan Mroke. I prepared my own script and vocalized it on AIR. Program- General Genetics: July 27, 2001, Genetic Syndromes: Jan 17, 2002

Contribution in Newspapers
The Tribune and The Indian Express (1999-2000)
To my credit I have a short-term experience as a journalist (part-time) under an unofficial name Pawan Mroke. *Articles can be shown on demand.*

- Organized stage and Anchored events for National Youth Festival, 2001, held on GNDU campus.

- Active participations made in sport events, cultural activities and arts on the GNDU campus.

- Sketching, Painting

- Basketball, trekking and snowboarding.

Int J Hum Genet, 8(4): 335-342 (2008)

Lymphocytic Chromosomal Instability in Sporadic Gastrointestinal Tract (GIT) Cancer Patients and their First-Degree Relatives

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KEYWORDS Sporadic; gastrointestinal cancer; first-degree relatives; chromosomal instability

ABSTRACT The present study was an attempt to assess utility of chromosomal instability in peripheral blood lymphocytes of first-degree relatives (FDR) of sporadic gastrointestinal tract (GIT) cancer patients for genetic surveillance. Standard lymphocyte culture technique was used for the purpose. Cultured peripheral blood lymphocyte metaphases were scored for aberrations in 10 sporadic cases of GIT cancer patients (6-esophageal, 1-gastric, 2-rectum and 1-cecum), 10 first-degree relatives and 10 healthy unrelated controls. There were significantly increased number of aberrations in cancer patients as compared to FDRs and controls. A perceptible increase in the level of metaphases with structural aberrations, including gaps, breaks, rings, centromeric separation and terminal deletions, was observed in first-degree relatives of cancer patients as compared to healthy unrelated controls taken from the same geographical area. There was high frequency of aberrations, mainly structural aberrations, involving specific chromosomes in first-degree relatives and in cancer patients. Majority of aberrations were at chromosomal loci harboring genes involved directly or indirectly in tumorigenesis, thus indicating the probability of a constitutional chromosomal instability in first-degree relatives of even sporadic GIT cancer patients.

INTRODUCTION

Instability of genome plays an important role in tumor initiation and progression. Aneuploidy is proposed as the primary cause of genome instability of neoplastic and preneoplastic cells (Langauer et al. 1997). Aneuploidy destabilizes the karyotype and thus the species, independent of mutation, because it corrupts highly conser-ved team of proteins that segregate, synthesize and repair chromosomes. Likewise it destabilizes genes. The transition of stable diploid to unstable aneuploid cell species is apparently the primary cause of preneoplastic and neoplastic genomic instability and of cancer, and the mutations are secondary (Duesberg et al. 2004). Chromosomal instability, leading to loss of crucial portion of DNA or loss of a complete chromosome, may participate in cancer predisposition by causing a further loss of closely linked group of genes producing proteins essential for controlled cell division. An increased risk of cancer has been seen in healthy individuals, with high levels of chromosomal aberrations in peripheral blood lymphocytes (Boffetta et al. 2007). A significantly elevated cancer risk was observed in a Nordic cohort for subjects with both high chromosome and chromatid-type aberrations in peripheral blood lymphocytes and these variables showed equally strong predictivity (Hagmar et al. 2004). There was no significant effect of modifications by age at test, gender, country or time since test observed on the association of cancer risk with increased chromosomal instability (Bonassi et al. 2000, 2004).

A wide range of chromosomal abnormalities and microsatellite instability have been reported in tumor tissue of gastrointestinal tract (GIT) cancer patients. These include disruption of APC gene, reciprocal translocations, gain and loss of chromosomes, and rearrangements in chromosomes 1, 2, 3, 4, 5, 8, 9, 10, 11, 16, 17, 18 and 19 (Bomme et al. 1996; Popat et al. 2003; Shiomi et al. 2003; Jin et al. 2004). Studies on family members and cancer patients from high risk families using peripheral blood lymphocytes, dermal fibroblast monolayer cultures and 8 blood markers did not yield any common biomarker shared by all family members at risk of developing cancer (DiLerina et al. 1987; Guanti et al. 1990). But increased in-vitro tetraploidy was reported in skin cultures derived from patients with colon cancer syndrome and some family members at risk (Danes, 1981). Increased endoreduplication and tetraploidy was also reported in lymphocytes

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