2008-09-03

Regulation of HSC Self-Renewal and Differentiation by Pumilio Proteins

Jennifer Zayas
University of Miami, jzayas@med.miami.edu

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
https://scholarlyrepository.miami.edu/oa_dissertations/300

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
Evolutionarily conserved Pumilio (Pum) RNA-binding proteins act as translational repressors during embryo development and cell fate specification. Previous work in the lab has shown that over-expression of Pum2 (Pum2-EML) supports maintenance and suppresses multilineage differentiation of murine multipotent HSC/MPP-like cell line EML. The subsequent analysis of HSC markers and functional analysis has revealed that wt EML cells share the LKS CD34\(^+\) phenotype, whereas the majority Pum2-EML cells are similar to LKS CD34\(^-\). The CD34\(^+\) wt EML cells can be divided into CD34\(^{low}\), CD34\(^{med}\) and CD34\(^{high}\) subpopulations, whereas Pum2-EML CD34\(^+\) cells correspond to CD34\(^{low}\) subpopulation. Colony forming assays have revealed that the overall multilineage differentiation of wt EML and Pum2-EML cells strongly correlates with the CD34 expression levels. Multiple experiments have revealed that purified CD34\(^-\) and CD34\(^+\) wt EML cells can generate each other and among CD34\(^-\) wt EML cells the CD34\(^{low}\) cells have the highest capacity to give rise to CD34\(^-\) EML cells. We have proposed a model in which CD34\(^-\) EML cells are more primitive cells in an “inactive” (differentiation inhibited) state, that give rise to CD34\(^{low}\) “active” (differentiation ready) EML cells. The CD34\(^{low}\) EML cells can revert back to the CD34\(^-\) state or give rise to CD34\(^{med/high}\) cells that can readily differentiate into multiple lineages. Based on that model, the over-expression of Pum2 leads to increased maintenance of cells in inactive
CD34 state, and blocks development of CD34^+ cells past the CD34^{low} stage. Cumulatively, these results support the notions that Pum2 could be involved in maintaining the balance between inactive and active state of multipotent hematopoietic cells.

The c-kit receptor plays a vital role in self-renewal and differentiation of hematopoietic stem cells (HSC) and multipotent progenitors (MPPs). We have discovered that besides c-kit, the murine multipotent HSC/MPP-like cell line EML expresses the transcript and protein for a truncated form of c-kit, called tr-kit. Notably, the tr-kit transcript and protein levels were down-regulated during cytokine induced differentiation of HSC/MPP-like cell line EML into myelo-erythroid lineages. RT-PCR results show tr-kit is transcribed solely in cell populations enriched for LTR-HSC, STR-HSC and MPPs. The observation that tr-kit is co-expressed with c-kit only in more primitive, HSC and MPP-enriched cell populations raises an exciting possibility that tr-kit functions either as a new component of SCF/c-kit pathway, or is involved in a novel signaling pathway, present exclusively in HSC and MPPs. These findings necessitate functional characterization of tr-kit, and analysis of its potential role in the self-renewal, proliferation and/or differentiation of HSC and multipotent progenitors.
Dedicated to my family
ACKNOWLEDGEMENTS

I thank my mentor Dr. Roland Jurecic from the bottom of my heart for the support and guidance in all these years. The complete training from him is going to benefit for my rest of life.

I thank the members of my committee: Dr. Becky Adkins, Dr. Robert B. Levy, Dr. Richard L. Riley, Dr. Pantelis Tsoufas, also Dr. Kelvin Lee for their guidance and suggestions throughout my graduate program.

Also, I am very grateful for Dr. James Palis for participating at my dissertation defense as an outside examiner.

I thank Vesna Jurecic for the help of real-time PCR, Dr. Eric McIntush (Bethyl Inc.) for provide the Ab for trkit, Jim Philips for technical help for flow cytometry.

I thank the members of the Jurecic Lab: Dr. Danislav S. Spassov, Dr. Xin Jing, Dr. Ronald Nachman, Dr. Snjezana Kutlesa for their support and thank for Kyle DiVito, Noelia Kunzevitzky, Ali Saeed, John George, Zahid Huq, Nadim Kassimali for their help.
TABLE OF CONTENTS

LIST OF TABLES........................................................................................................................ix
LIST OF FIGURES........................................................................................................................ix

I. INTRODUCTION

1.1. Development, phenotype and functional features of hematopoietic stem cells and progenitors.................................................................................................................................................................................1
1.2. HSC niches and regulation of dormancy and self-renewal.............................................6
1.3. Increasingly complex network of non-redundant pathways regulates HSC self renewal and differentiation.................................................................................................................................................................................9
1.4. The role of pumilio RNA-binding proteins in regulating stem cell self-renewal and maintenance.........................................................................................................................................................................................................14
1.5. The role of SCF/c-kit signal pathway in self-renewal and differentiation of HSC and progenitors ........................................................................................................................................................................................................23
1.6. Multipotent hematopoietic cell line EML as a model system to study early hematopoiesis.................................................................................................................................................................................26
1.7. Statement of the problem.................................................................................................29

II. MATERIALS AND METHODS

2.1. Cell, cell sorting and flow cytometry........................................................................31
2.2 Hematopoietic colony-forming assays.........................................................................32
2.3. Cytokines and antibodies ..........................................................................................33
2.4. RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR).....33
2.5. Western blotting

2.6. Expression vectors

2.7. shRNA-mediated knockdown of Pum2

2.8. Microarray expression analysis

III. RESULTS

3.1. The role of Pum2 RNA-binding protein in the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML

3.1.1. Pum2 over-expression leads to SCF-independent maintenance of EML cells and attenuates their differentiation

3.1.2. Pum2 over-expression changes the cell surface phenotype of EML cells

3.1.3. Multilineage differentiation of wt EML and Pum2-EML cells correlates with the level of CD34 expression

3.1.4. Developmental relationship between CD34⁻ and CD34⁺ wt EML and Pum2-EML cells

3.1.5. The level of CD34 expression on CD34⁺ EML cells correlates with their capacity to give rise to CD34⁻ cells

3.1.6. Cell cycle status of CD34⁻ and CD34⁺ wt EML and Pum2-EML cells

3.1.7. Molecular differences between wt EML and Pum2-EML cells and their CD34⁻ and CD34⁺ populations

3.1.8. The effect of Pum2 attenuation on the maintenance and differentiation of EML cells
3.2. The role of truncated c-kit receptor (tr-kit) in the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML………………………………..64

3.2.1. Pum2-EML cells express higher levels of new truncated form of c-kit receptor protein, called tr-kit..................................................64

3.2.2. EML cells co-expresses the full-length and truncated c-kit receptor...............67

3.2.3. Expression of tr-kit is restricted to populations enriched for HSC and MPPs...69

3.2.4. Expression of tr-kit is down-regulated during differentiation of EML cell line into myelo-erythroid lineages..............................................74

3.2.5. The impact of tr-kit over-expression on the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML……………………………….78

IV. DISCUSSION

4.1. The role of Pum2 RNA-binding protein in the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML........................................82

1. Over-expression of Pum2 in EML cells leads to reversal of CD34 expression pattern and maintains the cells in a more primitive status CD34 structure in hematopoietic cells.................................................................82

2. CD34 expression levels correlate with multilineage differentiation of EML and Pum2-EML cells .................................................................82

3. Cell surface CD34 expression is linked with “inactive” and “active” state of EML and Pum2-EML cells.................................................................83
4. Possible correlation between CD34 expression and localization of HSC in different
niches..................................................................................................................87

5. Concluding remarks.......................................................................................88

4.2. The role of truncated c-kit receptor (tr-kit) in the maintenance and differentiation of
multipotent hematopoietic cells............................................................................88

VI. REFERENCES..................................................................................................92
LIST OF TABLES AND FIGURES

Table 1. The sequence of the primers used for gene expression analysis………..34

Figure 1. Hematopoietic Stem Cells (HSC) have the capacity to regenerate entire hematopoietic system…………………………………………………………...1

Figure 2. Developmental Regulation of Hematopoiesis in the Mouse………………...2

Figure 3. Hematopoietic developmental hierarchy and cell surface markers on hematopoietic cells…………………………………………………………...4

Figure 4. Dormant and activated HSC bone marrow niches………………………….7

Figure 5. Migration and homing of cells between BM niches………………………..8

Figure 6. Intrinsic and extrinsic mechanisms…………………………………………9

Figure 7. Several conserved and non-redundant intrinsic and extrinsic pathways have been implicated in the control of HSC self-renewal…………………………….10

Figure 8. Phylogenetic dendrogram of Pumilio family proteins…………………………14

Figure 9. A schematic representation of the quaternary complex of Pumilio, nos, Brat and hb mRNA that leads to translational repression of hb in the fruitfly embryo…………………………………………………………………………15

Figure 10. Mutations in the Pumilio gene lead to loss of stem cells due to abolishment of the stem cell asymmetric self-renewal division……………………………..16

Figure 11. Pum proteins are characterized by a highly conserved C-terminal RNA-binding domain (also called PUM-HD domain)……………………………..18

Figure 12. A quaternary complex composed of Pum, Nos, Brat and Hb mRNA based on crystal structure and mutational analysis of Pum RNA-binding domain……..19
Figure 13. Pumilio RNA-binding domain (center) mediates various biological functions...................................................................................................22

Figure 14. SCF/c-kit signaling pathway in hematopoietic cells.................................................25

Figure 15. The model of the maintenance and multilineage differentiation of a multipotent hematopoietic cell line EML.........................................................27

Figure 16. The CFU-GM, CFU-Meg and BFU-E colony-forming progenitors among EML cells cultured in the presence of IL-3, Tpo and Epo.......................27

Figure 17. Flow cytometry analysis of HSC cell surface markers and RT-PCR expression analysis of HSC relevant genes on EML cells...........................................28

Figure 18. Schematic representation of murine stem cell virus (MSCV)-based, bicistronic retroviral vector for over-expression of tr-kit.........................................................36

Figure 19. Colony-forming capacity of wt EML, vector control EML and Pum2-EML cells..................................................................................................................39

Figure 20. Flow cytometry analysis of HSC cell surface markers expression on wt EML, EML/pcDNA cells, and Pum2-EML cells.........................................................41

Figure 21. Sorting of wt EML and Pum2-EML cells into CD34+ and CD34− populations and each population’s differentiation capacity measured by CFU assay........42

Figure 22. Morphology of purified wt EML CD34+ and CD34− cells.................................43

Figure 23. Sorting of CD34+ wt EML cells into CD34 low, CD34 med and CD34 high populations and each population’s differentiation capacity measured by CFU assay.................................................................44

Figure 24. Model of CD34− give rise to CD34+ cells in wt EML and Pum2-EML cells..................................................................................................................47
Figure 25. Flow cytometry analysis of CD34 expression on cultured FACS purified CD34⁻ and CD34⁺ wt EML cells.................................................................48

Figure 26. Flow cytometry analysis of CD34 expression on cultured FACS purified CD34⁻ and CD34⁺ Pum2-EML cells.................................................................49

Figure 27. Analysis of differentiation capacity of “secondary” CD34⁻ and CD34⁺ cells derived from culture of purified CD34⁺ and CD34⁻ EML cells..............................49

Figure 28. “Secondary” CD34⁻ and CD34⁺ EML cells..................................................50

Figure 29. The model of linear differentiation of CD34⁻ EML cells into CD34⁺ cells....51

Figure 30. Correlation between the level of CD34 expression and the capacity of CD34⁺ EML cells to generate CD34⁻ cells.................................................................52

Figure 31. Illustration of experiments in the murine system have shown that mouse CD34⁻ CD34⁺ is reversible.................................................................52

Figure 32. Models of activation; level of CD34 expression correlates with their capacity to give rise to CD34⁻ cells and the model that merges linear differentiation and the reversible CD34 expression and activation........................................53

Figure 33. The maintenance of EML cells correlates with decreasing levels, whereas differentiation of EML cells correlates with increasing levels of CD34 expression........................................................................................................54

Figure 34. The over-expression of Pum2 leads to increased maintenance of EML cells in inactive CD34⁻ state, and blocks development of CD34⁺ cells past the CD34⁺low stage........................................................................................................55

Figure 35. Cell cycle status of CD34⁻ and CD34⁺ wt EML and Pum2-EML cells and comparison with BM-derived HSC.................................................................55
**Figure 36.** RT-PCR analysis of Notch1 transcript and flow cytometry analysis of Notch1 protein expression in wt EML, vector control and Pum2-EML cells..............56

**Figure 37.** RT-PCR analysis of expression of several developmentally and functionally relevant genes in CD34− and CD34+ wt EML cells.............................................56

**Figure 38.** Representative data from Agilent 44K cDNA microarray expression analysis of wt EML and Pum2-EML cells, and their CD34+ and CD34− subpopulations.......................................................................................... 57-58

**Figure 39.** RT-PCR analysis of CD34, Bmi-1, and CD150 expression in wt EML and Pum2-EML cells, and their CD34+ and CD34− subpopulations.................59

**Figure 40.** Flow cytometry analysis of CD34 and CD150 co-expression on wt EML and Pum2-EML cells..............................................................................................61

**Figure 41.** Western analysis of Pum2 protein in wt EML cells, EML clone transduced with Non-Target shRNA Control virus, and EML cell clones transduced with Pum2 shRNA vectors 60-64.................................................................62

**Figure 42.** Western blot analysis of c-kit protein has shown that Pum2-EML cells express higher levels of new truncated form of c-kit receptor protein, called tr-kit.....64

**Figure 43.** Structure of c-kit and tr-kit protein.................................................................66

**Figure 44.** Western blot of wt EML, vector control and Pum2-EML cells (cultured in the condition of with or without SCF) expression of tr-kit and c-kit and tr-kit are phosphorylated at Y936...............................................................67

**Figure 45.** Western analysis of tr-kit and c-kit protein expression and phosphorylation in EML cells.................................................................................................69
Figure 46. Expression of tr-kit is restricted to cell populations enriched for HSC and MPPs.................................................................70, 72, 73

Figure 47. Tr-kit transcription and protein expression are down-regulated during differentiation of EML cell line into erythroid lineage.........................................75

Figure 48. Tr-kit transcription and protein expression are down-regulated during differentiation of EML cell line into myeloid lineages.......................................77

Figure 49. BaF3 cells transduced with MSCV/GFP or MSCV/trkit/GFP sorted into GFP+ and GFP populations and RT-PCR analysis of these populations..................78

Figure 50. Sorting of GFP+ EML cells transduced with the control MSCV/GFP virus or MSCV/trkit/GFP virus..........................................................79

Figure 51. Western analysis of tr-kit protein in wt EML and GFP+ EML cells transduced with MSCV/trkit/GFP virus..........................................................79

Figure 52. Flow cytometry analysis of HSC/MPP markers on wt EML and selected GFP+ EML cells, which were transduced either with the control MSCV/GFP virus or MSCV/trkit/GFP virus at different MOI..................................................80

Figure 53. EML cells over-expressing tr-kit exhibit increased CFC activity in comparison to wt and MSCV/GFP control EML cells.......................................80

Figure 54. EML cells over-expressing tr-kit produced larger hematopoietic colonies in comparison to wt and MSCV/GFP control EML cells.......................................81

Figure 55. Model of regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo.................................................................87

Figure 56. Hypothetical interaction of tr-kit and the full-length c-kit receptor in SCF-independent activation of c-kit.................................................................90
I. INTRODUCTION

1.1. Development, phenotype and functional features of hematopoietic stem cells and progenitors

Hematopoietic stem cells (HSC) are defined as multipotent cells that have the capacity to self-renewal and differentiate into all types of mature blood cells (Fig. 1).

Through self-renewal and differentiation, HSC maintain the homeostasis of the blood system by replacing differentiated blood cells lost through physiological turnover, disease, or injury. During murine embryonic development there are two waves of hematopoiesis: the first wave of hematopoiesis is observed in the yolk sac (E7.5) and the aorta-gonad-mesonephros (AGM) region (E10.5-E11), which last until E13. HSC activity is also detected in placenta parallel to the AGM region. The expansion of HSC pool in the placenta occurs prior to and during the initial expansion of HSCs in the fetal liver (1). The next wave of hematopoiesis begins in fetal liver (FL) at E12. HSC activity is subsequently detected in the bone marrow (BM) as early as E17.5 and is sustained throughout the life (Fig. 2) (2).
Figure 2. Developmental Regulation of Hematopoiesis in the Mouse. (A) Hematopoiesis occurs first in the yolk sac (YS) blood islands and later at the aorta-gonad mesonephros (AGM) region, placenta, and fetal liver (FL). (B) Hematopoiesis in each location favors the production of specific blood lineages. Abbreviations: ECs, endothelial cells; RBCs, red blood cells; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor. (C) Developmental time windows for shifting sites of hematopoiesis [Stuart H. Orkin and Leonard I. Zon, Cell, Vol 132 (4), 22 February 2008, P 631-644].
Discovery and characterization of numerous cell surface markers has enabled sorting, purification and functional characterization of various populations of hematopoietic cells.

Combining cell surface marker-based cell sorting and cell transplantation has helped to define specific stages of hematopoietic cell development and measure their self-renewal and reconstituting capacity (3). The capacity of HSCs to reconstitute the entire blood system is measured through transplantation, which has shown that a single HSC is capable of reconstituting the entire hematopoietic system (4).

Transplantation of phenotypically defined cell populations has helped to identify several different populations with different self-renewal and reconstituting capacities. Based on the capacity to repopulate hematopoietic system in irradiated recipients one can distinguish between long-term repopulating HSCs (LTR-HSC), short-term repopulating HSCs (STR-HSC) and multipotent progenitors (MPPs) (Fig. 3). These functionally different cell populations differ in their self-renewal capacity as well, with LTR-HSC having the long-lasting and MPPs having the short-term limited self-renewal capacity.

Phenotypically, LTR-HSCs are defined as being LKS (Lin$^{-}$c-kit$^{hi}$Sca-1$^{hi}$) CD34$^{Low/-}$ Flk2$^{Low/-}$; STR-HSCs are defined as LKS CD34$^{Hi}$ Flk2$^{Low/-}$; and MPP as LKS CD34$^{Hi}$ Flk2$^{Hi}$ (Fig. 3). The most immature HSC-derived myeloid cell is termed the common myeloid progenitor (CMP) and is defined by its Lin$^{-}$Sca-1$^{-}$IL-7R$^{-}$c-kit$^{+}$ FcγR$^{Lo}$ CD34$^{+}$ phenotype. CMP progeny includes granulocyte–macrophage (GMP) and megakaryocyte–erythroid (MEP) progenitors.
The most immature HSC-derived lymphoid cell is the early lymphoid progenitor (ELP), defined by its Lin⁻c-kit^hi^Sca-1^hi^Flt-3^+^ phenotype. The early T-lineage progenitor (ETP) is a cell whose principal developmental fate is to generate T cells. Following entry and/or production in the thymus, these Lin⁻CD44^+^c-kit^hi^IL-7Rα^lo/^– cells progress through the stages of development indicated that culminate in the production of single-positive CD4^+^ and CD8^+^ T cells. Lin⁻c-kit^lo^Sca-1^lo^ common lymphoid progenitors (CLPs) are the principal intermediates, through which B cells are produced (Fig. 3) (5, 6).

**Figure 3.** Hematopoietic developmental hierarchy and cell surface markers on hematopoietic cells (modified from references 5 and 6).
CD34 is a heavily glycosylated type I transmembrane protein, and is also a member of the sialomucin family. CD34 is expressed on hematopoietic cells, as well as other cell types, including small-vessel endothelial cells and embryonic fibroblasts. Hematopoietic cells express two forms of the CD34 protein, the full-length and truncated form, which arise from alternative splicing (7). The complete extracellular region is present in both forms of CD34. There is a cysteine-rich repeat (Ig-like domain) in the extracellular region. The full-length form of CD34 protein has an intracellular domain, which contains consensus sites for protein kinase C (PKC) phosphorylation. The truncated form of CD34 lacks most of the intracellular domain, including many of the potential phosphorylation sites.

While the precise function of CD34 remains unknown, involvement in either adhesion/homing or differentiation (or both) has been suggested. Interestingly, the CD34 KO mice exhibit significantly reduced content of hematopoietic progenitors (e.g. CFU-GM, BFU-E, CFU-GEMM), which are also unable to expand in vitro in response to cytokines. Hematopoietic recovery of CD34 KO mice from sub-lethal irradiation is identical to wild type mice, suggesting that lack of CD34 does not affect LTR-HSC function (8, 9, 10). Moreover, transplantation studies have shown that murine LTR-HSC are CD34^Low/- (4).

Recently, CD150 was identified as a new cell surface marker that defines HSC and MPP stages (11). The CD150 receptor (Slamf1) is a member of the SLAM family, and is expressed on activated T and B lymphocytes, dendritic cells and monocytes. Based on the co-expression pattern of CD150 and other HSC/MPP markers LTR-HSC were
defined as LKS CD34\(^-\) Flk2\(^-\) CD150\(^+\): STR-HSCs are LKS CD34\(^+\) Flk2\(^-\) CD150\(^+\), whereas MPPs have LKS CD34\(^+\) Flk2\(^+\) CD150\(^-\) phenotype (12).

Also, based on the efflux of Hoechst 33342 dye, the Hoechst 33342 side population (SP) BM cells were reported to be highly enriched for LTR-HSCs (13). However, Yohei Morita et al. recently pointed out that the SP phenotype does not specify all HSCs (14).

1.2. HSC niches and regulation of dormancy and self-renewal

HSC reside in special microenvironments called “niches” that are involved in their maintenance, self-renewal, and differentiation. Different cell types that constitute the niche can secrete different regulatory factors or contain specific ligands for cell-cell interaction with HSC, which can activate/inhibit specific signaling pathways.

In the adult BM, HSC reside in the trabecular endosteum (osteoblastic or endosteal niche) or sinusoidal perivascular areas (vascular niche). The osteoblastic niche contains osteoblasts, CXCL12-abundant reticular cells (CAR) and stromal fibroblasts, whereas the vascular niche consists of endothelial and CAR cells. The quiescent LTR-HSCs are thought to reside in the endosteal niche, and are unlikely to contribute substantially to the routine hematopoietic homeostasis (12). According to new proposed model, activated HSCs reside in the vascular niche, and are located adjacent to perivascular CAR cells near sinusoids forming the vascular niche. Upon an asymmetric self-renewing division, one activated HSC generates two daughter cells: STR-HSC and an activated HSC. The progeny of STR-HSC and MPPs enters the circulation through the fenestrated endothelium of sinusoidal microvasculature (Fig. 4) (12). The activated HSCs,
residing in the vascular niche seem more likely responsible for the normal hematopoietic turn over.

**Figure 4.** Dormant and activated HSC bone marrow niches. [Anne Wilson et al, 2007, Hematopoietic Stem Cells VI, Ann. N.Y. Acad. Sci. 1106: 64-75].

The vascular BM niche is thought to be involved in rapid and robust response such as stress or injury, and both niches are involved in the process of HSC migration and homing. These opposing biological processes are controlled by overlapping but distinct molecular mechanisms. Migration process is mediated by the release of neutrophil proteases, which lead to the degradation of niche-retention signals and adhesive connections (such as membrane-bound SCF, VCAM1 and CXCL12). On the contrary, homing is mediated by several cell-surface adhesion molecules, including selectins and integrins (15) (Fig. 5).
Migration of HSC between niches in different tissues also plays an important role in establishment of definitive embryonic and adult hematopoiesis. During embryonic blood cell formation, migration of cells from the yolk sac to AGM, and fetal liver to adult bone marrow, rather than de novo generation is thought to be critical (16). The CXCL12 and its receptor CXCR4 play critical role during this process, since the CXCR4 genetic deficient mice fail to transition to hematopoiesis in the marrow space. The recent evidence showed the vascular labyrinth region in the placenta should also be considered as a niche for HSCs during hematopoietic system development (1, 17).

Analogous to neuronal and immunological synapses, the crosstalk between HSCs and niche cells takes place through the 'stem-cell–niche synapse', which integrates intrinsic and extrinsic pathways that regulate biology of HSCs (Fig. 6).
1.3. Increasingly complex network of non-redundant pathways regulates HSC self-renewal and differentiation

Self-renewal is the process by which a stem cell divides to generate one (asymmetric division) or two (symmetric division) daughter stem cells with the same developmental potentials as the mother cells (18, 19).

Self-renewal involves both proliferation and inhibition of differentiation. Several conserved and non-redundant molecular pathways have been implicated in the increasingly complex molecular network that controls HSC self-renewal by inducing proliferation and/or inhibiting differentiation (Fig. 7). Some pathways regulate
proliferation, whereas others regulate developmental potential and/or inhibit
differentiation (20).

The machinery regulating HSCs self-renewal includes both extrinsic and intrinsic
signals. The extrinsic signals from the bone marrow niche can be mediated by cell-cell
interactions, classical cytokines and developmental fate determinants (Fig. 7).

![Figure 7](image-url)

**Figure 7.** Several conserved and non-redundant intrinsic and extrinsic pathways have
been implicated in the control of HSC self-renewal. [Omobolaji O Akala and Michael F
Clarke, Hematopoietic stem cell self-renewal, Current Opinion in Genetics and
Development 2006, 16:496–501].

Among intrinsic regulators, Meis1, a homeobox protein from the TALE family
may be involved in self-renewal expansion (21). Also, over-expression of HoxB4 leads to
HSC expansion (22). These observations suggest that Meis-1 and Hox genes may be
involved in the self-renewal process by regulating proliferation. This function could be
accomplished by forming trimeric nuclear complex of Pbx, Meis and Hox genes (23, 24).

However, the role of HoxB4 in HSC self-renewal is still controversial. HOXB4
over-expression induces unique in vivo and in vitro expansion of HSC without causing
leukemia. The DNA binding–incompetent HOXB4 mutant failed to enhance the
proliferation activity of transduced BM populations in vitro and HSC expansion in vivo.
In contrast, the HOXB4(W→G) mutant conferred a pronounced in vitro proliferation advantage to the transduced BM populations, and dramatically enhanced their in vivo regenerative potential (25). On the other hand, Hoxb4 knock out mice exhibit hypocellularity in hematopoietic organs and impaired proliferative capacity, however, Hoxb4 is not required for generation of HSC or maintenance of steady state hematopoiesis (26).

In adult HSCs, several additional factors contribute to self-renewal as intrinsic regulators (20) (Fig. 7). The polycomb gene Bmi-1 and the polycomb-group Mphl/Rae-28 gene product (a known nuclear partner of Bmi-1), GATA-2 and TEL all have a role in regulating the HSC maintenance.

Bmi1 is a Polycomb group repressor and is essential for the self-renewal of adult murine hematopoietic stem cells (HSCs). Postnatal mice lacking Bmi1 exhibit defects in hematopoiesis, skeletal patterning, neurological functions, and development of the cerebellum (27). The effects of Bmi-1 might be mediated through its repression of the genes encoding p16^{Ink4a} and p19^{Arf}, proteins that respectively inhibit cell proliferation and enhance cell death (28).

HoxA9 is important during embryogenesis and blood cell development. HOXA9-/- deficient mice display major proliferation defects in HSCs, and a variety of myeloid and lymphoid defects. Conversely, enforced HOXA9 expression is leukemogenic in mice, and HOXA9 is frequently activated in human acute myeloid leukemia (AML) (29).

Phosphatase and tensin homologue (PTEN) was recently found to have a role in HSC maintenance. PTEN is a negative regulator of the PI3K-Akt pathway, which has crucial roles in cell proliferation, survival, differentiation and migration (30). Several
studies have demonstrated that Pten-deficient HSCs, when transplanted into irradiated mice, were only capable of short-term multilineage reconstitution of the hematopoietic system and could not stably engraft irradiated recipients long-term. Pten normally maintains HSCs in a quiescent state; in the absence of Pten, HSCs are driven into the cell cycle, eventually leading to depletion of HSC reserves. Thus, increased cell cycle activity may associate with reduction of HSCs self-renewal (31). The impact of Pten loss on HSC self-renewal is independent of the role that Pten inactivation plays in driving leukemogenesis (32).

The forkhead O (FoxO) family of transcription factors participates in several processes, including induction of cell-cycle arrest, stress resistance, differentiation, apoptosis, and metabolism. Recent studies indicate that FoxO-dependent signaling is required for quiescence and survival of LTR-HSC, and that FoxOs are critical mediators of HSC resistance to oxidative stress. FoxO interact with PI3K/AKT and PTEN, and activated AKT inhibits FoxO function by phosphorylation of FoxOs at three conserved residues (33).

The self-renewal of HSC is also dependent on cell division. Several negative regulators of the cell cycle, such as the cyclin-dependent kinase inhibitors p21\(^{\text{Cip1}}\), p27\(^{\text{Kip1}}\), and p16\(^{\text{INK4a}}/\text{p19\text{ARF}}\), have been demonstrated to regulate HSC fate decisions, suggesting that regulation of the G\(_1\)–S phase transition can contribute to HSC self-renewal. HSCs express high levels of cyclin-dependent-kinase inhibitor p21 (also known as CIP1 and WAF1). Loss of p21\(^{\text{Cip1}}\) led to an increase in HSC cycling, and increased sensitivity to stress-induced HSC exhaustion, indicating that p21 is essential for maintenance of quiescence in HSC (12, 34).
Among extrinsic factors the Wnt and Notch pathways were thought to regulate HSC self-renewal (35). In vertebrates, there are two Wnt signaling pathways: canonical and noncanonical Wnt pathway. Canonical Wnt $\beta$-catenin signaling was initially proposed to increase HSC self-renewal (36). However, conditional inactivation of $\beta$-catenin did not affect hematopoiesis or HSC capacity (37). Thus, the precise role of canonical Wnt $\beta$-catenin signaling in HSC function and hematopoiesis is not clear. Wnt ligands can also activate one or more $\beta$-catenin–independent 'noncanonical' pathways. The noncanonical Wnt pathway is in a state of default repression at several levels (38). WNT signals are transduced to the canonical pathway for cell fate determination, and to the noncanonical pathway for control of cell movement and tissue polarity (39).

Notch signaling used to be considered as an important regulator of HSC self-renewal since Notch pathway is considered to be at the interface of osteoblasts and HSCs, where Jagged/Notch signaling might be important in the extracellular regulation of HSC self-renewal (40). Recent data show that inducible Cre-loxP–mediated inactivation of the Jagged1 gene in bone marrow progenitors and/or bone marrow (BM) stromal cells does not impair HSC self-renewal or differentiation (41). Most recently, it was reported that the canonical Notch signaling is dispensable for the maintenance of adult HSC (42).
1.4. The role of Pumilio RNA-binding proteins in regulating stem cell self-renewal and maintenance

**Pumilio RNA-binding proteins act as translational repressors**

Besides transcriptional component, asymmetric cell division is also regulated by translational repression, which is mediated by *cis*-acting signals in the 3’UTR of target mRNAs and *trans*-acting RNA-binding proteins (43-46). Pumilio proteins have emerged as a new fascinating family of translational repressors. A *Drosophila* Pumilio (Pum) gene is a founder member of this growing family of RNA-binding proteins that function as translational regulators during embryo development and cell fate specification (47-53). Pumilio proteins are evolutionarily highly conserved, and are present from yeast to humans and plants (52-55). Among vertebrates, the genomes of zebrafish, *Xenopus*, mouse and humans contain two highly conserved Pum genes (52-55) (Fig. 8).

![Figure 8. Phylogenetetic dendrogram of Pumilio family proteins.](image)


The typical feature of Pum proteins is a C-terminal RNA-binding domain (RBD), which consists of eight imperfect repeats of 36 aa (50, 52-55).
Studies in yeast, *Drosophila* and *C. elegans* have shown that Pum proteins (a) recognize specific nucleotide sequence, known as nanos response element (NRE), in the 3’ UTR of target mRNAs, and (b) establish anterior/posterior protein gradient or differential distribution of target proteins in daughter cells after cell division (52, 53, 78).

The *Drosophila* Pum is required for proper posterior segmentation and abdomen formation in the early embryo. Pum establishes the anterior/posterior gradient of the *hunchback* (*hb*) protein, whose absence at the posterior pole of the embryo allows the formation of eight abdominal segments. Pum binds the NRE within the 3’ UTR of *hb* mRNA, and represses *hb* translation only at the posterior part of the embryo because there it associates with *nanos* (*nos*) protein (56-67). Together with another protein called Brat, *nos* is recruited by Pum/NRE to form a quaternary complex, which promotes deadenylation of *hb* mRNA and inhibits *hb* protein synthesis (57, 61-67) (Fig. 9).

Remarkably, the Pum RBD was shown to be necessary and sufficient for the function of Pum, since alone it is capable of (a) RNA binding, *nos* and Brat recruitment and translational repression, and (b) complementing the posterior segmentation defect in the Pum mutant fruitfly embryos (67).

---

**Figure 9.** A schematic representation of the quaternary complex of Pumilio, *nos*, Brat and *hb* mRNA that leads to translational repression of *hb* in the fruitfly embryo. [Spassov DS, Jurecic R. (2003). IUBMB Life 55: 359-366].
Pumilio proteins are necessary for germline stem cell maintenance in *Drosophila* and *C. elegans*

In *Drosophila* ovary 2 to 3 germline stem cells (GSC) are positioned at the apical tip of a germarium. Through asymmetric division of GSCs the daughter cell proximal to the apical tip remains a stem cell, whereas the distal daughter cell becomes a progenitor that differentiates into mature eggs. The complete inactivation of *Drosophila* Pum leads to embryonic lethality, due to the posterior segmentation defect (56-67). However, attenuated Pum mutations, generated by transposon or chemical mutagenesis allow normal embryogenesis and GSC formation, but abolish their asymmetric division (Fig. 10). These Pum mutants are characterized by non self-renewing symmetric divisions of GSC and production of two progenitors that differentiate into mature eggs. As a consequence of that the GSC pool is quickly depleted and the production of new germ cells is abrogated (67).

![Diagram showing normal and Pumilio mutant division](image)

**Figure 10.** Mutations in the Pumilio gene lead to loss of stem cells due to abolishment of the stem cell asymmetric self-renewal division.

Failure of GSC maintenance was also observed after conditional inactivation of Pum in the germline (47). Although it remains unknown exactly how Pum supports self-
renewal of GSC, it is clear that stem cells require Pum intrinsically (47, 69), and that \( \text{hb} \) is not the target of Pum in GSC (48).

The *C. elegans* Pum protein FBF also controls germline stem cell maintenance (53, 68, 69). Similar to Pum, FBF is preferentially expressed in GSC and down-regulated in progenitors. FBF inhibits translation of fem-3 and promotes the hermaphroditic switch from spermatogenesis to oogenesis (68, 69). Thus, FBF mutants do not switch to oogenesis, and more importantly also fail to maintain GSC. Besides fem-3, FBF also inhibits the translation of gld-1, an RNA-binding protein from the STAR family, whose translational arrest is necessary to inhibit differentiation and promote GSC proliferation (68, 69).

**Vertebrate and mammalian Pumilio proteins**

We have previously reported the full-length coding sequence, protein sequence, gene structure and expression pattern of mouse and human Pum genes. The mouse and human genome contain two highly conserved Pum-related genes, named Pum1 and Pum2 (53-55). Overall, human PUM1 and PUM2 proteins share 75% identity, whereas their highly conserved RNA-binding domain is 91% identical. The mouse Pum1 and Pum2 are almost identical to their human orthologs (98% and 96%, respectively) (54, 55). The C-terminal RBD of human and mouse Pum proteins shows extraordinary evolutionary conservation, sharing 78-79% identity (86-88% similarity) with the RBD of the fruitfly Pum protein (54, 55). In contrast, the protein sequences upstream of the RBD are poorly conserved (53-55) (Figure 11).
Figure 11. Pum proteins are characterized by a highly conserved C-terminal RNA-binding domain (also called PUM-HD domain), which consists of eight tandem repeats and is sufficient and necessary for Pumilio function. Mouse and human genome contain two highly conserved Pumilio orthologs (Pum1 and Pum2).

We have reported previously that mouse Pum1 and Pum2 genes are transcribed in murine fetal liver and adult bone marrow cell populations highly enriched in HSC and multipotent progenitors (55). For example, during adult hematopoiesis Pum1 and Pum2 are both transcribed in the population of Rho-123\textsuperscript{low}Sca-1\textsuperscript{+}c-kit\textsuperscript{+}Lin\textsuperscript{−} bone marrow (BM) cells, highly enriched for HSC (55). However, in more heterogeneous populations of Lin\textsuperscript{−}Sca-1\textsuperscript{+} and Lin\textsuperscript{−}Sca-1\textsuperscript{−} BM cells, Pum1 transcript is not detectable, whereas the expression of Pum2 in these cells is significantly down-regulated (55). Interestingly, the expression pattern of mouse Pum1 and Pum2 during hematopoiesis closely resembles the expression pattern of 	extit{Drosophila} and 	extit{C. elegans} Pum proteins in adult GSC and their progeny (55).

**Recognition of mRNA targets and translational repression by Pum proteins**

The crystal structures of the Drosophila and human PUM1 RNA-binding domain showed that those repeats form an extended concave surface of the molecule (‘rainbow’ structure). Pum proteins bind the RNA via the concave surface of repeats. The most conserved amino acid residues reside in the middle of each repeat and interact with the corresponding RNA bases. Each repeat interacts with a single base therefore the Pum binding site consists of at least eight nucleotides and the binding is anti-parallel, which
means repeat 8 binds to the 5’ nucleotide and repeat 1 binds to the 3’ nucleotide (53) (Fig. 12). There are two boxes of conservative nucleotides on the DrPum target mRNA. However, most Puf proteins bind to only Box B (UGUAUUUA). The consensus binding site for Puf proteins is the core sequence UGUA.

Figure 12. A quaternary complex composed of Pum, Nos, Brat and Hb mRNA based on crystal structure and mutational analysis of Pum RNA-binding domain. Pum binds to the NRE sequence in the 3’UTR of Hb mRNA, so that the target RNA is located on its concave side (blue line). Brat and Nos are not required for binding of Pum to RNA, but are required for the function of Pum as translational repressor.

The mechanism of how Pum proteins regulate the target mRNA is still under investigation. Four possible mechanisms of Pum regulation of mRNA target are: (1) regulation of translation by direct sequestration; (2) regulation of the stability of target mRNA by RNA degradation; (3) regulation of mRNA through mechanisms in (1) and (2), and (4) trafficking RNA out of nucleus in some species (78). So far the budding yeast Puf protein MPT5 has been shown to inhibit translation by increasing the efficiency of deadenylase activity or increasing the degradation of target mRNA (76). Another article reported that human PUM2 binds CEP3 and sequesters CEP3 mRNA translation (79).

The specificity of RNA binding by Pum proteins can be used to rearrange and manipulate their RNA binding specificity. Recently, Cheom-Gil Cheong and Traci M.
Tanaka Hall engineered the structure of *Homo sapiens* Pumilio 1 homology domain (HsPUM1-HD) to bind to different RNA targets by the site-directed mutagenesis of side chains that make specific interactions with RNA bases. They found that mutagenesis of the two side chains that contact the Watson-Crick edge of the base is sufficient to alter sequence specificity. These findings have provided a unique scaffold to rationally design sequence-specific RNA-binding proteins, and have supplied another tool to regulate the translation of a transcript (80).

**The function of invertebrate and vertebrate Pumilio proteins**

Mammalian Pum proteins show biochemical properties typical for the Pum protein family (50, 52, 79, 81). For example, human PUM1 and mouse Pum2 RBD were shown to recognize and bind in vitro the NRE from *Drosophila* hunchback mRNA as well as other NREs (50, 77). This interaction is sequence-specific since point mutations in NRE can drastically impair the binding (50). The crystal structure of PUM1-RBD complexed with the fly NRE has shown that interaction is modular and each repeat interacts with a single base (52). Human PUM2 protein was shown to interact in a yeast two-hybrid system with DAZ (Deleted in azoospermia) protein, which is important for germ cell development (81). Another study has reported that PUM2 protein binds the mRNA for P2P-R, a nuclear protein that interacts with the p53 and Rb1 tumor suppressor proteins, and with potential roles in the control of gene expression and mitosis (82). One of the *Xenopus* Pumilio proteins (XPum) binds the NRE-like element in the 3’UTR of cyclin B1 mRNA in frog oocytes, and interacts with a *Xenopus* homolog of *nanos* Xcat-2.
Furthermore, XPum interacts with CPEB protein, which plays a key role in both translational repression and activation of mRNAs stored in oocytes (74).

Human nanos homolog NANOS1 (NOS1) interacts with human PUM2 protein via highly conserved domains to form a stable complex. In men, the NOS1 and PUM2 proteins are particularly abundant in germline stem cells, suggesting that interaction of PUM2 and NOS1 could have a conserved role in germ cell development (83). Recently, it was also reported that human PUM2 protein binds the 3' UTR region of Cdc42 effector 3 (CEP3) mRNA by mobility shift assay (79).

Overexpression of Pumilio in the nervous system of Drosophila reduces neuronal excitability, and pumilio mutants exhibit altered behavior and defective motor neuron excitability (84). Moreover, Pum is an important mediator of synaptic growth and plasticity at the neuromuscular junction (NMJ). Postsynaptic Pum negatively regulates expression of the translation factor eIF-4E at the NMJ by binding selectively to the 3'UTR of eIF-4E mRNA. This suggests that postsynaptic Pum modulates synaptic function via direct control of eIF-4E expression (85).

Another study found that lack of Pumilio-like protein PUF-8 in C. elegans leads to dedifferentiation of primary spermatocytes into germ cell tumors, suggesting that Pum proteins might function as tumor suppressors in the germline of other organisms (86).

Recently, it was reported that genes present at elevated levels in pum13 Drosophila mutants (27%) encode proteins involved in the antibacterial fungal immune response. Among them, five (AttB, AttC, AttD, CecC, CG13422) of the 20 mRNAs encode antibacterial peptides (76) (Fig. 13).
Taken together, these findings suggest that the function of Pum proteins as translational regulators may be widely conserved throughout evolution (52, 53), and that they could regulate various biological functions (76) (Fig. 13). Pum proteins could be important regulatory factors that act at a defined stage of development when the progeny of a cell has to adopt divergent fates (52, 53). The function of Pum proteins could be diverse in different cell types, stemming from the fact that in different cell types and developmental stages Pum proteins recruit different protein partners and target transcripts of various genes (52, 53).

![Diagram of Pumilio RNA-binding domain mediating various biological functions](image)

**Figure 13.** Pumilio RNA-binding domain (center) mediates various biological functions [Robin P. Wharton and Aneel K. Aggarwal, mRNA Regulation by Puf Domain Proteins, 2006, Sci. STKE, Vol. 2006 (354), 37].
1.5. The role of SCF/c-kit signal pathway in self-renewal and differentiation of HSC and progenitors

Stem cell factor (SCF) is a hematopoietic cytokine that triggers its biologic effects by binding to its 145 KD receptor c-kit. SCF is encoded by the Sl locus on mouse chromosome 10 and human chromosome 12q22-12q24. SCF is normally found in both soluble form and transmembrane form. The soluble and transmembrane forms of SCF are generated by alternative splicing that includes or excludes a proteolytic cleavage site. Both of these two forms are active biological forms. SCF/c-kit pathway mediates endosteal bone marrow HSC niche activity. A mutation in the gene encoding membrane bound SCF that is present in Sl/Sld mice (steel-Dickie mice) causes changes in the HSC niche and leads to the failure of bone-marrow HSC maintenance in vivo. This was probably the first study demonstrating that the microenvironment is essential for bone-marrow HSC function and/or maintenance (15, 88, 89). The c-kit (CD117), a SCF receptor, is a member of the type III receptor tyrosine kinase family. The c-fms receptor, the platelet-derived growth factor (PDGF) receptors and flk2/flt3 receptor also belong to the same tyrosine kinase family. The structure of these receptors includes an extracellular domain with five Ig-like motifs, a transmembrane domain and a cytoplasmic domain with tyrosine kinase activity. The kinase domain is separated into two parts: ATP binding part and phosphotransferase part by a kinase insert region. Binding of SCF (which circulates as a non-covalently associated dimer) triggers c-kit receptor homodimerization and intermolecular tyrosine phosphorylation of the receptor, creating docking sites for a number of SH2-containing signal transduction molecules (Fig.14). The c-kit pathway transfers the extrinsic signal (SCF) to these intrinsic pathways by activation of different
tyrosine residues (Fig. 14). Phosphorylation of murine c-kit at tyrosine 566 and 568 (human Tyr 568 and 570) allows binding and activation of Src family kinases which later on can activate MAPK pathway. Phosphorylation at murine tyrosine 719 (Tyr721 in humans) allows binding and activation of PI3-kinase. Phosphorylation at tyrosine 728 of murine c-kit (human Tyr 730) allows binding of PLCγ and signaling to multiple pathways, whereas phosphorylation at murine tyrosine 701 and 934 (human Tyr703 and Tyr936 accordingly) of c-kit allows binding of Grb2 and activation of MAPK pathway.

The duration and intensity of cytokine-induced signals are regulated by protein tyrosine phosphatases, phosohatidyl inositol lipid phosphatases and by the induction of the SOCS-family of JAK kinase pseudosubstrate inhibitors (90). The tyrosine phosphatase SHP1 may terminate c-kit signaling by dephosphorylating the downstream substrates of the activated c-kit receptor. The SOCS-1 SH2 domain binds to c-kit receptor kinase insert region and suppress downstream of constitutively active form of c-kit receptor. During embryogenesis, SCF and c-kit receptor RNA are expressed along the migratory pathways and in destinations of primordial germ cells and melanocytes, in sites of hematopoiesis (including the yolk sac, fetal liver, and bone marrow), gut and the central nervous system (91). Hematopoietic cells expressing the c-kit receptor protein were detected at E8 in the yolk sac and E10 in fetal liver, progressively increased until E15 and then decreased, paralleling the transition from yolk sac to fetal liver to BM hematopoiesis. The naturally occuring mutations at the Sl locus or at the W locus, which encode for SCF and c-kit receptor, respectively, have been shown to be embryonic lethal or to lead to perinatal death with severe macrocytic anemia. In vitro, SCF synergizes with other cytokines (Epo, Tpo, IL-3, GM-CSF, G-CSF) to increase the direct colony growth
of BFU-E, CFU-GM and CFU-GEMM. In the presence of cytokines, SCF increases both the size and number of colonies.

In summary, c-kit plays an important role in HSC self-renewal and differentiation by different mechanisms. The c-kit pathway links with FOXO3A to inhibit D-cyclins and activate p21 through PI3 Kinase pathway to promote HSCs self-renewal but at the same time could activate MAPK pathway to activate D-cyclins and use part of the PI3K pathway to activate p21. This dual c-kit downstream signaling could be controlled by the amount of SCF or other molecules associated with c-kit (92).

Figure 14. SCF/c-kit signaling pathway in hematopoietic cells [David Kent et al, Regulation of Hematopoietic Stem Cells by the Steel Factor/KIT Signaling Pathway, Clin Cancer Res 2008, Vol.14(7), 1926-30].
1.6. Multipotent hematopoietic cell line EML as a model system to study early hematopoiesis

Several murine hematopoietic progenitor cell lines that can differentiate into various blood cell lineages are being used instead of primary hematopoietic stem cells (HSC) and multipotent progenitors (MPPs) for extensive molecular and biochemical studies of mechanisms that regulate maintenance, lineage commitment and differentiation of primitive hematopoietic cells. One of these cell lines is the SCF-dependent multipotent hematopoietic progenitor cell line EML, which exhibits features of HSCs and MPPs, and has the capacity for multilineage (erythroid, myeloid, lymphoid) differentiation \textit{in vitro} (93). In the presence of SCF EML cells undergo proliferative self-renewal and remain undifferentiated, whereas in response to cytokines and/or stroma EML cells differentiate into lineage-committed progenitors for erythroid, granulocyte-macrophage and megakaryocytic cell lineages (Fig. 15) (93-96). For example, in colony-forming assays EML cells generate granulocyte-macrophage (CFU-GM), burst-forming unit erythroid (BFU-E) and megakaryocytic (CFU-Meg) colonies in the presence of IL-3 or GM-CSF, Epo, and IL-3 and Tpo, respectively (Fig. 16). In co-culture with W20 stromal cell line and IL-7, EML cells give rise to pro-B cells that express RAG-1 and undergo D-J rearrangements (Fig. 15) (93-96). Recently, our lab has shown that EML cells have the capacity to undergo T cell development \textit{in vitro} in co-cultures with OP9 stromal cells expressing Notch ligand Delta-like 1 (97).
Figure 15. The model of the maintenance and multilineage differentiation of a multipotent hematopoietic cell line EML. In the presence of SCF EML cells undergo proliferative self-renewal and remain undifferentiated, whereas in the presence of cytokines and/or stroma EML cells differentiate into erythroid, myeloid and lymphoid lineages.

Figure 16. The frequency of granulocyte-macrophage (CFU-GM), megakaryocytic (CFU-Meg) and erythroid (BFU-E) colony-forming progenitors among EML cells cultured in the presence of IL-3, Tpo and Epo. Microphotographs on the right show CFU-GM, BFU-E and CFU-Meg colonies formed by EML cells in colony-forming assays.

EML cell line shares phenotypic and functional features with murine Lin⁻ c-kit⁺ Sca-1⁺ (LKS) and LKS Flk2⁻ bone marrow (BM) cells, which are enriched for
hematopoietic stem cells (HSC) and multipotent progenitors (MPPs) (96, 98, 99). Similar to HSC and MPPs, almost all EML cells are Sca-1<sup>+</sup>c-kit<sup>+</sup>Flk2<sup>-</sup> (Fig. 17A), and express all functionally relevant hematopoietic genes (e.g. Hoxb4, Meis1, Rae28, SCL, GATA-2, AML1, AML2, Ikaros, PU.1, Notch1, Bmi-1 etc.) (Fig. 17B) (96, 100, 101).

Thus, EML cell line represents a unique and very useful in vitro model for studying molecular mechanisms of maintenance, lineage commitment and differentiation of multipotent and lineage-committed progenitors for most hematopoietic lineages (102-107).

**Figure 17.** (A) Flow cytometry analysis of c-kit, Sca-1, and Flk-2 HSC marker expression on EML cells. (B) RT-PCR expression analysis of several important hematopoietic genes in EML cells from three separate cultures. HPRT was amplified as a positive control.
1.7. Statement of the problem

Based on the evolutionarily conserved protein structure, and the known functions of Pum proteins in invertebrates and lower vertebrates, we and others have proposed that a primordial function of Pum proteins is to sustain proliferation and maintenance of stem cells (52, 53). We hypothesized that mammalian Pum proteins are part of evolutionarily conserved molecular network, necessary for self-renewal and maintenance of germline and diverse types of somatic stem cells (53).

The mammalian Pum proteins were found to be expressed in germline stem cells, murine and human embryonic stem (ES) cells and hematopoietic stem cells (53, 108). The recent study reported that human PUM2 protein in human ES cells binds 3'UTR elements in both Erk2 and p38alpha mRNAs, suggesting that the negative regulation of MAPK by Pum repression may be a conserved mechanism that influences both stem cell maintenance (109). However, no evidence that Pum proteins control mammalian stem cell self-renewal and maintenance is available yet.

Being interested in the mechanisms that regulate HSC self-renewal, we set out to determine the function of Pum proteins during hematopoiesis. In that regard, we have over-expressed murine Pum2 in a multipotent hematopoietic cell line EML. Remarkably, over-expression of Pum2 suppressed multilineage differentiation and induced growth factor-independent proliferation and survival of EML cells. These results suggested that Pum2 could be regulating the balance between maintenance and differentiation of stem cells and early progenitors.
Using a variety of experimental and technical approaches we proposed to analyze in depth the biological and molecular aspects of Pum2 role in the maintenance and differentiation of multipotent hematopoietic cell line EML.
II. MATERIALS AND METHODS

2.1. Cells, cell sorting and flow cytometry

SCF-dependent EML cell line was maintained in IMDM medium (Gibco), supplemented with 20% equine serum (HyClone), 2 mM L-glutamine (Cellgro), 100 U/ml Penicillin, 100 µg/ml Streptomycin (HyClone), and 10% conditioned media (a source of SCF) from BHK/MKL cell line, which constitutively expresses recombinant soluble SCF (95).

BHK/MKL cell line was cultured in DMEM medium (Gibco-Life Technologies), supplemented with 10% Fetal bovine serum (HyClone), 2 mM L-glutamine (Cellgro), and 100 U/ml Penicillin-Streptomycin (HyClone) (95). BHK cells were cultured in T75 flasks, and after 48 hours of culture the condition medium was collected, filtered and frozen in 50 ml conical tubes at -20°C.

EML cells were maintained at a relatively low density (usually 0.5-5x10^5 cells/ml, with peak density less than 5x10^5 cells/ml), and were passaged every second day. Only early passage cells were used for experiments. When necessary the cells were frozen in growth medium supplemented with 10% DMSO (Sigma) at -70°C overnight, and were then transferred to -135°C freezer the following day.

Mouse pro-B cell line BaF3 was grown in RPMI medium supplemented with 10% FCS (HyClone, Logan, UT) and 10% WEHI-3B conditioned medium. Mouse pre-B cell line 70Z3 was grown in RPMI 1640 medium supplemented with 10% FCS and 0.05 mM 2-mercaptoethanol. Mouse monocyte cell lines RAW 309 and J774A.1 were grown in DMEM (Gibco) with 10% FCS.
Bone marrow cells and thymocytes were isolated from 6-8 weeks old C57BL/6J mice. Populations of (a) BM Lin<sup>-</sup> c-kit<sup>+</sup> Sca-1<sup>+</sup> (LKS) Flk2<sup>-</sup> and LKS Flk2<sup>+</sup> cells, Lin<sup>-</sup> c-kit<sup>+</sup> CD34<sup>-</sup> and Lin<sup>-</sup> c-kit<sup>+</sup> CD34<sup>+</sup> cells, Lin<sup>-</sup> c-kit<sup>+</sup> Sca-1<sup>-</sup> and Lin<sup>-</sup> c-kit<sup>+</sup> Sca-1<sup>-</sup> cells, Lin<sup>-</sup> Sca-1<sup>-</sup> and Lin<sup>-</sup> Sca-1<sup>-</sup> cells, and Lin<sup>+</sup> cells, (b) double negative DN1 (CD44<sup>+</sup>CD25<sup>-</sup>) and DN2 (CD44<sup>+</sup>CD25<sup>+</sup>) thymocytes, and CD4<sup>+</sup> and CD8<sup>+</sup> T cells, (c) BM pro/pre-B cells (B220<sup>+</sup>CD43<sup>-</sup> and B220<sup>+</sup>CD43<sup>+</sup> cells), and (d) BM Ter119<sup>+</sup> erythroid cells were isolated by sorting on Aria cell sorter (Becton Dickinson). The cells were analyzed on LSR flow cytometer (Becton Dickinson). Cell cycle analysis was performed on cells stained with propidium iodide (PI).

Cell-specific cDNAs from fetal liver HSC-enriched AA4.1<sup>+</sup> LKS cells, fetal liver progenitors (AA4.1<sup>-</sup> Lin<sup>-</sup>), and adult HSC-enriched Rho-123<sup>lo</sup> Ho<sup>lo</sup> LKS bone marrow cells were a kind gift from Dr. Lemischka (Princeton University).

2.2. Hematopoietic Colony-forming Assays

To assess their myelo-erythroid differentiation, EML cells were cultured in 2 mls of 0.3% low melting temperature agarose (Cambrex, Bio Science) with 2X IMDM, 20% heat inactivated horse serum and 10% SCF-conditioned medium in replicate 6-well plates (10<sup>3</sup> cells/well). The media was supplemented with 0.1 ng/ml of recombinant mouse interleukin 3 (IL-3) (R&D Systems), 8U/ml of recombinant human erythropoietin (Epo) (Ortho Biotech), or 3ng/ml of recombinant mouse thrombopoietin (Tpo) (R&D Systems). The granulocyte/macrophage (CFU-GM), burst-forming unit erythroid (BFU-E) and megakaryocytic (CFU-Meg) colonies were counted 7-10 days after plating (124).
2.3. Cytokines and antibodies

The following recombinant cytokines were used: mouse interleukin 3 (IL-3) (R&D Systems), human erythropoietin (Epo) (Ortho Biotech), mouse thrombopoietin (Tpo) (R&D Systems), mouse GM-CSF and G-CSF (PeproTech). Goat α-c-kit M-14, α-c-kit H300 and rabbit α-c-kit antibodies were purchased from Santa Cruz and Cell Signaling. Rabbit α-c-kit [pY^{730}] and α-c-kit [pY^{936}] phospho-specific Abs were purchased from BioSource. FITC-conjugated α-B220, Mac-1, Gr-1, Ter119, CD4, CD8, CD25, PE-conjugated CD44 and CD43, and α-c-kit-APC, α-Sca-1-PE-Cy7, α-Flk-2-PE, and α-CD34-PE antibodies were purchased from BD Pharmingen or eBioscience. Two different α-Pum2 Abs were developed in collaboration with Dr. McIntush (Bethyl Inc.).

2.4. RNA extraction, reverse transcription and polymerase chain reaction (RT-PCR)

Total RNA from cell lines was isolated with Trizol (Life Technologies) and reverse transcribed using SuperScript II System (Invitrogen). The RNA from multiple batches of sorted cells (purity 98-99.5%) was isolated with RNeasy Micro Kit (Qiagen), and the RNA equivalents of 2 X 10^3 to 5 X 10^4 cells were reverse transcribed and amplified using SuperSCRIPT III System (Invitrogen). PCR reactions were performed in an Eppendorf Mastercyler for 35 cycles (95°C for 30 sec, 57-62°C for 45 sec, 72°C for 30 sec). The sequences of the primers for analyzed genes are provided in the Table 1.
<table>
<thead>
<tr>
<th>Gene</th>
<th>5' oligo</th>
<th>3' oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit</td>
<td>TCCTCACTCACGGGCGGATC</td>
<td>TGGAGGTGGGGTGGAAGAC</td>
</tr>
<tr>
<td>tr-kit</td>
<td>TTAGAGCCCCCATCCTGGTA</td>
<td>AGCAGCAAAAGCTGTTGGAC</td>
</tr>
<tr>
<td>tr-kit (nested primers)</td>
<td>AAATGAATGGCTGGTTGGCTGT</td>
<td>AACCACAGAAGCCAGAA</td>
</tr>
<tr>
<td>Ikaros</td>
<td>CACTACCTCTGGGACGAGCAA</td>
<td>CATAGGGCATGTCAGACAGGCA</td>
</tr>
<tr>
<td>Sca-1</td>
<td>CAATGTAGCAGTCCCTCAATG</td>
<td>CAGGGGCTATAAGGGGAAAA</td>
</tr>
<tr>
<td>GATA-1</td>
<td>GGGCCCCCCCTCAGCGCTCAGC</td>
<td>GACTTCTGAGTGGACCACCAGG</td>
</tr>
<tr>
<td>GATA-2</td>
<td>GGGCTCAAGTACCAAGTGTGAC</td>
<td>GAGAACTGACGACACCTCT</td>
</tr>
<tr>
<td>GATA-3</td>
<td>GGGCTCCTCAAGGTGTCAGCAC</td>
<td>TCGGGCACATAGGGGAGGATAG</td>
</tr>
<tr>
<td>PU.1</td>
<td>CCGATGACTTTGGTTACTTCG</td>
<td>TGGACGAGAAGTGGAAGGTA</td>
</tr>
<tr>
<td>Rae-28</td>
<td>GTGCTACATGGTGACAGCTT</td>
<td>AGCTAGGAAAGCTGACCTCT</td>
</tr>
<tr>
<td>Meis1</td>
<td>ATGGTTCCCTCGGTCAATGAC</td>
<td>ATGGTCTCTATTTCAAGAAGGGC</td>
</tr>
<tr>
<td>HoxA9</td>
<td>CCCACGCTTTGACACTCACAATTTTG</td>
<td>GAGTGAGCGAGCATGTAG</td>
</tr>
<tr>
<td>HoxB4</td>
<td>TCCAGTGCAGCCAAACAGGCGGG</td>
<td>GTGCCACCGAGGCTGACCTT</td>
</tr>
<tr>
<td>AML1</td>
<td>CCAGCAAGCTGAGGAGGCGGGG</td>
<td>CGGATTTGAAAAGGAGGTGA</td>
</tr>
<tr>
<td>AML2</td>
<td>CACTCAGTCATTTCCCTCAA</td>
<td>TTGGTCTTTCCTTCCTGTC</td>
</tr>
<tr>
<td>SCL</td>
<td>TATGAGATGGAGATTTCTGATG</td>
<td>GCTCTCTGAGTAACTGTCC</td>
</tr>
<tr>
<td>Notch1</td>
<td>CAATCCAGGGACACCTGAGGCCACAT</td>
<td>TAGAGGCGCTTGATGGGTTGGTCTGCG</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>AAGGAAGAGGTGAAATGATAAAGG</td>
<td>ACTACAAAGGAAAGCAACGTGGACC</td>
</tr>
<tr>
<td>CD34</td>
<td>TCGACAGTTGGAGCCCTACAG</td>
<td>ATACCACCCAGTTGCCCCACC</td>
</tr>
<tr>
<td>CD150</td>
<td>GGGGAGAGTTGAAAGAGTGTA</td>
<td>TGGCTGGCAGTATTTGTATT</td>
</tr>
<tr>
<td>Lactoferrin</td>
<td>AAGCCAAGGCTTGTCTCTCATAG</td>
<td>TCTCATCTCGTTTCTGCCACC</td>
</tr>
<tr>
<td>β-globin</td>
<td>CTGACAGATGCTCTTCTGGGG</td>
<td>CACAAACCCAGAAACAGACA</td>
</tr>
<tr>
<td>HPRT</td>
<td>GTTGAGAGAGTATCTCCACC</td>
<td>AGCGATGATGAACCCAGGTA</td>
</tr>
</tbody>
</table>

Table 1. The sequence of the primers used for gene expression analysis.
Quantitative real-time PCR reactions for tr-kit and HPRT were performed with LightCycler (Roche) under optimized conditions using SybrGreen, and analyzed with LightCycler software 3.5. A standard curve method was used for quantification of tr-kit and hypoxanthine phosphoribosyl transferase (HPRT) expression according to LightCycler instructions. A water control and melting curve analysis were also performed to confirm the specificity of PCR. The RNA content of samples was normalized based on the amplification of HPRT as a control gene. Statistical analyses were performed using the Student $t$ test. $P < 0.05$ was considered significant.

2.5. Western blotting

The cells were lysed in 100 μl of buffer (150 mM NaCl, 1% NP-40, 0.25 % DOC, 0.1% SDS and 50 mM Tris, pH 7.4, 5mM EDTA, 1mM PMSF), supplemented with protease inhibitor (Roche). Up to 50 μg of protein was run on the 12% SDS- PAGE gels (Gradipore), and transferred to PVDF membranes (Bio-Rad). The membranes were incubated at 4°C overnight with primary antibodies at recommended dilutions, washed and incubated with goat α-rabbit Ig-HRP or rabbit α-goat Ig-HRP secondary antibodies (Zymed) for 1 hour at room temperature (1:5,000 to 1:10,000 dilutions). The proteins were detected with ECL detection system (Amersham) or SuperSignal West system for detection of HRP (Pierce). Where indicated, the membranes were stripped and re-probed with α-actin antibody (Sigma).
2.6. Expression vectors

To over-express tr-kit in EML cells, we have inserted tr-kit cDNA into a murine stem cell virus (MSCV)-based, bicistronic retroviral vector containing GFP as a marker (Fig. 18). The MSCV-based vectors are optimized for introducing target genes into the pluripotent cells, including murine or human HSC and progenitors (113-118).

**Figure 18.** Schematic representation of murine stem cell virus (MSCV)-based, bicistronic retroviral vector for over-expression of tr-kit.

Production of replication-incompetent viral particles and determination of viral titer were performed according to standard protocols (118).

2.7. shRNA-mediated knockdown of Pum2

Sigma MISSION® shRNA Lentiviral vectors for long-term gene silencing were used to study the impact of shRNA-mediated Pum2 attenuation on the maintenance and differentiation of multipotent cell line EML. The MISSION™ mouse Pum2 shRNA Target Set consists of premade lentiviral transduction particles carrying five different (#60-64) shRNAs, which will target unique sequences in the CDS and 3’ UTR regions of murine Pum2 transcript. Multiple batches of EML cells were transduced with individual MISSION™ Pum2 shRNA lentiviral vectors, and with MISSION Non-Target shRNA Control lentiviral vector, carrying a non-targeting shRNA that will activate the RNAi pathway, but does not target any human or mouse genes. Transduced cells were selected
with puromycin for 2 weeks. The levels of Pum2 protein in selected transduced EML cell clones were analyzed by Western. The expression of HSC/MPP markers on wt EML, control EML and selected EML cell clones showing Pum2 protein knockdown was analyzed by flow cytometry. The impact of Pum2 knockdown on multilineage differentiation of EML cells was determined using colony-forming assays.

2.8. Microarray expression analysis

To continue elucidating molecular differences between wt EML and Pum2-EML cells, and their CD34⁺ and CD34⁻ subpopulations, microarray expression analysis was performed using multiple Agilent 44K microarrays. Multiple RNA samples were prepared from (a) total wt EML and Pum2-EML cells, and (b) independently sorted CD34⁺ and CD34⁻ subpopulations of wt EML and Pum2-EML cells. Additional purification of mRNA, sample labeling and hybridization to microarrays was performed by Ocean Ridge Biosciences. Microarray data clustering and analysis, and pathway analysis were performed using standard software.
III. RESULTS

3.1. The role of Pum2 RNA-binding protein in the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML

3.1.1. Pum2 over-expression leads to SCF-independent maintenance of EML cells and attenuates their differentiation

Several EML cell clones stably transfected with control pcDNA4 vector and Pum2/pcDNA4 vector (referred to as Pum2-EML cells), were established and analyzed independently. The survival and proliferation of EML cells absolutely depend on the presence of SCF (93), and without SCF >95% of wt EML and EML vector control cells die within 24 hours (data not shown). In contrast, Pum2-EML cells survive and proliferate in the absence of SCF, and have been continuously maintained without SCF and under selection with zeocin (data not shown). Pum2-EML cells are not transformed, and have not gained the capacity to produce SCF or any other soluble factor themselves, since the Pum2-EML cell-conditioned media does not support survival of wt EML cells.

Erythropoietin (Epo), interleukin 3 (IL-3), granulocyte-macrophage and granulocyte colony-stimulating factors (GM-CSF and G-CSF) induce differentiation of EML cells into myelo-erythroid progenitors, which give rise to erythrocytes, granulocytes and macrophages (93, 105). Myelo-erythroid differentiation of wt EML, vector control EML and Pum2-EML cells was examined using colony-forming (CFC) assays (120, 124, 125). Remarkably, in comparison to wt and vector control EML cells, two independently established Pum2-EML cell clones have produced significantly reduced number of erythroid (BFU-E), megakaryocyte (CFU-Meg) and granulocyte-macrophage (CFU-GM) colonies when plated at the same cell density, or with different concentrations of single
cytokines (Fig. 19A). Interestingly, even the treatment with multiple cytokines (IL-3, Epo, GM-CSF and Tpo) did not augment attenuated differentiation of Pum2-EML cells (Fig. 19B).

**Figure 19.** Colony-forming capacity of wt EML, vector control EML and Pum2-EML cells. **A.** Equal numbers of cells (10^3/well in replicate 6-well plates) were plated in semisolid agarose supplemented with 8 U/ml rEpo (for BFU-E), 3 ng/ml rTpo (for CFU-Meg), and 0.1 ng/ml rIL-3 (for CFU-GM). **B.** Pum2-EML cells were cultured for 48 hours with a cocktail of cytokines [Epo (8 U/ml), GM-CSF (2ng/ml), Tpo (3 ng/ml), and IL-3 (0.1 ng/ml)]. The colony number was determined seven days after plating. The data are representative of three separate experiments with a total of 15 wells per colony type, and are presented as mean ± s.e.m.

In summary, the over-expression of Pum2 leads to uncoupling of the survival / proliferation and differentiation signals in EML cells, and to their SCF-independent growth and significantly attenuated multilineage differentiation.
3.1.2. Pum2 over-expression changes the cell surface phenotype of EML cells

To study further the differences between wt EML and Pum2-EMl cells we examined the expression of Sca-1, c-kit, Flk-2 and CD34 HSC and MPP markers by flow cytometry. This approach yielded several interesting and important observations. First, we reproducibly found that almost all wt EML, vector control and Pum2-EMl cells are Sca-1+ c-kit+ Flk2- (Fig. 20A). However, the important observation was that both the wt EML and Pum2-EMl cells have reproducibly displayed heterogeneous and markedly different expression of CD34 marker (Fig. 20A). Among the wt EML and vector control EML cells 70-85% of cells are CD34+, and 15-30% of cells are CD34- (Fig. 20A). Moreover, among the CD34+ wt EML cells there is a further heterogeneity based on the level of CD34 expression (Fig. 20A).

Remarkably, the majority of cells in Pum2-EMl cell clones are CD34- (70-90%), with 10-30% of cells being CD34+, which express low levels of CD34 marker (Fig. 20A). Thus, the wt and vector control EML cells are a mixture of cells with Sca-1+ c-kit+ Flk2- CD34+ and Sca-1+ c-kit+ Flk2- CD34- phenotypes, whereas the majority of Pum2-EMl cells have Sca-1+ c-kit+ Flk2- CD34- phenotype.

In view of the heterogeneous CD34 expression, EML cells resemble Lin- c-kit+ Sca-1- Flk2- BM cells, which are highly enriched for HSC, and are also heterogeneous for the expression of CD34 (Fig. 20B). Transplantation studies have shown that long-term repopulating (LTR) HSC reside in the population of Lin- Sca-1+ c-kit+ Flk-2- CD34- BM cells, whereas the Lin- Sca-1+ c-kit+ Flk-2+ CD34+ BM cells lack LTR-HSC and contain short-term repopulating (STR) HSC and multipotent progenitors (MPPs) (126-130).
Figure 20. A. Flow cytometry analysis of Sca-1, c-kit, Flk-2 and CD34 marker expression on wt EML, EML/pcDNA cells, and Pum2-EML cells. B. Lin− c-kit+ Sca-1+ Flk2− BM cells, which are highly enriched for HSC, are also heterogeneous for the expression of CD34 marker.

Thus, based on the heterogeneous CD34 expression EML cells resemble Lin− c-kit+ Sca-1+ Flk2− CD34+ BM cells, highly enriched for LTR-HSC, and Lin− c-kit+ Sca-1+ Flk2− CD34− BM cells, highly enriched for STR-HSC and MPPs.

3.1.3. Multilineage differentiation of wt EML and Pum2-EML cells correlates with the level of CD34 expression

To determine the functional significance of this change in CD34 expression, wt EML and Pum2-EML cells were sorted into CD34+ and CD34− populations (>99% purity) (Fig. 21A), and their myelo-erythroid differentiation capacity assessed using colony-forming (CFC) assays (Fig. 21B).

We have repeatedly observed that CD34+ population of wt and vector control EML cells contains the majority of progenitors capable of forming BFU-E, CFU-Meg and
CFU-GM hematopoietic colonies. In contrast, the CD34+ population of wt and vector control EML cells differentiated poorly or not at all, and contained very small percentage of progenitors capable of differentiating into myelo-erythroid lineages (Fig. 21B, data for EML/pcDNA cells not shown). In addition, the colonies generated by CD34+ EML cells were quite small (data not shown).

**Figure 21.** A. Sorting of wt EML and Pum2-EML cells into CD34+ and CD34- populations. B. Differentiation capacity of CD34+ and CD34- populations of wt EML and Pum2-EML cells.

The CD34+ and CD34- populations of Pum2-EML cells displayed similar pattern of differentiation. The Pum2-EML CD34+ cell population repeatedly contained the majority
of cells capable of multilineage differentiation in response to cytokines, whereas the CD34^- subpopulation did not differentiate at all (Fig. 21B). However, the major difference observed was that the CD34^+ population of Pum2-EML cells differed from and differentiated poorly in comparison to CD34^+ population of wt EML cells.

These results support the notion that CD34^+ EML cell population contains the majority of progenitors which can readily undergo differentiation. On the other hand, CD34^- cell population differentiates poorly or not at all in response to cytokines.

Another interesting observation made through analysis of sorted CD34^+ and CD34^- cell populations is their different morphology. The total EML cells consist of both round and irregular-shaped cells with short pseudopodia. Interestingly, the majority of CD34^+ EML cells were irregularly shaped with short pseudopodia, whereas the majority of CD34^- cells are round cells without podia (Fig. 22). The majority of Pum2-EML cells were round cells without podia (data not shown).

**Figure 22.** Morphology of purified wt EML CD34^+ and CD34^- cells. Total EML cells consist of round and irregular-shaped cells with short pseudopodia. The majority of CD34^+ EML cells were irregularly shaped with short pseudopodia, whereas the majority of CD34^- cells are round cells without podia.

Based on the level of CD34 expression, CD34^+ wt EML cells can be divided into CD34^{low}, CD34^{medium} and CD34^{high} populations (Fig. 23A), representing on average
15%, 34% and 27% of the CD34+ EML cells. Thus, we sought to determine whether there is a correlation between the progenitor cell content and the level of CD34 expression on EML cells. The CD34−, CD34low, CD34med and CD34high wt EML cell populations were purified by FACS sorting (Fig. 23A), and their multilineage differentiation analyzed using CFC assays.

**Figure 23.** A. Sorting of CD34+ wt EML cells into CD34low, CD34med and CD34high populations. B. Analysis of multilineage differentiation of sorted CD34+, CD34low, CD34med and CD34high wt EML cell populations. The frequency of myelo-erythroid progenitors that form BFU-E, CFU-Meg and CFU-GM colonies correlates with the level of CD34 expression, with CD34high cells producing the highest colony number and the CD34low cells producing the lowest number of colonies.
Multiple CFC experiments have reproducibly shown that the frequency of myelo-erythroid progenitors that respond to cytokines by forming BFU-E, CFU-Meg and CFU-GM colonies correlates with the level of CD34 expression (Fig. 23B). In fact, among CD34+ EML cells the frequency of these progenitors declined with a decrease in the level of CD34 expression on EML cells, with CD34 high EML cells producing the highest colony number and the CD34 low EML cells producing the lowest number of colonies (Fig. 23B).

Similarly, since Pum2-EML cells consist of CD34- population and CD34+ cells that correspond to CD34 low wt EML cells, the low progenitor activity was accordingly detected only in the CD34+/low population of Pum2-EML cells (Fig. 21A and B, and data not shown).

Cumulatively, these data indicate that the overall multilineage differentiation and the colony-forming activity of wt EML and Pum2-EML cells strongly correlate with the level of CD34 expression, and that the CD34- subpopulation of EML cells is in a state where these cells do not respond well to cytokines by differentiation.

Remarkably, our observations about the correlation between the level of CD34 expression and differentiation of EML cells are very similar to previous study which examined the progenitor cell content in Lin- c-kit+ CD34high, Lin- c-kit+ CD34low, and Lin- c-kit+ CD34- BM cells, isolated from one to 16 week-old mice (131). This study reported that only Lin- c-kit+ CD34high cells differentiate efficiently in colony forming assays, whereas Lin- c-kit+ CD34low and Lin- c-kit+ CD34- BM cells differentiate poorly or not at all (131).
The finding that Pum2-EML cells consist of CD34− cells, and that CD34+ cells which based on the level of CD34 expression are equal to CD34low wt EML cells (Fig. 23B), explains at least in part why Pum2-EML cells display attenuated differentiation. Cumulatively, these results indicate that over-expression of Pum2 in EML cells leads to increased content of CD34− cells, which differentiate poorly or not at all in colony forming assays.

3.1.4. Developmental relationship between CD34− and CD34+ wt EML and Pum2-EML cells

The majority of LTR-HSC resides in the population of Lin− c-kit+ Sca-1+ Flk2− CD34− BM cells, whereas Lin− c-kit+ Sca-1+ Flk2− CD34+ BM cells contain STR-HSC and MPPs (126-130). The findings described in previous chapter suggest that similar to primary BM-derived cells, Sca-1+ c-kit+ Flk-2− CD34− population of wt EML and Pum2-EML cells could be more primitive than Sca-1+ c-kit+ Flk-2− CD34+ population. Thus, in that case CD34− EML cells could divide asymmetrically to maintain the CD34− cell pool and at the same time give rise to CD34+ EML cells (Fig. 24). If that scenario is correct then one could hypothesize that among Pum2-EML cells, the population of CD34− cells undergoes preferentially symmetric rather than asymmetric division, thus giving rise to increased CD34− cell content and diminishing frequency of CD34+ cells. In other words, normal endogenous levels of Pum2 in EML cells regulate the balance between the maintenance and generation of CD34+ and CD34− cell populations. In contrast, gain of Pum2 function could be skewing this balance towards increased maintenance of CD34− cell population through symmetric cell division, while suppressing generation of CD34+ cells (Fig. 24).
Figure 24. CD34⁺ EML cells could give rise to more differentiated CD34⁺ cells through asymmetric cell division. In contrast, gain of Pum2 function could lead to increased maintenance of CD34⁻ cell population through symmetric cell division, while suppressing generation of CD34⁺ cells.

To determine developmental relationship between CD34⁻ and CD34⁺ wt EML cells, equal numbers (10⁵) of purified CD34⁻ and CD34⁺ wt EML cells (99.9% pure) were cultured separately in EML cell medium for 3 days. Both populations of wt EML cells grew and proliferated equally well. An aliquot of cells from these cultures was analyzed each day by flow cytometry for CD34 expression (Fig. 25A).

Four separate experiments with 99.9% pure CD34⁻ cells have shown that CD34⁻ wt EML cells can maintain the CD34⁻ population and reproducibly generate CD34⁺ EML cells (Fig. 25B). The high purity of starting CD34⁻ cells and the kinetics of CD34⁺ cell appearance and expansion in CD34⁻ EML cell cultures make it unlikely that this observation is due to the presence of 0.1% of contaminating CD34⁺ cells.

Remarkably, four separate experiments with 99.9% pure CD34⁺ wt EML cells have revealed that CD34⁺ cells reproducibly give rise to CD34⁻ cells also (Fig. 25C). The high purity of starting CD34⁺ EML cells, and the kinetics of CD34⁻ EML cell appearance and expansion in CD34⁺ EML cell cultures make it unlikely that this observation is due to the presence of 0.1% of contaminating CD34⁻ EML cells.
There is a subpopulation of CD34+ cells with the capacity to give rise to CD34- cells as well.

In an identical set of experiments equal numbers of purified CD34- and CD34+ Pum2-EML cells were cultured for 3 days, and aliquots of cells from these cultures analyzed by flow cytometry for CD34 expression (Fig. 26). Notably, these experiments have revealed that CD34- Pum2-EML cells maintain CD34- population and generate a subpopulation of CD34+ cells with the capacity to give rise to CD34- cells as well.

**Figure 25.** Flow cytometry analysis of CD34 expression on cultured FACS purified CD34- and CD34+ wt EML cells.

Taken together, these results suggest that although the CD34- population of wt EML cells could be more primitive than CD34+ cells and gives rise to CD34+ population, there is a subpopulation of CD34+ cells with the capacity to give rise to CD34- cells as well.

In an identical set of experiments equal numbers of purified CD34- and CD34+ Pum2-EML cells were cultured for 3 days, and aliquots of cells from these cultures analyzed by flow cytometry for CD34 expression (Fig. 26). Notably, these experiments have revealed that CD34- Pum2-EML cells maintain CD34- population and generate...
small population of CD34^{+/low} cells, which in turn can generate CD34^- cells (Fig. 26).

These results suggest that in contrast to wt EML cells, Pum2-EML cells are maintained predominantly as CD34^- cells that generate a small population of CD34^{+/low} cells.

Figure 26. Flow cytometry analysis of CD34 expression on cultured FACS purified CD34^- and CD34^- Pum2-EML cells.

To further confirm the findings from above, EML cells were sorted into CD34^- and CD34^- cells, and cultured separately for seven days. After seven days the cells from CD34^- and CD34^- EML cell cultures were analyzed for CD34 expression and sorted into “secondary” CD34^- and CD34^- cell populations (Fig. 27).

Figure 27. Analysis of differentiation capacity of “secondary” CD34^- and CD34^- cells derived from culture of purified CD34^- and CD34^- EML cells (arrow).

The myelo-erythroid differentiation capacity of these “secondary” CD34^- and CD34^- EML cells was analyzed by CFC assays (Fig. 27). Remarkably, similar to previous results,
the secondary CD34\(^+\) cells, derived either from the cultures of primary CD34\(^-\) or CD34\(^+\) EML cells, contained the majority of BFU-E, CFU-Meg and CFU-GM progenitors. On the other hand, the secondary CD34\(^-\) cells, derived either from the cultures of primary CD34\(^-\) or CD34\(^+\) EML cells, produced less BFU-E, CFU-Meg and CFU-GM colonies (data not shown).

Next we sought to analyze whether the secondary CD34\(^-\) and CD34\(^+\) EML cells can generate each other in culture. Sorted secondary CD34\(^-\) and CD34\(^+\) EML cells were cultured for 6-10 days and an aliquot of cells from these cultures was analyzed every two days by flow cytometry for CD34 expression (Fig. 28).

![Diagram](image)

**Figure 28.** EML cells were sorted into CD34\(^-\) and CD34\(^+\) cells, cultured separately for 7 days, and then sorted again into CD34\(^-\) and CD34\(^+\) cell populations. These “secondary” CD34\(^-\) and CD34\(^+\) EML cells were then cultured for 6-10 days and an aliquot of cells from these cultures analyzed every two days by flow cytometry for CD34 expression.

These experiments have shown that secondary CD34\(^-\) and CD34\(^+\) cells can generate the other population, although CD34\(^-\) cells gave rise to CD34\(^+\) cells more rapidly (data not shown). These data have confirmed that while the CD34\(^-\) EML cells do give rise to CD34\(^+\) subpopulation, at least a portion of CD34\(^+\) cells can give rise to CD34\(^-\) EML cells as well.
3.1.5. The level of CD34 expression on CD34⁺ EML cells correlates with their capacity to give rise to CD34⁻ cells

Obviously, the observation that CD34⁻ and CD34⁺ wt EML and Pum2-EML cells can generate each other cannot be fully explained by the model of linear differentiation of CD34⁻ cells into CD34⁺ population (Fig. 29).

Figure 29. The model of linear differentiation of CD34⁺ EML cells into CD34⁺ cells.

Since the population of CD34⁺ EML cells contains the cells capable of giving rise to CD34⁻ cells, we investigated the possible correlation between the level of CD34 expression, and the capacity of CD34⁺ EML cells to generate CD34⁻ cells (Fig. 30A).

FACS purified CD34⁻, CD34 med and CD34 high wt EML cell populations (Fig. 30B) were cultured for 3-6 days, and the cells from each culture analyzed for CD34 expression to determine which of the three CD34⁺ EML cell subpopulations can most efficiently give rise to CD34⁻ cells.

Remarkably, four separate experiments have revealed that CD34 low EML cells have the highest capacity to give rise to CD34⁻ EML cells (Fig. 30C), whereas CD34 high EML cells had the least capacity to give rise to CD34⁻ EML cells (Fig. 30C).
Figure 30. A. Is there a correlation between the level of CD34 expression and the capacity of CD34+ EML cells to generate CD34− cells? B. FACS sorting of CD34low, CD34med and CD34high wt EML cell populations. C. Analysis of CD34 expression on cultured CD34low, CD34med and CD34high wt EML cell populations from two separate experiments.

Several studies have shown that mouse CD34+ LTR-HSC became CD34+ after mobilization with G-CSF or 5-FU treatment, and that after transplantation these “activated” CD34+ LTR-HSC reverted back to CD34− “inactive” state (132-134) (Fig.31).

Figure 31. Experiments in the murine system have shown that mouse CD34+ LTR-HSC became CD34+ after mobilization with G-CSF or 5-FU treatment, and that after transplantation this “activated” CD34+ LTR-HSC reverted back to CD34− “inactive” state.

These studies were the first to report a link between CD34 expression and activation of adult murine HSC, and have shown (a) that mouse LTR-HSC can alternate between
CD34− “inactive” and CD34+ “activated” state, and (b) that the heterogeneous population of LKS CD34+ BM cells contains activated LTR-HSC, STR-HSC and MPPs (Fig. 32A). The existence of human CD34− HSC, and the generation of CD34+ cells from CD34− cells were reported also (134-139).

**Figure 32.** A. The model of LTR-HSC activation. B. The level of CD34 expression on CD34+ EML cells inversely correlates with their capacity to give rise to CD34− cells. C. New proposed model that merges linear differentiation of CD34+ EML cells into CD34− EML cells, and the reversible CD34 expression and activation.

In view of these reports and our findings we have proposed a new model that merges linear differentiation of CD34− EML cells into CD34+ cells, and the reversible CD34 expression and activation (Fig. 32B and C). In this model the CD34− EML cells are more primitive cells in an “inactive” state (differentiation inhibited) that give rise to CD34+ EML cells. On the other hand, the CD34+ population of EML cells is heterogeneous and consists of CD34med/high cells that can readily differentiate into multiple lineages, and CD34low cells that are in “activated” state and can revert back to the CD34− state. In that
model, CD34− inactive EML cells would undergo asymmetric cell division and give rise to CD34− and CD34+/low daughter cells. The portion of CD34+/low daughter cells would give rise to CD34+/med-high cells that can differentiate into multiple lineages in response to different cytokines. The remaining CD34+/low daughter cells, arising from asymmetric division of CD34− cells, could represent activated cells which through asymmetric division could “revert” back to CD34− inactive state (Fig. 32B).

Cumulatively, these observations support the notion that the varying levels of CD34 expression correlate with the maintenance and differentiation of HSC/MPP-like cell line EML, and that EML cells can alternate between CD34− inactive and CD34+/low active state (Fig. 33). Thus, EML cell line could represent a powerful in vitro model to study the linear differentiation of CD34− cells into CD34+ cells, and the mechanisms of reversible CD34 expression and activation of primitive hematopoietic cells (133, 141, 142).

**Figure 33.** The maintenance of EML cells correlates with decreasing CD34 levels, whereas differentiation of EML cells correlates with increasing levels of CD34 expression.

Based on the proposed model, the over-expression of Pum2 leads to increased maintenance of cells in inactive CD34− state, and blocks development of CD34+ cells past the CD34low stage (Fig. 34). This model also supports the hypothesis that Pum2 protein could be regulating stem cell self-renewal through inhibition of differentiation and induction of proliferation, and could be involved in maintaining the balance between inactive and active state of multipotent hematopoietic cells.
Figure 34. The over-expression of Pum2 leads to increased maintenance of EML cells in inactive CD34⁻ state, and blocks development of CD34⁺ cells past the CD34⁺low stage.

3.1.6. Cell cycle status of CD34⁻ and CD34⁺ wt EML and Pum2-EML cells

Cell cycle analysis with PI staining revealed that similar percentages of wt EML and Pum2-EML CD34⁺ cells are in G0/G1, S and G2/M cell cycle phase. In contrast, the majority of wt EML and Pum2-EML CD34⁻ cells are in G0/G1 or S phase (Fig. 35A), with Pum2-EML CD34⁻ cells being predominantly in G0/G1 phase. These data suggest that in terms of the cell cycle status, CD34⁻ EML cell subpopulations resemble BM-derived HSC-enriched BM cells (Fig. 35B).

Figure 35. A. Cell cycle status of CD34⁻ and CD34⁺ wt EML and Pum2-EML cells. B. The majority of BM-derived HSC are in G0/G1 or S phase of the cell cycle (143).
3.1.7. Molecular differences between wt EML and Pum2-EML cells and their CD34− and CD34+ populations

To determine if there were molecular differences between wt EML and Pum2-EML cells, and their CD34+ and CD34− subpopulations, various types of expression analyses were performed. For example, RT-PCR and flow cytometry analysis with α-Notch1 A6 Ab (NeoMarkers) has shown that Pum2 over-expression did not affect transcription of Notch1, or change the expression of Notch1 protein on Pum2-EML cells as compared to wt EML and vector control EML cells (Fig. 36).

![Figure 36. RT-PCR analysis of Notch1 transcript and flow cytometry analysis of Notch1 protein expression in wt EML, vector control and Pum2-EML cells.](image)

Also, RT-PCR analysis has shown that CD34− and CD34+ wt EML cells express similar levels of transcripts for various developmentally and functionally relevant hematopoietic genes (e.g. Rae28, GATA-2, c-kit etc.) (Fig. 37, and data not shown).

![Figure 37. RT-PCR analysis of expression of several developmentally and functionally relevant hematopoietic genes in CD34− and CD34+ wt EML cells.](image)
To continue investigating molecular differences between wt EML and Pum2-EML cells, and their CD34⁺ and CD34⁻ subpopulations, we performed microarray expression analysis using multiple Agilent 44K microarrays and multiple RNA samples prepared from independently sorted cells (Fig. 38). Out of 14,868 expressed genes, 760 exhibited significant differences in expression level between wt EML and Pum2-EML cells, and included Mmp14, Mpp1, Ripk3, Calmodulin 5, DDEF2 (Development and differentiation enhancing factor 2), C/EBPbeta, TXK tyrosine kinase, Egr3, Ermap, Eraf, TNFR1a, Jam4 and others (Fig. 38).

Among 760 differentially expressed genes, 146 genes exhibited >10-fold higher expression in wt EML cells, and 85 exhibited >10-fold higher expression in Pum2-EML cells (Fig. 38). The results of these studies will shed more light on molecular differences between these cell populations, and will provide additional directions for undertaking further research.

**Figure 38.** Representative data from Agilent 44K cDNA microarray expression analysis of wt EML and Pum2-EML cells, and their CD34⁺ and CD34⁻ subpopulations.
**Figure 38 (continued).** Representative data from Agilent 44K cDNA microarray expression analysis of wt EML and Pum2-EML cells, and their CD34\(^+\) and CD34\(^-\) subpopulations.
Fackler et al. (144, 145) demonstrated that phosphorylation of preformed CD34 protein determines whether it is expressed on the cell surface or retained inside the cell. These studies showed the importance of not relying on FACS data for cell surface CD34 expression alone to determine whether cells are truly CD34\(^-\) versus CD34\(^+\) ones. The analysis of CD34 mRNA or intracellular protein should also be done to determine whether CD34 is present, but not yet localized to the cell surface (144, 145).

Interestingly, Agilent microarray analysis has revealed the presence of CD34 transcript in CD34\(^-\) and CD34\(^+\) wt and Pum2-EML cells (Fig. 38). RT-PCR analysis has confirmed that both CD34\(^-\) and CD34\(^+\) wt EML cells express transcripts for the full-length and truncated CD34, although CD34\(^-\) EML cells expressed fewer transcripts for both forms (Fig. 39A). Similarly, both CD34\(^-\) and CD34\(^+\) Pum2-EML cells transcribe CD34, although both cell populations expressed more transcripts for the full-length than truncated CD34 (Fig. 39B). Notably, CD34 transcript was detected also in LKS CD34\(^-\) BM cells (146).

**Figure 39.** RT-PCR analysis of CD34, Bmi-1, and CD150 expression in wt EML (A) and Pum2-EML cells (B), and their CD34\(^+\) and CD34\(^-\) subpopulations.
Since the Agilent microarray and RT-PCR analysis revealed that CD34⁻ wt EML and Pum2-EML cells transcribe CD34, the possibility of some CD34⁻ EML cells expressing CD34 protein cytoplasmically could not be excluded. Furthermore, these observations are consistent with the model in which EML cells may alternate between CD34⁻ inactive and CD34⁺/low active state, which would involve either rapid translation of CD34 protein or only the transport of cytoplasmic CD34 protein to the cell surface.

The Agilent microarray (Fig. 38) and RT-PCR analysis (Fig. 39) have also revealed that Pum2-EML cells transcribe CD150/Slamf1 (a member of the SLAM family and new HSC marker) (147-149) at a higher level than wt EML cells.

The flow cytometry analysis of CD34 and CD150 co-expression on wt EML cells has shown that ~20% of EML cells are CD150⁺, and that the majority of CD34⁺ and CD34⁻ wt EML cells are CD150⁻ (Fig. 40A). In contrast, around 80% of Pum2-EML cells are CD150⁺, and the majority of CD34⁻ cells are CD150⁺ as well (Fig. 40B). In the light of CD150 being shown to be a rather specific marker for LTR-HSC (147-149), our findings indicate that besides CD34 pattern, Pum2 over-expression changes the pattern of CD150 expression as well, and leads to expansion of EML cells with the Sca-1⁺ c-kit⁺ Flk2⁻ CD34⁻ CD150⁺ primitive phenotype.

Sorting and functional analysis of CD34⁻ CD150⁺, CD34⁻ CD150⁻, CD34⁺ CD150⁺ and CD34⁺ CD150⁻ wt EML and Pum2-EML cells could help to further elucidate functional differences between them.
We have transduced multiple batches of EML cells with: (1) Sigma’s MISSION® shRNA Lentiviral vector target set for long-term silencing of Pum2, which consists of premade lentiviral transduction particles carrying five different (#60-64) shRNAs that will target unique sequences in the CDS and 3’ UTR regions of murine Pum2 transcript, and (2) MISSION Non-Target shRNA Control lentiviral vector, carrying a non-targeting shRNA that will activate the RNAi pathway, but does not target any human or mouse genes. Transduced cells were selected with puromycin.

We have transduced EML cells with MISSION Non-Target shRNA control virus, and have generated two stably transduced EML clones through selection with puromycin. The Western analysis has shown no Pum2 protein knockdown in these cell clones in

**Figure 40.** Flow cytometry analysis of CD34 and CD150 co-expression on wt EML (A) and Pum2-EML cells (B).

**3.1.8. The effect of Pum2 attenuation on the maintenance and differentiation of EML cells**

To study the impact of shRNA-mediated Pum2 attenuation on the maintenance and differentiation of multipotent cell line EML we have transduced multiple batches of EML cells with: (1) Sigma’s MISSION® shRNA Lentiviral vector target set for long-term silencing of Pum2, which consists of premade lentiviral transduction particles carrying five different (#60-64) shRNAs that will target unique sequences in the CDS and 3’ UTR regions of murine Pum2 transcript, and (2) MISSION Non-Target shRNA Control lentiviral vector, carrying a non-targeting shRNA that will activate the RNAi pathway, but does not target any human or mouse genes. Transduced cells were selected with puromycin.

We have transduced EML cells with MISSION Non-Target shRNA control virus, and have generated two stably transduced EML clones through selection with puromycin. The Western analysis has shown no Pum2 protein knockdown in these cell clones in
comparison to wt EML cells (Fig. 41 and data not shown). Furthermore, control shRNA vector EML cell clones and wt EML cells have exhibited the same pattern of HSC and MPP marker expression (data not shown).

EML cells were next transduced with five different Sigma’s MISSION™ Pum2 shRNA lentiviruses 60-64, and multiple EML cell clones were selected with puromycin. Multiple Westerns and densitometry measurement of Pum2/actin ratio have reproducibly identified a 40-50% knockdown of Pum2 protein only in EML clones transduced with shRNA vectors 63 and 64 (Fig. 41). However, the flow cytometry analysis has revealed no significant changes in the expression of HSC/MPP markers on EML clones 63 and 64 in comparison to wt EML and Non-Target shRNA Control EML cells. Also, the CFC assays have revealed no significant difference between the multilineage differentiation of EML clones 63 and 64, and wt EML and Non-Target shRNA Control EML cells. Taken together, these results suggest that partial Pum2 knockdown does not have a discernible impact on EML cell surface phenotype and differentiation.

Figure 41. Western analysis of Pum2 protein in wt EML cells, EML clone transduced with Non-Target shRNA Control virus, and EML cell clones transduced with Pum2 shRNA vectors 60-64.
To achieve at least 70% of Pum2 protein knockdown, we are in the process of generating EML clones transduced with Dharnacon’s three different SMARTvector shRNA Lentiviral vectors for Pum2, which became just recently available. These vectors will allow selection of transduced cells via sorting for GFP^+ cells and puromycin selection, and will enable us to analyze the impact of both transient and long-term Pum2 knockdown on the maintenance and differentiation of EML cells.
3.2. The role of truncated c-kit receptor (tr-kit) in the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML

3.2.1. Pum2-EML cells express higher levels of new truncated form of c-kit receptor protein, called tr-kit

EML cell line requires SCF for survival and proliferation (93), whereas Pum2-EML cells survive and proliferate in the absence of SCF. Thus, we examined expression of c-kit, receptor for SCF by Western in wt EML, EML/pcDNA and Pum2-EML cells. Surprisingly, we discovered that in addition to the full-length c-kit protein, wt EML and Pum2-EML cells express novel truncated (30 kDa) form of c-kit protein called tr-kit, and that in comparison to wt and EML/pcDNA cells Pum2-EML cells express increased levels of tr-kit (Fig. 42).

![Western blot analysis of c-kit protein](image)

**Figure 42.** Western blot analysis of c-kit protein has shown that Pum2-EML cells express higher levels of new truncated form of c-kit receptor protein, called tr-kit.

This intracellular truncated form of c-kit was first detected in mouse spermatids (150). Tr-kit is encoded by a 3.2 kb alternative transcript (GenBank Accession Number X65997), that originates from a cryptic promoter in intron 16 of the mouse c-kit gene (Fig. 43A). The tr-kit transcript contains a unique 415 bp long 5’ untranslated region
(5’UTR) and a unique 36 bp long start of the coding sequence, which differ from c-kit cDNA. The remaining coding and 3’UTR sequence are identical with the full-length, 5.5 kb long c-kit transcript. Besides the extracellular, transmembrane and juxtamembrane region, the 202 amino acid (aa) long tr-kit protein (Mw. 30 kDa) also lacks the ATP binding part of the kinase domain, as well as the hydrophilic kinase insert (Fig. 43A and B). Instead, tr-kit contains a unique 12 aa long hydrophobic region (derived from translation of intronic sequences), which is in frame with the 190 aa long C-terminal part of c-kit protein that encompasses the phosphotransferase domain and C-terminal tail relevant for c-kit interaction with phospholipase Cγ (PLCγ) (Fig. 43A and B) (152).

When injected into mouse oocytes, tr-kit protein induces their activation by stimulating phosphorylation of PLCγ (152, 153). Although it lacks intrinsic kinase activity, in mouse oocytes and Hek293 cells tr-kit becomes phosphorylated on the C-terminal tyrosine Y936 through interaction with the Src kinase Fyn and PLCγ. A model was proposed whereby tr-kit promotes the formation of a complex composed of Fyn, PLCγ and Sam68 protein. Phosphorylation of tr-kit is necessary for these interactions to occur (Fig. 43A) (154).
Figure 43. Structure of c-kit and tr-kit protein. (A) The 202 aa long tr-kit protein (Mw. 30 kDa) lacks the extracellular, transmembrane and juxtamembrane region, and the ATP binding part of the kinase domain. Instead of the typical hydrophilic kinase insert tr-kit contains a unique 12 aa long hydrophobic region, which is in frame with the C-terminus of c-kit that contains the phosphotransferase domain and C-terminal tail. Epitopes in the c-kit and tr-kit protein for three different α-c-kit Abs used in this study, and the tyrosines (Y) phosphorylated in the c-kit protein are shown also. (B) ClustalW alignment of amino acid sequence of c-kit and tr-kit proteins (GenBank Accession Number CAA46798). Twelve N-terminal amino acids specific for tr-kit are underlined.
Since tr-kit can be phosphorylated on the C-terminal tyrosine Y936, we used pY936 Ab to examine the phosphorylation of c-kit and tr-kit in wt EML and Pum2-EML cells. Western analysis has revealed that both c-kit and tr-kit are phosphorylated in Pum2-EML cells in the absence of SCF (Figure 44A and B).

![Figure 44](image)

**Figure 44.** (A) Western blot shows that in addition to c-kit protein, wt EML, vector control and Pum2-EML cells express novel truncated (30 kDa) form of c-kit protein called tr-kit, with tr-kit expression level being higher in Pum2-EML cells. (B) Western blot shows that c-kit and tr-kit are phosphorylated at Y936 in wt EML, vector control and Pum2-EML cells. The phosphorylation of c-kit and tr-kit is SCF-independent in Pum2-EML cells.

The significant differences in the expression level and phosphorylation of tr-kit between wt EML and Pum2-EML cells prompted us to study further the function of tr-kit in hematopoiesis.

### 3.2.2. EML cells co-expresses the full-length and truncated c-kit receptor

The expression of tr-kit protein in EML cells was confirmed by Western using goat α-c-kit (M-14, Santa Cruz) and rabbit α-c-kit (Cell Signaling) antibodies (Abs) that recognize the C-terminus of c-kit, and the phosphotransferase part of the c-kit kinase domain (Fig. 45). Both Abs have detected the full length c-kit protein (160/145 kDa) and
tr-kit protein (30 kDa) in EML cells, with the expression level of tr-kit being consistently lower than the level of c-kit protein (Figs. 45A and B). However, c-kit and tr-kit proteins were not detected in pre-B cell line 70Z3, pro-B cell line BaF3, and 293T and NIH3T3 cell lines, which are known not to express c-kit (Fig. 45B) (155, 156). In contrast, the H-300 α-c-kit Ab (Santa Cruz), that recognizes the N-terminus of c-kit, did not detect tr-kit protein in EML cells (Fig. 45A).

Transcription of tr-kit in EML cells was confirmed by RT-PCR, using a primer set (tr-kit’, Table 1) that specifically amplifies 800 bp fragment of tr-kit cDNA, and spans several introns in the c-kit gene. The 5’ oligo is specific for the unique 5’ UTR of tr-kit, whereas the 3’ oligo is specific for the beginning of the 3’UTR, common for tr-kit and c-kit (Fig. 45C). The identity of amplified tr-kit PCR product was confirmed by sequencing and secondary PCR using a set of nested tr-kit (tr-kit”) primers (Fig. 45C and Table 1).

Previous studies have reported that the exogenously expressed tr-kit protein in oocytes and Hek293 cells becomes phosphorylated on the C-terminal tyrosine Y936 (154). Notably, Western analysis with α-c-kit [pY936] phosphospecific Ab (BioSource) has shown that both c-kit and tr-kit protein in EML cells are phosphorylated at the C-terminal tyrosine Y936 (Fig. 45D). On the other hand, Western analysis with α-c-kit [pY730] Ab (BioSource) has demonstrated that only the c-kit protein is phosphorylated on tyrosine Y730, which is not present in the tr-kit protein (Figs. 45A and D).
Figure 45. **A.** Western analysis of tr-kit and c-kit protein expression in EML cells. The goat and rabbit α-c-kit Abs have detected the full length c-kit protein (160/145 kDa) and tr-kit protein (30 kDa) in EML cells. H-300 α-c-kit Ab, that recognizes the N-terminus of c-kit, did not detect tr-kit protein in EML cells. The blots were stripped and re-probed with α-actin antibody. **B.** Western analysis of c-kit and tr-kit protein expression in EML cells, 70Z3 pre-B cell line, BaF3 pro-B cell line, and 293T and NIH3T3 cell lines with rabbit α-c-kit Ab. **C.** RT-PCR analysis of c-kit and tr-kit expression in EML cells, BaF3 pro-B cell line, and mouse testis as a positive control. The expression of tr-kit was analyzed with two different sets of primers (tr-kit' and tr-kit'', Table 1). **D.** Western analysis with rabbit α-c-kit [pY936] phosphospecific Ab has shown that c-kit and tr-kit protein in EML cells are both phosphorylated at the C-terminal tyrosine Y936. In contrast, the analysis with α-c-kit [pY73b] phosphospecific Ab has demonstrated that only the c-kit protein is phosphorylated on tyrosine Y730, which is absent in tr-kit protein.

3.2.3. Expression of tr-kit is restricted to populations enriched for HSC and MPPs

The presence of tr-kit transcript and protein in EML cells indicated that besides germline cells, the cells of the hematopoietic origin express tr-kit as well. Thus, we examined the pattern of tr-kit expression during murine blood cell development.
The expression of tr-kit and c-kit was analyzed first by RT-PCR in FACS-purified
(a) Lin⁻ c-kit⁺ Sca-1⁻ Flk2⁻ (LKS Flk2⁻) BM cells, highly enriched for long-term
repopulating (LTR) HSC, (b) Lin⁻ c-kit⁺ Sca-1⁺ Flk2⁺ (LKS Flk2⁺) BM cells, highly
enriched for short-term repopulating (STR) HSC and MPPs, (c) more heterogeneous
populations of Lin⁻ c-kit⁺ CD34⁻, Lin⁻ c-kit⁺ Sca-1⁻ and Lin⁻ Sca-1⁺ BM cells, containing
HSC and progenitors, (d) Lin⁻ c-kit⁺ CD34⁺, Lin⁻ c-kit⁺ Sca-1⁻ and Lin⁻ Sca-1⁻ BM cells,
enriched for progenitors but lacking LTR-HSC, and (e) Lin⁺ BM cells, containing
immature and mature blood cells (Fig. 46A).

**Figure 46**. Expression of tr-kit is restricted to cell populations enriched for HSC and
MPPs. (A) Multi-parameter sorting of Lin⁻ c-kit⁻ CD34⁻ and Lin⁻ c-kit⁺ CD34⁺ cells, Lin⁻
c-kit⁺ Sca-1⁻ and Lin⁻ c-kit⁺ Sca-1⁺, and Lin Sca-1⁺ c-kit⁺ (LKS) Flk2⁻ and LKS Flk2⁺
cells from the bone marrow of C57BL6/J mice.
Interestingly, tr-kit transcript was reproducibly detected in cDNA prepared from LKS Flk2−, LKS Flk2+ Lin− c-kit+ CD34−, Lin− c-kit+ CD34+, and Lin− c-kit+ Sca-1+ BM cells (equivalent to 5 -12 X 10^3 cells), which also express c-kit (Fig. 46B). Tr-kit expression was detectable in Lin− c-kit+ Sca-1− cells also, but only when cDNA equivalent of 3 X 10^4 cells or more was used as a template for amplification (Fig. 46B). However, the tr-kit transcript was not found in the Lin− Sca-1+, Lin− Sca-1− and Lin− BM cells, even after using increasing numbers of sorted cells (ranging from 5 X 10^3 to 10^5 cells) to isolate and transcribe RNA (Fig. 46B and data not shown).

Transcription of tr-kit and c-kit was also examined by PCR in cDNA libraries prepared from (a) fetal liver (FL) AA4.1+ Lin− Sca-1+ c-kit+ (AA4.1+ LKS) cells highly enriched for fetal HSC, (b) FL AA4.1+ Lin− cells lacking HSC but enriched for progenitors, and (c) Rho-123lo Ho-1+ Lin− Sca-1+ c-kit+ (Rho123lo Ho-1 LKS) BM cells, highly enriched for HSC and MPPs (24-26). Although the c-kit was detected in all three cell populations, only the HSC-enriched AA4.1+ LKS and Rho123lo Ho-1 LKS cells transcribe tr-kit (Fig. 46C). Moreover, tr-kit exhibits identical expression pattern with GATA-2 and HoxA9 in these cell populations (Fig. 46C) (157, 158).
Figure 46 (continued). Expression of tr-kit is restricted to cell populations enriched for HSC and MPPs. (B) RT-PCR analysis of tr-kit and c-kit expression in (a) LKS Flk2− BM cells, highly enriched for LTR-HSC, (b) LKS Flk2− BM cells, highly enriched for STR-HSC and MPPs, but lacking LTR-HSC, (c) more heterogeneous populations of Lin− c-kit+ CD34+, Lin− c-kit+ Sca-1+ and Lin Sca-1+ BM cells, containing HSC and progenitors, (d) Lin− c-kit+ CD34+, Lin− c-kit+ Sca-1+ and Lin Sca-1− BM cells, enriched for progenitors but lacking LTR-HSC, and (e) Lin+ BM cells, containing immature and mature blood cells. The expression of tr-kit was analyzed with two different sets of primers (tr-kit’ and tr-kit"), Table 1. Bmi-1, required for maintenance of HSC, and HPRT were amplified as controls. (C) The analysis of tr-kit and c-kit expression in cDNA libraries prepared from fetal liver (FL) AA4.1+ LKS cells highly enriched for fetal HSC, FL AA4.1− Lin+ cells lacking HSC but enriched for progenitors, and BM-derived Rhö(lo) Ho(lo) LKS cells, highly enriched for LTR-HSC. The expression of c-kit and tr-kit in these cell populations was compared with the expression of GATA-1, GATA-2, HoxA9, and Meis1.

Next, we examined tr-kit expression in primary hematopoietic cells and cell lines representing more advanced stages of differentiation into lymphoid and myelo-erythroid lineages. The c-kit transcript was detected by RT-PCR in the thymus, spleen, and double negative DN1 (CD44+CD25−) and DN2 (CD44+CD25+) thymocytes, but none of these tissues and cells expressed tr-kit (Fig. 46D). Although CD4+ and CD8+ T cells express GATA-3 as a control, they do not express c-kit or tr-kit (Fig. 46D and data not shown). The BM pro/pre-B cells (B220− CD43− and B220+CD43+ cells), and J774A.1 monocyte cell line express c-kit, while the BaF3 pro-B cell line, RAW 309 monocyte cell line, and
erythroid Ter119+ BM cells do not (155). However, tr-kit was not expressed in any of these cell types (Fig. 46D). These results have demonstrated that during murine blood cell development tr-kit expression is restricted to cell populations enriched for HSC and MPPs.

**Figure 46 (continued).** Expression of tr-kit is restricted to cell populations enriched for HSC and MPPs. (D) The expression of tr-kit in primary cells and cell lines representing various stages of differentiated into lymphoid and myeloid-erythroid lineages. The expression of tr-kit and c-kit was analyzed by RT-PCR in the thymus, spleen, double negative DN1 (CD44+CD25−) and DN2 (CD44−CD25+) thymocytes, CD4 and CD8 T cells from thymus, BM-derived pro/pre-B cells (B220+CD43− and B220−CD43+ cells), BaF3 pro-B cell line, 70Z3 pre-B cell line, J774A.1 and RAW 309 monocyte cell lines, and BM-derived erythroid Ter119+ cells. GATA-3 was amplified as a control for CD4 and CD8 cells, and β-globin as a control for Ter119+ cells.
3.2.4. Expression of tr-kit is down-regulated during differentiation of EML cell line into myelo-erythroid lineages

To analyze tr-kit expression during their differentiation into erythroid lineage, EML cells were cultured in triplicate with recombinant Epo (8 U/ml) for 72 and 96 hours. Tr-kit transcription in undifferentiated and differentiated EML cells was analyzed by RT-PCR and quantitative real-time PCR, whereas the tr-kit protein expression was analyzed by Western using rabbit α-c-kit Ab.

In contrast to undifferentiated cells, the EML cells cultured with Epo express β-globin as an indicator of their differentiation into erythroid lineage (158) (Fig. 47A). More importantly, in comparison to EML cells maintained with SCF, the cells cultured for 72 hours with Epo have significantly down-regulated expression of tr-kit, whereas in the cells cultured with Epo for 96 hours tr-kit transcript is barely detectable (Fig. 47A). The β-globin was amplified in the BM Lin+ cells as a positive control.

The quantitative real-time PCR analysis has revealed that the levels of tr-kit transcript (normalized against HPRT) have decreased >5-fold in EML cells cultured with Epo for 72 hours, and >8-fold in cells cultured with Epo for 96 hours in comparison to undifferentiated EML cells (Fig. 47B). Remarkably, the Western analysis has demonstrated a significant decrease of tr-kit protein level in EML cells cultured for 72 and 96 hours with Epo (Fig. 47C). Similarly, the expression of c-kit protein was also significantly down-regulated during erythroid differentiation of EML cells (Fig. 47C).
The levels of tr-kit transcript were normalized based on the amplification of HPRT as a control gene. Student t test was used for statistical analysis, where P < 0.05 was considered significant.

Western analysis of tr-kit and c-kit protein expression during EML cell differentiation into erythroid lineage. The blots were stripped and re-probed with α-actin Ab.

Next, EML cells were cultured in triplicate with the combination of recombinant G-CSF (0.1 ng/ml) and GM-CSF (2 ng/ml) for 72 and 96 hours, and tr-kit expression analyzed during their differentiation into granulocyte and macrophage lineages. After 72 hours of culture in the presence of G-CSF and GM-CSF (G/GM-CSF), tr-kit transcription was only slightly down-regulated in EML cells (Fig. 48A). However, after 96 hours tr-kit expression was significantly down-regulated in comparison to undifferentiated EML cells (Fig. 48A). In addition, EML cells cultured with G/GM-CSF transcribed lactoferrin as a marker of their differentiation into granulocyte-macrophage lineage (158) (Fig. 48A).

Figure 47. Tr-kit transcription and protein expression are down-regulated during differentiation of EML cell line into erythroid lineage. (A) RT-PCR analysis of tr-kit expression during differentiation of EML cells into erythroid lineage. EML cells were cultured in triplicate with Epo (8 U/ml) for 72 and 96 hours. The expression of β-globin demonstrates differentiation of EML cells into erythroid lineage. β-globin was amplified in BM Lin+ cells as a positive control. (B) Quantitative real-time PCR analysis of tr-kit expression in undifferentiated EML cells and EML cells that are undergoing differentiation into erythroid lineage in the presence of Epo. The levels of tr-kit transcript were normalized based on the amplification of HPRT as a control gene. Student t test was used for statistical analysis, where P < 0.05 was considered significant. (C) Western analysis of tr-kit and c-kit protein expression during EML cell differentiation into erythroid lineage. The blots were stripped and re-probed with α-actin Ab.
Real-time PCR analysis has shown that the levels of tr-kit transcript (normalized against HPRT) in EML cells cultured with G/GM-CSF for 72 and 96 hours have decreased about 2- and 2.5-fold when compared with undifferentiated EML cells (Fig. 48B). Similarly, the expression of tr-kit and c-kit protein was only slightly decreased in EML cells cultured with G/GM-CSF for 72, but was significantly down-regulated in EML cells treated with G/GM-CSF for 96 hours (Fig. 48C). Nevertheless, the down-regulation of tr-kit expression in EML cells differentiated with G/GM-CSF was not as prominent as in EML cells differentiated with Epo.

Simultaneous treatment of EML cells with increased concentration of GM-CSF and high concentration of retinoic acid (10⁻⁵ M RA) has been reported to dramatically augment their differentiation into granulocyte-macrophage progenitors and granulocytes and macrophages (6). Hence, in the next set of experiments the expression of tr-kit was examined in EML cells cultured in the presence of G-CSF (0.1 ng/ml), GM-CSF (10 ng/ml) and retinoic acid (10⁻⁵ M) for 72 and 96 hours, respectively.

The RT-PCR analysis has shown that in comparison to undifferentiated EML cells and cells differentiated with G/GM-CSF (Fig. 48A-C), EML cells cultured with G/GM-CSF and RA for 72 and 96 hours do not express detectable levels of tr-kit transcript (Fig. 48D and E). More importantly, the expression of tr-kit protein in these cells is significantly down-regulated at 72 hours and barely detectable at 96 hours (Fig. 48D and E). Moreover, in comparison to EML cells differentiated with G/GM-CSF alone (Fig. 48A), the EML cells treated with G/GM-CSF and RA also show elevated expression of lactoferrin (Fig. 48D).
Figure 48. Tr-kit transcription and protein expression are down-regulated during differentiation of EML cell line into myeloid lineages. (A) RT-PCR analysis of tr-kit expression during differentiation of EML cells into granulocyte-macrophage lineages. EML cells were cultured in triplicate with the combination of G-CSF (0.1 ng/ml) and GM-CSF (2 ng/ml) for 72 and 96 hours. The expression of lactoferrin demonstrates differentiation of EML cells into granulocyte-macrophage lineages. Lactoferrin was amplified in the BM Lin^- cells as a positive control. (B) Quantitative real-time PCR analysis of tr-kit expression in undifferentiated EML cells and EML cells that are undergoing differentiation into granulocyte-macrophage lineages in the presence of GM-CSF and G-CSF. The levels of tr-kit transcript were normalized based on the amplification of HPRT as a control gene. (C) Western analysis of tr-kit and c-kit protein expression during EML cell differentiation into granulocyte-macrophage lineages. The blots were re-probed with α-actin Ab. (D) RT-PCR analysis of tr-kit expression in EML cells differentiating into granulocyte-macrophage lineages in the presence of G-CSF (0.1 ng/ml), GM-CSF (2 ng/ml) and retinoic acid (RA, 10^-5 M) for 72 and 96 hours. The expression of lactoferrin demonstrates differentiation of EML cells into granulocyte-macrophage lineages. (E) Western analysis of tr-kit and c-kit protein expression in EML cells differentiating into granulocyte-macrophage lineages in the presence of G-CSF, GM-CSF and RA for 72 and 96 hours. The blots were re-probed with α-actin Ab. Previous studies have shown that GM-CSF cannot induce differentiation of all EML cells into granulocyte-macrophage progenitors and mature granulocytes and macrophages (93). Thus, the residual expression of tr-kit transcript and protein seen in EML cells cultured with G/GM-CSF is most likely due to the presence of remaining undifferentiated EML cells in these cultures.
Overall, these results demonstrate that the expression of tr-kit is rapidly and significantly down-regulated in EML cells undergoing terminal differentiation into erythroid and myeloid lineages.

3.2.5. The impact of tr-kit over-expression on the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML

To over-express tr-kit in EML cells, we have inserted tr-kit cDNA into a murine stem cell virus (MSCV)-based, bicistronic retroviral vector containing GFP as a marker (Fig. 49A).

Mouse pro-B cell line BaF3, which does not express c-kit or tr-kit (Fig. 46D and 49B), was first transduced with the control MSCV/GFP virus or MSCV/tr-kit/GFP virus. Transduced cells were selected based on expression of GFP, and GFP+ BaF3 cells were sorted after one week in culture (Fig. 49A). RT-PCR analysis has detected tr-kit transcript only in BaF3 cells transduced with MSCV/tr-kit/GFP virus, but not in wt BaF3 cells or BaF3 cells transduced with control MSCV/GFP virus (Fig. 49B).

Figure 49. A. Sorting of GFP+ BaF3 cells transduced with the control MSCV/GFP virus or MSCV/tr-kit/GFP virus. B. RT-PCR analysis of tr-kit transcript in wt BaF3 cells, BaF3 cells transduced with control MSCV/GFP virus, and BaF3 cells transduced with MSCV/tr-kit/GFP virus.
Next, multiple batches of EML cells were transduced with the control MSCV/GFP virus or MSCV/tr-kit/GFP virus. Transduced GFP+ EML cells were sorted (Fig. 50), maintained in culture, and analyzed for the expression of tr-kit.

![Figure 50](image)

**Figure 50.** Sorting of GFP+ EML cells transduced with the control MSCV/GFP virus or MSCV/tr-kit/GFP virus.

Western analysis has detected increased amount of tr-kit protein in GFP+ EML cells transduced with MSCV/tr-kit/GFP virus (Fig. 51).

![Figure 51](image)

**Figure 51.** Western analysis of tr-kit protein in wt EML and GFP+ EML cells transduced with MSCV/tr-kit/GFP virus.
Flow cytometry analysis has revealed that there is no significant difference in the expression of HSC/MPP markers between wt EML cells and selected GFP+ EML cells, which were transduced either with the control MSCV/GFP virus or MSCV/tr-kit/GFP virus at different MOI (Fig. 52).

![Figure 52](image)

**Figure 52.** Flow cytometry analysis of HSC/MPP markers on wt EML cells and selected GFP+ EML cells, which were transduced either with the control MSCV/GFP virus or MSCV/tr-kit/GFP virus at different MOI.

On the other hand, colony forming assays have shown that over-expression of tr-kit increases the frequency of myelo-erythroid progenitors in EML cells. GFP+ EML cells transduced with the MSCV/tr-kit/GFP virus at two different MOI exhibit increased CFC capacity in comparison to wt EML cells and GFP+ EML cells transduced with the control MSCV/GFP virus (Fig. 53).

![Figure 53](image)

**Figure 53.** EML cells over-expressing tr-kit exhibit increased CFC activity in comparison to wt and MSCV/GFP control EML cells.
In addition, the colonies formed by MSCV/tr-kit/GFP+ EML cells were significantly larger than colonies generated by wt EML and MSCV/GFP+ EML cells (Fig. 54, and data not shown).

![Image](image-url)

**Figure 54.** EML cells over-expressing tr-kit produced larger hematopoietic colonies in comparison to wt and MSCV/GFP control EML cells.

Interestingly, MSCV/tr-kit/GFP+ EML cells, that over-express tr-kit, have shown the same sensitivity to SCF withdrawal as wt EML cells, suggesting that increased levels of tr-kit do not confer SCF independence. Cumulatively, these results suggest that elevated levels of tr-kit protein enhance proliferation of EML cells, and lead to (a) expansion of multipotent progenitors that in response to cytokines differentiate into myelo-erythroid progenitors, and (b) formation of enlarged myelo-erythroid colonies with increased cell content. Notably, similar outcomes of tr-kit over-expression in germline cells and in hematopoietic cells indicate that tr-kit affects cell proliferation. More importantly, the over-expression experiments in EML cells have provided the evidence that tr-kit could have important role in proliferation and differentiation of primitive hematopoietic cells.
IV. DISCUSSION

4.1. The role of Pum2 RNA-binding protein in the maintenance and differentiation of multipotent hematopoietic progenitor cell line EML

1. Over-expression of Pum2 in EML cells leads to reversal of CD34 expression pattern and maintains the cells in a more primitive status

Previously we have reported that over-expression of RNA-binding protein Pum2 supports proliferative self-renewal and severely attenuates multilineage differentiation of murine HSC-like cell line EML (172).

Flow cytometry analysis of HSC/MPP marker expression has revealed that almost all EML cells are Sca-1⁺c-kit⁺Flk2⁻, but exhibit heterogeneous expression of CD34, with 20-40% of cells being CD34⁻, and 60-80% of cells being CD34⁺. In contrast, EML cells over-expressing Pum2 have exhibited reversal of CD34 expression pattern, with vast majority of cells (80-95%) having Sca-1⁺c-kit⁺Flk2⁻CD34⁻ phenotype.

CD34 is one of the very important cell surface markers used for distinguishing LTR-HSCs from STR-HSCs. In the mouse model, the LTR-HSCs are LKS Flk2⁻CD34⁻ cells, whereas the STR-HSC reside in LKS Flk2⁻CD34⁺ cell population. Thus, in terms of the expression of HSC/MPP markers, the EML cell line mimics BM LKS Flk2⁻ cells which consist of LKS Flk2⁻CD34⁻ and LKS Flk2⁺CD34⁺ subpopulations.

2. CD34 expression levels correlate with multilineage differentiation of EML and Pum2-EML cells

To examine the functional impact of this significant change in CD34 expression pattern, wt EML and Pum2-EML cells were separated into CD34⁺ and CD34⁻ populations, and their differentiation capacity assessed using colony-forming assays. These experiments have revealed clear link between CD34 expression and multilineage
differentiation of EML cells. The colony forming capacity resided almost exclusively within the CD34\(^+\) population of wt EML and Pum2-EML cells. In contrast, the CD34\(^-\) population of wt EML and Pum2-EML cells differentiated poorly or not at all.

Flow cytometry analysis has reproducibly shown that based on the level of CD34 expression, CD34\(^+\) wt EML cells can be divided into CD34\(^\text{low}\), CD34\(^\text{medium}\) and CD34\(^\text{high}\) populations, whereas CD34\(^+\) Pum2-EML cells correspond to CD34\(^\text{low}\) cells. Remarkably, the analysis of multilineage differentiation capacity of purified CD34\(^\text{low}\), CD34\(^\text{med}\) and CD34\(^\text{high}\) wt EML cell populations has revealed direct correlation between the level of CD34 expression and the extent of cell differentiation into myeloid, erythroid and megakaryocytic lineages.

Importantly, our observations are in accordance with the findings of the previous study which examined the progenitor cell content in Lin\(^-\) c-kit\(^+\) CD34\(^\text{high}\), Lin\(^-\) c-kit\(^+\) CD34\(^\text{low}\), and Lin\(^-\) c-kit\(^+\) CD34\(^\text{low}\) BM cells and reported that only Lin\(^-\) c-kit\(^+\) CD34\(^\text{high}\) cells differentiated efficiently in colony forming assays, whereas Lin\(^-\) c-kit\(^+\) CD34\(^\text{low}\) and Lin\(^-\) c-kit\(^+\) CD34\(^\text{low}\) BM cells differentiated poorly or not at all (131).

Cumulatively, these data indicate that the overall multilineage differentiation of wt EML and Pum2-EML cells strongly correlates with the level of CD34 expression and that CD34\(^-\) subpopulation of EML and Pum2-EML cells are in a state where they do not respond to cytokines by differentiation.

3. Cell surface CD34 expression is linked with “inactive” and “active” state of EML and Pum2-EML cells

Distinct differences in the phenotype and differentiation have raised the question about developmental relationship between CD34\(^-\) and CD34\(^+\) EML and Pum2-EML cells.
Since LKS Flk2\(^-\) CD34\(^-\) BM cells are more primitive and give rise to LKS Flk2\(^+\) CD34\(^+\) BM cells, the first prediction would be (a) that similar to primary BM cells CD34\(^-\) and CD34\(^+\) cell populations represent different developmental stages, and (b) that EML cells follow the linear differentiation model in which Sca-1\(^+\) c-kit\(^+\) Flk-2\(^-\) CD34\(^-\) population of wt EML and Pum2-EML cells is more primitive and generates Sca-1\(^+\) c-kit\(^+\) Flk-2\(^-\) CD34\(^+\) population. In that case by dividing asymmetrically CD34\(^-\) EML cells could maintain CD34\(^-\) cell pool and at the same time generate CD34\(^+\) EML cells. If that hypothesis is correct then among Pum2-EML cells the population of CD34\(^-\) cells undergoes more frequent symmetric division, thus giving rise to increased CD34\(^-\) cell content and diminishing frequency of CD34\(^+\) cells.

Surprisingly, the analysis of developmental relationship between CD34\(^-\) and CD34\(^+\) EML cells has reproducibly shown that CD34\(^-\) and CD34\(^+\) cells can generate each other, albeit with different kinetics. These findings suggested that although the CD34\(^-\) population of wt EML cells could be more primitive than CD34\(^+\) cells and gives rise to CD34\(^+\) population, there is a subpopulation of CD34\(^+\) cells with the capacity to give rise to CD34\(^-\) cells as well. Also these findings did not support the model of linear cell differentiation.

In an attempt to determine which subpopulation of CD34\(^+\) EML cells can generate CD34\(^-\) cells, purified CD34\(^{high}\), CD34\(^{medium}\) and CD34\(^{low}\) EML cells were cultured separately and the generation of CD34\(^-\) cells monitored and quantified by flow cytometry. These experiments have reproducibly shown that the lowest level of CD34 expression correlates with the highest capacity to generate CD34\(^-\) cells.
Identical experiments with purified CD34\(^{-}\) and CD34\(^{+}/\text{low}\) Pum2-EML cells have revealed that CD34\(^{-}\) Pum2-EML cells maintain CD34\(^{-}\) population and generate small population of CD34\(^{+}/\text{low}\) cells, which in turn can generate CD34\(^{-}\) cells.

Several studies have established the link between CD34 expression and activation of adult murine HSC, and have shown that mouse LTR-HSC can alternate between CD34\(^{-}\) “inactive” and CD34\(^{+}\) “activated” state (132-134).

In view of that we proposed a new model that merges linear differentiation of CD34\(^{-}\) EML cells into CD34\(^{+}\) cells, and the reversible CD34 expression and activation (Fig. 33B and C). In this model the CD34\(^{-}\) EML cells are more primitive cells in an “inactive” state (differentiation inhibited) that give rise to CD34\(^{+}\) EML cells. On the other hand, the CD34\(^{+}\) population of EML cells is heterogeneous and consists of CD34\(^{\text{med/high}}\) cells that can readily differentiate into multiple lineages, and CD34\(^{\text{low}}\) cells that are in “activated” state and can most efficiently revert back to the CD34\(^{-}\) state.

Taken together our results support the notion that upregulation of CD34 cell surface expression coincides with increased readiness of EML cells to differentiate and decreased capacity to revert back to inactive CD34\(^{-}\) state. Our results also suggest that Sca-1\(^{+}\) c-kit\(^{+}\) Flk2\(^{-}\) CD34\(^{-}\) population of EML cells is in “inactive” self-renewal state and differentiates poorly or not at all, whereas the CD34\(^{\text{low}}\) population could represent “activated” state at which the cells are ready to undergo differentiation.

In steady state, EML cell culture a balance is maintained between CD34\(^{-}\) inactive and CD34\(^{\text{low}}\) active state of EML cells. In contrast, Pum2 over-expression tips that balance and maintains the cells in the CD34\(^{-}\) inactive state, thus promoting proliferation and inhibiting differentiation (172), two critical components of self-renewal.
The studies that have established the link between CD34 expression and activation of adult murine HSC have also brought to attention the fact that heterogeneous population of LKS Flk2− CD34+ BM cells contains not only STR-HSC but also the population of activated LTR-HSC (Fig. 32A). Up until now there was no attempt to try to distinguish and separate these two populations based on cell surface marker expression.

Our EML cell results suggest that one putative way to distinguish between activated LTR-HSC and STR-HSC would be through the difference in the level of cell surface CD34 expression. In that sense activated LTR-HSC would be characterized by the lowest level of CD34 expression, which would be upregulated as cells transition through STR-HSC and MPP stages.

Thus, EML cell line could represent a very useful in vitro model to study the linear differentiation of CD34− cells into CD34+ cells, and the mechanisms of reversible CD34 expression and activation of primitive hematopoietic cells (133, 141, 142).

Examples similar to HSC alternating between CD34− “inactive” and CD34+ “activated” states, are the models of germline stem cell compartments in Drosophila and mouse (160-162). For example, in the mouse during normal spermatogenesis the stem cell potential is not limited to the cells that actually self-renew in the stem cell niche (actual stem cells). Upon loss of the actual stem cells, the transit-amplifying cells can switch their mode from amplification to self-renewal, resulting in the genesis of new actual stem cells (Fig. 55).
Figure 55. Model of regeneration of male germline stem cells by spermatogonial dedifferentiation in vivo (162).

4. Possible correlation between CD34 expression and localization of HSC in different niches

As suggested by Wilson et al. the dormant LTR-HSC are located in the osteoblastic niche and are in inactivate state, whereas the self-renewing LTR-HSCs, that are in activated state, are found in vascular niche in close contact to the circulation (Fig. 4) (12). One of the morphological differences between primary CD34+ and CD34- BM cells and CD34+ and CD34- EML cells is that CD34+ cells have the podia whose purpose could be to enable migration of cells from the osteoblastic niche to the vascular niche.

Based on our data and the model of HSC activation, we are proposing a new hypothetical model in which both inactive and activated LTR-HSC reside in an osteoblast niche, and HSC activation takes place in the same niche. Once activated, HSC would migrate to the vascular niche, where they give rise to STR-HSC and MPPs whose progeny can be than released into circulation.
5. Concluding remarks

(A) Over-expression of the RNA-binding protein Pum2 supports proliferative self-renewal and severely attenuates multilineage differentiation of the murine HSC-like cell line EML.

(B) CD34 expression levels on EML and Pum2-EML cells correlate with their multilineage differentiation.

(C) Cell surface CD34 expression is linked with “inactive” and “active” state of EML and Pum2-EML cells.

(D) Over-expression of Pum2 leads to reversal of CD34 expression pattern on EML cells and locks most cells in an inactive CD34^− state.

(E) These results support the notion that Pum2 is regulating the balance between self-renewal and differentiation of primitive hematopoietic cells by promoting proliferation and attenuating differentiation, two critical components of self-renewal.

4.2. The role of truncated c-kit receptor (tr-kit) in the maintenance and differentiation of multipotent hematopoietic cells

Discovery of tr-kit in multipotent HSC/MPP-like cell line EML prompted us to determine its expression pattern during mouse hematopoiesis. Remarkably, tr-kit transcripts were detected only in the FL and BM-derived cell populations that are highly enriched for LTR-HSC, STR-HSC and MPPs, and are expressing c-kit as well. Notably, an online search of several public HSC databases generated through differential gene expression profiling (158, 163-166), has revealed that tr-kit was found in the cDNA library from HSC-enriched Thy-1.1^lo LKS BM cells, which was subtracted with cDNA from Lin^+ BM cells (165).
In contrast, the expression of tr-kit was not detected in (a) FL and BM cell populations in which HSC and MPPs are either present at a much lower frequency or are absent altogether, and (b) in cells representing more advanced stages of differentiation into lymphoid and myeloid lineages. These findings were corroborated by the observation that the expression of tr-kit transcript and protein is rapidly and significantly down-regulated in EML cells as they differentiate into erythroid and myeloid lineage cells in response to Epo, G-CSF, GM-CSF, and retinoic acid.

Taken together, our studies have revealed that in contrast to c-kit, whose expression is more widespread during murine hematopoiesis (91), tr-kit expression is restricted to cell populations enriched for HSC and MPPs. The absence of tr-kit in later stages of blood cell development and down-regulation of tr-kit expression during myelo-erythroid differentiation of EML cells suggest that tr-kit expression is regulated in a developmental stage-specific manner.

The remaining questions are whether tr-kit expression is heterogeneous in HSC and MPP-enriched populations, and whether it correlates with LTR-HSC, STR-HSC and/or MPP cell activity. Intracellular staining with tr-kit specific Ab would be one approach to determine whether e.g. LKS Flk2<sup>−</sup>, LKS Flk2<sup>+</sup>, Lin<sup>−</sup> c-kit<sup>+</sup> CD34<sup>−</sup> and Lin<sup>−</sup> c-kit<sup>−</sup> CD34<sup>+</sup> BM cells can be divided into tr-kit<sup>+</sup> and tr-kit<sup>−</sup> subpopulations. If so, the creation of the tr-kit-GFP knock-in mouse model could enable purification of tr-kit<sup>+</sup> and tr-kit<sup>−</sup> cells from HSC and MPPs, and their functional analysis through colony-forming and competitive repopulation assays.

Despite the documented importance for self-renewal and differentiation of HSC and MPPs, the mechanisms through which c-kit regulates these processes are still not
well understood (91, 167). The novel finding that HSC and MPP-enriched cell populations co-express c-kit and tr-kit raises an interesting possibility that tr-kit functions either as a new component of canonical SCF/c-kit pathway, or it participates in a novel signaling pathway, present exclusively in HSC and MPPs.

Pum2 over-expression in EML cells resulted in their SCF-independent growth, which was accompanied by elevated expression of tr-kit and SCF-independent phosphorylation of c-kit protein. This observation posed the question about the putative role of tr-kit in SCF-independent phosphorylation of c-kit and growth of Pum2-EML cells.

Interestingly, similar to previous report (154), in EML cells tr-kit is phosphorylated at the C-terminal tyrosine Y936, indicating that tr-kit is activated in primitive hematopoietic cells through a yet unidentified process. Putative indirect or direct interaction of tr-kit and the full-length c-kit receptor through hetero-dimerization could lead to ligand-binding independent c-kit activation (Fig. 56).

![Figure 56](image)

**Figure 56.** Hypothetical interaction of tr-kit and the full-length c-kit receptor in SCF-independent activation of c-kit.
Thus, it could be very important to elucidate whether tr-kit interacts with c-kit in HSC and MPPs, and participates in the c-kit signaling pathway. Interactions between the full-length and naturally occurring or engineered truncated forms of tyrosine kinase receptors (e.g. EGF-R, trkB and c-kit) have been observed before, and can lead to ligand-independent receptor dimerization and activation (168-170).

The results of tr-kit over-expression studies in EML cells have revealed that increased levels of tr-kit alone do not confer SCF independence. However, the results suggest that an increase of tr-kit protein enhances differentiation of EML cells into myelo-erythroid and megakaryocytic progenitors in response to cytokines in colony-forming assays. This observation could be due to proliferative effect of tr-kit on EML cells, and tr-kit-mediated expansion of multipotent progenitors which in turn will generate lineage-committed progenitors with increased frequency.

Nevertheless, along with c-kit, tr-kit could still be involved in the regulation of self-renewal of HSC and MPPs. For example, the W-41 and W-42 mutations in the c-kit phosphotransferase domain that affect c-kit function, could affect the function of tr-kit as well. Hence, the HSC functional defects found in mice heterozygous for W-41 and W-42 mutations (167, 171), could be due to attenuated activity of both c-kit and tr-kit.
VI. REFERENCES


9. Akira Suzuki, David P. Andrew, Jose-Angel Gonzalo et al. (1996), CD34-Deficient mice have reduced eosinophil accumulation after allergen exposure and show a novel crossreactive 90-kD protein, *Blood*, 87 (9), 3550-3562.


37. Monica Cobas, Anne Wilson, Bettina Ernst, Stéphane J.C. Mancini, H. Robson MacDonald, Rolf Kemler, and Freddy Radtke (2004), ß-Catenin Is Dispensable for Hematopoiesis and Lymphopoiesis, JEM, 199 (2), 221-229.


93. Tsai S, Bartelmez S, Sitnicka E, Collins S. Lymphohematopoietic progenitors immortalized by a retroviral vector harboring a dominant-negative retinoic acid receptor can recapitulate lymphoid, myeloid, and erythroid development. Genes Dev. 1994; 8:2831-2841.


