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Identification of the Downstream Effector Genes Involved in HOXB4-Induced Expansion of Hematopoietic Progenitor Cells

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Abstract

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**IDENTIFICATION OF THE DOWNSTREAM EFFECTOR GENES
INVOLVED IN HOXB4-INDUCED EXPANSION OF
HEMATOPOIETIC PROGENITOR CELLS**

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirement for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Jie Jiang
December 2009

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DEDICATION

This dissertation is dedicated to
My parents, Mali Wu and Zhiyuan Jiang
My husband, Ji Zhang
And My daughter Ariel Q. Zhang
for their love and support

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ABSTRACT

Overexpression of HOXB4, a member of Homeobox transcription factor family, promotes expansion of hematopoietic stem and progenitor cells both *in vivo* and *in vitro*. However, the molecular mechanisms underlying this effect are not well understood. In order to identify direct target genes of HOXB4 in primary murine hematopoietic progenitor cells, we induced HOXB4 function in lineage-negative, murine bone marrow cells, using a tamoxifen-inducible HOXB4-ER^{T2} fusion protein. Seventy seven genes with differentially changed expression in early response to HOXB4 have been identified as candidate target genes. Among them, we show that *Hemogen (Hemgn)*, encoding a nuclear protein specifically expressed in hematopoietic stem and progenitor cells, is a direct transcriptional target of HOXB4, and that HOXB4 binds to the promoter region of *Hemgn*. More importantly, when overexpressed in bone marrow cells, Hemgn promotes expansion of 5-fluorouracil (5-FU) treated bone marrow cells in both liquid and semi-solid cultures, recapitulating the effects of HOXB4. Furthermore, both Hemgn and HOXB4 can protect bone marrow cells from apoptosis. Our results identify an important direct transcriptional target of HOXB4 that can confer expansion of primitive myeloid progenitor cells.

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CHAPTER 1. INTRODUCTION

1.1 Hematopoiesis

Hematopoiesis is a process of successive formation and development of blood cells derived from hematopoietic stem cells.¹ Hematopoietic stem cells can generate multi-lineage or single-lineage committed hematopoietic progenitor cells for mature blood cells production. Within multi-lineage hematopoietic progenitor cells, common lymphoid progenitors (CLPs) can give rise to B and T lymphocytes; on the other hand, common myeloid progenitors (CMPs) can give rise to various multi- or single-lineage hematopoietic progenitor cells to produce platelets, erythrocytes, granulocytes and monocytes (Figure 1-1).²⁻⁶ In humans, hematopoiesis originates in blood islands in the embryonic yolk sac; then switches to fetal liver, and then to spleen as adult hematopoietic stem cells migrate to these locations. In adults, bone marrow is the major site of hematopoiesis.

Hematopoietic cells are short-lived, thus, to maintain cellular homeostasis, continuous generation of hematopoietic cells is required. This process is regulated by a complex balance between cell self-renewal, differentiation and apoptosis.⁷⁻¹⁰

1.2 Hematopoietic Stem and Progenitor Cells

Hematopoietic stem cells were originally identified by Till and McCulloch in mouse transplantation assays.¹¹ Hematopoietic stem cells are self-renewing cells that have the ability to differentiate into all of the blood cell lineages.^{12;13}

There are two kinds of hematopoietic stem cells that can be isolated in mice — embryonic hematopoietic stem cells and adult hematopoietic stem cells.^{14;15} Embryonic hematopoietic stem cells develop in the yolk sac at day 9.0 of embryogenesis (E9.0) and can reconstitute hematopoiesis after injection into neonatal mice. Adult hematopoietic stem cells emerge in the aorta, gonads, and mesonephros (AGM) region around E10.0 and can reconstitute hematopoiesis in lethally-irradiated adult mice. The most stringent method to evaluate and quantify hematopoietic stem cells is to transplant them into lethally-irradiated recipient animals. *In vivo* studies show that one single hematopoietic stem cell is sufficient to restore the whole blood system in lethally irradiated recipient mice.¹⁶ Hematopoietic stem cells can be isolated from bone marrow by various methods. One approach is to use antibodies that enrich for cells expressing hematopoietic stem cell-associated cell surface markers (e.g., c-kit, Sca-1, Thy1.1) and are depleted of cells expressing mature lineage markers (e.g., Ter119, B220, CD3, Mac-1, Gr-1). Hematopoietic stem cell activity resides in a very small proportion of bone marrow cells, characterized by the Lin⁻CD41⁻CD48⁻CD150⁺ or

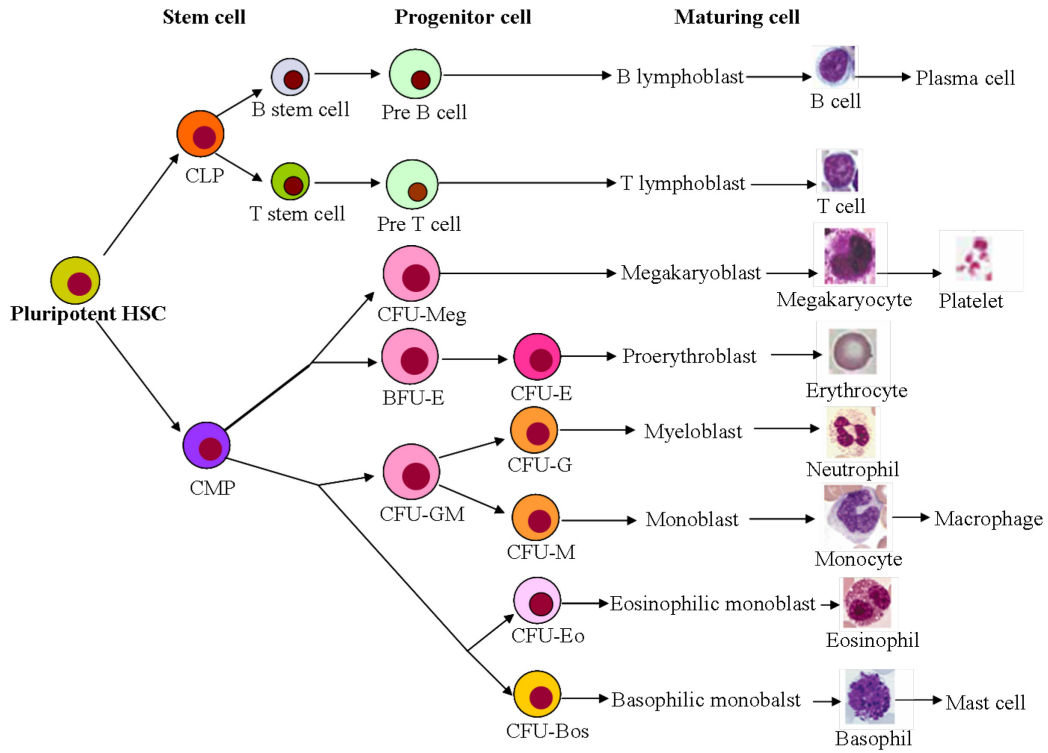


Figure 1-1. Hematopoiesis.

Lin⁻Sca1⁺CD117⁺ (LSK) phenotype.¹⁷ After injection into lethally-irradiated recipient mice, these cells migrate to appropriate microenvironmental niches and undergo rapid expansion and differentiation. In the short term, they provide radioprotection by generating mature erythroid and myeloid cells that are necessary for survival. Hematopoietic stem cells also self-renew to generate more stem cells and sustain steady-state hematopoiesis during the lifetime of an organism.^{18;19} Hematopoietic stem cells are less well-defined in humans due to the lack of an equivalent repopulating assay suitable for quantitative experiments, although xenograft studies in immunodeficient mice are increasingly used for this purpose.

Under homeostatic conditions *in vivo*, most hematopoietic stem cells and some hematopoietic progenitor cells are in the G₀ phase of cell cycle. Many of the late and more committed hematopoietic progenitor cells are actively proliferating to generate mature offsprings. Once hematopoietic stem and progenitor cells are stimulated to enter cell cycle, several outcomes are possible (Figure 1-2).²⁰ First, they can divide into committed daughter cells in the process of differentiation. Under stressful conditions *in vivo*, such as bleeding or infection, this process can generate mature blood cells to replenish the loss or to increase the immune response. Under unfavorable *in vitro* culture conditions, hematopoietic stem and progenitor cells can also undergo differentiation and lose their original stem and progenitor cell characteristics. Hematopoietic stem cells can also undergo self-renewal by dividing into two daughter cells which retain the biological properties of the original cell. Third, under homeostatic conditions *in vivo*, apoptosis can occur and serve to balance the proliferation and differentiation processes.^{21;22}

Committed hematopoietic progenitor cells can enter a transient state of rapid cell expansion. After exhausting their expansion potential, these cells withdraw from cell cycle and progressively acquire characteristics of a predetermined blood cell type.²⁰ The commitment of hematopoietic progenitor cells depends on specific growth factors and particular microenvironmental signals. Hematopoietic stem cells reside and develop on stroma cells, such as fat cells, endothelial cells, fibroblasts, and macrophages. These nonhematopoietic cells provide microenvironmental signals that support the growth and differentiation of hematopoietic cells.²³⁻³⁰

1.3 Factors in Hematopoietic Stem and Progenitor Cell Self-Renewal

While the phenotypic and functional properties of hematopoietic stem cells have been extensively characterized, a fundamental question that remains is how self-renewal is regulated. One of the most essential issues in stem cell research is to understand the mechanism of hematopoietic stem and progenitor cells self-renewal, due to its significance in both basic research and clinical applications. Identifying the molecules for promoting hematopoietic stem cells self-renewal may eventually allow the generation of expanded populations of

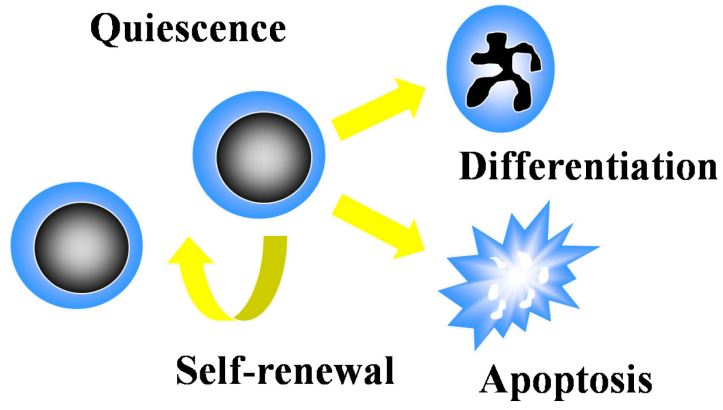


Figure 1-2. Hematopoietic stem cell outcomes.

hematopoietic stem cells *in vitro* that can be used therapeutically. Self-renewal requires the combination of both survival and proliferation pathways, and inhibition of apoptosis and differentiation signals, which appears to be controlled both by extrinsic signals from the microenvironment and by intrinsic genetic programs.^{31,32} For cell-extrinsic pathways, recent results suggest that the osteoblastic niche could impact hematopoietic stem cell function. It has been proposed that hematopoietic stem cells reside in a specific microenvironment, osteoblastic niche, which is composed of the non-hematopoietic stroma cells, locally released cytokines, and the extracellular matrix. All these factors act together to sustain hematopoietic stem cells in bone marrow.^{24;26-30;33;34} For cell-intrinsic genetic programs involved in hematopoietic stem and progenitor cell self renewal, cell cycle regulators, anti-apoptotic proteins, signal transducers, and transcription factors have been proven to play essential roles.

1.3.1 Microenvironment

Genetic studies have demonstrated that osteoblasts are a crucial cellular element of the hematopoietic stem cell niche in bone marrow.^{35;36} Hematopoietic stem cells receive critical signals from stroma cells and extracellular matrix (ECM) for expansion and differentiation. Some components of the ECM can bind to growth factors produced by stroma cells and also can bind to glycoproteins expressed on the cell surface of hematopoietic stem and progenitor cells. The interaction between hematopoietic stem cells and a component of ECM—fibronectin (FN) can stimulate growth and survival.³⁷ This interaction is augmented by angiopoietin 1 (Ang-1) which is expressed by both osteoblasts and hematopoietic stem cells. This cytokine acts through the hematopoietic stem cell-surface receptor Tie-2 for maintaining the self-renewal ability.^{38;39} The direct interaction between hematopoietic stem cells and stroma cells or ECM in the niche can also promote hematopoietic stem cell self-renewal. For example, activation of Notch transmembrane receptors expressed in hematopoietic stem cells by their corresponding ligands expressed on stroma cells such as Jagged 1 and Jagged 2, can promote the self-renewal process.⁴⁰⁻⁴⁴ Inhibition of Notch signaling in adult hematopoietic stem cells led to accelerated differentiation *in vitro* and depletion of stem cells *in vivo*.⁴⁵ Retroviral overexpression of a dominant active form of Notch established immortalized hematopoietic stem cells that were dependent on a cytokine cocktail of SCF, IL-6, IL-11 and Flt3 ligand. Enhanced activation of Notch1 or Notch4 (also known as Int3) contributed to increased self-renewal ability and consequent differentiation inhibition of hematopoietic stem cells, providing further evidence of the role of Notch signaling.^{46;47} The Wnt pathway, which functions during development for initiating cellular events such as cell proliferation and differentiation, is also shown to be involved in this process. For example, it has been shown that Wnt3A and β -catenin play key roles in hematopoietic stem cell self-renewal.^{48;49}

1.3.2 Cytokines

Cytokines are a broad family of proteins that mediate positive and negative signals that control cellular quiescence, apoptosis, proliferation, and differentiation. In general, cytokines function by engaging a specific receptor and activating a variety of signaling pathways.⁵⁰ Among the different cytokines, stem cell factor (SCF), interleukin-3(IL-3), IL-6, Flt3 ligand, thrombopoietin (TPO) have been shown to modestly expand hematopoietic stem cells *in vitro*.⁵¹⁻⁵⁴ The combination of SCF, IL-6, TPO and Flt3 ligand can promote transient hematopoietic stem cell expansion *in vitro*.⁵⁵ Particularly, the combination of SCF, IL-3 and IL-6 was demonstrated to increase the retrovirus-mediated gene transduction efficiency in murine hematopoietic stem cells.^{56;57} Some cytokines such as IL-3 and SCF induce cell proliferation of hematopoietic stem and progenitor cells, while others such as Flt-3 ligand and kit ligand protect cells from apoptosis and sensitize them to the effects of growth promoting cytokines.⁵⁸⁻⁶⁰ In contrast, TGF- β 1 can inhibit the growth of hematopoietic stem cells.^{61;62} The inhibition of TGF- β 1 production by antisense oligos can promote the release of hematopoietic stem cells from quiescence.^{63;64} Another TGF- β family member, bone morphogenetic protein-4 (BMP4) and its upstream regulator sonic hedgehog (shh) is able to induce hematopoietic stem cell self-renewal.^{65;66}

1.3.3 Cell Cycle and Apoptotic Regulators

A key mediator of hematopoietic stem cell maintenance is a G1 checkpoint regulator, the cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ ($p21$).^{67;68} In $p21^{-/-}$ mice, the number of hematopoietic stem cells was increased at steady state conditions but hematopoietic failure occurred under conditions of stress. Hematopoietic stem cell self-renewal failure had been observed during the serial transplantation of $p21^{-/-}$ cells. Another example is a cell cycle inhibitor, $p18^{INK4C}$. The absence of $p18^{INK4C}$ led to increased hematopoietic stem cell self-renewal.⁶⁹ Therefore, intricate control of the cell cycle machinery is required for self-renewal regulation.

A large body of evidence suggests that suppression of apoptosis is required for hematopoietic stem cell survival. Studies using transgenic mice constitutively expressing BCL2 (B-cell lymphoma 2) in all hematopoietic tissues provided evidence directly supporting this hypothesis. The forced expression of the *Bcl2* oncogene resulted in increased numbers of transgenic hematopoietic stem cells *in vivo* and those cells showed a competitive advantage over wild type cells in competitive reconstitution experiments *in vivo*,^{10;21;22} suggesting that cell death plays a role in regulating the homeostasis of hematopoietic stem cells. Recently, Mcl1 (Myeloid cell leukemia 1), another anti-apoptotic Bcl2 family member, has also been shown to be required for hematopoietic stem and progenitor cell survival.⁷⁰ These results indicate that anti-apoptotic signals may contribute to regulating hematopoietic stem cell numbers.

1.3.4 Signal Transducer and Transcriptional Factors

The Pten (phosphatase and tensin homologue deleted on chromosome ten) tumor suppressor, a modulator of several major signaling pathways, has recently been implicated as a regulator of hematopoietic stem cell self-renewal and an initiator of leukemogenesis. Pten functions by inhibiting signaling through the AKT pathway.^{71;72} Although *Pten* deletion initially led to a transient expansion of hematopoietic stem cell numbers, the hematopoietic stem cell pool in mutant mice become depleted over time. *Pten*-deficient hematopoietic stem cells engrafted normally in recipient mice, but were unable to sustain multilineage hematopoietic reconstitution over time.^{71;72}

Many transcription factors play important roles in regulating hematopoietic stem cell self-renewal. c-Myb can promote the growth of hematopoietic stem cells possibly through the induction of c-myc and the upregulation of c-kit and Flt3.^{73;74} c-Myb deficient mice die at embryonic day 15.5 due to a defect in definitive hematopoiesis.⁷⁵ PcG (polycomb group) genes are involved in the maintenance of cellular memory through epigenetic chromatin modifications. Recent studies have shown that PcG proteins are important in the regulation of hematopoietic stem cell self-renewal, a process in which cellular memory is maintained through cell division. Members of the PRC1 (Polycomb repression complex 1), such as Bmi1 (Bone marrow l-1), rae28 and Mel-18, which form DNA-binding protein complexes with gene-suppressing activity, were shown to be involved in lympho-hematopoiesis.⁷⁶ Among these genes, Bmi1 plays an important role in regulating the proliferative activity of stem and progenitor cells.⁷⁷⁻⁷⁹ Bmi1 was able to enhance symmetrical expansion of the stem cell pool through self-renewal, induce a marked *in vitro* expansion of multipotent progenitors, and increase the ability of stem cells to repopulate bone marrow *in vivo*.⁸⁰ Rae28-deficient hematopoietic stem cells were defective in long-term repopulating ability in serial transplantation experiments.^{81;82} Loss or knockdown of *Mel18* leads to increased expression of *Hoxb4*, and transplanted *Mel18*-deficient bone marrow showed increased hematopoietic stem cell numbers, but a decrease in activity due to arrest in the G₀ phase of the cell cycle.⁸³ Gfi1 (Growth factor independence 1), a Zinc-finger repressor of transcription, has been recently implicated as a regulator of hematopoietic stem cell self-renewal. It was determined that Gfi1 controls self-renewal by restraining stem cell proliferative potential.^{84;85} Gfi1-deficient hematopoietic stem cells displayed increased proliferation rates and were functionally compromised in competitive repopulation and serial transplantation assays. Gfi1 might exert these effects by regulating the cell cycle inhibitor p21^{CIP1/WAF1}, as p21 mRNA expression levels were dramatically lower in the Gfi1-deficient hematopoietic stem cells.⁸⁵ p21^{CIP1/WAF1} itself was also implicated in the regulation of hematopoietic stem cells.⁶⁸ In its absence, hematopoietic stem cells had impaired serial transplantation capacity. Activation of the Stat5 transcription factor in hematopoietic stem cells led to the dramatic expansion of multipotent progenitors and promoted hematopoietic stem cell self-renewal *in vitro*.⁸⁶ Deletion of *Stat5* resulted in profound defects in

hematopoiesis and markedly reduced ability of the mutant cells to reconstitute in lethally irradiated mice.⁸⁷ Transduction of adult mouse bone marrow cells with a constitutively activated form of Stat3 increased their regenerative activity in lethally irradiated recipients, whereas the transduction of these cells with a dominant negative form of Stat3 suppressed their regenerative activity.⁸⁸ These studies suggest that Stat proteins play a role in hematopoietic stem cell self-renewal. The *Hox* (homeobox) genes encode transcription factors that regulate embryonic body patterning and organogenesis. They play important roles in the regulation of hematopoiesis. Overexpression of HOXB4 in bone marrow cells, which we will discuss in further detail, led to dramatic expansion of hematopoietic stem cells *in vivo* and *in vitro*.⁸⁹⁻⁹¹

1.4 Expanding Hematopoietic Stem Cells for Transplantation

Understanding the ability of hematopoietic stem cells to replenish themselves and identifying molecules that promote their expansion *in vitro* is an important clinical goal, since several clinical applications for hematopoietic stem cell expansion could be realized if effective expansion can be achieved.^{3;91} For example, the use of cord blood as a source of hematopoietic stem cells for allogeneic transplantation has been limited by the relatively small number of stem cells in a single harvest.⁹² However, due to their low frequency and the difficulties in culturing stem cells *in vitro*, effective expansion has not yet been achieved. *In vitro* culture systems are insufficient to establish the self-renewing capacity of hematopoietic stem cells in general, and often result in differentiation and apoptosis and thus loss of repopulating activity.⁹¹ Thus, new approaches for the *in vitro* expansion of hematopoietic stem cells are needed.⁹¹ Although progress has been made in identifying conditions that maintain hematopoietic stem cell activity in culture for a brief period of time,⁹³ it has proven difficult to identify combinations of growth factors that cause significant expansion in culture of hematopoietic stem cells. In most cases, combinations of growth factors that can induce proliferation are unable to prevent differentiation of hematopoietic stem cells in long-term cultures.⁹⁴

Assorted signaling molecules and transcription factors have been introduced into hematopoietic stem cells to induce *in vitro* expansion or immortalization of cells. However, many attempts were unsuccessful because of the inadequate expansion or the unintended induction of leukemias. Ectopic expression of the transcription factor HOXB4 has been shown to dramatically increase the numbers of transplantable hematopoietic stem cells both *in vivo* and *in vitro*, and may be the most promising approach.^{90;95}

Genetic manipulation of hematopoietic stem and progenitor cells is one important tool for experimental hematology. Successful generation of hematopoietic cells from embryonic stem (ES) cells could provide safe and histocompatible hematopoietic stem cells.⁹⁶ However, achieving stable long-term

blood engraftment of ES cell-derived hematopoietic stem cells in irradiated mice has proven difficult. To improve the hematopoietic activity of induced hematopoietic cells from ES cells, genetic manipulation was attempted using transcription factors, such as Lhx-2 and GATA-2, or the oncogenic chimeric gene *bcr-abl*. However, these trials produced limited improvement in ES cell-induced hematopoietic activities. In contrast, enforced HOXB4 expression brought about the best results by promoting the generation of expanded hematopoietic stem cells with reconstitution ability.⁹⁷ Considering the multitude of biological and ethical challenges facing the use of human ES cells at present, expansion of hematopoietic stem cells represents a more realistic and feasible goal.⁹⁸

Four factors (*Oct3/4*, *Sox2*, *c-Myc*, and *Klf4*) transferred in the mouse oocyte can reprogram mouse embryonic or adult fibroblast cells into pluripotent stem cells that exhibit the essential characteristics of embryonic stem cells.⁹⁹ Subcutaneous transplantation of these induced pluripotent stem (iPS) cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPS cells contributed to mouse embryonic development. Generation of hematopoietic stem cells from the iPS cells should be useful in the applications in transplantation.

1.5 Characteristics of *Hox* Gene Family Members

Mammalian *Hox* genes encode homeodomain transcription factors which play important roles in embryonic body patterning and organogenesis. These genes were initially identified by homology with fruit fly *Drosophila* Hom genes.^{100;101} In human and mouse, the *Hox* gene family contains 39 members arranged in 4 distinct clusters (A, B, C, D) on different chromosomes (human 7, 17, 12 and 2; mouse 6, 11, 15, and 2 respectively) and 13 paralog groups (Figure 1-3).¹⁰²⁻¹⁰⁴ The 3' to 5' chromosomal organization of the genes in each *Hox* cluster corresponds to their anterior-posterior axis expression pattern in the body, that is, 3' genes affect development of cephalic structures and 5' genes control caudal organs.¹⁰² *Hox* genes also show temporal colinearity in expression during development from anterior to posterior direction. The 3' genes are expressed earlier than 5' genes. In general, members of the same homology group are expressed at the same time and have the same anterior boundary of expression.¹⁰³ This orderly activation of *Hox* genes is also essential for normal hematopoietic development.¹⁰⁵ The *Hox* genes we described here belong to the class I group. There is another group of genes other than *Hox* genes in the homeobox gene family, and they are named as nonclustered or divergent homeobox genes.^{106;107} The divergent homeobox genes are scattered throughout the genome and classified into different small groups based on the similarity in their sequence. In this dissertation, we only focus on the class I, clustered homeobox genes (*Hox* genes).

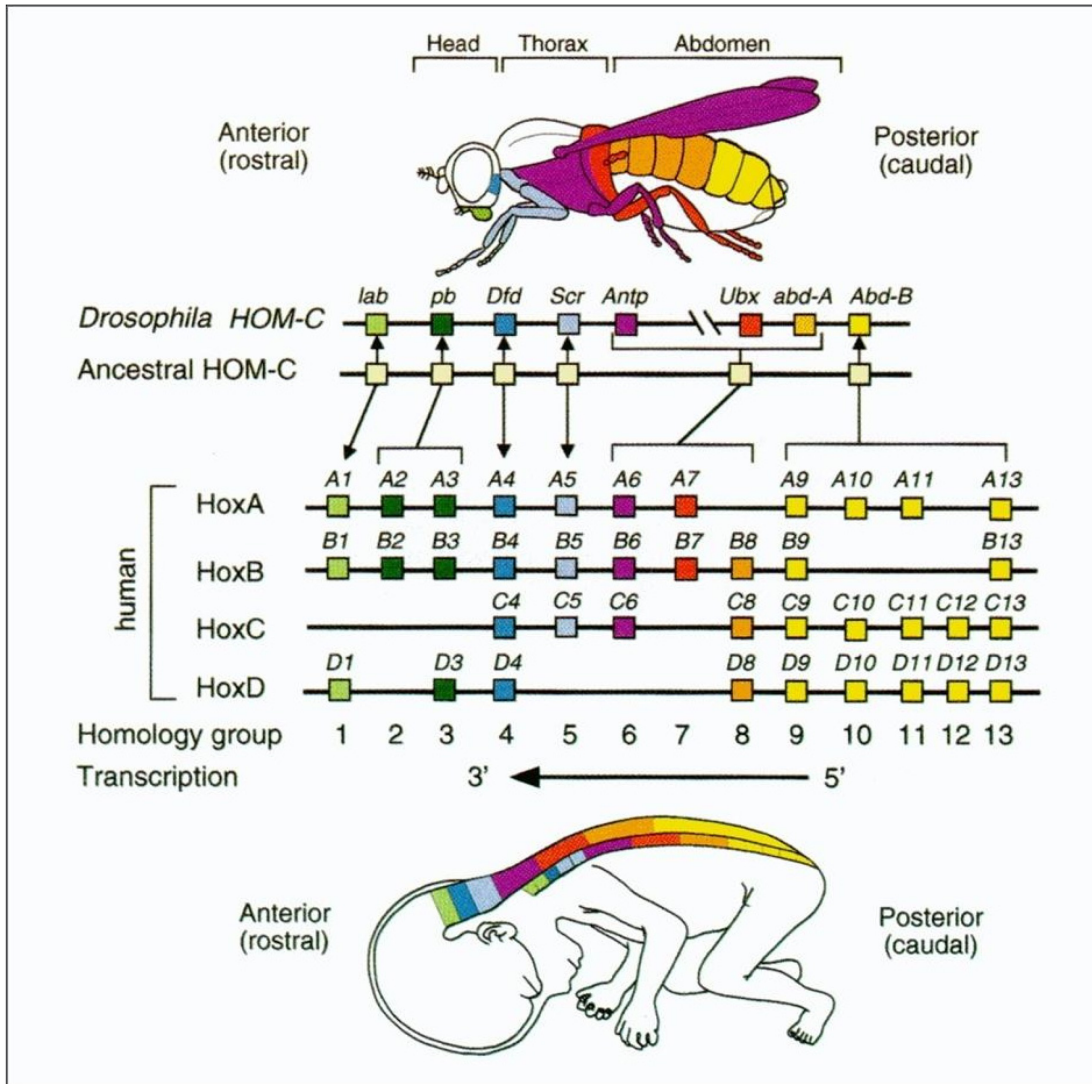


Figure 1-3. Genomic organization and collinear expression patterns of *Drosophila* Hom genes and mammalian Hox genes.

Reprinted with permission from Pediatric Research. Mark M, Rijli FM, Chambon P. Homeobox genes in embryogenesis and pathogenesis. *Pediatr.Res.* 1997;42:421-429.

1.5.1 Functions of Hox Family Members in Hematopoiesis

The Hox family members of transcription factors are functionally involved in both normal and leukemic hematopoiesis. Genes 3' in the *Hox* cluster are maximally expressed in hematopoietic stem cells; in contrast, genes 5' in the cluster from *Hox7* to *Hox11* are maximally expressed in more committed hematopoietic progenitor cells.¹⁰⁵ Furthermore, expression of *Hox7* to *Hox11* decreases during differentiation but is maintained in mature blood cells.¹⁰⁵ Perturbation of orderly *Hox* gene expression can result in hematologic abnormalities.¹⁰⁸ Elevated expression of HOX7-11, HOXB6 and HOXB8 has been reported in human acute myeloid malignancies.¹⁰⁹⁻¹¹² Disregulation of these Hox proteins in mice also causes hematological abnormalities.^{113;114} *Hox* genes are also involved in chromosomal translocations seen in human leukemias. For instance, the NUP98 gene at chromosome band 11p15 can undergo chromosomal translocation and be fused to a number of hox family members including HOXA9, HOXA11, HOXA13, HOXC11, HOXC13, HOXD9, HOXD11 and HOXD13 in various hematological malignancies such as myelodysplastic syndromes and acute myeloid leukemia.¹¹⁵⁻¹¹⁷

The functions of several *Hox* genes, especially those that are highly expressed in immature hematopoietic cells (e.g. *Hoxa9*, *a10*, *b3* and *b4*), have been studied extensively. For example, overexpression of *Hoxb3* impairs lymphoid development but enhances myeloid development.¹¹⁸ HOXB4 enhances the *in vivo* repopulating activity of hematopoietic stem cells.⁹⁵ HOXB4 also induces a 40-fold *in vitro* expansion of mouse hematopoietic stem cells in cultures containing IL-3, IL-6 and SCF.⁹⁰ HOXB4 enhances hematopoietic stem cell proliferation or the probability of self-renewal, although the gene is not leukemogenic. The genes of trans-paralogs which occupy the same relative location along the 3' to 5' axis along the chromosomes are named with same number, and they share a relatively high degree of sequence.¹⁰⁴ Thus, it is suggested that paralogous genes can act as redundant factors with similar functions. For example, *Hoxa3* and *Hoxd3* provide equivalent function in gene swapping experiments.¹¹⁹ Another example is *Hoxa4*, *Hoxb4*, *Hoxc4* and *Hoxd4*.¹²⁰ Overexpression of *Hoxc4*, a paralog of *Hoxb4*, also induces *in vitro* expansion of human immature hematopoietic progenitors.¹²¹ These findings suggest the importance of the four paralog groups of *Hox* genes for hematopoietic stem cell expansion. Although there are no obvious hematological abnormalities in mice deficient in *Hoxb4* alone, hematopoietic stem cells and progenitor cells deficient in *Hoxb3* and *Hoxb4* displayed mildly impaired activity both *in vitro* and *in vivo*.¹²²

1.5.2 Biochemical Characteristics of HOXB4 and Other Hox Family Members

Mammalian *Hox* genes usually have relatively small coding sequences with only two exons and a single intron. All contain a highly conserved 60 amino acid common homeobox domain (homeodomain) which contains a helix-turn-helix DNA binding motif encoded by the second exon of *Hox* genes.¹¹¹ In HOXB4, the homeodomain contains 3 α -helices (Figure 1-4A), among which, helix 3 contacts the major groove of DNA recognizing a conserved "TAAT" core. Helix 1 and 2 do not contact the DNA but stabilize the protein-DNA interaction. The flexible N-terminal domain located just adjacent to helix 1 in the homeodomain extends from the packed helical structure and interacts with the minor groove of DNA to recognize 5' and 3' flanking sequences adjacent to the core consensus sequence.^{123;124} There is a c-terminus acidic domain and a conserved YPWM region upstream of homeodomain in *Hox* proteins. The YPWM region binds to TALE (three amino acid loop extension) transcription factor family members which act as cofactors of *Hox* to increase DNA binding specificity and activity of *Hox*-cofactor complexes.¹²⁵⁻¹²⁸

Functional specificity of *Hox* proteins depends on a number of factors including their specific expression pattern, the particular interacting proteins present in a cell, and the specificity of the particular homeodomain region. Due to the high degree of homology within the homeodomain, a core DNA sequence "TAAT" is recognized and bound by a majority of *Hox* proteins,¹²⁸ raising the question of how the transcriptional and thus functional specificity of these *Hox* family members is realized. *Hox* proteins can elicit distinct and variable effects on downstream target genes to regulate variety aspects of developmental fates. Indeed, the DNA binding specificity, affinity and transregulating potential of a specific *Hox* protein can be modified by the flanking DNA sequence or interaction with cofactors.¹²⁹⁻¹³¹ *Hox* family members bind to their specific preferred DNA sequence as monomers or as heterodimers with other cofactors (Figure 1-4B), although some deficiencies are thought to exist in monomer binding activity of *Hox* proteins to DNA. DNA binding affinity of *Hox* proteins can also be regulated by posttranslational modifications. For example, PKC (protein kinase C) - dependent serine and threonine phosphorylation can decrease the binding affinity of *Hoxa9* to its specific DNA consensus sequence.¹³² Phosphorylation of conserved homeodomain tyrosine residues decreases the DNA binding ability of *Hoxa10* to some DNA sequences, but increases the binding affinity of *Hoxa9* to other DNA sites.^{133;134} *Hox* proteins form heterodimers with *Pbx* or *Meis* members of the TALE family of transcription factors (Figure 1-4B).¹³⁵ *Hox* homologs 1 to 10 can cooperatively bind to DNA through interaction with *Pbx* in the core YPWM residues 5' upstream of homeodomain of HOXB4. *Hox* homologs 9 to 13 can directly interact with *Meis*.¹³⁶ *Hox* homologs 9 and 10 can interact with both *Pbx* and *Meis*, and functional trimeric interactions between them have been reported.¹³⁷ Interaction with these TALE family cofactors is hypothesized to increase the DNA binding ability of some *Hox* family members to their target

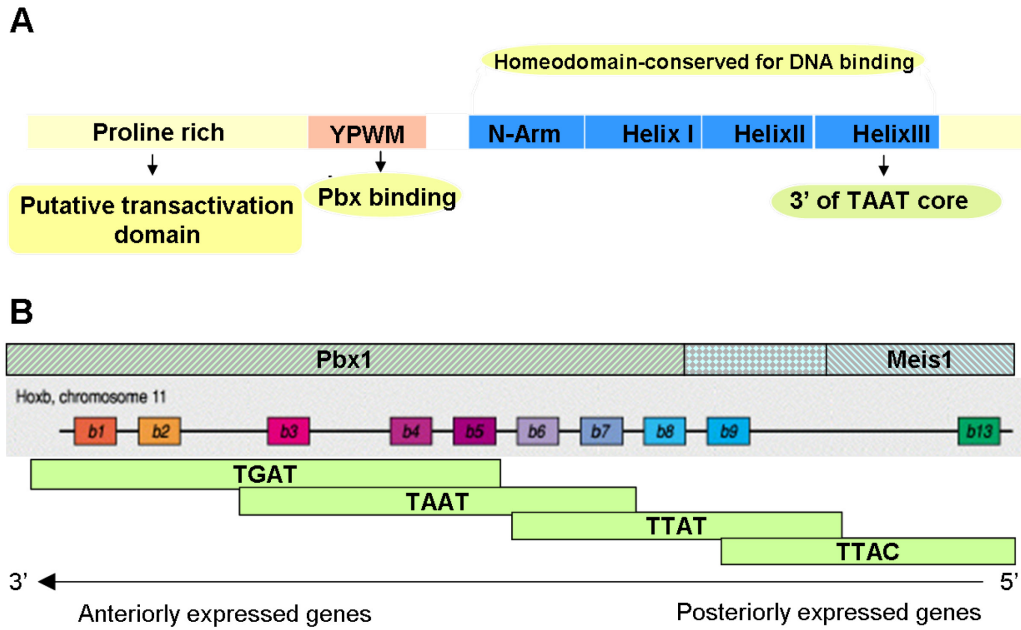


Figure 1-4. Biochemical characteristics of Hox proteins. (A) Functional domains of HOXB4. (B) Cofactors and DNA binding specificity of Hox family members.

genes.¹³⁸ In addition, the transcriptional specificity of Hox proteins also lies in their ability to act both as transcriptional activators and repressors in different conditions. The complexity of Hox regulation of downstream target genes is underscored by the observation that they can regulate different target genes either dependent or independent of TALE family cofactors.^{133;139}

It is known that HOXB4 regulates hematopoietic stem and progenitor cell expansion through a transcription-dependent mechanism.¹⁴⁰ A HOXB4 mutant incompetent for DNA binding failed to cause the proliferation of transduced bone marrow *in vitro* and hematopoietic stem cell expansion *in vivo*.¹⁴⁰ Similar to other Hox family members, HOXB4 contains a homeodomain that is required for DNA binding. In this homeodomain, the helix 3 region is essential for recognizing and interacting with the consensus DNA regulatory element containing “TAAT” or “ATTA” core sequence in the promoters of Hox target genes. *In vitro* studies have proven that HOXB4 has preference for specific flanking nucleotides (Figure 1-5).¹²⁹ HOXB4 can cooperatively bind to 5’ TGATTAAT 3’ sequences with its cofactor Pbx through its “YPWN” residues. However, it was demonstrated that a HOXB4 mutant that cannot bind Pbx was functional and caused *in vitro* proliferation and elevated *in vivo* regenerative potential in bone marrow cells.¹⁴⁰ The proline-rich N-terminus region of HOXB4 is conserved in other proteins such as p53, AP2, HOXD4 and HOXA13, in which it functions as a transactivation domain.

1.5.3 Hox Downstream Target Genes

Hox transcription factor family members can act as key masters for controlling cellular identity and play critical roles in controlling embryogenesis and organ development. Although much is now known about their regulation and function, very little is known of their effector genes. Recent studies have focused on identifying their common or unique downstream target genes to facilitate understanding of their functions, especially in the process of normal and malignant hematopoiesis. Such understanding may provide means for hematopoietic stem cell engineering approaches to hematopoietic. For the purpose of identifying downstream target genes, various approaches have been taken including high throughput genetic screens, in which, the gene expression profiles of cells overexpressing specific Hox proteins are analyzed.

A small number of Hox target genes have been identified in different biological systems that play roles in cellular identity and function. However, the experimental procedures used for identification of each target gene sometimes make it difficult to determine whether they are truly direct targets. For instance, for some direct targets, the DNA sites bound by Hox factors have not been confirmed experimentally. A summary of identified Hox target genes which are involved in a variety of cellular processes have been reported.¹⁴¹ In the list outlined in this review,¹⁴¹ the identified target genes of Hox proteins participate in

Nucleotides preferred 5' Nucleotide preferred 3'
A, G > C, T C > G, T > A **TAAT** T, G > A > C G, A > T, C

Figure 1-5. HOXB4 has preference for the nucleotides flanking the “TAAT” core binding sequence.

Source: Pellerin I, Schnabel C, Catron KM, Abate C. Hox proteins have different affinity for a consensus DNA site that correlate with the positions of their genes on the hox cluster. Mol.Cell Biol. 1994;14:4532-4545.

organogenesis and cellular differentiation, cell adhesion and migration, cell cycle and apoptotic pathways.

The TALE class homeodomain transcription factor *Irx5* and the small GTPase *Rap1* were reported to be regulated by *Hoxb4* in development.^{142;143} However, no specific target genes of *HOXB4* have been identified in hematopoietic cells until recently. There is only one report that addresses the targets of *HOXB4* in hematopoietic cells.¹⁴⁴ In this report, the downstream pathways ectopically expressed *HOXB4* were identified in adult and embryo-derived hematopoietic stem cells. Gene expression profiling assays were performed in combination with functional assays using enriched adult hematopoietic stem cells and embryonic derivatives overexpressing an inducible form of *HOXB4*. A set of overlapping genetic changes was generated from these systems and selected to represent common targets of *HOXB4* in embryonic and adult hematopoietic stem cells.¹⁴⁴ In functional assays, *HOXB4* activity was found to protect adult hematopoietic stem cells from the detrimental effects of the proinflammatory cytokine *TNF- α* , which likely contributes to the competitive repopulation advantage of *HOXB4*-overexpressing cells *in vivo*.¹⁴⁴ It was also demonstrated that *HOXB4* activity and *FGF* signaling were intertwined, since *HOXB4*-mediated expansion of adult and ES cell-derived hematopoietic stem cells was enhanced by inhibition of *FGF* receptors.¹⁴⁴ In this report, however, no evidence was provided to prove that any of the target genes was a direct transcriptional target of *HOXB4*. Instead, their data suggest that multiple pathways downstream of *HOXB4* could be primary or secondary changes related to *HOXB4* overexpression.

1.6 Role of *HOXB4* in Hematopoietic Stem and Progenitor Cell Expansion

Hematopoietic stem cells or repopulating cells are able to self-renew and differentiate into cells of all hematopoietic lineages, and because of this unique property, hematopoietic stem cells have been used for stem cell transplantation and gene therapy applications. However, the inability to expand hematopoietic stem cells has been a significant limitation for clinical applications. To date, the most successful hematopoietic stem cell expansion method involves the overexpression of the *HOXB4* gene.^{145;146}

1.6.1 Overexpression of *HOXB4* In Vivo

Retroviral-mediated overexpression of *HOXB4* enhances hematopoietic repopulation, through the increased self-renewal of hematopoietic stem cells.^{90;95;147} Serial transplantation studies revealed a greatly enhanced ability of *HOXB4*-transduced bone marrow cells to regenerate the most primitive hematopoietic stem cell compartment, resulting in 50 fold higher numbers of transplantable totipotent hematopoietic stem cells in primary and secondary recipients (Figure 1-6).^{95;145} After transplantation into primary recipient mice,

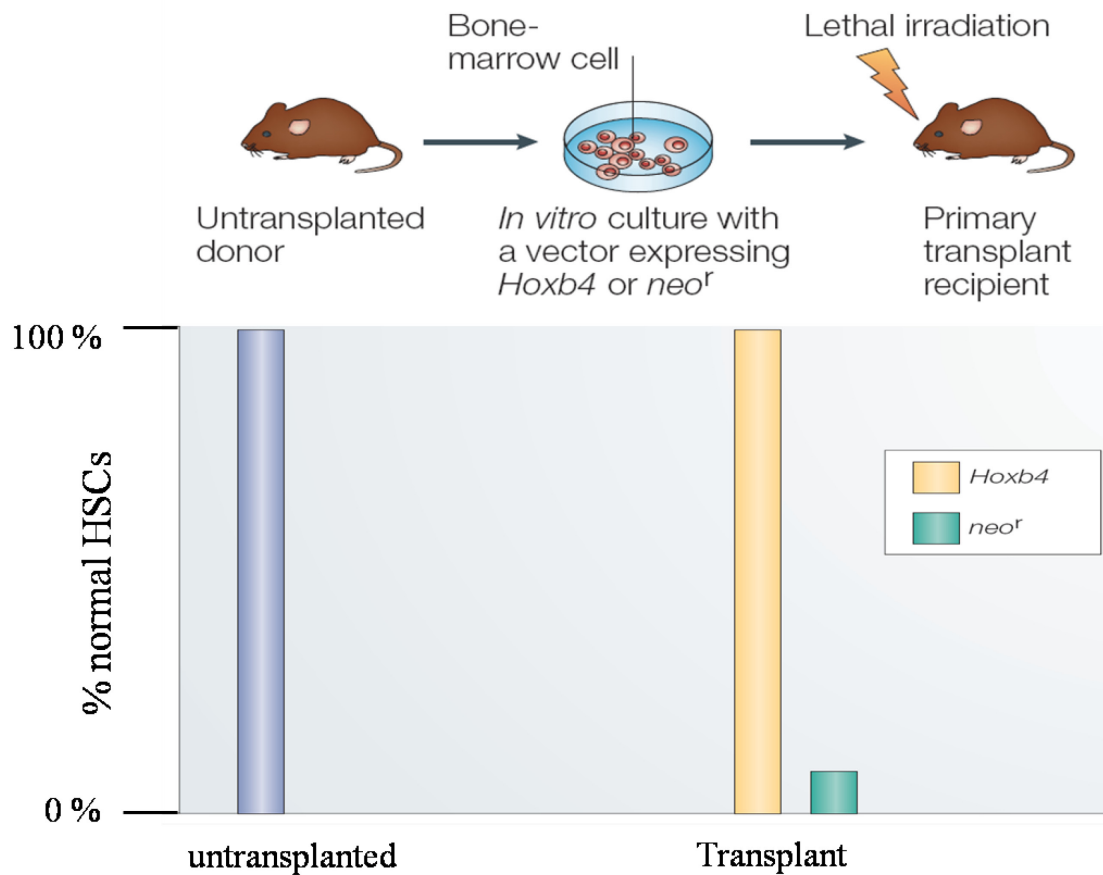


Figure 1-6. HOXB4 induces expansion of hematopoietic stem cells *in vivo*.

Reprinted with permission from Nature Publishing Group. Sorrentino BP. Clinical strategies for expansion of haematopoietic stem cells. *Nat.Rev.Immunol.* 2004;4:878-888.

there was a rapid regeneration to normal pretransplantation levels in the numbers of hematopoietic progenitors and mature blood cells in the mice transplanted with control vector-transduced bone marrow cells, whereas hematopoietic stem cell numbers recovered to only 5% to 10% of normal levels. This finding demonstrates that hematopoietic stem cells were significantly restricted in their self-renewal activity during the process of hematopoietic repopulation (Figure 1-6).¹⁴⁷ In contrast, HOXB4 vector-transduced cells were capable of fully reconstituting the stem cell compartment, and resulted in a 14 fold more hematopoietic stem cells than the control-transduced bone marrow cells in primary transplant recipients. Furthermore, HOXB4-overexpressing hematopoietic stem cells did not expand above the normal level observed in unmanipulated mice, indicating that its overexpression did not override the regulatory mechanisms that maintain normal hematopoietic stem cell pool size.¹⁴⁷

In addition, recovery of stem cell numbers in HOXB4 recipients was reported to be extremely rapid, reaching 25% of normal by 14 days posttransplant, some 80 fold greater than that seen in control transplanted recipients.⁸⁹ The heightened *in vivo* expansion of HOXB4-overexpressing hematopoietic stem cells was not associated with identifiable anomalies in the peripheral blood of the transplanted mice.^{95;145;146} In a clinically relevant nonhuman primate model, HOXB4 overexpression in CD34⁺ cells was shown to dramatically promote expansion and engraftment of short-term repopulating cells and a significant, but less pronounced, expansion of long-term repopulating cells.¹⁴⁸

1.6.2 Overexpression of HOXB4 In Vitro

In addition to its function in promoting stem cell expansion *in vivo*, HOXB4-overexpression also confers stem cell expansion in bone marrow cell cultured *in vitro*. Cultures of HOXB4-transduced bone marrow cells resulted in rapid, extensive, and highly polyclonal hematopoietic stem cell expansion, with over 1000 fold greater numbers of stem cells relative to control vector-transduced cells, which instead underwent stem cell loss during a 14 day culture period with a corresponding 40 fold net stem cell decrease relative to input levels (Figure 1-7).⁹⁰ Importantly, those *in vitro* expanded cells retained full lympho-myeloid repopulating ability and enhanced regenerative potential *in vivo*, indicating the feasibility of achieving *in vitro* expansion of hematopoietic stem cells without functional impairment *in vivo*.⁹⁰ Enhanced proliferation of day 12 CFU-S and immature mixed clonogenic progenitors *in vitro* was also documented in HOXB4-overexpressing bone marrow cells.^{95;149}

The cellular effects and mechanisms of HOXB4-induced cell expansion were studied *in vitro*. HOXB4-overexpressing bulk bone marrow cells were reported to show rapid dominance in mixed culture and shortened population doubling time *in vitro*.⁸⁹ HOXB4 overexpression *in vitro* resulted in a stabilization of the

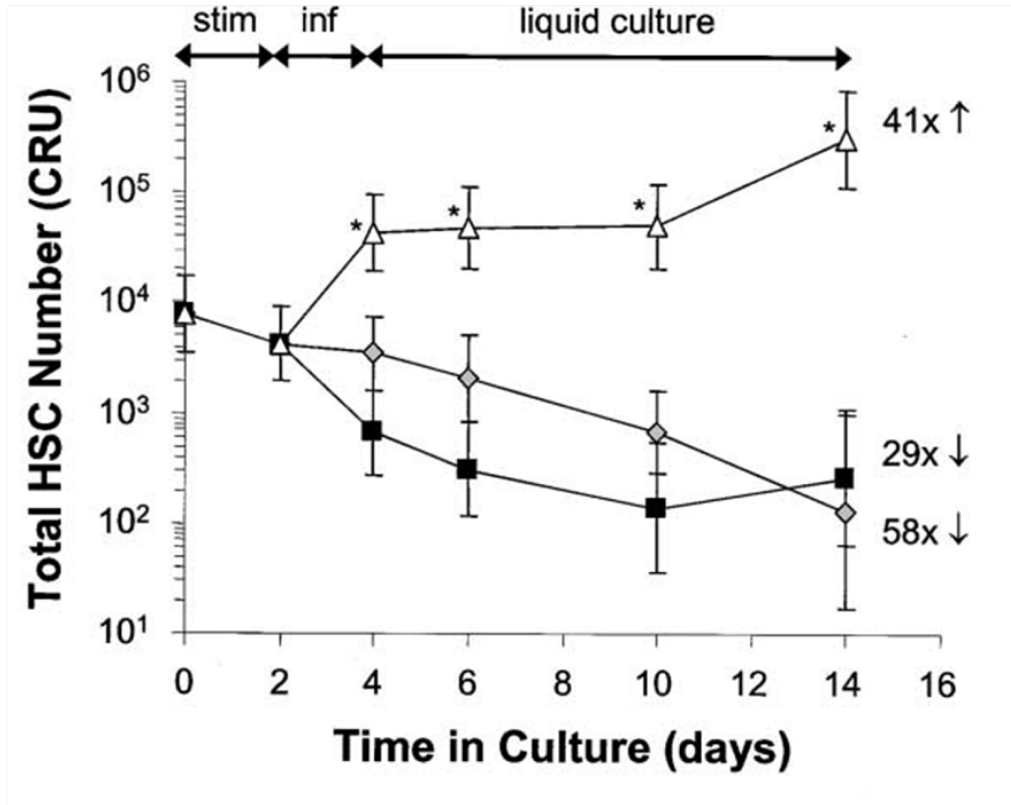


Figure 1-7. HOXB4 induces expansion of hematopoietic stem cells *in vitro*.

Reprinted with permission from Elsevier. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells *in vitro*. *Cell* 2002;109:39-45.

proliferation rate, while the proliferation rate of control-transduced bone marrow cultures gradually declined.¹⁵⁰ In addition, HOXB4-overexpressing bone marrow cells displayed a more homogenous morphology and contained increased numbers of hematopoietic progenitor cells capable of generating primitive colonies *in vitro*. In contrast, control-transduced bone marrow cells in long-term cultures showed hallmarks of myeloid differentiation and a reduced secondary colony forming activity.¹⁵⁰

1.6.3 ES Cell Specification

HOXB4 also plays a role in the specification of hematopoiesis at the earliest stages of development by enhancing the hematopoietic differentiation potential of ES cells.^{146;151} Overexpression of HOXB4 in murine ES cell-derived primitive progenitors combined with culture on hematopoietic stroma cells induced a switch to the definitive hematopoietic stem cell phenotype.¹⁴⁶ The induction of differentiation was initiated with EB (embryoid body) formation, and subsequent differentiation and maintenance of hematopoietic cells was achieved by co-culture with OP9 cells.¹⁴⁶ The transient overexpression of HOXB4 for only several weeks in hematopoietic progenitor cells initiated conversion of progenitor cells to immature transplantable stem cells.¹⁴⁶ Leukocytes derived from ES cells ectopically expressing HOXB4 were immunologically functional, implying the potential use of ES cell-derived hematopoietic progenitor cells in the treatment of hematologic and immunologic diseases.¹⁵² These results provide new evidence implicating HOXB4 in the control of very early development of the hematopoietic system.

1.6.4 Potential Toxicity of HOXB4 Overexpression

Ectopic HOXB4 expression was reported to have a concentration-dependent effect on lineage differentiation in mouse and human hematopoietic cells. The existence of threshold levels were suggested for HOXB4 activity that control the differentiation and self-renewal behavior of hematopoietic stem and progenitor cells.¹⁵³ Enforced HOXB4 expression caused a significant reduction of erythroid and myeloid progenitors and B lymphoid compartment *in vitro* and *in vivo* when using a strong viral promoter for expression.¹⁵⁴ Transducing CD34⁺ cells from large animals including nonhuman primates, dogs, and humans with HOXB4-retroviral vectors can cause increased colony formation ability in dog cells compared to human peripheral blood, human cord blood, and baboon cells.¹⁵⁵ High levels of HOXB4 overexpression resulted in immortalization of primitive hematopoietic cells from mice and dogs; in contrast, the cells from monkeys and humans were not immortalized due to the relative low expression level of HOXB4.¹⁵⁵ In differentiated ES cells ectopically expressing HOXB4 *in vitro*, myeloid development can be enforced and T lymphoid development can be suppressed over a wide range of expression levels of HOXB4.¹⁵⁶ These findings

suggest that the growth promoting effects of HOXB4 are critically dependent on its expression levels, which can result in important species-specific differences in potency. Based on these facts, the therapeutic window of HOXB4 expression levels must be carefully defined before initializing clinical trials.

Retroviral vector-mediated insertional mutagenesis can occur with HOXB4 overexpression. Hematopoietic stem cell gene therapy has been used to treat patients with a number of life-threatening diseases. However, some patients have developed retroviral vector-mediated leukemia after treatment.¹⁵⁷ Myeloid leukemia was reported to occur in large animals (dogs and macaques) 2 years after transplantation with HOXB4-overexpressing cells, but not in control retroviral vector transplanted large animals.¹⁵⁷ However, based on the activation of nearby genes known to be involved in leukemogenesis, the integration sites observed in the leukemic clones containing HOXB4 were very likely a combination consequence of sustained *in vivo* proliferation through the activated oncogenes and expansion effects by HOXB4.¹⁵⁷

When using short hairpin RNAs to knockdown HOXB4 expression in a cell line from the leukemic cells, profound growth inhibition and cell death occurred with downregulation of the *HOXB4* transgene. This evidence indicates that HOXB4 overexpression using retroviral vectors can pose a significant risk of leukemogenesis in large animals. Thus, safety issues related to HOXB4-mediated gene therapy must be considered. Newer vectors based on lentivirus are now available that may circumvent this limitation.¹⁵⁸

1.6.5 *Hoxb4* Knockout and Other Hox-Deficient Mice

As overexpression of HOXB4 leads to dramatic hematopoietic phenotypes, it is a surprise that *Hoxb4*^{-/-} mice show only a defect in mild hematopoietic stem cell proliferation.¹⁵⁹ In *Hoxb4*^{-/-} mice, the cellularity in spleen and bone marrow, red blood cell counts, and hemoglobin values were minimally reduced. However, the hematopoietic lineage distribution was normal and in bone marrow and fetal liver, only a mild reduction of hematopoietic stem and progenitor cell numbers were found.¹⁵⁹ The conclusion from this study was that *Hoxb4* is not required for generation of hematopoietic stem cells and steady state maintenance of hematopoiesis.¹⁵⁹ c-Kit⁺ fetal liver cells from *Hoxb4*^{-/-} mice expressed higher levels of several other *Hoxb* cluster genes than control wild type cells by quantitative PCR assay, indicating that normal hematopoietic stem cell activity in *Hoxb4*^{-/-} mice might be due to a compensatory upregulation of other *Hox* genes. In order to determine whether this hypothesis is true, the entire *Hoxb* cluster was deleted.¹⁶⁰ However, again, this did not lead to major defects in hematopoiesis.¹⁶⁰ *Hoxb1-b9*^{-/-} fetal liver cells retained full competitive repopulation potential and the ability to regenerate all myeloid and lymphoid lineages.¹⁶⁰ The gene expression profile in purified c-Kit⁺ *Hoxb1-b9*^{-/-} fetal liver cells revealed expression changes in *Hoxa4*, *a11*, and *c4*. Primitive

c-kit⁺Scal1⁺Lin⁻ hematopoietic stem and progenitor cells lacking Hoxb3 and Hoxb4 also displayed impaired proliferative capacity *in vitro* and *in vivo*.¹²² As no defects in homing were observed, a slower regeneration of mutant hematopoietic stem cells was suggested. Furthermore, treatment with cytostatic drugs demonstrated slower cell cycle kinetics of hematopoietic stem cells deficient in Hoxb3 and Hoxb4, resulting in increased tolerance to antimetabolic drugs.

In the *Hoxa9*^{-/-};*b3*^{-/-};*b4*^{-/-} triple deficient mice, the reconstitution defect in *hoxa9* deficient hematopoietic stem cells was not enhanced further in the triple deficient cells. Therefore, none of these hox proteins was proved to play absolutely essential role for generation or maintenance of all major blood lineages.¹⁶¹ Some other Hox knockout mice have been reported to show defects in embryonic development. *Hoxa4*^{-/-} or *Hoxa6*^{-/-} mice showed homeotic transformation of cervical vertebrae in development. However, defects were not observed in other tissues that normally express these genes.¹⁶² *Hoxc4* is a mouse homeobox gene located at the 3' end of the HoxC cluster. Of the Hoxc genes, *Hoxc4* is expressed in the most anterior regions of the central nervous system and prevertebral column. *Hoxc4*^{-/-} mice were viable and fertile, and various abnormalities in the cervical and thoracic regions were documented.¹⁶³ However, no studies have been performed on the hematopoietic system yet. Similarly, *Hoxd4*^{-/-} mice also had defects in skeleton development with homeotic transformations of the second cervical vertebrae (C2) to the first cervical vertebrae (C1) and malformations of the neural arches of C1 to C3 and of the basioccipital bone.¹⁶⁴ These phenotypic similarities and differences between *Hoxd4* and previously reported *Hoxa4*, *Hoxb4* and *Hoxc4* deficient mice suggest that Hox gene paralogs have both redundant and unique functions.

Pbx1, described above as a cofactor of Hox, is a homeodomain transcription factor that was originally identified as the product of a proto-oncogene in acute pre-B-cell leukemia, and is also a global regulator of embryonic development. Pbx1 and a subset of homeodomain proteins collaboratively bind to DNA as molecular complexes with potential consequences for mammalian development. Due to embryonic lethality in its absence, caused by severe hypoplasia or aplasia of multiple organs and widespread patterning defects of the axial and appendicular skeleton,^{165,166} *Pbx1*^{-/-} embryonic stem cells were studied instead and failed to generate common lymphoid progenitors, resulting in a complete lack of B and NK cells, and a partial impairment of T-cell development in transplanted mice.¹⁶⁵ B-cell development was rescued from common lymphoid progenitors following restoration of Pbx1 expression in its deficient ES cells. This critical functions of Pbx1 occurred at a stage between hematopoietic stem cell development and B cell commitment as one earliest-acting transcription factor to regulate de novo B lineage lymphopoiesis.¹⁶⁵

With the information generated from Hox deficient mice, it seems like no single Hox gene plays an essential physiological role in hematopoiesis maintenance or hematopoietic stem cell generation. Instead, a redundant network of genetic

interactions between different Hox genes in primitive hematopoietic cells is most likely. It was recently reported that Hoxb4 and its other three paralogs: Hoxa4, Hoxc4 and Hoxd4 had the similar effect in promoting hematopoietic stem and progenitor cells expansion *in vitro*.¹⁶⁷ Our lab also demonstrated that when overexpressed in bone marrow cells, HOXA4, B4, C4 and D4 showed similar effect in promoting engraftment in the transplanted mice (Figure 1-8). This redundancy may explain the mild defect phenotype in *Hoxb4*^{-/-} mice,¹⁵⁸ and suggests that Hox4 paralogs may be required in hematopoiesis and hematopoietic stem cell functions.

1.6.6 Mechanisms of HOXB4 Function

Although it is well-known that enforced expression of HOXB4 can induce stem cell self-renewal and expansion. Relatively little is known about the molecular mechanisms by which this occurs. Key domains in the HOXB4 protein were mutated to investigate their roles in protein function and stem cell expansion.¹⁴⁰ The *in vitro* and *in vivo* expansion capacity of DNA-binding HOXB4 mutants was completely lost, indicating that HOXB4 must bind DNA in order to promote self-renewal.¹⁴⁰ In contrast, a point mutation in “YPWM” motif, which disrupts Pbx cofactor binding, still conferred a pronounced *in vitro* expansion advantage in transduced bone marrow populations, and those cells also showed dramatically enhanced regenerative potential *in vivo*.¹⁴⁰ This finding suggests that the capacity of HOXB4 to induce hematopoietic stem and progenitor cell expansion is DNA-binding dependent but does not require direct Hox and Pbx interaction. In another report, when *Pbx* expression was knocked down in HOXB4 overexpressing bone marrow cells, the hematopoietic stem cell generation ability was >20 times more competitive than the wild type cells overexpressing HOXB4.¹⁶⁸ Furthermore, the myeloid and lymphoid lineage differentiation activity and the hematopoietic stem cell pool were normal *in vivo* in mice transplanted with *Pbx* knocked down cells.¹⁶⁸ This result suggests the existence of a distinct mechanism that limits the expansion of HOXB4 transduced hematopoietic stem cells in mice.^{168;169}

The specific mechanisms by which HOXB4 promotes hematopoietic stem cell expansion are still a mystery. No direct targets of HOXB4 have been defined *in vivo* or even in primary cells so far. Although as discussed above, the TNF- α pathway was recently shown to be downstream of HOXB4, using gene expression profiling in long-term cultured bone marrow cells. However, it is unclear whether any of the genes on this pathway are direct transcriptional targets of HOXB4.¹⁴⁴

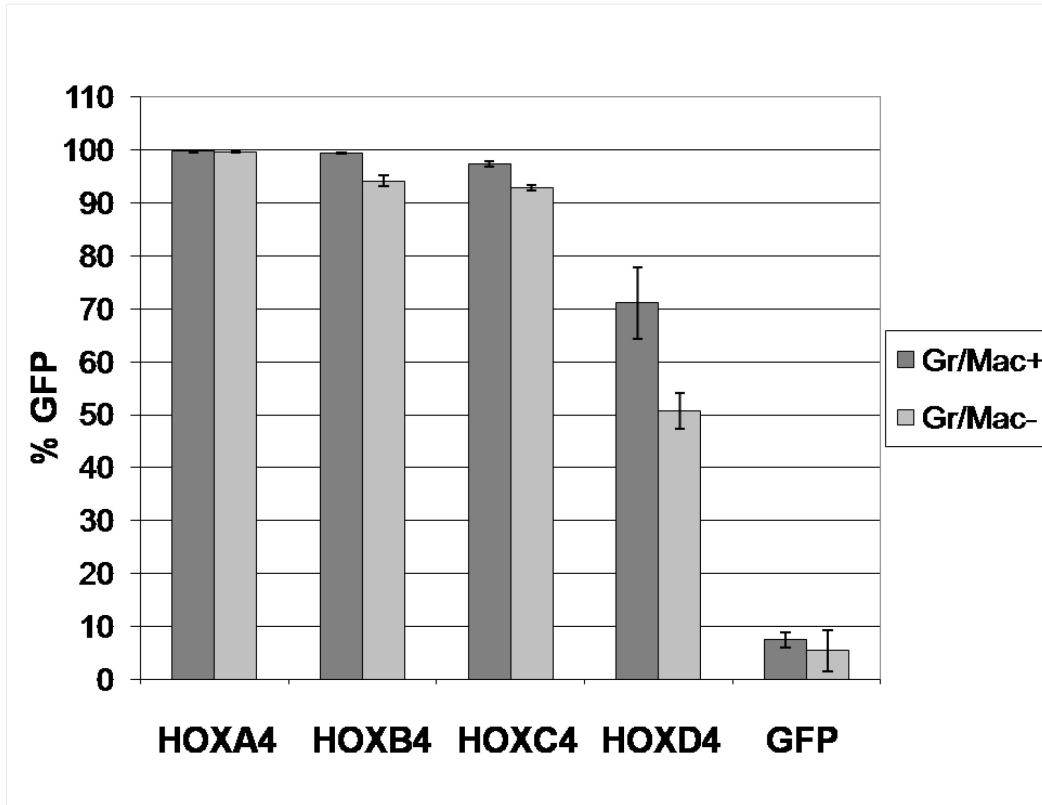


Figure 1-8. Redundancy of HOX4 paralogs in promoting hematopoietic stem and progenitor cell expansion. Flow cytometry-detected GFP percentage of the peripheral blood cells from 12 weeks-transplanted mice with bone marrow cells transduced by MSCV-HOX4-IRES-GFP or MSCV-IRES-GFP control vectors. Gr and Mac: markers for rapid turnover myeloid cells.

Source: Unpublished data received from Brian.P. Sorrentino's lab on October 1st, 2009.

1.7 Hemgn

1.7.1 Expression Pattern and Biochemical Characteristics of Hemgn

In my study, I have focused on a new target gene of HOXB4 in hematopoietic cells called Hemogen (Hemgn) or EDAG. Hemgn is a recently discovered nuclear protein and expresses in testis and hematopoietic tissues; specifically, it is highly expressed in hematopoietic stem and progenitor cells and is downregulated as hematopoietic cells differentiate.¹⁷⁰ The expression of Hemgn during embryogenesis shows a spatial and temporal pattern corresponding to the ontogeny of hematopoiesis. The expression switches from yolk sac blood islands, to fetal liver hematopoietic cells during embryogenesis to bone marrow and spleen in adult mice.¹⁷⁰ In adult mouse tissues, Hemgn mRNA expression is restricted to spleen and bone marrow cells (Figure 1-9A). In bone marrow cells, relatively high levels of Hemgn mRNA were detected in Lin^{lo}c-kit⁺ Scd1⁺, CD34⁺, and Lin⁻ hematopoietic stem and progenitor cell populations (Figure 1-9B).¹⁷⁰ In adult mouse spleen, Hemgn was expressed specifically in the erythrocytes red pulp regions but not in the white pulp which contains B and T lymphocytes (Figure 1-9C).¹⁷⁰ Immunostaining studies showed that Hemgn was localized in nuclei and absent in the cytoplasm (Figure 1-9D).¹⁷⁰ Moreover, hemgn is also expressed in testis in a variant splice form using an alternative promoter, suggesting a complex transcriptional regulation.¹⁷¹

A human homolog of *Hemgn*, *HEMGN*, also called *EDAG*, maps to chromosome 9q22, which contains a cluster of important genes involved in hematopoiesis. The expression pattern of EDAG is similar to its mouse homolog.¹⁷² Like Hemgn, EDAG exhibited specific expression in human hematopoietic cells in the adult bone marrow and fetal liver. No EDAG transcripts were detected in peripheral blood cells or other non-hematopoietic tissues, such as adult liver, heart, brain, skeletal muscle, kidney, spleen, pancreas, tonsil, and colon.¹⁷²

Sequence analysis shows that Hemgn and EDAG contain a nuclear localization motif and a coiled-coil domain, but no other recognizable protein motifs. At the nucleotide level, human and mouse Hemgn homologs show 43% identity, although the sequence conservation between the nuclear localization signal and the coiled-coil domains are higher. Coiled-coil domains of a protein are protein-protein interaction domains containing multiple α -helices twisting around each other to form a supercoil. A protein containing coiled-coil domains can usually interact with other coiled-coil proteins for protein-protein recognition and interaction.

Based on the nuclear localization of EDAG, the role of EDAG as a transcription factor has been investigated.¹⁷³ Using an yeast and mammalian two-hybrid system, it was suggested that that EDAG has transactivation

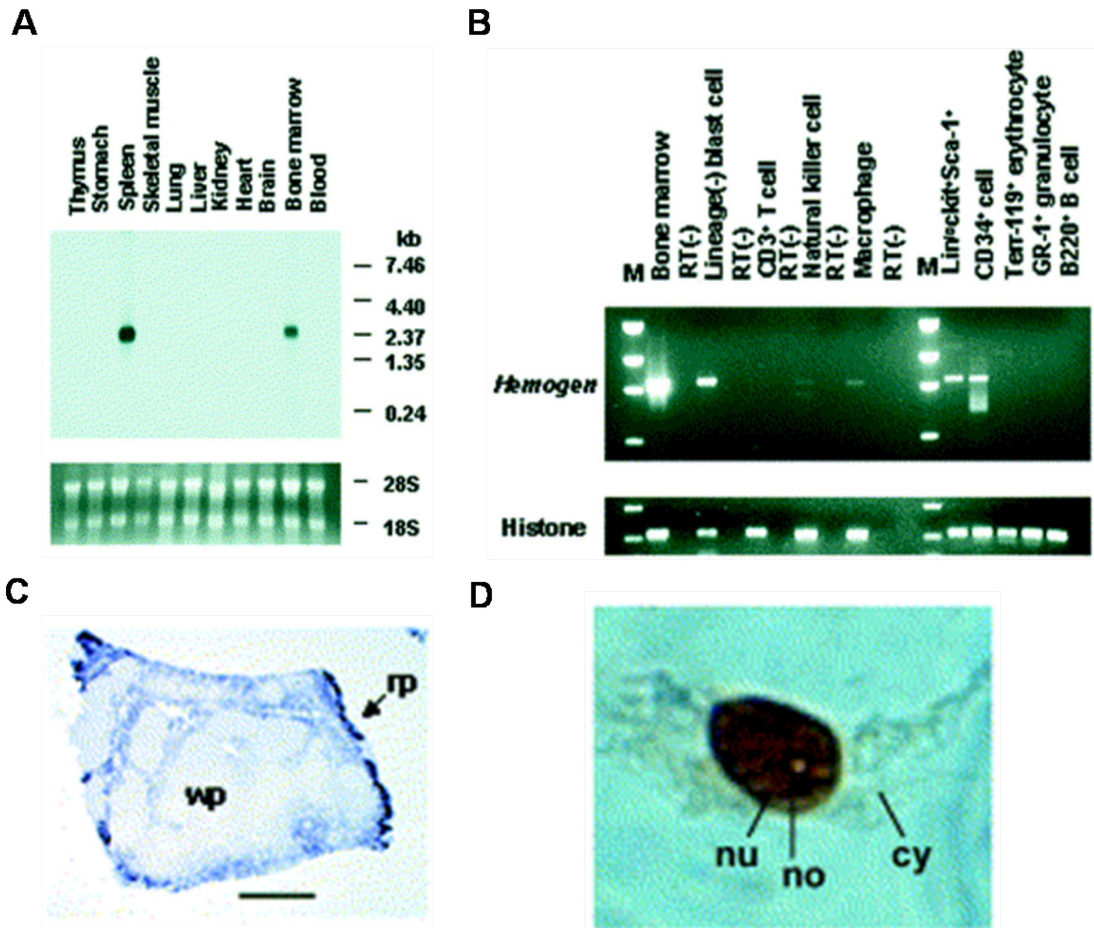


Figure 1-9. Expression and localization of Hemgn. (A) Northern blotting for mRNA of *Hemgn* in indicated tissue. A 2.4 kb message was detected in the spleen and bone marrow. (B) RT-PCR detected mRNA expression of *Hemgn* in indicated tissue. (C) By hybridization with antisense RNA probe, *Hemgn* transcripts were localized in the red pulp (rp) but not in the white pulp (wp) in mouse spleen. (D) The Hemgn-FLAG fusion protein was transfected into COS-7 cells and detected with anti-FLAG antibody. The signal was localized in the cell nuclei (nu) but not in the nucleoli (no) or cytoplasm (cy).

Reprinted with permission from Elsevier. Yang LV, Nicholson RH, Kaplan J, Galy A, Li L. Hemogen is a novel nuclear factor specifically expressed in mouse hematopoietic development and its human homologue EDAG maps to chromosome 9q22, a region containing breakpoints of hematological neoplasms. *Mech.Dev.* 2001;104:105-111.

activity, indicating that EDAG could be a transcriptional regulator.¹⁷³ EDAG cDNA fused with the Gal4 DNA-binding domain (DBD) was transformed into yeast cells and found to transcriptionally activate a chromosomally integrated lacZ reporter gene. This result was also confirmed in mammalian cells through luciferase assay.

1.7.2 Functions of Hemgn

EDAG expression is very high in leukemia cell lines such as K562, HEL and Mo7e and megakaryoblast leukemia cell lines DAMI, MEG-01.¹⁷⁴ Moreover, overexpression of EDAG has been reported in a variety of human leukemia and lymphoma cells,¹⁷⁵ suggesting that EDAG may play a role in hematopoietic transformation and leukemogenesis.

Enforced expression of EDAG in hematopoietic cell lines increased proliferation, enhanced survival, and inhibited differentiation.¹⁷² Overexpression of EDAG in NIH3T3 cells results in malignant transformation of cells, as characterized by cell morphology, anchorage-independent growth and tumorigenicity in nude mice.¹⁷⁶ Overexpression of EDAG in HL-60 cells blocked monocyte and macrophage differentiation after pentahydroxytiglia myristate acetate induction.¹⁷² Overexpression of EDAG in pro-B Ba/F3 cells prolonged survival and increased the expression of c-Myc, Bcl-2 and Bcl-xL in the absence of IL-3.¹⁷² In contrast, downregulation of EDAG protein in K562 cells inhibited growth and colony formation, and enhanced sensitivity to erythroid differentiation induced by hemin.¹⁷² EDAG was shown to enhance the transcriptional activity of NF-κB (nuclear factor kappa B), and DNA-binding activity of NF-κB was sustained in Ba/F3 cells overexpressing EDAG after IL-3 withdrawal. Inhibition of NF-κB activity resulted in promoting EDAG-induced Ba/F3 cell death.¹⁷² These results indicate that EDAG can block apoptosis, possibly through the activation of NF-κB.¹⁷² These results suggest that EDAG is an important regulator for proliferation and differentiation of hematopoietic cells, making its involvement in the HOXB4 phenotype very interesting.

An EDAG transgenic mouse was generated, using the human CD11a promoter to selectively express EDAG in hematopoietic tissues.¹⁷³ EDAG overexpression in transgenic mice resulted in disrupted hematopoietic homeostasis. Myelopoiesis was enhanced with a progressive myeloid hyperplasia in the spleen and increased peripheral blood granulocytes in circulation. Lymphopoiesis was severely inhibited in the transgenic mice with B lymphopoiesis blocked at the transition from pro/pre-B I to pre-B II stage in the bone marrow. Thymocyte development was also blocked at the most immature double negative stage. These data suggest that EDAG can act as a key regulator in primitive hematopoietic cells and its downregulation during maturation of blood cells may be necessary for maintaining homeostasis of the hematopoietic lineages. In the same report, EDAG was shown to be a transcriptional regulator

which had transactivation activity and regulated the expression of several key transcription factors such as PU.1 and Pax5 in transgenic hematopoietic stem cells,¹⁷³ suggesting that EDAG may positively or negatively regulate transcription of specific target genes. However, whether the effect of EDAG on these genes is direct needs further determination, since there is no evidence now that EDAG can bind DNA directly.

CHAPTER 2. IDENTIFICATION OF EARLY AND POTENTIAL DIRECT TARGET GENES OF HOXB4 USING AN INDUCIBLE HOXB4-ER^{T2} SYSTEM

2.1 Summary

In order to understand the mechanism of HOXB4 mediated expansion of hematopoietic stem cells, we focused on identifying early and direct transcriptional targets of HOXB4 in primary murine hematopoietic progenitor cells. For this purpose, a HOXB4-ER^{T2} fusion protein was created by molecular subcloning and introduced into undifferentiated bone marrow cells. Twelve hours after tamoxifen (TAM) exposure, HOXB4-ER^{T2} transduced cells were functionally evaluated in myeloid progenitor expansion assays. Extracted RNA was analyzed by Affymetrix 430V2 chips, comparing expression patterns in HOXB4-ER^{T2} versus GFP control vector-transduced cells. Four biologically independent experiments were performed to assess reproducibility. Stringent criteria were applied for mining microarray data using bioinformatics algorithms. Seventy seven probesets were identified that were differentially expressed (>1.5 fold, paired t-test $p < 0.01$) in this early response to HOXB4 induction and were prioritized as candidate targets. Quantitative PCR was performed to validate the microarray data for specific genes of interest.

2.2 Introduction

Transcriptional regulation of gene expression is an essential function in all eukaryotes and is central to complex developmental control. Transcription factors are proteins that can regulate the expression of many physiologically important target genes including those that regulate proliferation and differentiation. The identification of transcription factors, transcription factor-target genes and transcriptional regulatory networks is therefore of fundamental importance in biology, because unraveling the logic of these gene regulation networks is necessary for a complete understanding how transcription factors contribute to biological phenotype. In my study, identifying molecules that promote hematopoietic stem cell expansion is a central goal, since significant expansion of hematopoietic stem cells would allow new transplant-based therapies.^{3;91} However, *in vitro* expansion of hematopoietic stem cells has been difficult to achieve and often results in differentiation and apoptosis of developmentally primitive hematopoietic cells. Human HOXB4, a member of the homeobox transcription factor family, can promote hematopoietic stem and progenitor cell expansion both *in vivo* and *in vitro* when ectopically expressed in murine bone marrow cells.^{90;95} HOXB4 overexpression also promotes specification of definitive hematopoietic stem cells from differentiating murine embryonic stem cells.^{146;156} Since very little is known about the mechanisms of how HOXB4 promotes expansion of hematopoietic stem and progenitor cells, and because no specific

direct target genes have yet been identified in hematopoietic cells, one objective of this study was to identify the downstream genetic pathways of HOXB4. In addition, direct target identification could also provide new factors for promoting hematopoietic stem and progenitor cell expansion.

There is only one published report identifying the targets of HOXB4 in hematopoietic cells.¹⁴⁴ In this report, the downstream pathways of ectopic expressed HOXB4 were studied. Gene expression profiling assays were performed using enriched adult hematopoietic stem cells and embryonic derivatives that had been transduced with an inducible form of HOXB4. A set of overlapping gene expression change was generated from these two cellular systems and selected to represent common targets of HOXB4.¹⁴⁴ These results showed that HOXB4 protected adult hematopoietic stem cells from the inhibitory effects mediated by the proinflammatory cytokine tumor necrosis factor alpha (TNF- α), which likely inhibits the competitive repopulation advantage in the absence of HOXB4 overexpression.¹⁴⁴ It was also demonstrated that HOXB4 activity and FGF signaling were related, since HOXB4-mediated expansion of adult and ES cell-derived hematopoietic stem cells was enhanced by inhibition of FGF receptors.¹⁴⁴ In this report, however, no evidence was provided to show that any target genes from the generated candidate list was a direct transcriptional target of HOXB4. It was not clear if these pathway effects were primary versus secondary effects. Instead, their data suggest that multiple pathways downstream of HOXB4 could be regulated and they intertwined to modulate the self-renewal of hematopoietic stem cells promoted by HOXB4.

In my study, I utilized a traditional method for identifying the target genes of HOXB4 by an unbiased screen of its potential targets in a large scale gene expression profile analysis. Indeed, the activity of many nuclear localized proteins can be induced with hormones when these proteins are fused to hormone binding domains of steroid receptors such as estrogen receptor (ER). This allows pulsing of activity and enables study of acute effects. We established a HOXB4-ER^{T2} inducible system to regulate the activity of HOXB4 in transduced hematopoietic cells by controlling the nuclear entry of HOXB4 with exogenous application of tamoxifen. When tamoxifen is absent, the HOXB4-ER^{T2} fusion protein is sequestered by HSP90 in the cytosol. When tamoxifen is added, it diffuses into cells and binds to the ligand binding domain of ER^{T2} and thus releases the fusion protein from HSP90, which then can translocate into the nucleus and regulate the cis- elements of downstream genes for their activation or repression. Although the function of HOXB4 is most important in hematopoietic stem cell expansion, it also has a profound effect on hematopoietic progenitor cell expansion. It is relatively difficult to purify enough hematopoietic stem cells for microarray screening analysis, and there exists an established simple *in vitro* assay for evaluating HOXB4 function in hematopoietic myeloid progenitor cell expansion promptly.⁹⁵ Therefore, I used this assay as a surrogate for hematopoietic stem cells in this study of HOXB4 downstream target identification.

2.3 Materials and Methods

2.3.1 Vector Construction

Generation of MSCV-IRES-GFP vector was described previously.¹⁷⁷ The human *HOXB4* cDNA⁹⁵ was kindly provided by Dr. Keith Humphries (Terry Fox Laboratory, Vancouver, Canada), and was cloned upstream of the IRES cassette into MSCV-IRES-GFP^{177;178} backbone. For the MSCV-HOXB4-ER^{T2}-IRES-GFP vector, the mutated ligand binding domain of estrogen receptor (ER^{T2}) was fused to the C-terminus of the *HOXB4* cDNA with a linker with the following sequence: CLQNSDQRNE. The fusion *HOXB4-ER^{T2}* gene was then cloned into the EcoRI and XhoI sites of MSCV-IRES-GFP vector.

2.3.2 Retroviral Vector Preparation

A polyclonal population of ecotropic retroviral producer GPE+86 cells¹⁷⁹ was generated using a previously described approach.^{180;181} Briefly, 2 µg pCAGGS-VSVG and 4 µg pEQ-PAM3(-E) helper plasmids were co-transfected with 4 µg MSCV-cDNA-IRES-GFP vector into 8x10⁶ cycling 293T cells using FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Retroviral vector supernatants were harvested 48 hours post transfection, pooled, filtered through 0.45 µm PVDF filter (Millipore, Billerica, MA), and used for transducing the cycling GPE+86 cells in the presence of 6 µg/ml polybrene. The transduced GFP positive GPE+86 cells were sorted and expanded in culture for establishing stable retroviral vector producer lines. The helper-free recombinant retroviral vector supernatants freshly collected from the GPE+86 producer cells were filtered through 0.45 µm pore filter for transducing bone marrow cells.

2.3.3 Separation, Transduction of Lin⁻ Bone Marrow Cells and Analysis

Bone marrow cells were harvested from the tibias and femurs of 7 to 12 weeks old female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) as previously described,¹⁸² and were incubated with phycoerythrin (PE)-conjugated lineage antibody mixture (anti-mouse Mac-1, Gr-1, B220, CD4, CD8, Ter-119, Pharmingen, BD Biosciences, Franklin Lakes, NJ). Lin⁻ bone marrow cells were enriched by depleting PE-labeled cells through anti-PE magnetic microbeads (Miltenyi Biotec, Auburn, CA) on autoMACSTM separator (Miltenyi Biotec) according to the manufacturer's instructions. The Lin⁻ bone marrow cells were prestimulated for 2 days with 20 ng/ml recombinant mouse IL-3, 50 ng/ml human IL-6, and 50 ng/ml mouse SCF (R&D Systems, Minneapolis, MN) in Dulbecco's modified Eagle medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine (R&D

systems, Minneapolis, MN). The cells were then centrifuged at 2000 rpm for 30 minutes at room temperature by using plates preloaded with 25 µg/ml RetroNectin (Takara, Madison, WI) and retroviral vector supernatant generated as described above at an multiplicity of infection (MOI) of 10 to 20. After 3 to 4 rounds of transduction in 2 consecutive days, cells were collected, washed with PBS, and then resuspended in the cytokine-supplemented medium described above. Proportions of the transduced GFP positive cells were determined by flow cytometry. Cells were cultured in the presence of TAM for additional time before plating into methylcellulose media for CFU-C assay,⁹⁵ and RNA extraction for microarray.

2.3.4 TAM Induction of HOXB4-ER^{T2} and Myeloid Progenitor Assays

15.2 mg of tamoxifen citrate (equivalent to 10 mg of tamoxifen-TAM) tablet (Barr Laboratories, Pomona, NY) was dissolved in 20 ml ddH₂O to obtain the stock 0.5 mg/ml TAM solution. The TAM solution was added directly into the bone marrow cells culture medium at the concentration 300 nM. TAM was withdrawn, and cells were plated for myeloid progenitor CFU-C assays established for HOXB4 expansion activity as previously described.⁹⁵ In details, bone marrow cells were plated at 500 cells/ml in into MethoCult GF M3434 methylcellulose medium (StemCell Technologies, Vancouver, BC, Canada) containing various cytokines supporting growth of clonogenic myeloid progenitor cells. After 7 days, primary (1°) colonies were scored, collected, resuspended, and re-plated at 10⁵ cells/ml density into the M3434 methylcellulose medium for 10 days culture. The generated secondary (2°) colonies were scored to quantitate the expansion of myeloid progenitors.

2.3.5 Western Blotting

Whole cell extracts were prepared from GPE+86 cells by M-PER Mammalian protein extraction reagent (Pierce, Thermo Scientific, Rockford, IL) supplemented with the protease inhibitors (HaltTM protease inhibitor cocktail kit, Thermo Scientific, Rockford, IL; Pefabloc SC Plus, Roche Applied Science, Indianapolis, IN). The proteins were then resolved on a 4-12% gradient NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, California) and transferred to 0.45 µm PVDF membrane. The primary monoclonal antibody (clone I12) (The Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA) was used for HOXB4 and HOXB4-ER^{T2} detection.

2.3.6 Immunofluorescence Staining

1x10⁵ Lin⁻ bone marrow cells were cytopspined and fixed in 3.7% formaldehyde for 30 minutes at room temperature, washed and permeabilized in 1% Triton X-

100 for 5 minutes at room temperature. Anti-HOXB4 antibody (clone I12) was added at 37°C for 1 hour, and after PBS washing, secondary antibody Alexa Fluor 594 goat anti-rat IgG(H+L) (Invitrogen, Carlsbad, California) was added to the cells for at room temperature for 1 hour. The nucleus was stained with TOTO-1 iodide (Invitrogen, Carlsbad, California). The stained slides were imaged with a Nikon TE2000-E inverted microscope equipped with a C1Si confocal system and an X40 Plan Apo objective.

2.3.7 RNA Isolation, Affymetrix Array Hybridization and Analysis

Total RNA was extracted from TAM-treated Lin⁻ bone marrow cells using RNA STAT-60™ reagent (Tel-Test, Friendswood, TX) according to the manufacturer's instructions. The RNA concentration and purity was determined by spectrophotometric analysis, integrity was confirmed by electrophoresis analysis using Agilent 2100 Bioanalyzer Lab-on-a-chip system (Agilent Technologies, Santa Clara, CA). Total RNA (100 ng) was processed in the Hartwell Center microarray core according to the Affymetrix eukaryote two-cycle target labeling protocol

(http://www.affymetrix.com/support/technical/manual/expression_manual.affx). Biotin-labeled cRNA (20 µg) was hybridized overnight at 45°C to the Mouse-430v2 GeneChip array. After staining and washing, arrays were scanned and expression values were summarized using the MAS5 algorithm as implemented in the GCOS v1.4 software (Affymetrix, Santa Clara, CA). Signals were normalized for each array by scaling to a 2% trimmed mean of 500. Detection calls (Present, Absent and Marginal) were determined using the default parameters of the software. Signal values were log₂-transformed prior to analysis. Differential expression between HOXB4-ER^{T2} transduced and GFP only-transduced cells was determined from four independent experiments using the paired t-test (S-Plus 6.2 software, TIBCO, Palo Alto, CA). Hierarchical clustering was performed using the Spotfire DecisionSite 9.0 software (TIBCO, Palo Alto, CA). Probeset annotations were obtained from the Affymetrix website (<http://www.affymetrix.com/analysis/index.affx>).

2.3.8 Gene Expression Validation

The RNA was first reversely transcribed with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California) by using oligo-dT primers. All gene-specific PCR primers were ordered from Quanti Tect Primer Assay kit (Qiagen, Valencia, CA) (Table 2-1).

The real-time PCR reactions were performed using QuantiTect™ SYBR Green PCR kit (Qiagen, Valencia, CA) following the manufacturer's instructions. The relative expression of each candidate gene was performed at least in triplicate and calculated using the comparative quantitative comparative C_T (2^{-ΔΔCT})

Table 2-1. The ordered gene-specific PCR primers used in real-time PCR validation analysis.

Gene Symbol	QuantiTect Primers	Cat. No.
<i>Gapdh</i>	Mm_Gapdh_3_SG QuantiTect Primer Assay	QT01658692
<i>Actb</i>	Mm_Actb_2_SG Quanti Tect Primer Assay	QT01136772
<i>Thy1</i>	Mm_Thy1_1_SG QuantiTect Primer Assay	QT00245287
<i>Ephx2</i>	Mm_Ephx2_1_SG QuantiTect Primer Assay	QT00109165
<i>Slc22a3</i>	Mm_Slc22a3_1_SG QuantiTect Primer Assay	QT00131894
<i>Hemgn</i>	Mm_Hemgn_1_SG QuantiTect Primer Assay	QT00106099
<i>Ms4a4c</i>	Mm_Ms4a4c_1_SG QuantiTect Primer Assay	QT00175763
<i>Pltp</i>	Mm_Pltp_2_SG QuantiTect Primer Assay	QT01053311
<i>Mettl1</i>	Mm_Mettl1_1_SG QuantiTect Primer Assay	QT00153433
<i>Cldn11</i>	Mm_Cldn11_1_SG QuantiTect Primer Assay	QT00104195

method with normalization to *Gapdh* internal control. The PCR primers of *Actb* were used for normalization in the real-time PCR reactions for detecting Hemgn expression in *Hoxb4*^{-/-} and wild type (WT) mice.

2.4 Results

2.4.1 Retroviral Constructs and Expression of HOXB4-ER^{T2} Fusion Protein

To identify early and direct transcriptional targets of HOXB4 in murine bone marrow cells, a HOXB4-ER^{T2} fusion gene was developed to allow temporally regulated HOXB4 activity by TAM.¹⁸³ As controls, I used a retroviral vector expressing GFP protein as negative control, HOXB4 wild type gene as positive control under control of the MSCV promoter (Figure 2-1A). Expression of HOXB4 or HOXB4-ER^{T2} fusion protein were verified in established virus producer GPE+86 cells using western blotting analysis (Figure 2-1B).

2.4.2 TAM Inducible Nuclear-Translocation of HOXB4-ER^{T2}

To test whether HOXB4-ER^{T2} fusion protein can translocate into the nucleus after TAM treatment, TAM was added into the cultures of Lin⁻ bone marrow cells at a concentration 300 nM for different time periods. TAM-induced nuclear translocation of HOXB4-ER^{T2} in transduced Lin⁻ bone marrow cells was confirmed by immunofluorescence staining 6 hours after TAM treatment (Figure 2-2). This experiment showed no nuclear localization of HOXB4 in the absence of TAM, implying that function would not be “leaky” in the absence of induction.

2.4.3 TAM Inducible HOXB4-ER^{T2} Activity Promoted Hematopoietic Progenitor Cell Expansion In Vitro

An established expansion assay for myeloid progenitors was utilized for verifying TAM-dependent function.⁹⁵ Lin⁻ bone marrow cells were purified and transduced with the HOXB4-ER^{T2} vector or the control GFP vector. The transduced cells were exposed to 300 nM TAM in the culture medium for 12, 24, and 48 hours, washed, and then plated into semi-solid methylcellulose medium for myeloid progenitor colony forming unit cells (CFU-Cs) assay (Figure 2-3). TAM treatment for only 12 hours resulted in the generation of larger myeloid colonies, and more importantly, resulted in an increase in secondary colony number after re-plating (Figure 2-4A and B). Fluorescent microscope studies verified that the majority of secondary colonies were GFP positive and therefore contained and expressed the vector (Figure 2-5). These results prove that activation of HOXB4-ER^{T2} by TAM resulted in expansion of myeloid progenitor cells *in vitro*. This experiment also verified that no myeloid expansion occurred in

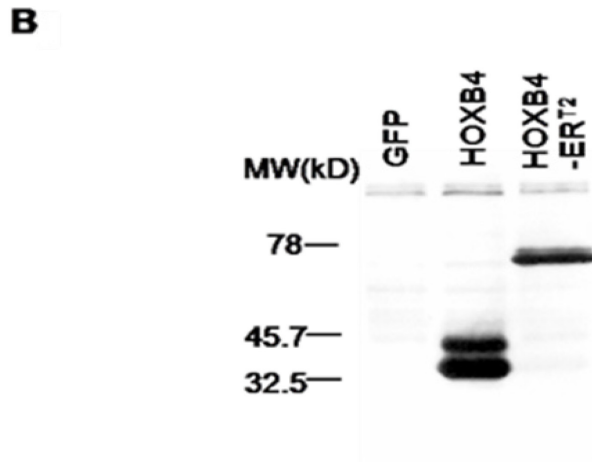
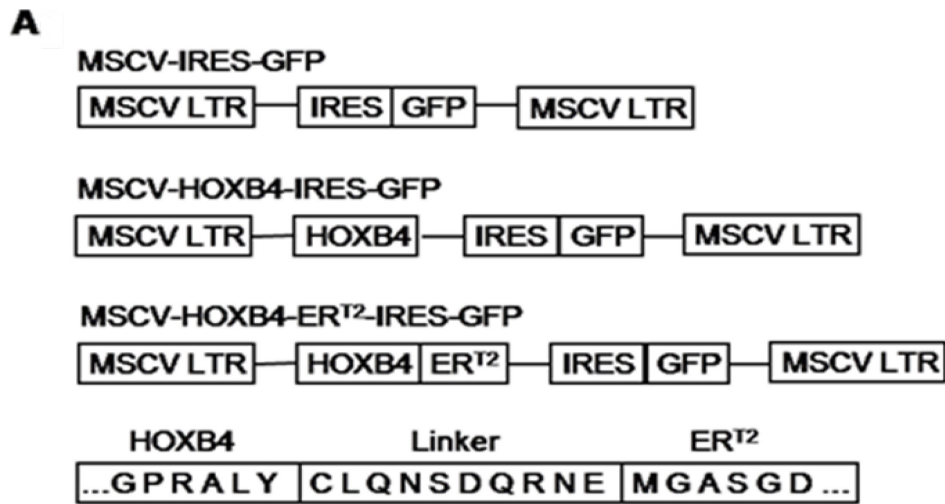


Figure 2-1. Retroviral constructs and expression of HOXB4 and HOXB4-ER^{T2} protein. (A) cDNA of HOXB4 and HOXB4-ER^{T2} was cloned upstream of the IRES cassette in MSCV-IRES-GFP backbone; The mutated ligand binding domain of estrogen receptor (ER^{T2}) was fused to the C-terminus of the HOXB4 cDNA with a linker CLQNSDQRNE. (B) Overexpression of HOXB4 or HOXB4-ER^{T2} was confirmed in retroviral vector producer GPE+86 cells by western blotting with a monoclonal anti-HOXB4 antibody (clone I12).

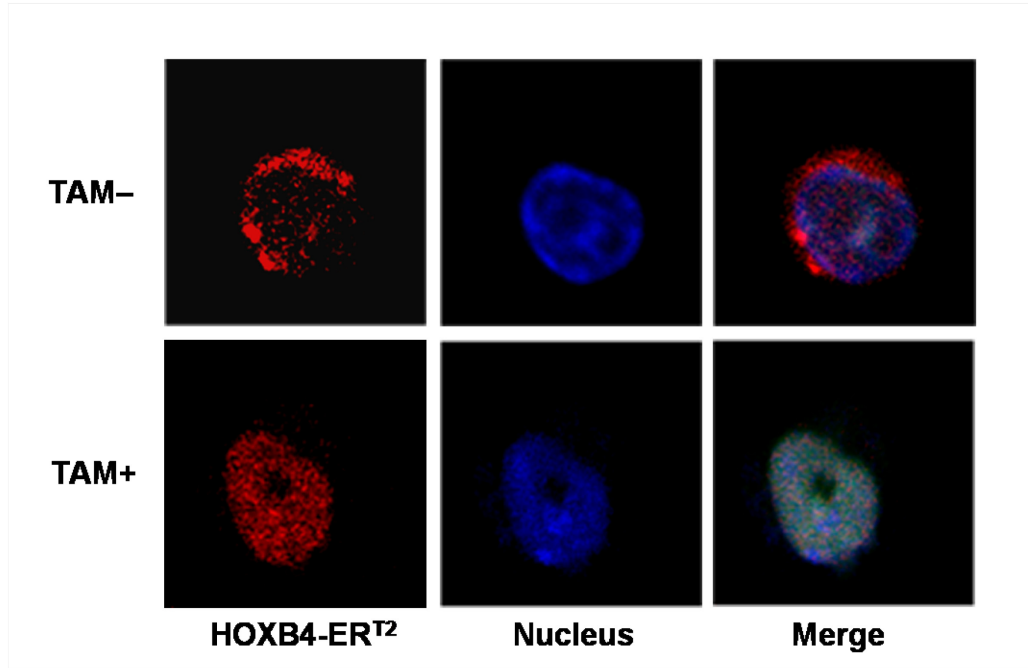


Figure 2-2. TAM-induced cytosol to nucleus translocation of HOXB4-ER^{T2} in Lin⁻ bone marrow cells confirmed by confocal microscopy. Red: HOXB4-ER^{T2}; Blue: nucleus dye.

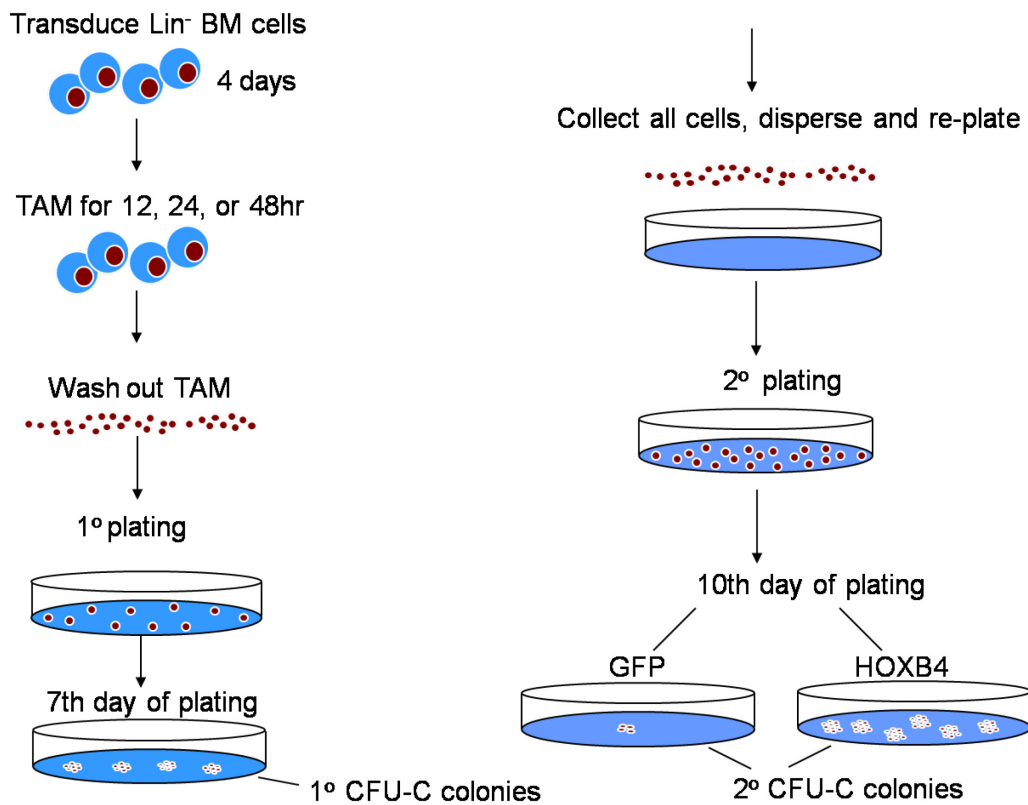


Figure 2-3. Schematic illustration of myeloid progenitor secondary CFU-C expansion assays.

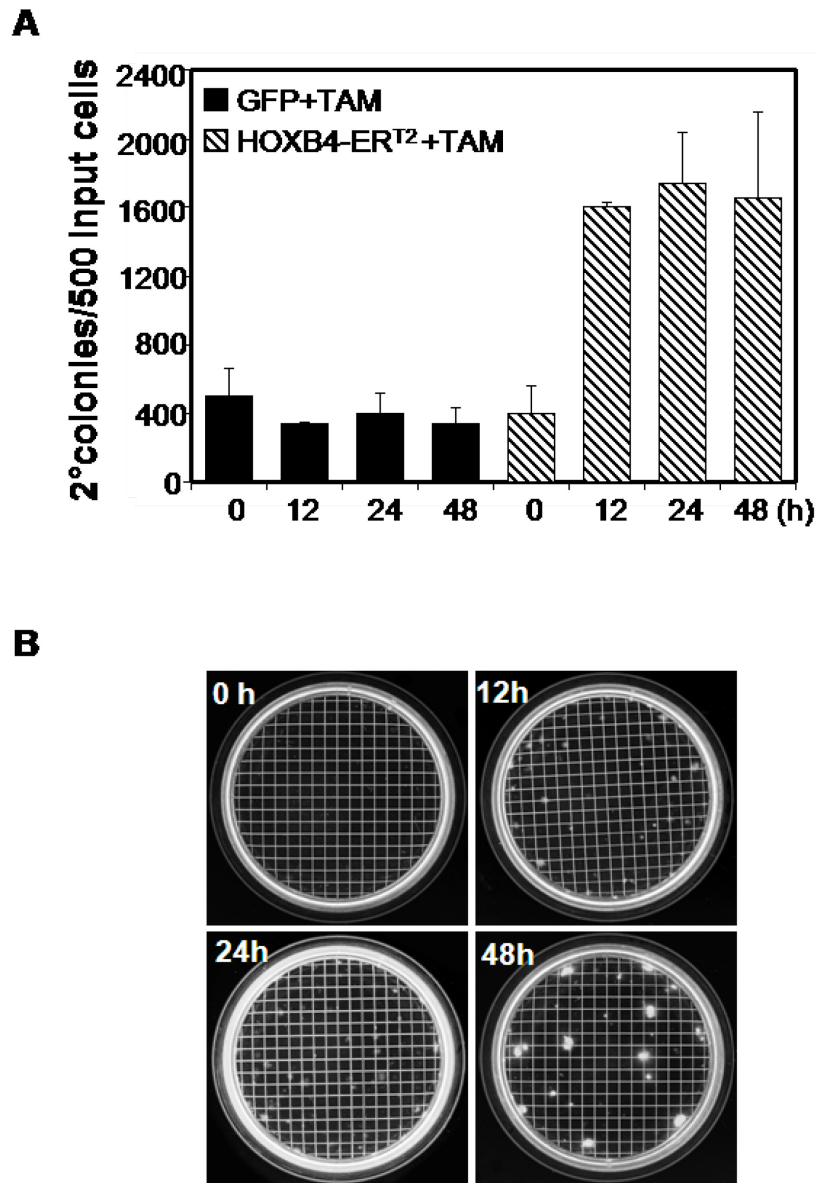


Figure 2-4. Effect of TAM-induced HOXB4-ER^{T2} activity *in vitro*. (A) TAM treatment for 12, 24 and 48 hours induced increase in secondary myeloid progenitor colony formation in HOXB4-ER^{T2}-overexpressing Lin⁻ bone marrow cells *in vitro*. (B) Pictures of the secondary myeloid progenitor colony plates from HOXB4-ER^{T2}-overexpressing cells treated with TAM for 0, 12, 24 and 48 hours.

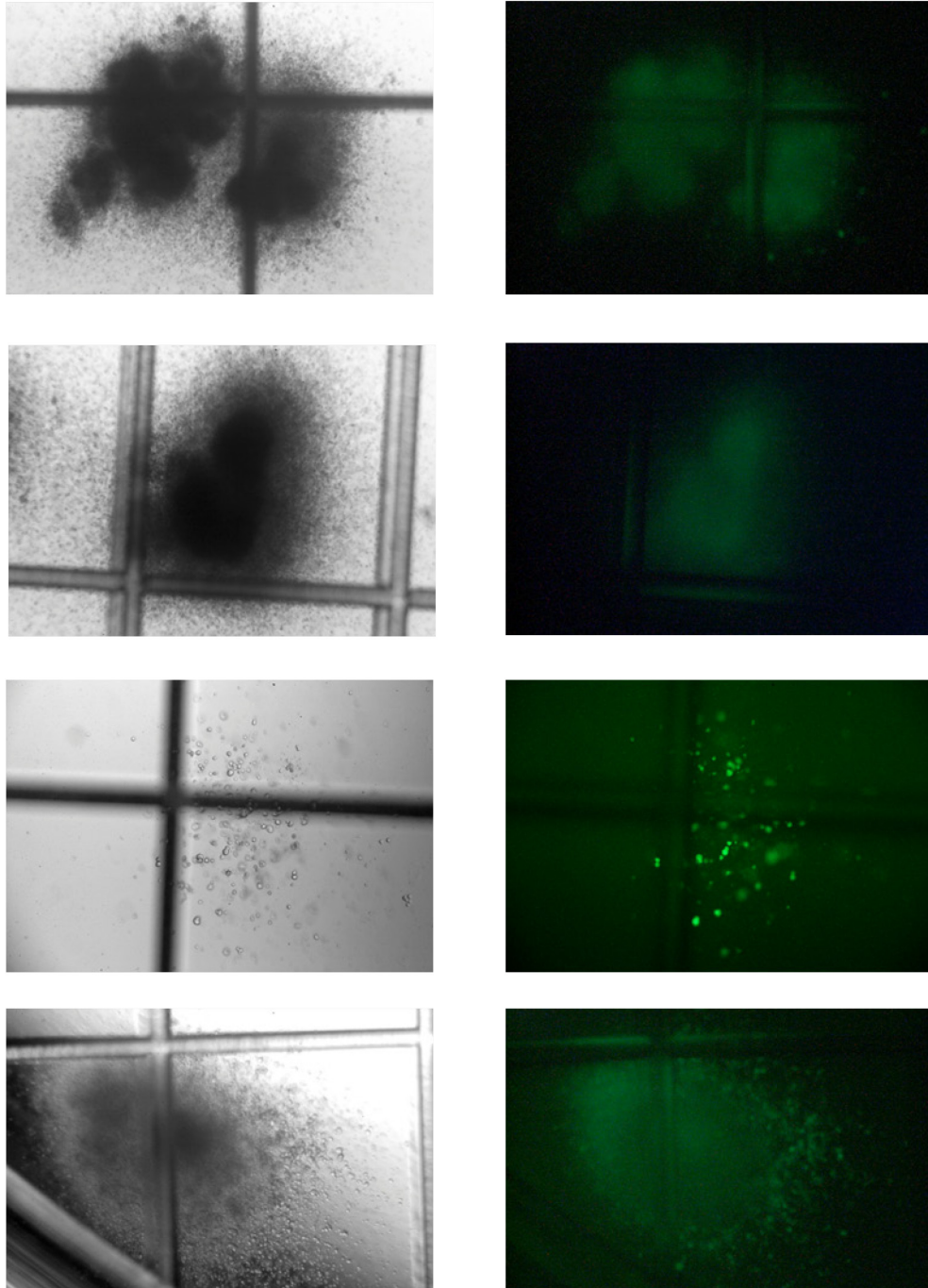


Figure 2-5. Secondary myeloid progenitor colonies from HOXB4-ER^{T2} overexpressing Lin⁻ bone marrow cells were GFP positive under fluorescence microscopy.

the absence of TAM, given that secondary CFU-C colony formation was low and equivalent to that seen with the GFP-only negative control vector.

2.4.4 Identification of HOXB4 Early Downstream Targets by Expression Microarrays

To identify early and direct downstream HOXB4 target genes involved in expansion of hematopoietic progenitor cells, RNA was extracted 12 hours after TAM-treatment of Lin⁻ bone marrow cells transduced with either the HOXB4-ER^{T2} vector or with the GFP control vector, and was analyzed for differential gene expression using Affymetrix 430v2 chips (Figure 2-6). The transduction efficiency ranged from 60% to 70% in multiple experiments. Analysis of 4 independent transduction experiments showed good reproducibility in gene expression changes. The Pearson correlation coefficient between all experimental samples ranged from 0.91 to 0.97 among probesets with more than one “Present” calls (Table 2-2), indicating that there was low experimental variation and that HOXB4 overexpression did not cause a change in global gene expression. First, we manually excluded those probesets showing more than 4 “Absent” calls in a total of 8 samples from the 4 experiments. A probeset was considered a potential downstream target of HOXB4 if the difference between the 2 mean expression values (HOXB4-ER^{T2} vs. GFP) was at least 1.5 fold change, and the extent of the expression difference was statistically significant ($p < 0.01$) as evaluated by paired t-test method. We chose a 1.5 fold threshold due to the fact that 30~40% of the cells in a given sample were not transduced, hence the 1.5 fold change measured from the samples would underestimate what actually occurred in the transduced cells.

Seventy seven probesets were identified as being significantly changed by this analysis. Among them, 44 were upregulated (Table 2-3), and 33 were downregulated (Table 2-4) by HOXB4 induction. Hierarchical clustering analysis and heat map was generated for these 77 probesets from the 4 independent experiments (Figure 2-7 and Figure 2-8). Functional classification of those probesets by Gene Ontology annotations denoting different biological processes (Figure 2-9) showed a relatively high percentage of probesets involved in metabolism (20, 17%), signal pathways (15, 13%), and development and differentiation (14, 12%).

2.4.5 Validation of the Selected HOXB4 Target Genes

To validate the expression change seen in the microarray assays with HOXB4 induction, eight genes which showed a relatively large change in expression were selected for quantitative real time-PCR confirmation (Figure 2-10).

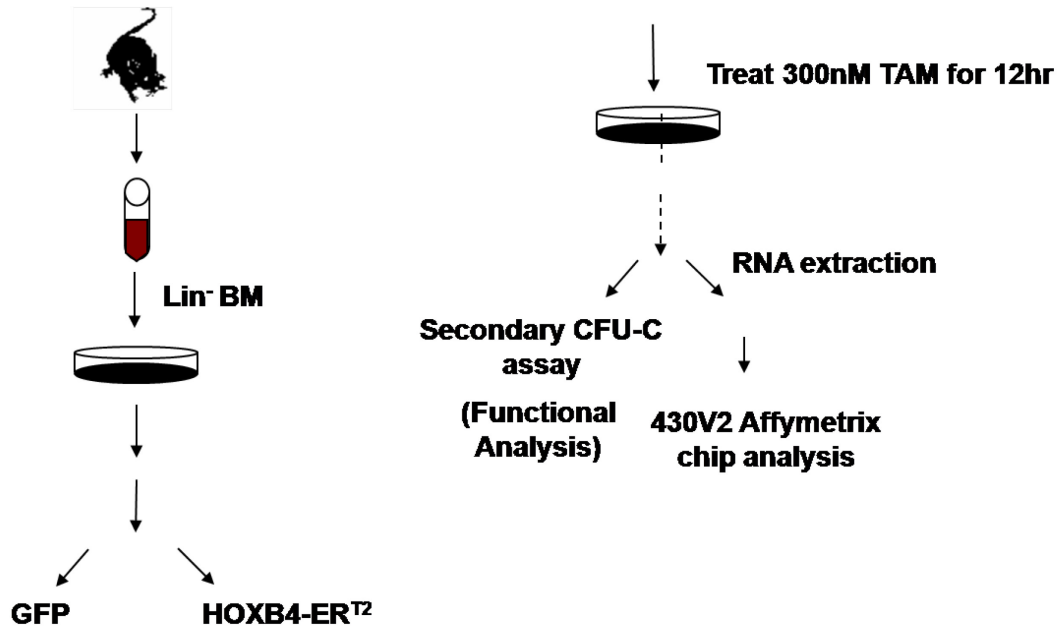


Figure 2-6. Schematic illustration of the inducible $\text{HOXB4-ER}^{\text{T2}}$ system for target screening in Lin^- bone marrow cells.

Table 2-2. Pearson correlation coefficients between the 8 experimental samples with probesets >1 present call in microarray.

Array	Exp1 GFP	Exp1 HOXB4- ER ^{T2}	Exp2 GFP	Exp2 HOXB4- ER ^{T2}	Exp3 GFP	Exp3 HOXB4- ER ^{T2}	Exp4 GFP	Exp4 HOXB4- ER ^{T2}
Exp1 GFP		0.95	0.94	0.94	0.95	0.94	0.92	0.91
Exp1 HOXB4- ER ^{T2}	0.95		0.94	0.94	0.94	0.95	0.94	0.93
Exp2 GFP	0.94	0.94		0.94	0.94	0.94	0.93	0.92
Exp2 HOXB4- ER ^{T2}	0.94	0.94	0.94		0.94	0.93	0.92	0.91
Exp3 GFP	0.95	0.94	0.94	0.94		0.95	0.94	0.93
Exp3 HOXB4- ER ^{T2}	0.94	0.95	0.94	0.93	0.95		0.95	0.95
Exp4 GFP	0.92	0.94	0.93	0.92	0.94	0.95		0.95
Exp4 HOXB4- ER ^{T2}	0.91	0.93	0.92	0.91	0.93	0.95	0.95	

Table 2-3. The 44 probesets upregulated by HOXB4-ER^{T2}.

Probeset	Gene Symbol	Fold Chng	Paired t-Test p Value	Gene Description
1421244_at	Esr1	14.42	5.56E-04	estrogen receptor 1 (alpha)
1445285_at	9230107O19	2.47	7.77E-03	Similar to histamine receptor H3
1423135_at	Thy1	2.34	8.99E-03	thymus cell antigen 1, theta
1448499_a_at	Ephx2	2.25	4.85E-03	epoxide hydrolase 2, cytoplasmic
1437260_at	Mmrn1	1.94	6.56E-03	multimerin 1
1420444_at	Slc22a3	1.91	3.74E-03	solute carrier family 22, member 3
1460130_at	EG433632	1.90	4.95E-05	predicted gene, EG433632
1418199_at	Hemgn	1.85	8.05E-03	hemogen
1459262_at	Mterfd1	1.80	9.14E-03	MTERF domain containing 1
1425506_at	Mylk	1.80	7.37E-03	myosin, light polypeptide kinase
1429642_at	Anub1	1.79	4.35E-03	AN1, ubiquitin-like, homolog (Xenopus laevis)
1446745_at	EG433637	1.78	5.59E-03	predicted gene, EG433637
1419550_a_at	Stk39	1.78	8.12E-03	serine/threonine kinase 39, STE20/SPS1 homolog (yeast)
1436419_a_at	1700097N02	1.77	5.82E-03	RIKEN cDNA 1700097N02 gene
1446470_at	Rik			
1446470_at	ENSMUSG0000045520	1.77	6.65E-03	predicted gene, ENSMUSG0000045520
1441747_at	A330024P14	1.76	5.30E-03	RIKEN cDNA A330024P14 gene
1441747_at	Rik			
1422317_a_at	Il1rl1	1.75	2.60E-03	interleukin 1 receptor-like 1
1421182_at	Clec1b	1.73	1.38E-03	C-type lectin domain family 1, member b
1435594_at	Arl6ip2	1.71	9.15E-03	ADP-ribosylation factor-like 6 interacting protein 2
1417872_at	Fhl1	1.70	9.35E-03	four and a half LIM domains 1
1418892_at	Rhoj	1.69	1.12E-04	ras homolog gene family, member J
1429691_at	5430405N12	1.66	1.07E-03	RIKEN cDNA 5430405N12 gene
1429691_at	Rik			

Table 2-3. Continued.

Probeset	Gene Symbol	Fold Chng	Paired t-Test p Value	Gene Description
1434869_at	Tdrd3	1.65	5.62E-03	tudor domain containing 3
1458505_at	LOC552901	1.65	8.95E-03	hypothetical LOC552901
1429960_at	4930438A08	1.63	6.62E-03	RIKEN cDNA 4930438A08 gene
1418186_at	Rik			
1418186_at	Gstt1	1.62	5.36E-03	glutathione S-transferase, theta 1
1451038_at	Apln	1.61	2.22E-03	apelin
1443633_at	1700086O06	1.60	7.26E-03	RIKEN cDNA 1700086O06 gene
	Rik			
1438575_a_at	3974589	1.60	6.23E-03	Transcribed locus
1444869_at	H3145H06	1.60	7.05E-03	Transcribed locus
1422927_at	Yipf7	1.60	4.81E-03	Yip1 domain family, member 7
1442884_at	Hgf	1.58	5.55E-03	hepatocyte growth factor
1435710_at	AI661384	1.58	1.86E-04	expressed sequence AI661384
1430522_a_at	Vamp5	1.57	4.61E-03	vesicle-associated membrane protein 5
1440227_at	Slc5a3	1.57	9.71E-03	solute carrier family 5 (inositol transporters), member 3
1439755_at	Sipa1l1	1.55	3.44E-03	Signal-induced proliferation-associated 1 like 1
1438664_at	Prkar2b	1.54	2.84E-03	protein kinase, cAMP dependent regulatory, type II beta
1444433_at	30077393	1.53	9.38E-04	Transcribed locus
1420688_a_at	Sgce	1.52	1.24E-03	sarcoglycan, epsilon
1457110_at	Pank1	1.52	6.25E-03	pantothenate kinase 1
1450235_at	Fgd3	1.52	1.33E-03	FYVE, RhoGEF and PH domain containing 3
1432362_at	Cenpp	1.52	1.66E-03	centromere protein P
1431874_at	4931429L15	1.51	4.89E-03	RIKEN cDNA 4931429L15 gene
	Rik			
1446719_at	Atad2b	1.51	7.03E-03	ATPase family, AAA domain containing 2B

Table 2-4. The 33 probesets downregulated by HOXB4-ER^{T2}.

Probeset	Gene Symbol	Fold Chng	Paired t-Test p Value	Gene Description
1416003_at	Cldn11	-3.85	4.11E-03	claudin 11
1447683_x_at	Mettl1	-2.44	2.39E-03	methyltransferase-like 1
1435529_at	OTTMUSG0000016644	-2.33	8.63E-03	predicted gene, OTTMUSG00000016644
1417963_at	Pltp	-2.27	1.92E-03	phospholipid transfer protein
1435792_at	Csprs	-2.27	1.90E-03	component of Sp100-rs
1420671_x_at	Ms4a4c	-2.22	1.55E-03	membrane-spanning 4-domains, subfamily A, member 4C
1450616_at	Ear5	-2.04	4.27E-03	eosinophil-associated, ribonuclease A family, member 5
1443956_at	Zfp397	-2.00	5.84E-03	zinc finger protein 397
1416023_at	Fabp3	-2.00	7.63E-03	fatty acid binding protein 3, muscle and heart
1429984_at	5730455O13 Rik	-1.96	8.95E-03	RIKEN cDNA 5730455O13 gene
1417500_a_at	Tgm2	-1.85	6.51E-03	transglutaminase 2, C polypeptide
1423963_at	LOC100045629	-1.79	1.60E-03	similar to myocardial ischemic preconditioning upregulated protein 2
1452361_at	Rnf20	-1.75	2.32E-04	ring finger protein 20
1430388_a_at	Sulf2	-1.72	2.84E-03	sulfatase 2
1437347_at	Ednrb	-1.72	9.05E-04	endothelin receptor type B
1416289_at	Plod1	-1.69	4.00E-04	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1
1449195_s_at	Cxcl16	-1.67	3.40E-03	chemokine (C-X-C motif) ligand 16

Table 2-4. Continued.

Probeset	Gene Symbol	Fold Chng	Paired t-Test p Value	Gene Description
1421604_a_at	Klf3	-1.64	8.60E-04	Kruppel-like factor 3 (basic)
1428535_at	9430020K01	-1.64	6.12E-03	RIKEN cDNA 9430020K01 gene
1460648_at	Rik			
1460648_at	Nr2f6	-1.64	8.25E-03	nuclear receptor subfamily 2, group F, member 6
1424986_s_at	Fbxw7	-1.61	2.52E-03	F-box and WD-40 domain protein 7, archipelago homolog (Drosophila)
1425658_at	Cd109	-1.56	3.26E-03	CD109 antigen
1450488_at	Ccl24	-1.56	8.04E-03	chemokine (C-C motif) ligand 24
1417865_at	Tnfaip1	-1.54	5.89E-03	tumor necrosis factor, alpha-induced protein 1 (endothelial)
1424498_at	5730596K20	-1.54	8.06E-03	RIKEN cDNA 5730596K20 gene
1454676_s_at	Rik			
1454676_s_at	Ticam1	-1.54	9.53E-03	toll-like receptor adaptor molecule 1
1446528_at	Scyl2	-1.52	8.18E-03	SCY1-like 2 (S. cerevisiae)
1452014_a_at	Igf1	-1.52	8.07E-03	insulin-like growth factor 1
1422054_a_at	Skil	-1.52	9.93E-03	SKI-like
1416654_at	Slc31a2	-1.52	3.57E-03	solute carrier family 31, member 2
1426847_at	Sirt4	-1.52	1.38E-03	sirtuin 4 (silent mating type information regulation 2 homolog) 4 (S. cerevisiae)
1439348_at	S100a10	-1.52	7.14E-03	S100 calcium binding protein A10 (calpactin)
1416044_at	Flii	-1.52	1.40E-03	flightless I homolog (Drosophila)

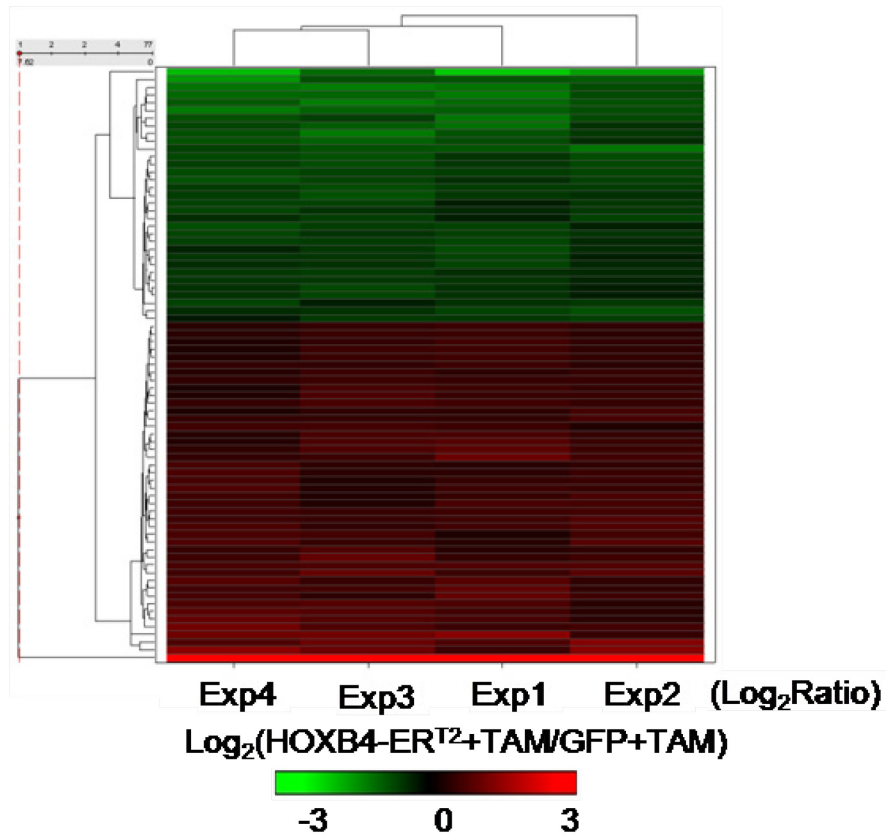


Figure 2-7. Hierarchical cluster diagram and heat map of the 77 candidate probesets selected from 4 biologically-independent experiments using the log₂-ratio of TAM-induced HOXB4-ER^{T2} vs. TAM-induced GFP expression values observed within each of the 4 experiments.

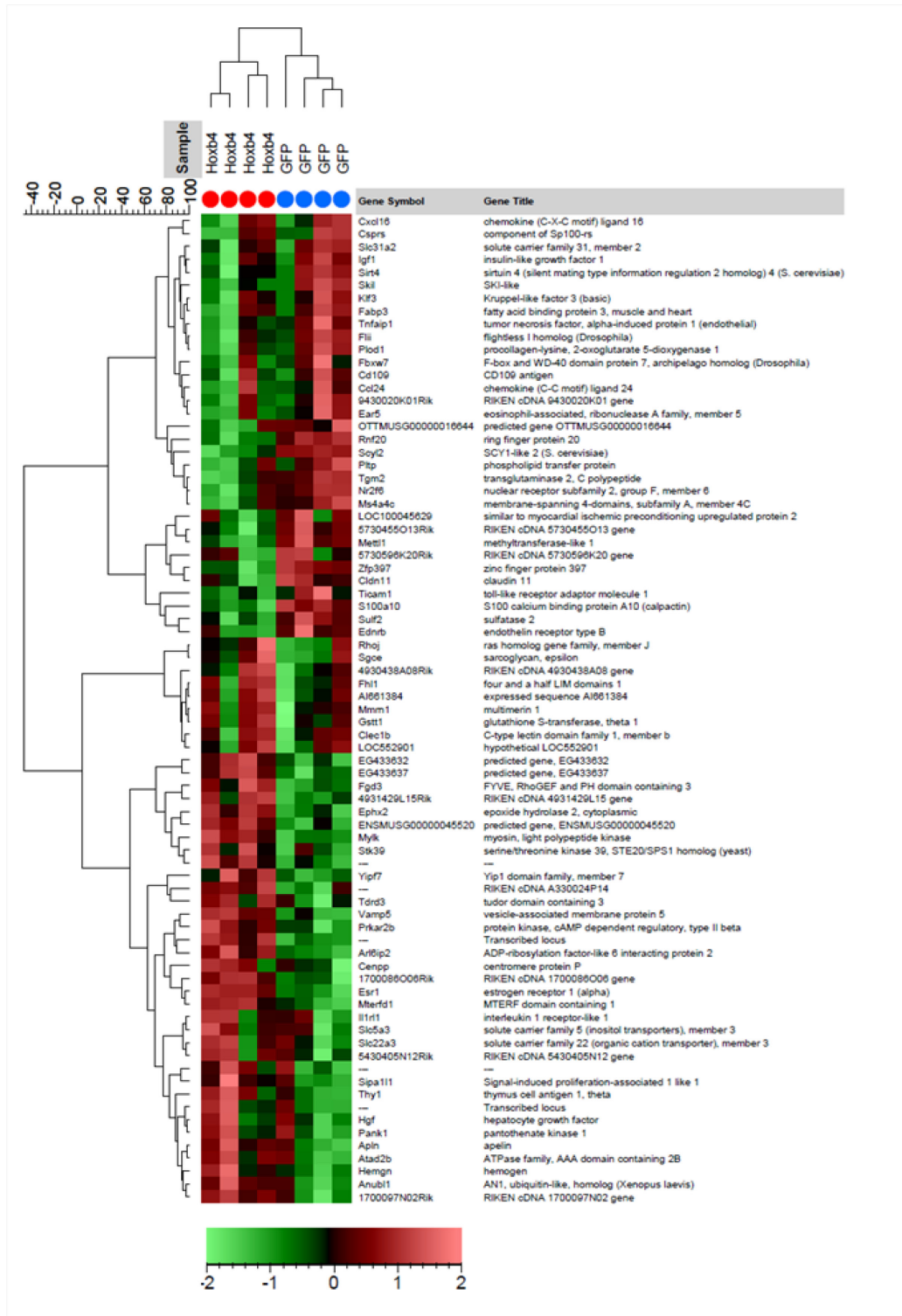


Figure 2-8. Hierarchical cluster diagram and heat map of the 77 candidate probesets selected from 4 biologically-independent experiments using the normalized z-score.

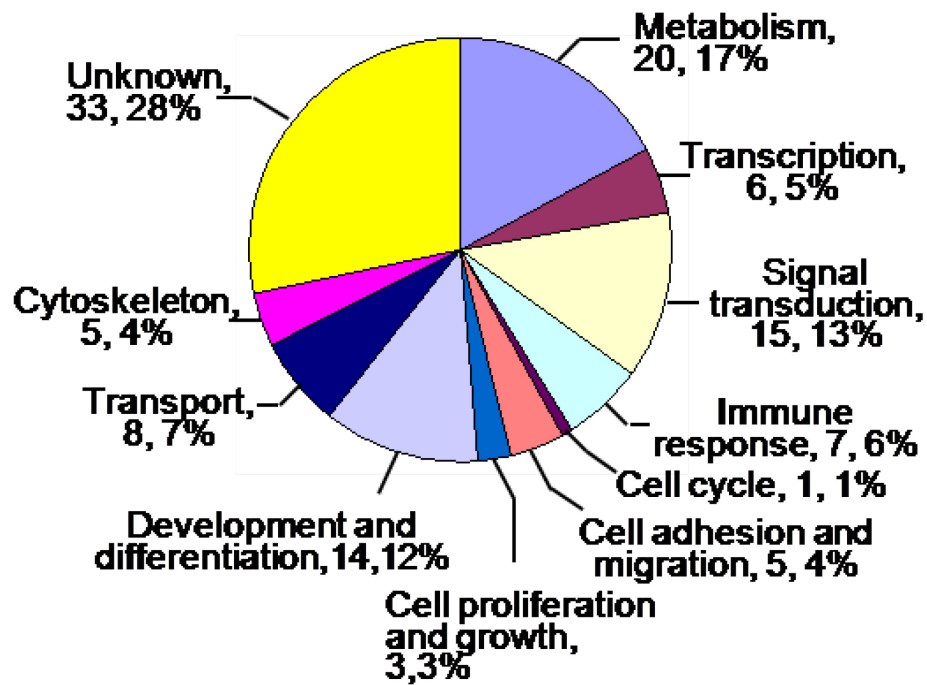


Figure 2-9. Functional classification of the 77 probesets by using gene ontology annotations. Some probesets were classified into more than one category, thus the sum of all 11 groups is more than 77.

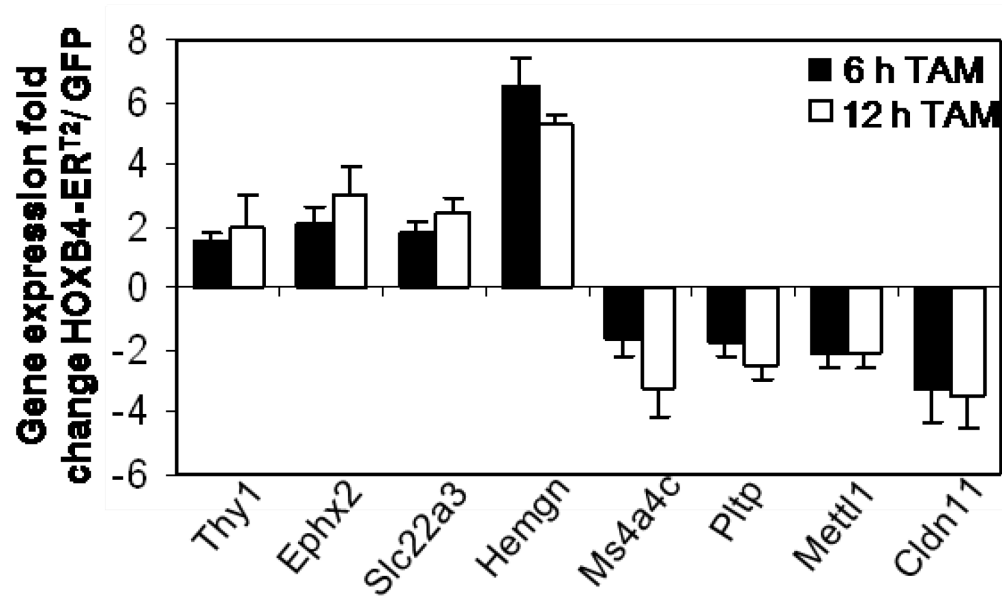


Figure 2-10. Quantitative real-time PCR confirmation of the expression change of the selected 8 candidate target genes from independent experiments with TAM treatment for 6 hours or 12 hours in HOXB4-ER^{T2} vector-transduced and GFP control vector-transduced Lin⁻ bone marrow cells.

$\Delta\Delta C_T$ method was used for quantification of relative gene expression levels. The housekeeping gene *Gapdh* was used as an internal control for gene expression normalization. The plot of template cDNA dilution vs. ΔC_T was close to zero, verifying the approximately equal PCR amplification efficiencies of the tested genes and the endogenous *Gapdh* control. Then the C_T values of the tested target gene and *Gapdh* control in HOXB4-ER^{T2} sample were compared to the C_T values of them in the GFP sample to generate a relative expression change of this specific target gene between the 2 samples.

The quantitative PCR results correlated fairly well with the microarray data and also showed that changes in the gene expression present in 12 hours TAM-treated samples were also present at 6 hours after TAM treatment, consistent with an immediate transcriptional effect.

2.4.6 *Hemgn* mRNA Expression Level in Bone Marrow and c-kit⁺Scal-1⁺Lin⁻ Cells from *Hoxb4*^{-/-} Mice

Hemgn was one most interesting gene that was upregulated at a relatively high level compared to other genes and appeared to be an interesting gene for further study. *Hemgn* is a nuclear protein specifically expressed in hematopoietic stem and progenitor cells that appears to protect cells from apoptosis and differentiation.^{170:172} We obtained whole bone marrow and c-kit⁺scal-1⁺Lin⁻ bone marrow cells from *Hoxb4*^{-/-} mice and showed by real-time PCR that *Hemgn* mRNA was significantly decreased relative to that seen in control cells from wild type mice (Figure 2-11), indicating that *Hemgn* may also be regulated by endogenous *Hoxb4*. The $\Delta\Delta C_T$ method was used for quantification of the relative gene expression level.

2.5 Discussion

The function of transcription factors may be confounded by factors such as redundancy, pleiotropic overlap, sequential downstream pathway activation due to the ectopic expression. The use of inducible expression cassettes or inducible nuclear targeting of transcription factors may overcome these limitations, and such systems have been widely used for direct target identification of transcription factors. In our study, an inducible HOXB4-ER^{T2} system was generated and proved to work well *in vitro*, recapitulating the functional phenotype caused by HOXB4 overexpression. Most importantly, the inducible construct did not cause any detectable leakiness in the absence of TAM treatment. Our use of the inducible HOXB4-ER^{T2} system allowed the identification of very early and potential direct downstream effectors of HOXB4, with the controlled application of TAM. A significant advantage of this system is that it ensures the detected gene expression changes are based on biological coherence instead of random events, since the myeloid progenitor CFU-C

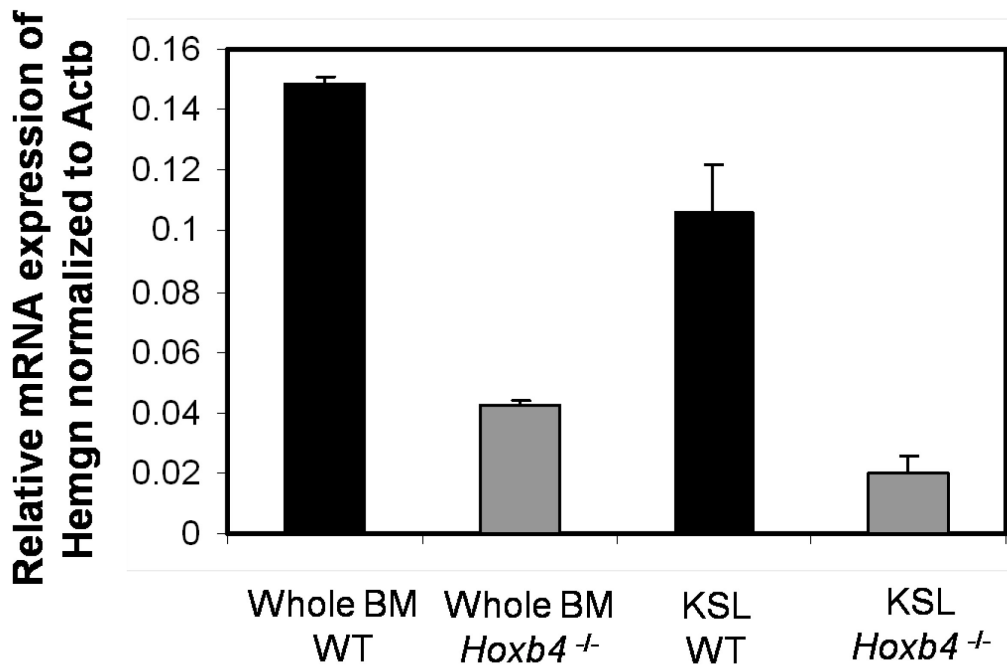


Figure 2-11. Quantitative real-time PCR detection of relative expression level of *Hemgn* mRNA in whole bone marrow or c-kit⁺Scal-1⁺Lin⁻ (KSL) cells from *Hoxb4*^{-/-} mice compared to wild type (WT) mice. The calculation is based on the quantitative comparative C_T (2^{-ΔΔCT}) method with normalization to *Actb* internal control. Four biological independent experiments were applied in this assay.

assays were validated in advance to reflect the function of induced HOXB4 in promoting hematopoietic progenitor cell expansion.

The gene profiling assay for the expression change between the HOXB4-ER^{T2} plus TAM samples versus the GFP plus TAM samples is a reasonable comparison method to exclude the effect caused by TAM itself. The criteria for screening the targets of HOXB4 were stringent in our study. A 1.5 fold threshold for gene differential change was selected since only 60 to 70% of the cells in a given sample were transduced, hence the 1.5 fold change measured from the samples would underestimate what actually occurred in the transduced cells. I tried once to sort transduced bone marrow cells for the gene screening assay, but due to the variance generated from the sorting process to the cells, I decided to use non-sorted cells in this study. We found HOXB4 activation did not induce a global gene expression change, as the gene expression showed high reproducibility between and within each experiment, indicated by the fact that the Pearson correlation coefficients between the 8 experimental samples ranged from 0.91 to 0.97. This data indicate that the chromatin structure was not altered to initiate a global change in gene expression, and only a small subset of specific genes were impacted by HOXB4 activation. Hierarchical clustering analysis and heat map generated from the identified 77 probesets from the 4 independent experiments further validated the high reproducibility between experiments. In this small target gene list, we found that 44 genes were reproducibly upregulated and 33 of them were downregulated by HOXB4, indicating that HOXB4 can both activate and repress downstream gene expression, which is consistent with previous results.¹⁴⁰ The identified target genes were shown to be involved in a variety of cellular processes, of which, the ones classified in signaling pathways, development and differentiation, cell proliferation and growth might be the most significant targets for further focus.

Further PCR confirmation of the selected genes with highest fold change between HOXB4-ER^{T2} and GFP samples validated the quality of the data generated from microarray screening. As 12 hours TAM treatment was used in microarray assays, to discern whether the detected gene expression change also exists at an earlier time point, samples from 6 hours of TAM treatment were also included and showed that most of the studied genes were also altered at 6 hours.

A gene called *Hemgn* was pursued further based on its relative high degree of expression change. After reviewing the literature, we found that relatively little is known about *Hemgn*, which was cloned in 2001.¹⁷⁰ Its expression is restricted to testis and hematopoietic tissues. In this regard, its spatial and temporal expression pattern is similar to endogenous *Hoxb4*. Overexpression of human *HEMGN* in transgenic mice caused disruption of the balance of myelopoiesis and lymphopoiesis,¹⁷³ suggesting an important role in hematopoiesis. This promoted us to study *Hemgn* further as an important HOXB4 target gene.

We recognize that other potential HOXB4 target genes identified from microarray screening are also likely to contribute to the downstream signaling network of HOXB4 in conveying its function. This 77 candidate list can serve as a resource for further research on HOXB4-promoted expansion effect. We did select several genes with relative high degrees of changes in expression between HOXB4-ER^{T2} and GFP samples based on microarray and real-time PCR results. I cloned their cDNA upstream of the IRES cassette in the MSCV-IRES-GFP retroviral vector for overexpression study. Stable GPE+86 vector producer cell lines overexpressing those individual target genes were generated to provide vector supernatant for bone marrow transduction. Bone marrow cells overexpressing individual target genes were tested for myeloid progenitor expansion ability in the secondary CFU-C assay as a prompt *in vitro* evaluation method for their function. In this assay, however, only Hemgn showed ability to promote myeloid progenitor expansion *in vitro*. Two of the upregulated genes by HOXB4: Thy1 or Ephx2, when overexpressed, did not cause expansion of myeloid progenitor cells. In contrast, one of the downregulated genes by HOXB4: Ms4a4c, inhibited expansion of myeloid progenitor cells when it was overexpressed. This suggests that downregulation of Ms4a4c could be a mechanism of HOXB4's expansion ability. In general, the data provided us an additional practical reason to focus on investigating *Hemgn* instead of other candidates in our study.

Interestingly, the mRNA expression of Hemgn also showed correlation with endogenous Hoxb4 expression. A 5-fold decrease in Hemgn mRNA was found in Hoxb4-deficient c-kit⁺Scal-1⁺Lin⁻ bone marrow cells compared to wild type cells. This data further strengthens the hypothesis that Hemgn is regulated by HOXB4. Because the expression of Hemgn can still be detected in the absence of Hoxb4, the residual amount of Hemgn expression possibly still effectively maintains the normal hematopoietic stem and progenitor cell expansion. Since Hox4 paralog members show redundancy in their functions,¹⁶⁷ investigation of whether Hemgn could be further downregulated in *Hoxa4;b4;c4;d4* quadruplex knockout mice would be interesting.

CHAPTER 3. DEMONSTRATION THAT HEMGN IS A DIRECT TRANSCRIPTIONAL TARGET OF HOXB4

3.1 Summary

Among the 77 candidate target genes identified by microarray screening using the HOXB4-ER^{T2} inducible system, *Hemogen (Hemgn)*, a newly discovered nuclear protein specifically expressed in hematopoietic cells with reported functions in blocking apoptosis, inhibiting differentiation, and promoting survival, is a particularly interesting one for us to pursue further. Here we show that Hemgn is indeed the first identified direct transcriptional target of HOXB4 in primary bone marrow cells. Promoter activity of *Hemgn* was increased by HOXB4 in MEL cells in transient co-activation assays, implying direct transactivation of Hemgn by HOXB4. Furthermore, we show HOXB4 directly bound to the promoter fragment of Hemgn in electrophoresis mobility shift assay (EMSA). Most importantly, we validated that HOXB4 was recruited *in vivo* to the Hemgn promoter region in primary 5-FU treated hematopoietic progenitor cell-enriched bone marrow cells, through chromatin immunoprecipitation (ChIP) assay.

3.2 Introduction

Traditionally, experimental approaches have sought to identify target genes of transcription factors by measuring gene expression in loss- or gain-of-function assays, while transcription factor-binding to transcriptional regulatory regions has been evaluated using EMSA, co-transfection or ChIP assays. Classic microarray for gene expression profiling or genome-wide location data for transcription factor binding from ChIP-chip experiments does not provide definitive evidence of target regulation. These screening methods should always be combined with functional biological experiments for targets confirmation.

Among the potential candidates from the microarray screening, we decided to focus on *Hemgn*, based on its relatively high degree of upregulated expression induced by HOXB4, its known hematopoietic expression pattern, and given the fact that relatively little is known about this gene and its potential function in hematopoiesis. Hemgn is a recently discovered nuclear protein that is restrictively expressed in testis and hematopoietic tissues.^{170;171} It shows a spatial and temporal expression pattern in hematopoietic sites during embryogenesis, and is expressed in the blood islands of yolk sac, then in fetal liver, and finally in the adult spleen and bone marrow.¹⁷⁰ In adult bone marrow, Hemgn is predominantly expressed in primitive progenitor and stem cell populations, but not in terminally differentiated mature blood cells.¹⁷⁰ Human *HEMGN*, also called *EDAG*, shows a similar expression pattern in bone marrow cells and interestingly, maps to chromosome 9q22 which contains well known

leukemic breakpoints.¹⁷⁰ Relatively high levels of *EDAG* expression have been observed in human acute myeloid leukemia bone marrow cells.¹⁷⁵ In transgenic mice in which *EDAG* was driven by human CD11a promoter, enhancement of myelopoiesis and suppression of lymphopoiesis was found, suggesting its regulatory role in hematopoiesis.¹⁷³ To our knowledge, *Hemgn* has not been knocked out in a mouse genetic model so that the null phenotype is unknown.

The activity of the *Hemgn* promoter containing 2975 bp of 5' flanking sequence and 196 bp of 5' UTR was tested in a luciferase assay and was reported to be active in a hematopoietic cell line K562, but not in nonhematopoietic cell lines.¹⁸⁴ This promoter region was also able to drive the transcription of a lacZ transgene in hematopoietic tissues in transgenic mice.¹⁸⁴ GATA1 was demonstrated to bind to 2 GATA binding sites in this promoter region of *Hemgn* in the K562 cells and to transactivate it. EMSA showed direct binding of GATA1 to the *Hemgn* promoter in MEL cells. CHIP assay showed the recruitment of GATA1 to *Hemgn* promoter in Meg-01 AML cell line. This study suggests that the *Hemgn* promoter contains critical regulatory elements for its transcription in hematopoietic tissues and *Hemgn* is a direct target of GATA1 in leukemia cell line K562.¹⁸⁴ Except for the data from this report, there is no other literature on the transcriptional regulation of *Hemgn* and no information exists for its regulation in primary hematopoietic cells.

In this study, after using the traditional method for identifying the target genes in a gain-of-function model of HOXB4, we then focused on validating a specific target, *Hemgn*, with supplemental co-transfection, EMSA, and CHIP assays.

3.3 Materials and Methods

3.3.1 Transient Cotransfection Luciferase Assay

In the promoter region of *Hemgn*, -1887bp ~ +213bp region containing the 4 putative HOXB4 binding sites based on sequence analysis. The transcription start site (TSS) was cloned upstream of a luciferase reporter gene in pGL4.12-Luc2CP vector (Promega, Madison, WI). 1 µg pGL4.12-Luc2CP vector and 0.5 ng renilla control pGL4.75-Rluc/CMV vector were transiently transfected with either 1 µg MSCV-IRES-GFP control or 1 µg MSCV-HOXB4-IRES-GFP vector into 2X10⁵ MEL cells with 4 µl Fugene 6 reagent (Roche Applied Science, Indianapolis, IN). Luciferase activity was measured by dual-luciferase reporter assay system (Promega, Madison, WI) 48 hours after transfection and was normalized to renilla activity.

3.3.2 Electrophoretic Mobility Shift Assay (EMSA)

A GST-tag was fused to the N-terminus of HOXB4 protein and the GST-HOXB4 fusion protein was expressed and purified through pET-GST Fusion System 42 (Novagen, EMD Chemicals Inc, San Diego, CA). Synthetic single strand DNA oligos were biotin-labeled with Biotin 3' END DNA labeling kit (Pierce, Holmdel, NJ) and annealed. The reactions were processed using LightShift Chemiluminescent EMSA Kit (Pierce, Holmdel, NJ) according to the manufacturer's instructions. Briefly, 20 fmol biotin-labeled probes were incubated with 5 μ g GST or GST-HOXB4 protein, together with 2 μ l 10x binding buffer, 1 μ g poly(dI.dC), HBC loading buffer (20% Glycerol, 20 mM HEPES (PH7.8), 0.2 mM EDTA, 1 mM DTT) and ultrapure water to a final 20 μ l reaction volume. For the cold probe competition reactions, 4 pmol unlabelled target oligos were additionally added in the reactions. For supershift analysis, 1 μ g Anti-HOXB4 antibody (clone I12) (The Developmental Studies Hybridoma Bank, the University of Iowa, Iowa City, IA) was incubated with 5 μ g GST or GST-HOXB4 for 30 minutes on ice preceding the DNA binding reaction. The DNA-protein complexes were separated by 6% TBE DNA retardation gel (Invitrogen, Carlsbad, California) and transferred to Biodyne B Nylon Membrane (Pierce, Rockford, IL). The probe containing complex in the crosslinked membrane was recognized by streptavidin-HRP and incubated with the substrate. The luminescent signal was captured by X-ray film. The sequence of the WT probe for the promoter of *Hemgn* is: 5' ACACTCTGCTAATTACAGCCTTT 3' (-1562bp ~ -1540bp); and sequence of the mutant probe is: 5' ACACTCTGCAGCATACAGCCTTT 3'.

3.3.3 Separation and Transduction of 5-FU Treated Bone Marrow Cells

Bone marrow cells were harvested 4 days after intraperitoneal injection of mice with 150 mg/kg 5-FU^{185,186} from the tibias and femurs of 7 to 12 weeks old female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) as previously described.¹⁸² Red blood cells were lysed with BD pharm Lyse™ lysing buffer (BD Biosciences, San Jose, CA). The 5-FU treated bone marrow cells were prestimulated for 2 days with the cytokine-supplemented DMEM medium described in Chapter 2. The method for transduction of the cells with retroviral vector supernatant collected from the GPE+86 producer cells were described in Chapter 2. After transduction, cells were collected for ChIP assay.

3.3.4 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed using ChIP-IT™ Express kit (Active Motif, Carlsbad, CA) following the manufacturer's instructions. Briefly, the transduced 5-FU treated bone marrow cells were fixed with 1% formaldehyde for 10 minutes at room temperature, and the chromatin from 2×10^7 cells was sonicated into fragments (~500bp). The chromatin-protein complex derived from 2.5×10^6 cells

was immunoprecipitated with either 1 µg anti-HOXB4 antibody (I12) or 1 µg IgG2A isotype control antibody (R&D Systems, Minneapolis, MN). The semi-quantitative PCR (Tag PCR core kit, Qiagen, Valencia, CA) was set as follow: 94°C 5 minutes; 35 cycles alternating between 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute; 72°C 10 minutes. The quantitative real-time PCR was performed with SYBR Green DNA incorporation method by using QuantiTect™ SBBR Green PCR kit (Qiagen) and was carried out on StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA), and was analyzed with quantitative comparative C_T (2^{-ΔΔCT}) method normalized to input control samples using StepOne Software v2.0 (Applied Biosystem, Foster City, CA). The PCR reaction program was set up as follow: 95°C 10 min; 40 cycles alternating between 95°C 15 seconds and 60°C 1 minute. The melting curves were generated by the program to validate one single PCR product for each reaction. The results were analyzed in quantitative comparative C_T (2^{-ΔΔCT}) method normalized to input control samples using StepOne Software v2.0 (Applied Biosystem, Foster City, CA).

The PCR primers are listed below:

1. Semi-quantitative and quantitative real time PCR primers for amplifying -54bp ~ +89bp in the promoter of *Actb*:

Forward: 5' CGCGAGCACAGCTTCTTTG 3'

Reverse: 5' GGTGTCCGTTCTGAGTGATCC 3'

2. Semi-quantitative PCR primers for amplifying regions in *Hemgn* promoter:

- (1). -1568bp ~ -1447bp region:

Forward: 5' GCCACCACACTCTGCTAATTAC 3'

Reverse: 5' CGGAGAGGAACTAGGAAAG 3'

(Also used in quantitative real time PCR)

- (2). -543bp ~ -228 bp region:

Forward: 5' GGTGAGCAGTGGAGATTAGC 3'

Reverse: 5' AGGGAGATCCCAGGGTTTAG 3'

3. Quantitative real-time PCR primers for amplifying regions in *Hemgn* promoter:

- (1). -1731bp ~ -1542bp region

Forward: 5' AGGATGGTGAGTTGAGGTCGGC 3'

Reverse: 5' AGGCTGTAATTAGCAGAGTGTGGTGG 3'

- (2). -1485bp ~ -1336bp region

Forward: 5' ACATTTCAAATGCTAGCCCCTTTCC 3'

Reverse: 5' CACCTTCTCTCTTTTATGCACACATGT 3'

- (3). -436bp ~ -332bp region
Forward: 5' GTGAAAACATAGCCACACCTAGGAAGAT 3'
Reverse: 5' CCACTCTTGCCTGTTTCATACACTTCTT 3'

3.4 Results

3.4.1 *The Promoter of Hemgn Contains Potential HOXB4 Binding Motifs*

Inspection of the promoter region (-1887bp ~ +213bp) of *Hemgn* revealed 4 potential HOXB4 binding sites with a “TAAT” core and preferred flanking nucleotides (Figure 3-1). We know that the “TAAT” core sequence is relatively common and could appear in any potential cis-regulatory elements, which could be in the promoter region 5' to *Hemgn*, or in an intron, or in the 3' untranslated region of *Hemgn*. Here we only examined the ~2 kb promoter region of *Hemgn*, and all the following functional evaluation were based on this region. The existence of multiple “TAAT” sites in this ~2 kb fragment suggests a potential increased abundance of HOXB4 on this promoter region due to the widespread binding sites. The specificity of potential HOXB4 binding is possible based on preferred flanking nucleotides of the 4 “TAAT”.

3.4.2 *The Promoter of Hemgn Was Transcriptionally Activated by HOXB4*

The -1887bp ~ +213bp region containing the 4 potential HOXB4 sites and the transcription start site (TSS) was cloned upstream of a luciferase reporter gene and this construct was tested in a transient transfection assay in mouse erythroleukemia (MEL) cells. When this construct was co-transfected with an internal renilla control vector and an MSCV vector expressing either HOXB4 or a GFP control protein, HOXB4 significantly increased the luciferase activity in MEL cells (Figure 3-2), indicating that the promoter of *Hemgn* was directly activated by HOXB4. However, the limitation of this assay is that first, the *Hemgn* promoter fragment used in the transfected luciferase reporter plasmid may not mimic the true transcriptional regulation of the endogenous gene. Second, both plasmid DNA and HOXB4 protein expression are introduced transiently and at high episomal copy numbers; far beyond the physiological level, potentially forcing non-physiologic the DNA-protein interactions. Third, a cell line instead of primary cells in this assay based on transfection efficiency considerations. Therefore, we sought independent confirmation of this putative interaction.

3.4.3 *HOXB4 Directly Bound to the Promoter Fragment of Hemgn In Vitro*

Among the 4 putative HOXB4 binding sites (Figure 3-1), site 1 (-1555bp~-1548bp) contains the most favored flanking nucleotides for high affinity HOXB4

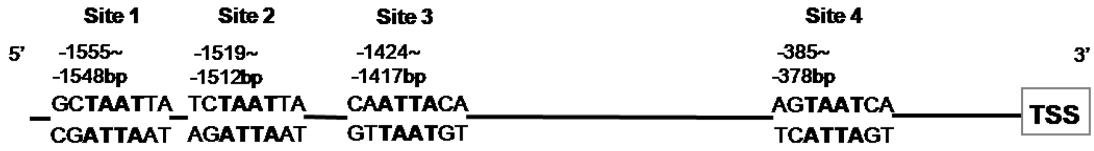


Figure 3-1. Schematic illustration of the multiple putative HOXB4 binding sites on the promoter region of *Hemgn* (site 1, 2, 3 and 4).

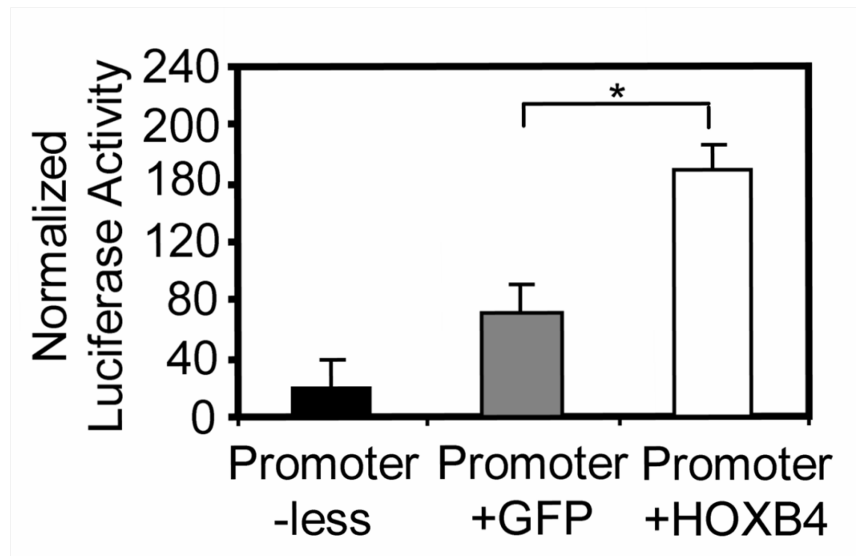


Figure 3-2. HOXB4-dependent increase in promoter activity of *Hemgn* in MEL cells. * p<0.05

binding.¹²⁹ To directly test whether HOXB4 can bind to this site, we designed DNA oligonucleotide probes spanning this specific site (-1562bp ~ -1540bp) to examine the binding of HOXB4 by using EMSA. Indeed, HOXB4 binding caused a band shift that was detected when the biotin-labeled wild type (WT) probe was incubated with purified GST-HOXB4 protein (Figure 3-3, lane 2). No band shift was detected in the reactions with a competitive unlabeled probe (lane 4), or a probe containing mutated “TAAT” core sequence (lane 6), or with control GST protein (lane 1, 3, 5, and 7). Addition of a HOXB4 antibody resulted in supershifting of the specifically retarded fragment (lane 8). These results demonstrate that HOXB4 can directly bind to site 1 in the promoter region of *Hemgn* *in vitro*. The limitation of this assay lies in the fact that excessive probes and purified HOXB4 protein were incubated together *in vitro* in reaction tube, and therefore do not directly test for *in vivo* occupancy of sites.

3.4.4 HOXB4 Was Recruited to the Promoter Region of Hemgn In Vivo

To discern whether HOXB4 can be recruited to the promoter region of *Hemgn* in bone marrow cells *in vivo*, we developed the first reported ChIP assay for HOXB4 binding in transduced bone marrow cells by using an anti-HOXB4 antibody (clone I12) that we first showed can be used to efficiently immunoprecipitate HOXB4 from a HOXB4-overexpressing cell line (Figure 3-4). The advantage of the ChIP assay is that the detected DNA-protein interaction occurs in the living cells *in vivo* instead of in artificial established *in vitro* system, faithfully reflecting the true biological process in a physiological microenvironment. Primary bone marrow cells were used here in which enforced expression of HOXB4 was shown to induce expansion of myeloid progenitor cells. PCR primers were designed to span several regions in the cis-regulatory elements of *Hemgn* that contain each of the four putative HOXB4 binding sites.

Cellular extracts from transduced bone marrow cells were immunoprecipitated with the I12 antibody and DNA fragments of an average 500bp in size were released for PCR detection (Figure 3-5). We designed semi-quantitative PCR primer pairs to span the -1568bp ~ -1447bp region containing 2 putative HOXB4 binding sites (site 1 and site 2, Figure 3-1) or to span the -543bp ~ -228bp region which contains 1 putative site (site 4, Figure 3-1). These two regions were specifically enriched by the I12 anti-HOXB4 antibody in the HOXB4-transduced bone marrow cells (Figure 3-6). In contrast, neither of these two regions was enriched in cells transduced with the GFP control vector, or in HOXB4 vector-transduced cells immunoprecipitated using an isotype control antibody. PCR primers amplifying an irrelevant region in *Actb* promoter containing no “TAAT” sequence showed no detectable enrichment with either antibody (Figure 3-6). These results verify that HOXB4 bound to the promoter of *Hemgn* *in vivo* in primary bone marrow cells that overexpressed HOXB4.

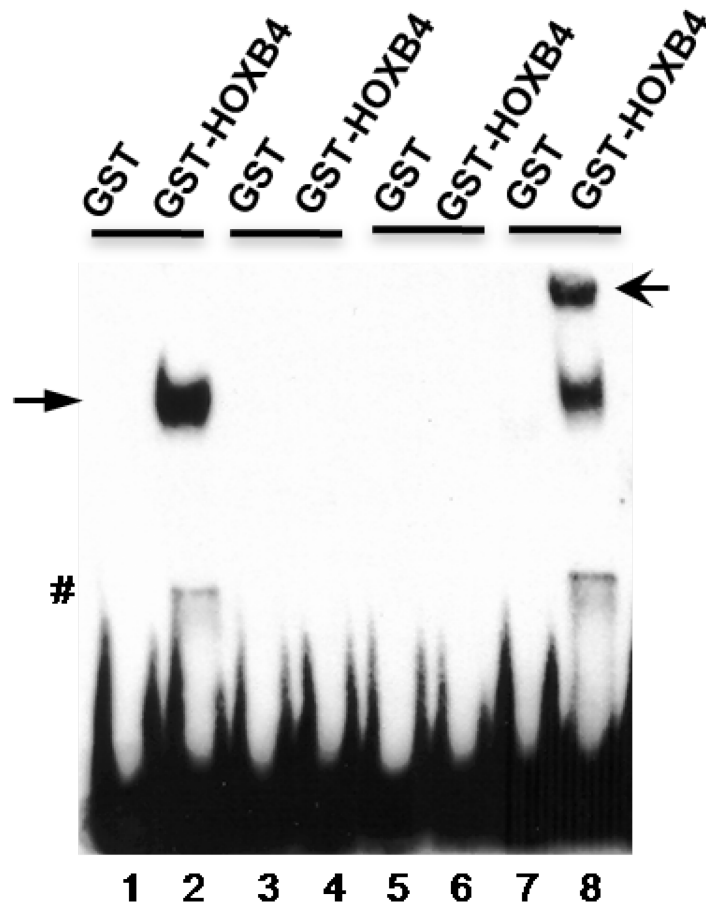


Figure 3-3. HOXB4-specific binding caused band shift of probes containing the putative HOXB4 binding site (site 1) in EMSA. 1-2: biotin-labeled WT probe (-1562~-1540bp of *Hemgn* promoter); 3-4: labeled WT probe: competitive unlabeled probe=1:200; 5-6: probe with “TAAT” core sequence mutated to “AGCA”; 7-8: labeled WT probe + anti HOXB4 antibody (I12); solid arrow: band shift by HOXB4; open arrow: supershift by I12; #: degraded GST-HOXB4 caused band shift.

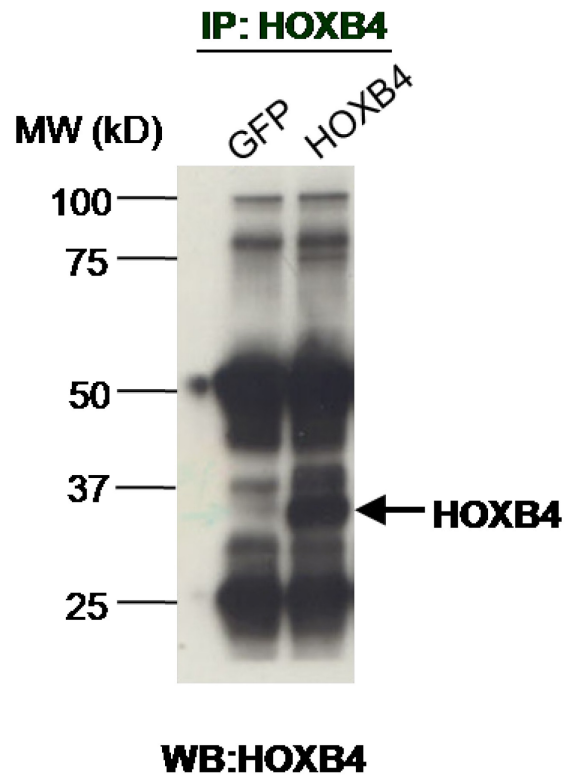


Figure 3-4. Immunoprecipitation and western blotting detection of HOXB4 protein from GPE+86 cells overexpressed with GFP control or HOXB4 with monoclonal anti-HOXB4 antibody (I12).

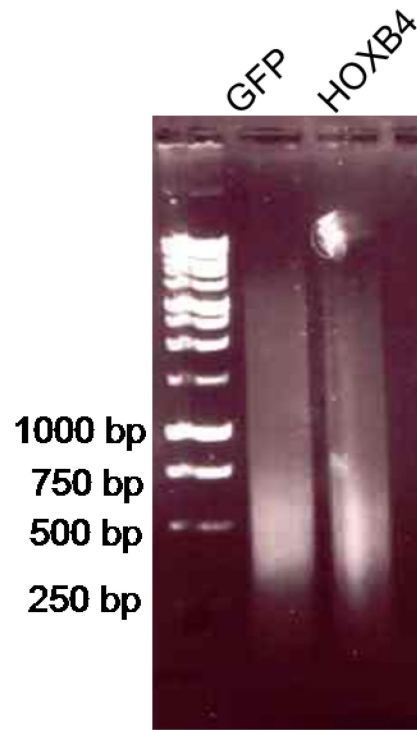


Figure 3-5. Sonicated chromatin fragments from GFP or HOXB4-transduced bone marrow cells for CHIP assay.

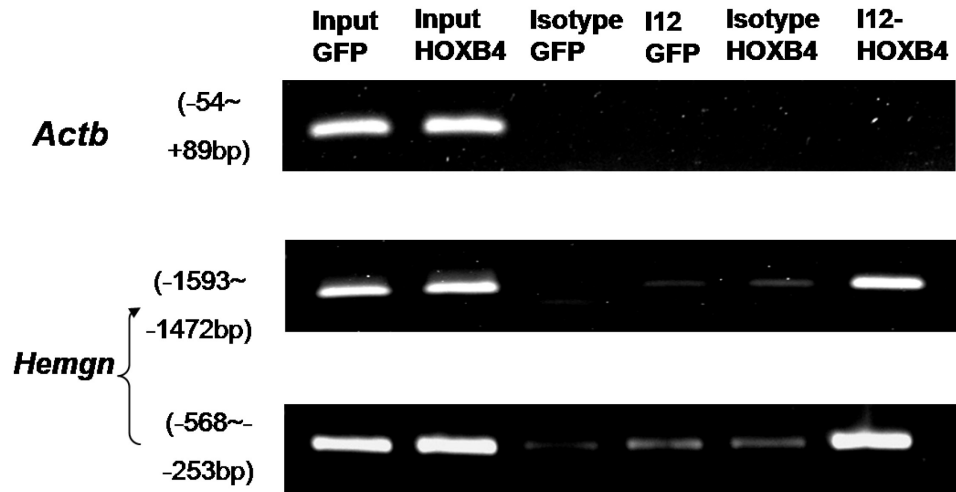


Figure 3-6. Semi-quantitative PCR detected enrichment of the promoter fragments of *Hemgn* by HOXB4 specific binding following CHIP assay in transduced bone marrow cells.

To further quantify the fold enrichment of HOXB4 on the individual “TAAT” site, quantitative real-time PCR primers were designed to amplify the individual putative HOXB4 binding site in a 5’ to 3’ order in the promoter of *Hemgn* (Figure 3-7). The -1731bp~-1542bp (site 1, Figure 3-1) and the -436bp~-332bp (site 4, Figure 3-1) regions were enriched at relatively higher levels (16.1 fold and 34.8 fold). As sites 1, 2 and 3 are very close to each other within 500bp, and due to the sonicated chromatin being around 500bp, probably all the 4 sites contributed to direct binding by HOXB4.

3.5 Discussion

Classic microarray analysis for gene expression profiling does not provide definitive evidence of target gene relationships and needs to be combined with DNA binding data in order to identify direct target genes. In our study, the binding of the HOXB4 transcription factor to a target promoter has been measured using transient co-activation assays, EMSA, and ChIP assays. We selected a specific candidate target gene, called *Hemgn*, for these studies for the reasons discussed in Chapter 2, but this could in principle be done with any of the genes in our list.

Multiple “TAAT” core sequences with HOXB4-preferred flanking nucleotides were found within the 2kb promoter region of *Hemgn*. As mentioned previously in Chapter 1, Hox regulation of downstream target genes occurs through binding to specific conserved DNA sequences in their regulatory elements. However, other Hox family members in addition to HOXB4 may also bind to the same “TAAT” core sequence through the homeodomain. Discrepancy exists between the relatively promiscuous binding of Hox monomers to DNA and the high specificity of Hox protein functions. It is hypothesized that the specificity of the downstream target regulation of Hox strongly depends on the flanking nucleotides surrounding the core sequence and on the cofactors which interact with Hox proteins to further increase the selective binding of Hox and cofactor heterodimers to DNA. However, prior studies have shown that one cofactor of HOXB4, Pbx, is not required, and even limits, HOXB4-induced expansion of hematopoietic stem and progenitor cells *in vitro* and *in vivo*.^{140;168;169} Thus, the binding specificity of HOXB4 to regulatory elements in its target genes are more likely dependent on the flanking DNA sequence surrounding the “TAAT” core. The binding affinity possibly could be enhanced with existence of multiple “TAAT” core sequence other than single one in the regulatory elements.

We proved that at least some of the “TAAT” containing promoter sequences of *Hemgn* can be transcriptionally activated by HOXB4 *in vitro* by luciferase reporter assays, implying that this promoter is regulated by HOXB4 *in vivo*. Furthermore, direct binding of purified HOXB4 to a promoter fragment of *Hemgn* was confirmed *in vitro* by EMSA. This finding suggests that the protein-DNA complex can be formed with purified GST-HOXB4 and that other cofactors of HOXB4 such as Pbx are not required for the direct binding of HOXB4 to these sequences.

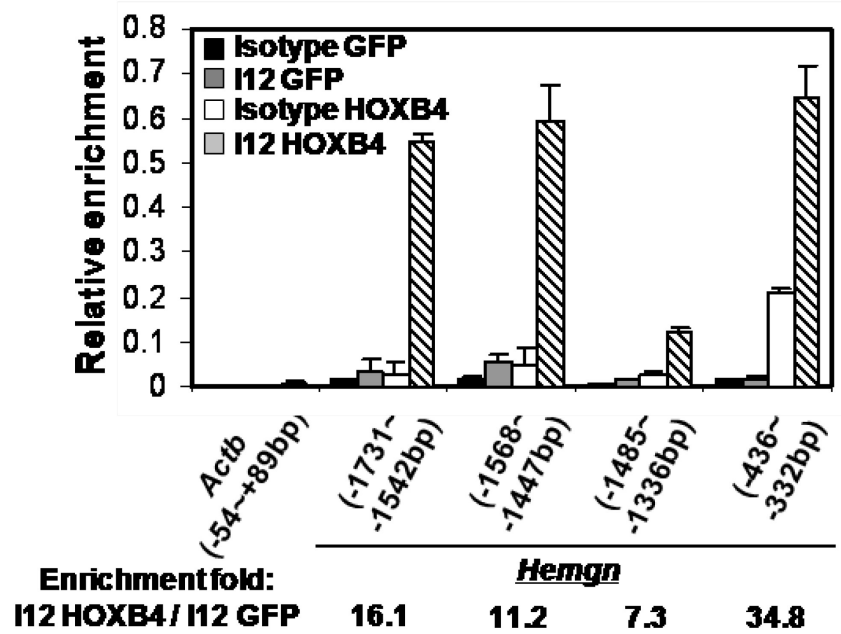


Figure 3-7. Quantitative real-time PCR detected relative enrichment of individual regions in *Hemgn* promoter by HOXB4 following ChIP assay in transduced bone marrow cells. All values were normalized to the corresponding input control sample.

For the first time, we have established a ChIP assay for HOXB4 binding and show that *Hemgn* is indeed a direct target gene of HOXB4 *in vivo*. This validated *in vivo* HOXB4 binding region encompasses multiple “TAAT” sites identified in the promoter region of *Hemgn*. Intriguingly, each of the four “TAAT” sites in the promoter region of *Hemgn* was enriched to a similar extent in immunoprecipitated samples. This is possibly due to the fact that those “TAAT” sites are close in location, thus multiple “TAAT” sites within the 500bp sonicated chromatin fragments were immunoprecipitated and detected by PCR. It is necessary to mention again that when we mutated the “TAAT” core on site 1 which contains the most favored flanking nucleotides of HOXB4, the GST-HOXB4-DNA interaction was disrupted *in vitro* in EMSA. However, to determine exactly which site contributes most to the binding and downstream functional effects, site-mutagenesis assays could be performed in future studies.

In ChIP assays, we used constitutively overexpressed HOXB4 instead of inducible HOXB4-ER^{T2}, based on the fact that although *Hemgn* is upregulated by HOXB4-ER^{T2} as early as 6 hours after TAM treatment, expression of *Hemgn* is also maintained at a high level in constitutively overexpressed HOXB4 samples by microarray (Brian P. Sorrentino’s lab, data not shown). These data indicate that the direct regulation of *Hemgn* by HOXB4 is persistent after the initial binding and activation.

CHAPTER 4. EXPRESSION OF HEMGN EXPANDS BONE MARROW AND HEMATOPOIETIC PROGENITOR CELLS AND PROTECTS AGAINST APOPTOSIS

4.1 Summary

To discern whether Hemgn is a downstream functional target of HOXB4, we constructed a MSCV retroviral vector for Hemgn overexpression. When overexpressed in 5-FU treated bone marrow cells, Hemgn promoted expansion of bone marrow cells and myeloid progenitor cells in *in vitro* liquid cultures and in secondary colony forming unit-culture (CFU-C) assays, recapitulating the effect of overexpressed HOXB4. Furthermore, both Hemgn and HOXB4 were antiapoptotic in transduced 5-FU treated bone marrow cells following cytokine withdrawal, suggesting a possible cellular mechanism for understanding the role of Hemgn in promoting expansion of primitive hematopoietic cells.

4.2 Introduction

Hemgn is a recently discovered nuclear protein that is expressed in testis and hematopoietic tissues.^{170;171} In bone marrow, Hemgn is predominantly expressed in primitive progenitor and stem cell populations, but not in terminal differentiated mature blood cells.¹⁷⁰ In transgenic mice in which *EDAG*, the human homolog of *Hemgn*, was driven by human CD11a promoter, enhancement of myelopoiesis and suppression of lymphopoiesis was found, suggesting a regulatory role in hematopoiesis.¹⁷³ Since HOXB4 overexpression can cause bone marrow cell expansion during extended culture with myeloid cytokines,⁹⁵ and because *Hemgn* is a direct transcriptional target of HOXB4 as discussed in Chapter 3, it is necessary to study the functional effects of Hemgn and whether Hemgn overexpression could recapitulate the expansion phenotype of HOXB4.

4.3 Materials and Methods

4.3.1 Vector Construction

The murine *Hemgn* cDNA (NCBI Reference Sequence: NM_053149.2) was cloned into XhoI sites in MSCV-IRES-GFP backbone including the following sequences from the 5' UTR: 5' AAGCTTTTAGGCTGCTTGTGAAGTCAAAGCCAATACCAAGAAGGCATCGT GGCAAG 3' and the 3' UTR: 5' CAGTGCTCAGT 3' (Figure 4-1). A polyclonal population of ecotropic retroviral producer GPE+86 cells¹⁷⁹ was generated using a previously described approach.^{180;181} Briefly, 2 µg pCAGGS-VSVG and 4 µg

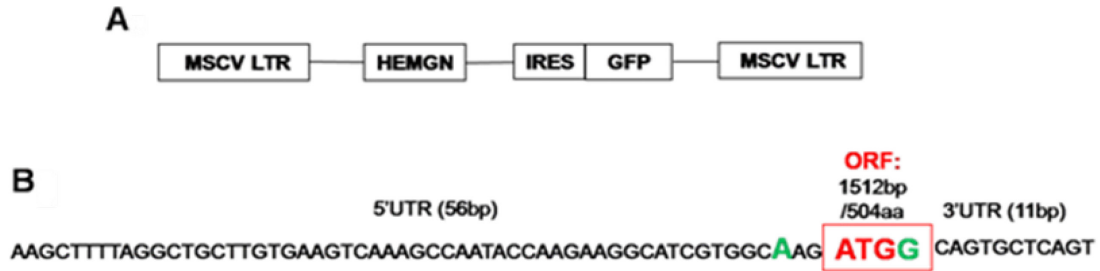


Figure 4-1. Hemgn-expressing retroviral vector. (A) MSCV-retroviral vector construct with cDNA of *Hemgn* inserted upstream IRES cassette. (B) DNA sequence of the cloned 5'UTR together with 3'UTR of the cDNA of *Hemgn* in the MSCV vector.

pEQ-PAM3(-E) helper plasmids were co-transfected with 4 µg MSCV-cDNA-IRES-GFP vector into 8×10^6 cycling 293T cells using FuGENE 6 reagent (Roche applied Science, Indianapolis, IN) according to the manufacturer's instructions. Retroviral vector supernatant were harvested 48 hours post transfection, pooled, filtered through 0.45 µm PVDF filter (Millipore, Billerica, MA), and used for transducing the cycling GPE+86 cells in the present of 6 µg/ml polybrene. The transduced GFP positive GPE+86 cells were sorted and expanded in culture for establishing stable retroviral vector producer lines. The helper-free recombinant retroviral vector supernatants freshly collected from the GPE+86 producer cells were filtered through 0.45 µm pore filter for transduction of bone marrow cells.

4.3.2 Southern Blotting

GPE+86 retroviral vector producer cells transduced with MSCV-IRES-GFP, MSCV-HOXB4-IRES-GFP, or MSCV-Hemgn-IRES-GFP vector were used in these assays. The genomic DNA extracted from the cells was digested with SacI to release the cDNA-IRES-GFP fragment between the 2 LTRs of the MSCV-cDNA-IRES-GFP vectors. A probe targeting GFP was used for detecting the liberated fragment.

4.3.3 Western Blotting

Whole cell extracts were prepared from GPE+86 cells by M-PER Mammalian protein extraction reagent (Pierce, Thermo Scientific, Rockford, IL) supplemented with the protease inhibitors (Halt™ protease inhibitor cocktail kit, Thermo Scientific, Rockford, IL; Pefabloc SC Plus, Roche Applied Science, Indianapolis, IN). The proteins were then resolved on a 4-12% gradient NuPAGE Bis-Tris gel (Invitrogen) and transferred to 0.45 µm PVDF membranes. The primary rabbit polyclonal IgG antibody for detecting Hemgn of mouse origin (M-180, sc-68360, Santa Cruz Biotechnology, INC, Santa Cruz, CA) was used for Hemgn detection.

4.3.4 Transduction of 5-FU Treated Bone Marrow Cells

The 5-FU treated bone marrow cells were prepared as described in Chapter 3. Briefly, bone marrow cells were harvested 4 days after intraperitoneal injection of mice with 150 mg/kg 5-fluorouracil (5-FU)^{185;186} from the tibias and femurs of 7 to 12 weeks old female C57BL and 6J mice (The Jackson Laboratory) as previously described.¹⁸² Red blood cells were lysed with BD pharm Lyse™ lysing buffer (BD Biosciences, San Jose, CA). The 5-FU treated bone marrow cells were prestimulated for 2 days with the cytokine-supplemented DMEM medium as described in previous Chapters. The cells were then spin-infected at 2000 rpm for 30 minutes at room temperature by using plates preloaded with 25 µg/ml RetroNectin (Takara, Madison, WI) and retroviral vector supernatant generated

as described above at an multiplicity of infection (MOI) of 10 to 20. After 3 to 4 rounds of transduction in 2 consecutive days, cells were collected, washed with PBS, and then resuspended in the cytokine-supplemented medium. Proportions of the transduced GFP positive cells were determined by flow cytometry.

4.3.5 In Vitro Expansion Assays

After retroviral vector transduction, the transduction efficiencies obtained were typically 30% for the GFP control and HOXB4 vectors, but around 13% for the Hemgn vector. For liquid culture and CFU-C assays, cells were mixed with non-transduced cells to have the same GFP percentage (13%), and then equal numbers of normalized cells were cultured in cytokine-supplemented medium or M3434 methylcellulose medium as described previously. GFP percentage was determined by flow cytometry during the culture for evaluating growth advantage.

4.3.6 Apoptosis and Cell Cycle Assays

The transduced 5-FU treated bone marrow cells were cultured in the cytokine-supplemented Dulbecco's modified Eagle medium (DMEM) containing 20 ng/ml recombinant mouse IL-3, 50 ng/ml human IL-6, 50 ng/ml mouse SCF (R&D Systems, Minneapolis, MN), 15% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (R&D Systems, Minneapolis, MN). After 6 days, cells were washed with PBS and cultured in the medium containing 1% FBS and no cytokines. Sixteen hours later, 3×10^5 cells were stained with Annexin V-allophycocyanin (APC) (BD Biosciences, Franklin Lakes, NJ). Flow cytometry was used to detect GFP and APC percentage. For cell cycle analysis, the transduced cells were cultured for 6 and 16 days in the cytokine-supplemented medium and then were sorted to separate GFP positive and negative population, which were respectively stained with PI for DNA content analysis. The ModFit program (Verity Software House, Topsham, MI) was used to determine the percentage of cells at the G₁, S, and G₂-M phases of the cell cycle.

4.4 Results

4.4.1 Expression of Hemgn in Transduced Cells

In order to answer the question whether overexpression of Hemgn could contribute to the expansion phenotype of HOXB4, a full length cDNA of murine *Hemgn* ordered from ATCC was cloned into the MSCV-IRES-GFP retroviral vector. Integration and protein expression of *Hemgn* were confirmed in GPE+86 vector producer cells by southern and western blotting (Figure 4-2A and B). However, the GFP expression from the MSCV-Hemgn-IRES-GFP vector was

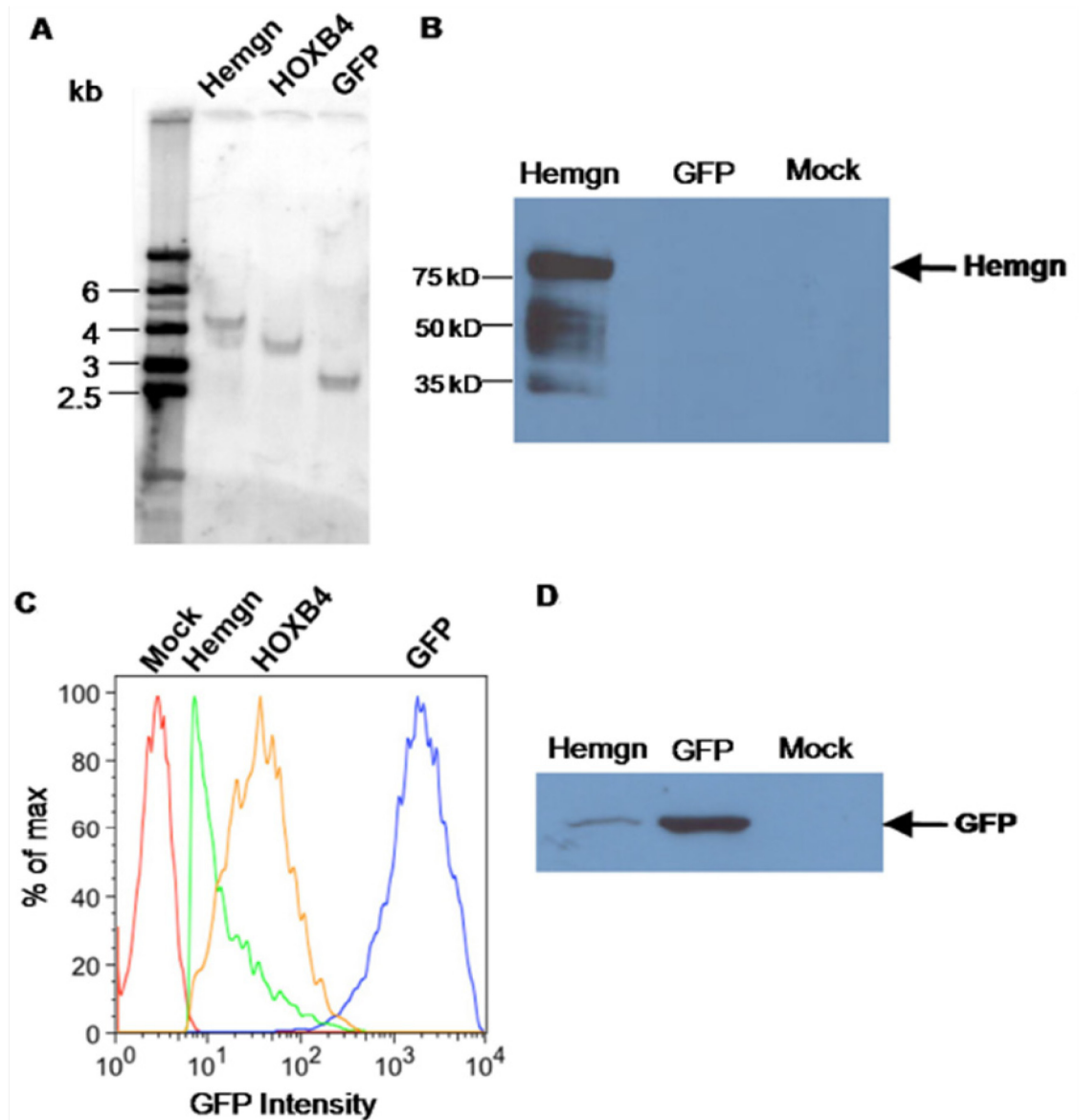


Figure 4-2. Integration, and protein expression of *Hemgn* and its GFP reporter in GPE+86 vector producer and bone marrow cells. (A) Southern blot detected the expected fragment size of integrated MSCV-cDNA-IRES-GFP vector: Hemgn vector-4547bp; HOXB4 vector-3576bp; GFP-only vector-2881bp. Enzyme: *SacI*; Probe: GFP. (B) Western blot with polyclonal anti-Hemgn antibody (M-180) for Hemgn protein detection in GFP-only or Hemgn vector-transduced GPE+86 cells. (C) FACS analysis plot for GFP signal in bone marrow cells transduced with GFP control, Hemgn or HOXB4 vector. (D) Western blot detected GFP protein expression in GFP-only or Hemgn vector-transduced GPE+86 cells. Smear bands around 50kd and 35kd: degraded Hemgn.

relatively low compared to control vectors as indicated by the mean fluorescence intensity for GFP signal in the transduced bone marrow cells (Figure 4-2C) and by the western blotting for GFP in the GPE+86 vector producer cells (Figure 4-2D). Furthermore, the titer of the MSCV-Hemgn-IRES-GFP vector was relatively low ($\sim 1 \times 10^5$ virus/ml compared to $\sim 1 \times 10^6$ virus/ml from HOXB4 producer cells). These problems with the Hemgn vector might be due to instability of the vector coded mRNA or the complex structure of the inserted cDNA of Hemgn. This deficiency resulted in relatively low transduction efficiency when bone marrow cells were transduced with this vector supernatant.

4.4.2 Hemgn Promoted Hematopoietic Cell Expansion in Liquid Culture

Despite the problems with the MSCV-Hemgn-IRES-GFP vector, I next tested the effects of enforced overexpression of Hemgn in primary hematopoietic cells. 5-FU was used to generate a hematopoietic progenitor cell-enriched population for vector transduction. Because the transduction efficiency of MSCV-Hemgn-IRES-GFP vector was 13%, we diluted the GFP control and HOXB4-overexpressing bone marrow cells with mock cells to the same proportion to examine whether there will be a growth advantage in subsequent liquid culture and CFU-C experiments (Figure 4-3).

We found that cells transduced with MSCV-Hemgn-IRES-GFP vector had a significant growth advantage over non-transduced cells in liquid culture, similar to the results seen with the HOXB4 vector (Figure 4-4). This was demonstrated by the increased GFP percentage of Hemgn-overexpressing and HOXB4-overexpressing cells at day 6 (Hemgn: 31%; HOXB4: 44%), day 11 (Hemgn: 50%; HOXB4: 78%) and day 16 (Hemgn: 77%; HOXB4: 91%) from 3 biological independent experiments (Figure 4-4). In contrast, cells transduced with GFP control vector did not show any growth advantage during this time. These results show that, Hemgn and HOXB4 promote expansion of hematopoietic cells in liquid culture.

4.4.3 Hemgn Promoted Myeloid Progenitor Cell Expansion In Vitro

As HOXB4 overexpression can also cause dramatic expansion of myeloid progenitor cells,⁹⁵ we examined whether Hemgn has similar effect in CFU-C assays. The 13% cell mixtures were plated in semi solid medium and re-plated 7 days later to generate secondary CFU-C colonies. This assay allowed quantitation of myeloid progenitor expansion. We found that Hemgn-overexpressing bone marrow cells generated significantly more secondary CFU-C colonies than mock (4.9 fold) and the GFP control cells (6.1 fold) (Figure 4-5). As a positive control, the HOXB4-overexpressing bone marrow cells generated 7.9 fold and 9.9 fold more secondary colonies than mock and GFP control cells, respectively. To confirm that the detected increase of secondary CFU-C colonies

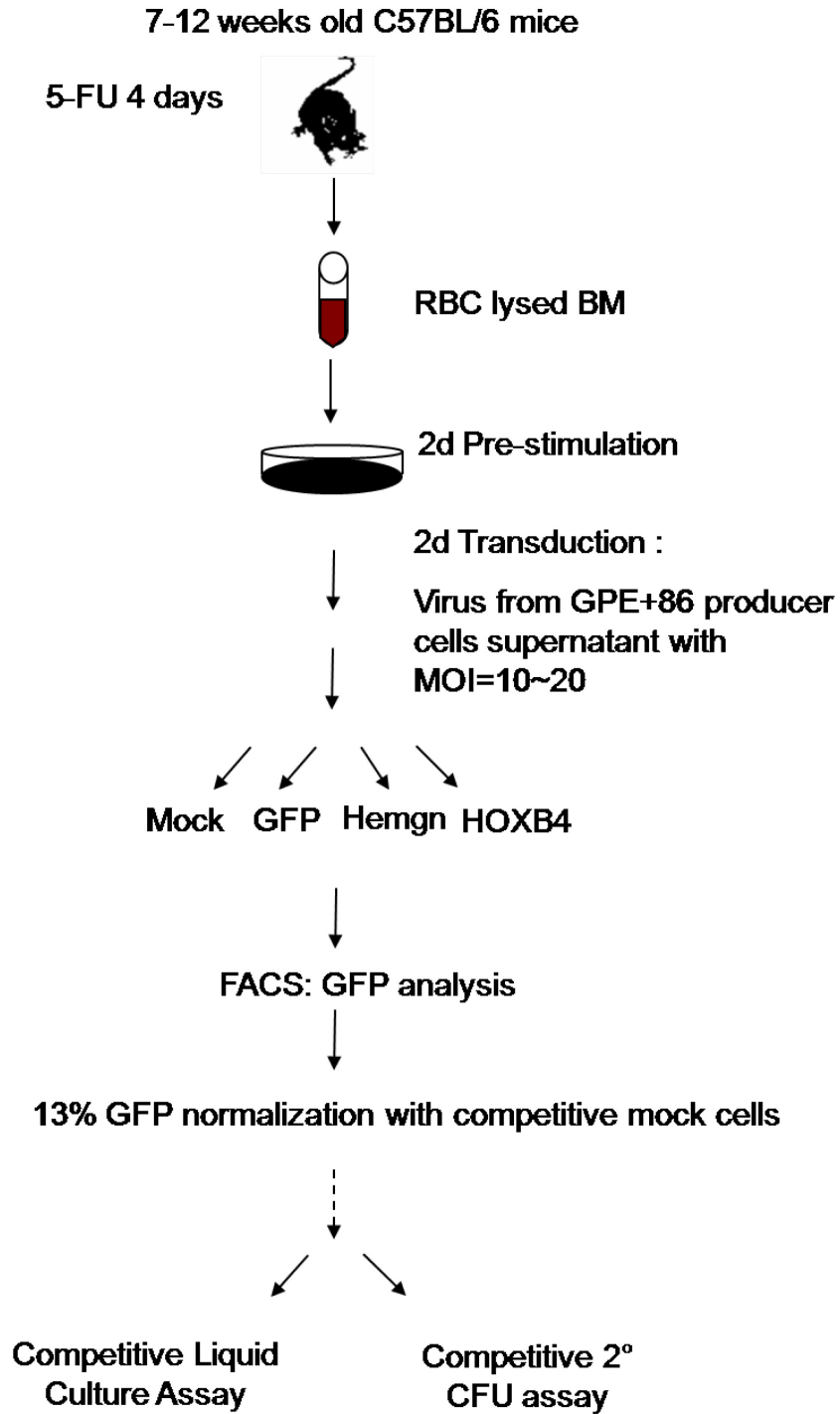


Figure 4-3. Experimental design of *in vitro* expansion assays.

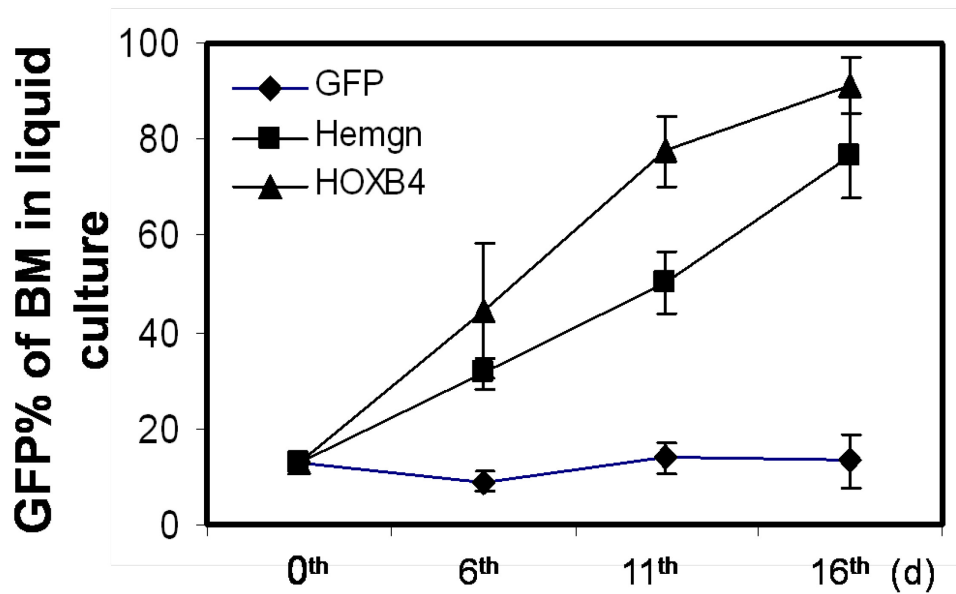


Figure 4-4. Growth advantage of Hemgn-overexpressing bone marrow cells in liquid culture. Flow cytometry-detected GFP percentage of the transduced bone marrow cells at 6th, 11th, 16th day in liquid culture post-transduction. 0th day: the day virus transduction was finished, when GFP percentage of each sample was normalized to 13% with mock cells. Three biological independent experiments were applied for statistics.

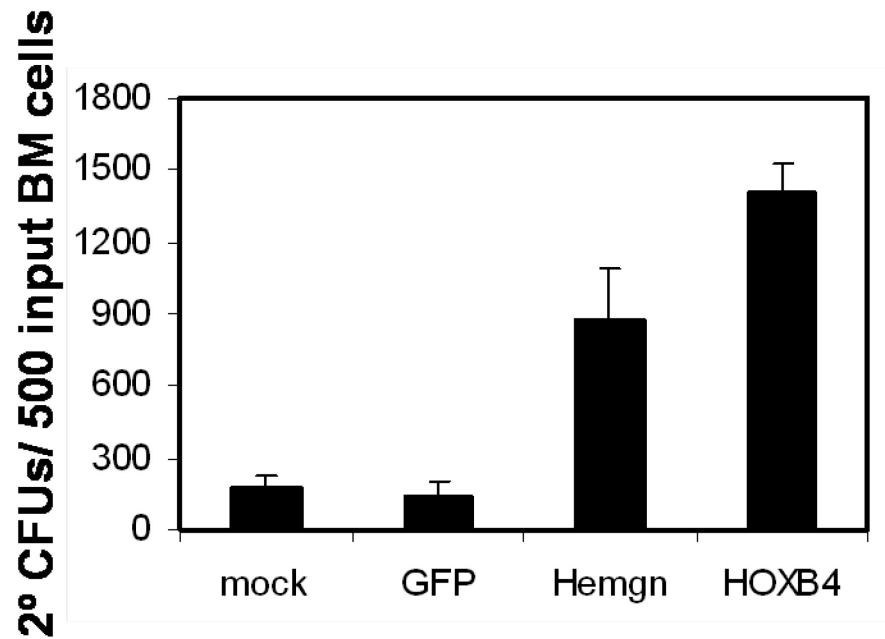


Figure 4-5. Growth advantage of Hemgn-overexpressing hematopoietic progenitor cells in methylcellulose culture. Secondary CFU-C colony number generated from the 13% GFP positive cell mixture. Three biological independent experiments were applied for statistics.

were indeed caused by expansion of the initial sub-population of Hemgn or HOXB4-transduced bone marrow cells, the proportion of GFP positive cells were scored by flow cytometry both from primary and secondary CFU-C dishes (Figure 4-6). We found that in Hemgn-overexpressing cells, the GFP percentage increased from the pre-plating 13% to 32% in cells collected from primary colonies and to 69% in cells collected from secondary colonies, respectively (Figure 4-6). As a positive control, GFP percentage in HOXB4-overexpressing cells increased from the pre-plating 13% to 58% in primary colony cells and to 91% in secondary colony cells, respectively. GFP control bone marrow cells did not show any expansion advantage. This result reveals that, Hemgn and HOXB4 transduced cells had a selective advantage during growth both in primary and in secondary semi-solid CFU-C assays.

4.4.4 Hemgn Protected Bone Marrow Cells from Apoptosis

To explore the potential cellular mechanism of how Hemgn promotes expansion of bone marrow cells *in vitro*, we first examined apoptosis phenotypes in Hemgn-overexpressing bone marrow cells. Transduced bone marrow cells were cultured for 6 days in cytokine-supplemented DMEM medium containing 20 ng/ml IL-3, 50 ng/ml IL-6 and 50 ng/ml SCF. The majority of those cells were viable (89%), and only a very small population was Annexin V+/ Propidium Iodide(PI)- (7%). The principle of this assay is based on the fact that the phosphatidylserine components of the phospholipid bilayers are externalized during the apoptotic process and can be detected by fluorescence labeled Annexin V, which is a member of the annexin family of calcium-dependent phospholipid-binding proteins. Under these conditions in liquid culture, Hemgn and HOXB4 decreased the proportion of Annexin V+/PI- cells from 7% to 2% and 1% respectively (data not shown). In order to induce more cells to undergo apoptosis, we then transferred these cells into DMEM medium containing 1% FBS and no cytokines. Sixteen hours later, cells were stained for Annexin V and PI. Flow cytometry was used to gate on the GFP positive and negative fractions for apoptosis detection.

A representative experiment (Figure 4-7) shows that both Hemgn and HOXB4 transduction resulted in a much smaller fraction of Annexin V+/PI- cells than seen in non-transduced cells or GFP control-transduced cells. Statistic analysis from 3 biologically-independent experiments (Figure 4-8) demonstrated that GFP control-transduced cells had a similar percentage of Annexin V+/PI- cells (45%) as non-transduced cells (43%) as expected. In contrast, HOXB4 transduction protected cells from apoptosis, demonstrated by the decrease of the percentage of Annexin V+/PI- cells in the transduced GFP positive population (3%). Hemgn transduction also resulted in an anti-apoptosis effect as seen by gating on transduced cells (17% Annexin V+/PI-). This was significantly different than that seen with non-transduced GFP negative cells (53% Annexin V+/PI-) (Figure 4-8).

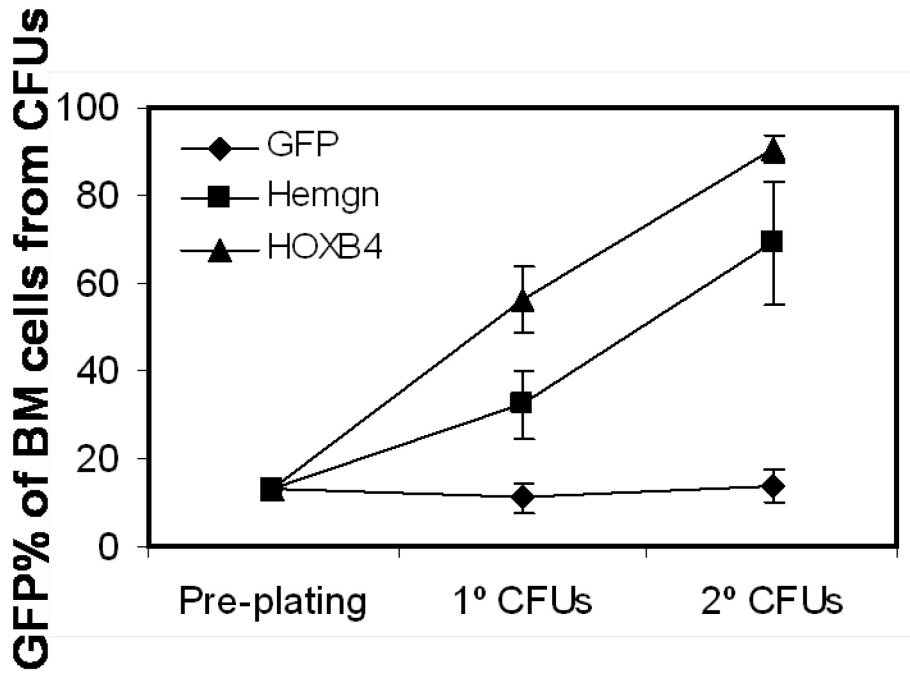


Figure 4-6. Flow cytometry-detected GFP percentage of bone marrow cells collected from primary and secondary CFU-C colonies. Three biological independent experiments were applied for statistics.

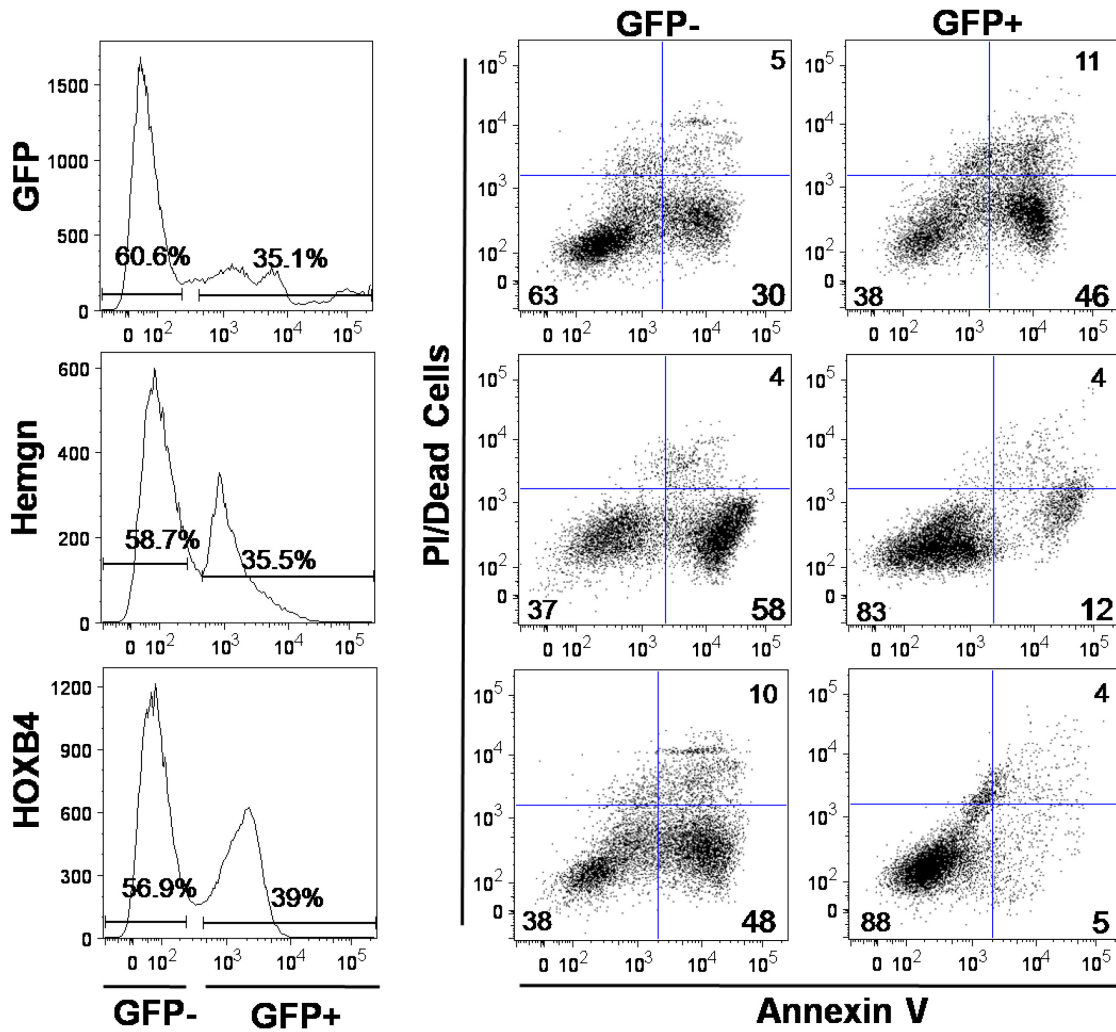


Figure 4-7. Flow cytometry analysis for apoptosis in GFP positive vs. negative population of transduced bone marrow cells in cytokine-depleted and serum-reduced medium.

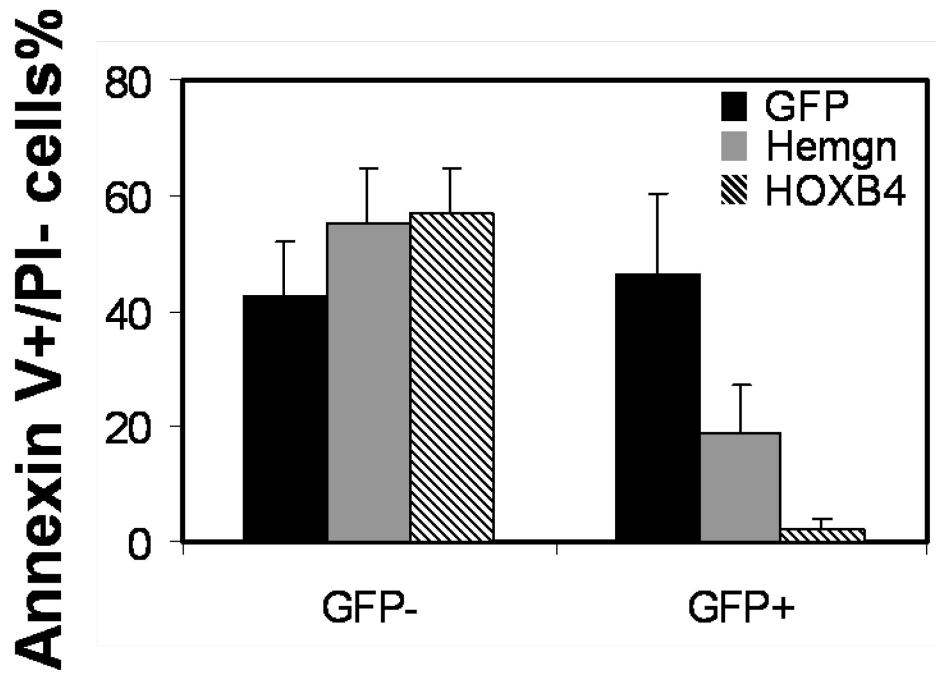


Figure 4-8. Quantification and statistics of three independent experiments for apoptosis in GFP positive vs. negative transduced bone marrow cells in cytokine-depleted and serum-reduced medium.

4.4.5 Hemgn Overexpression Has No Direct Effects on Cell Cycle in Hematopoietic Cells

We have already shown that Hemgn can protect transduced cells from apoptosis, which could be one potential cellular mechanism of the way Hemgn promotes expansion of bone marrow cells *in vitro*. To explore another potential cellular mechanism, that is, whether Hemgn increases cell proliferation by altering cell division rates, cell cycle analyses were performed. For this assay, the transduced cells were cultured for 6 days in cytokine-supplemented medium and then were sorted to separate GFP positive and negative populations, which were then stained with PI for DNA content analysis. The percentage of cells at the G₁, S, and G₂-M phases of the cell cycle was determined (Figure 4-9). We found that the percentage of cells in S phase was similar in Hemgn-transduced cells versus cells transduced with the control vector (38% and 42% respectively), indicating that the cell proliferation rate was not altered in Hemgn-overexpressed cells. The percentage of cells in G₁ and G₂-M phases were also not changed in Hemgn samples. The percentage of cells in S phase in the HOXB4-overexpressing sample was slightly higher than the control (49% and 42% respectively) (Figure 4-9), suggesting that HOXB4 could promote some cells to undergo division.

4.5 Discussion

Our results in overexpression studies show that Hemgn recapitulates the phenotype of HOXB4 in promoting expansion of bone marrow cells and myeloid progenitor cells *in vitro*. These results indicate that increased Hemgn expression may play a direct role in the mechanism by which HOXB4 induces myeloid progenitor expansion. Our results also suggest that Hemgn may play a role in hematopoietic stem cell self-renewal; however, transplant experiments will be required to prove this hypothesis.

Self-renewal that results in expansion of hematopoietic stem and progenitor cells requires the combination of both survival and proliferation signals together with the inhibition of apoptosis and differentiation. A large body of evidence suggests that suppression of apoptosis is required for hematopoietic stem cell survival and thus self-renewal.^{21;22;187;188} EDAG has been reported to enhance survival of Ba/F3 cells by protecting them from apoptosis following IL-3 withdrawal.¹⁷² HOXB4 has also been shown to protect bone marrow cells from the apoptosis-inducing effects of the TNF- α pathway.¹⁴⁴ In this work, we have found that both Hemgn and HOXB4 can protect bone marrow cells from apoptosis in liquid culture, particularly following cytokine withdrawal, suggesting an anti-apoptotic role of Hemgn and HOXB4 that may be important for self-renewal of myeloid progenitors and/or hematopoietic stem cells.

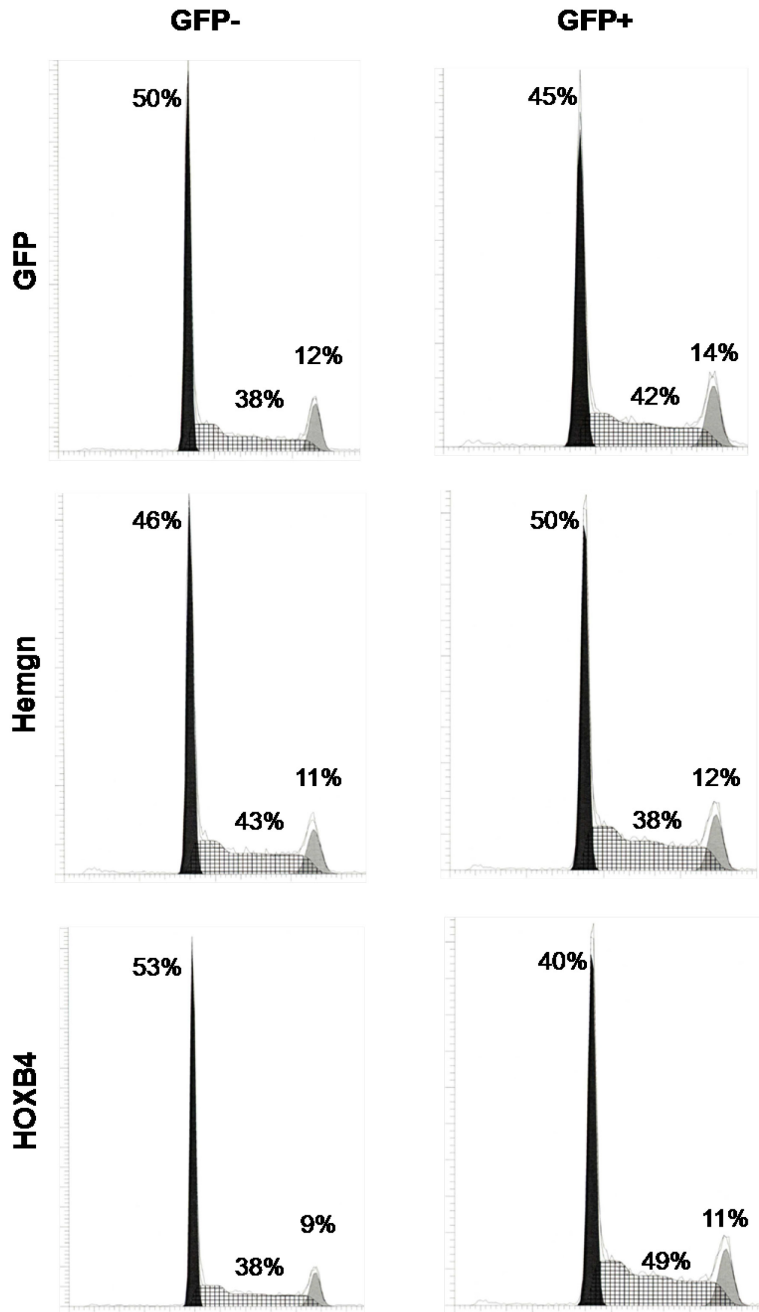


Figure 4-9. DNA content of vector-transduced bone marrow cells cultured for 6 days post transduction. DNA content was represented as the percentage of cells in G₁, S or G₂ and M phases. Left panel: non-transduced population; Right panel: transduced population; Black: G₁ phase; Grid: S phase; Grey: G₂ and M phase.

The cell cycle was not altered in Hemgn overexpressed cells, but was altered in HOXB4 overexpressed cells 6 days after transduction. These results suggest that the enhanced cell proliferation in HOXB4 samples was probably due to downstream events independent of Hemgn induction. We did observe an increased cell number in Hemgn and HOXB4 bone marrow samples compared to mock and vector control samples in the liquid culture assays. This is consistent with the result that the proportion of cells in S phase in Hemgn and HOXB4 samples was higher than GFP or mock control samples 16 days after transduction. As day 16 is a very late stage in the liquid culture, the observed consequence could be due to multiple causes. For example, Hemgn could block differentiation of cells in culture, resulting in enhanced cell number, a possibility suggested by the observation that EDAG overexpression can block the differentiation of HL-60 cells.¹⁷² There is also some evidence that ectopic HOXB4 expression can cause a delay in myeloid differentiation.^{154;189}

These *in vitro* results suggest further investigations to study whether Hemgn can cause expansion of hematopoietic stem cells in *in vivo* transplantation assays. We did transplant lethally irradiated recipient mice with donor-derived bone marrow cells transduced with Hemgn vector containing the GFP reporter gene. The reconstitution of GFP positive cells was poor in recipient mice 6 weeks post transplantation. The low titer and modest expression of the retroviral MSCV-Hemgn-IRES-GFP vector limited the ability to perform transplant assays. Further work is required to generate new lentiviral vectors for high and stable protein expression of Hemgn for use in transplant assays and to solve these technical issues.

CHAPTER 5. DISCUSSION

5.1 General Summary and Discussion

5.1.1 Target Genes Downstream of HOXB4

By using the inducible HOXB4-ER^{T2} system, screening by expression microarray analysis identified a relatively small subset of potential downstream target genes (n=77) of HOXB4. The high correlation of gene expression patterns obtained with the GFP control samples and the HOXB4-ER^{T2} samples demonstrates no global change of gene expression following HOXB4 activation, thereby indicating that only a few key mediators exist downstream of HOXB4 and function in hematopoietic cell expansion. In eukaryotes, activation of gene expression involves an ordered assembly of transcriptional regulators, chromatin-modifying factors, RNA polymerase II, and associated general transcription factors onto *cis*-regulatory elements embedded in chromatin. Chromatin-remodeling factors play an integral role in gene activation by perturbing chromatin structure and making specific loci permissive for transcription. Change of chromatin-remodeling factors usually causes the following global gene expression change. For example, knockdown of Bmi-1 and Mel-18, which belong to Polycomb group of transcriptional regulators, causes global changes in gene expression.^{190;191} Our data indicates that epigenetic alterations and chromatin reorganization are not induced by HOXB4, at least at a global level. Thus, chromatin-remodeling factors are probably not direct targets of HOXB4. This inference is consistent with a recent study when HOXB4 was overexpressed.¹⁴⁴

Of the 77 candidate target genes, 44 were upregulated and 33 were downregulated by HOXB4. This suggests that either gene activation or repression can be mediated by HOXB4, which is consistent with previous reports showing that HOX family members can activate or repress gene expression.¹⁴¹ The choice between activation and repression of target genes by HOXB4 is not well-understood and is probably context specific. To distinguish the trans-activation activity of HOXB4 from its repressive function, an engrailed repression domain-fused HOXB4 was introduced into hematopoietic stem and progenitor cells and was shown to completely block their expansion.⁹⁵ In contrast, a VP16 activation domain-HOXB4 fusion protein was fully active in conferring expansion, suggesting the requirement of trans-activation function, but the dispensability of repressive function, of HOXB4 in hematopoietic stem and progenitor cell expansion. Therefore, the genes that were downregulated by HOXB4 in our microarray analysis may be less functionally relevant to the biological activity of HOXB4 in stem cell expansion. On the other hand, it is still possible that some of the repressive events are important. For example, Ms4a4c (membrane-spanning 4-domains, subfamily A, member 4C), a trans-membrane protein that is expressed in hematopoietic stem and progenitor cells with unknown function,

was downregulated following HOXB4 activation. When it was overexpressed in bone marrow cells through retroviral vector-mediated gene transfer, Ms4a4c robustly suppressed secondary CFU-C colony formation compared to the mock and the empty vector controls (Jie Jiang, unpublished data). These data indicate that downregulation of Ms4a4c could be involved in HOXB4-mediated expansion of hematopoietic progenitor cells. To test this hypothesis, one could knockdown Ms4a4c expression in the hematopoietic stem and progenitor cell through shRNA to investigate its effect on hematopoietic stem and progenitor cell expansion.

5.1.2 The Mechanism of Hemgn as a Functional Direct Target of HOXB4

We show that when overexpressed, Hemgn can partially recapitulate the phenotype of HOXB4 in promoting the expansion of bone marrow cells and myeloid progenitor cells in *in vitro* assays. These results suggest that Hemgn can potentially play a mechanistic role in HOXB4-induced myeloid progenitor expansion. The mechanism of Hemgn as a functional target of HOXB4 is most likely due to the fact that Hemgn can protect cells from apoptosis. This anti-apoptotic effect was most apparent when the three cytokines: SCF, IL-3 and IL-6 were withdrawn and serum was reduced from culture medium, which is a common assay for inducing apoptosis in hematopoietic cells. However, we did note that the Hemgn overexpression phenotype only partially recapitulated the HOXB4 overexpression phenotype. Here I propose several explanations for this discrepancy.

First, retroviral vector-mediated expression of Hemgn usually results in high level expression in bone marrow cells due to strong retroviral promoter. In contrast, the mRNA level of Hemgn was increased merely 2 to 5 fold by HOXB4 overexpression. It is possible that different levels of transgene expression can result in quantitative or even qualitative differences in phenotype. For instance, the enforced expression of Hemgn may cause cytotoxicity which diminishes myeloid expansion and therefore results in a net effect that only partially resembles HOXB4. I hypothesize that a threshold of Hemgn expression level exists for optimal expansion of hematopoietic progenitor cells, like the case of HOXB4 itself.¹⁵³ When Hemgn is expressed at a high level, the toxic effects of Hemgn may inhibit further expansion of hematopoietic progenitor cells. To test this hypothesis, overexpressing Hemgn in different doses using retroviral vectors or generation of a TAT-Hemgn fusion protein could be applied to detect such a threshold.

The second explanation of the functional difference between HOXB4 and Hemgn is that other target genes regulated by HOXB4 are likely to be co-required for the maximal effect of HOXB4. In support of this notion, we found that although Hemgn can protect bone marrow cells from apoptosis like HOXB4, it cannot promote S phase entry and proliferation compared to the cells transduced by HOXB4. Thus, there probably are other target genes of HOXB4 that contribute

to the expansion phenotype by promoting cell division rate. To test this hypothesis, one could overexpress Hemgn together with other direct targets of HOXB4 in bone marrow cells. In detail, one could transduce bone marrow cells with both MSCV-Hemgn-IRES-GFP and MSCV-new target cDNA-IRES-RFP retroviral vectors. The GFP and RFP double positive cells could then be sorted as a source of cells transduced with both genes for further functional studies to test whether overexpression of both genes can fully recapitulate the phenotype of HOXB4 overexpression, particularly on its effects in both anti-apoptosis and cell cycle progression.

5.1.3 Relationship between Hemgn Expression and Endogenous Hoxb4

Our results showed that the endogenous Hemgn mRNA was increased about 2 to 5 fold following HOXB4 activation in expanded Lin⁻ bone marrow cells. Furthermore, the exogenous expression of Hemgn induces expansion of murine bone marrow and myeloid progenitor cells *in vitro*. One important question suggested by my results is “What is the normal function of Hemgn?”. The expression of Hemgn is restricted to the testis and hematopoietic tissues.^{170;171} This pattern of expression is similar to Hoxb4 in hematopoietic tissues, with a spatial and temporal pattern in hematopoietic sites during embryogenesis. Hemgn is first expressed in blood islands of yolk sac, then in fetal liver, and finally in the adult spleen and bone marrow.¹⁷⁰ In adult bone marrow, Hemgn is predominantly expressed in developmentally primitive stem and progenitor populations, and its expression decreases during differentiation.¹⁷⁰ These facts are consistent with the hypothesis that endogenous Hemgn may play a role in regulating normal hematopoiesis. To address this question, we are generating mice in which both alleles of *Hemgn* are disrupted to test its role in development, particularly hematopoiesis.

To understand whether the expression of Hemgn can be regulated by endogenous Hoxb4, we performed quantitative real-time PCR assay in Hoxb4-deficient bone marrow cells¹⁵⁸ and detected a 5-fold decrease in *Hemgn* mRNA level in c-kit⁺Scal-1⁺Lin⁻ cells when compared to wild type cells. These results suggest that Hemgn may also be regulated by endogenous Hoxb4 as opposed to overexpression constructs. However, the detectable expression of Hemgn in the absence of Hoxb4 indicates the presence of some degree of Hoxb4-independent regulation of Hemgn expression. One possibility is that other HOXB4 paralog members could provide redundant function through upregulating Hemgn expression.

It is important to emphasize that, in this study, we were interested in defining the targets in the context of overexpressing an ectopic *HOXB4* gene in mice, given that this is the biological setting in which expansion of hematopoietic progenitor cells have been noted. We recognize that these data may not necessarily represent target genes of endogenous Hoxb4 that is expressed at

significantly lower levels. To further explore the genes regulated by endogenous Hoxb4, we could use microarray analysis to compare the gene expression difference in c-kit⁺Scal-1⁺Lin⁻ cells between wild type and *Hoxb4*^{-/-} mice. The disadvantage of this system is that the activity of Hoxb4 could not be temporally induced and thus the candidate list may not represent immediate early response genes. To overcome this disadvantage, one could replace the endogenous *Hoxb4* gene with *Hoxb4-ER*^{T2} fusion gene. Hoxb4-ER^{T2} is inactive unless the mice are treated with tamoxifen, which can temporally induce Hoxb4-ER^{T2} activation. Microarray analysis can be applied to compare the gene expression profile in c-kit⁺Scal-1⁺Lin⁻ cells generated from tamoxifen treated Hoxb4-ER^{T2} mice and from non-treated Hoxb4-ER^{T2} mice to identify the early response genes regulated by Hoxb4.

5.1.4 Upstream Regulation of Hemgn: Other Mechanisms

Although HOXB4 was proved to be recruited to its consensus binding motifs in Hemgn promoter by ChIP assay in hematopoietic progenitor cells in our study, it does not exclude the possibility of involvement of other regulatory elements of Hemgn. HOXB4 could regulate *Hemgn* expression by recruiting other hematopoietic regulators that bind directly or indirectly to the *Hemgn* promoter. Alternatively, the recruitment of other transcription factors could be HOXB4-independent but could be necessary for maximal HOXB4 activity. The *Hemgn* promoter contains binding sites of multiple transcription factors in addition to HOXB4, including GATA-1, AML-1, Evi-1 and ETS.¹⁸⁴ HOXB4 may directly interact with AML-1, Evi-1 or ETS proteins. To test this hypothesis, one could perform co-immunoprecipitation assay using HOXB4 antibodies. If a positive interaction partner can be found, one can map the interaction domain and test its function in HOXB4-mediated hematopoietic cell expansion.

GATA-1, a member of the GATA family of transcription factors, is expressed in primitive and definitive erythroid cells, megakaryocytes, eosinophils, and mast cells. It is essential for normal erythroid and megakaryocytic development. GATA-1 was shown to be recruited to the promoter of Hemgn in an AML cell line, and to transactivate *HEMGN* in K562 cells.¹⁸⁴ *Gata1*^{-/-} mice die during early embryogenesis due to severe anemia with erythroid differentiation block at the proerythroblast stage and rapid apoptosis of those cells. Intriguingly, human HEMGN protects K562 cells from apoptosis and prevents their differentiation. Therefore, Hemgn may play a positive role in cell survival and a negative role in erythroid terminal differentiation as a consequence of GATA-1 upregulation in erythroid cells.

AML1 (Acute myeloid leukemia 1) is a transcription factor essential for hematopoiesis. *AML1* gene is the most common target of chromosomal translocations in human leukemias. AML1-Evi-1 fusion protein can transform hematopoietic stem cells.¹⁹² AML1 binds to sequences present in the regulatory

regions of a number of hematopoiesis-specific genes, including certain cytokines such as GM-CSF (granulocyte macrophage colony-stimulating factor).¹⁹³ Evi-1 has been recognized as a dominant oncogene associated with murine and human myeloid leukemia. It is a common and essential regulator for proliferation of embryonic and adult hematopoietic stem cells and transformed leukemic cells.¹⁹⁴ Members of the ETS family have been implicated in a wide variety of functions including the regulation of cellular differentiation, cell cycle control, cell migration, cell proliferation, apoptosis and angiogenesis. Multiple ETS factors have been found to be associated with cancer through gene fusion. These transcription factors which show potential binding sites in the promoter of *Hemgn* possibly provide other upstream signals in regulating *Hemgn* expression.

5.2 Short-Term Future Directions

Although overexpression of *Hemgn* partially recapitulated the effect of HOXB4 in mediating hematopoietic progenitor cell expansion *in vitro*, it is unknown whether *Hemgn* upregulation by HOXB4 is required for the observed hematopoietic stem and progenitor cell expansion. To complement our studies, we will use short hairpin RNA (shRNA) to knockdown endogenous *Hemgn* in cells overexpressing HOXB4 to determine if this will lead to reduced hematopoietic stem and progenitor cell expansion. We have designed a set of different shRNA sequences targeting different regions of *Hemgn*, including the cDNA coding region and 3' UTR. Those shRNA nucleotides were individually cloned into a lentiviral vector containing the MSCV promoter. Lentiviral vector supernatant will be collected from transiently transfected 293T cells and used to transduce GPE+86 cells that constitutively overexpress exogenous *Hemgn*. The knockdown efficiency of different shRNAs will be tested by western blotting using an anti-*Hemgn* antibody. Preliminary experiments have identified at least two of the short hairpin RNAs that efficiently knockdown the protein level of exogenous *Hemgn*. We will transduce bone marrow cells with both the *Hemgn*-shRNA lentiviral vector and the MSCV-HOXB4-IRES-YFP vector. The cells successfully transduced by both vectors can be sorted for functional assays to evaluate the effects of *Hemgn* deficiency on HOXB4-induced hematopoietic progenitor cell expansion.

The disadvantages of any knockdown strategy are the potential off-target effects of the short hairpins and the inability to fully suppress target protein expression. To circumvent these drawbacks, generation of *Hemgn*-null mice by targeted gene disruption would be the optimal strategy and is ongoing. To study the physiological role of *Hemgn* in hematopoietic tissues, and to avoid the potential failure of germline transmission due to the lack of *Hemgn*, we have generated a conditional allele of *Hemgn* flanked by loxP sites, which can be removed through a hematopoietic tissue specific Cre (MMTV-Cre) during development or an inducible Cre (Mx1-Cre) in adult mice. The effects of *Hemgn* deficiency on hematopoiesis, particularly on hematopoietic stem and progenitor

cells expansion, will be carefully investigated. We will perform liquid culture and CFU-C assays *in vitro* and competitive bone marrow transplantation *in vivo*. If the endogenous Hemgn is critical to hematopoietic stem cell function, I might expect to see a defect in self-renewal of Hemgn-deficient hematopoietic stem and progenitor cells. To further explore whether Hemgn is a key target of HOXB4, we can overexpress HOXB4 in Hemgn-deficient cells and assess its biological consequence similarly as I mentioned before.

5.3 Long-Term Future Directions

5.3.1 *Biochemical and Functional Characterization of Hemgn*

Hemgn is a newly identified protein and its biochemical and functional characterization is far from well-understood. A nuclear-localization signal together with a coiled-coil domain, but no other recognizable motifs, is present in the sequence of Hemgn.¹⁷⁰ Careful characterization of this protein at biochemical level will be important for future studies. The lack of a DNA-binding domain reasonably lowers the possibility that Hemgn is a transcription factor. We will fuse Hemgn with a known DNA-binding motif and test its ability to activate or repress reporter gene expression. If we see no activity of this fusion protein in regulating gene expression, it would suggest that Hemgn has no transactivation domain by itself. Otherwise, if we see some activity of this fusion protein in regulating reporter gene expression, it would suggest that Hemgn may function as a cofactor that can recruit other transcriptional regulators through direct interaction. If the second situation occurs, it will be interesting to identify the interaction partners of Hemgn. Since coiled-coil proteins usually interact with other coiled-coil proteins for protein-protein recognitions and interactions, the present coiled-coil domain in Hemgn should play an essential role in interacting with other proteins for conveying the function of Hemgn. We can first make a deletion mutation in the coiled-coil domain of Hemgn. I expect to see a loss of Hemgn function in promoting hematopoietic progenitor cell expansion and in protecting cells from apoptosis when its coiled-coil domain is deleted. If this is the case, I will immunoprecipitate Hemgn in cells overexpressing wild type Hemgn or mutant Hemgn with an anti-Hemgn antibody and then perform proteomics analysis in order to identify its interaction partners. Since human HEMGN was shown to regulate the expression of some key genes in hematopoietic stem cell function,¹⁷³ mouse Hemgn probably also acts as a transcription regulator through interaction with other transcription factors. Thus, microarray analysis can be conducted using wild type Hemgn and mutant Hemgn-overexpressing cells to identify differentially expressed genes. For example, I expect to see expression changes of genes that are involved in apoptotic pathways due to the fact that Hemgn shows profound effect in protecting bone marrow cells from apoptosis.

5.3.2 Potential Clinical Use of Hemgn

Successful *ex vivo* hematopoietic stem cell expansion has been a long-sought goal in hematopoietic stem cell transplantation and hematopoietic stem cell gene therapy. Hemgn could potentially be used to achieve this goal. Since constitutive expression of Hemgn by retroviral vectors may have unforeseen side effects, such as insertional mutagenesis, transient exposure of hematopoietic stem cells to Hemgn may offer a better approach. Recently, progress has been made to directly deliver proteins into cells by fusing the protein with plasma membrane penetrating peptide sequences. Several proteins and peptides are able to pass through plasma membranes independent of classical receptor- or endocytosis-mediated process. An example is human immunodeficiency virus (HIV)-1 transactivator of transcription (TAT) protein. The small protein transduction domain of TAT was fused to HOXB4 and was used to deliver TAT-HOXB4 protein into bone marrow cells.¹⁹⁵ The TAT-HOXB4 protein is fully active in supporting hematopoietic stem and progenitor cells expansion when added into the culture medium.¹⁹⁵ This process of TAT-based transportation is dependent on heparan sulfate proteoglycan on the cell surface. The advantage of using this strategy is to avoid conventional gene transfer approaches, which could be tumorigenic due to gene integration into the genome. Furthermore, the TAT-based protein transportation process is rapid, efficient and the dose is easy to control. Since Hemgn could have dose-dependent effects or side effects due to continuous constitutive expression as I discussed above, using a TAT-Hemgn fusion protein would be a promising approach to determine the optimal dose of TAT-Hemgn to mediate hematopoietic cell expansion *in vitro*. However, the biological activity of TAT-Hemgn should be determined first because TAT-Hemgn could potentially be inactivated when penetrating the plasma membrane, a phenomena that has been seen in some TAT-fusion proteins.

5.3.3 Identification of Other Direct Target Genes of HOXB4

The partial recapitulation of HOXB4 mediated hematopoietic stem and progenitor cell expansion by overexpression of Hemgn may suggest either that some other targets we identified using our gene expression profile approach, or some yet to be identified new targets are necessary for fully executing the HOXB4 downstream activity. In addition to studying other candidate genes in the generated 77 target list using the similar methods as we used before, other strategies such as ChIP-seq (Chromatin immunoprecipitation followed by sequencing) assay could be used to complement our microarray data. By using ChIP-seq, we can obtain genome-wide profiling of HOXB4 binding sequences. ChIP-seq offers higher resolution, less noise and greater coverage than its array-based predecessor ChIP-chip, which necessarily introduces some bias, as an array is restricted to a fixed number of probes.¹⁹⁶ Another important advantage of ChIP-seq over the expression array is that only a small amount of DNA (10-50 ng) is required for generating reliable signal,¹⁹⁶ which would allow us

to use a purified pool of hematopoietic stem cells, instead of a mixed population of hematopoietic progenitor cells to obtain enough DNA sample.

5.4 Detailed Consideration of These Results and Their Impact on Understanding the Molecular Mechanisms of Hematopoietic Stem Cell Expansion

5.4.1 Hematopoietic Stem Cell Self-Renewal and the Role of HOXB4

Self-renewal or expansion of hematopoietic stem and progenitor cells requires the coordinated proliferation signals together with inhibition of apoptosis and differentiation programs.

5.4.1.1 Survival Signals

Inhibition of apoptosis is essential for hematopoietic stem and progenitor cell survival.^{197;198} Quiescent hematopoietic stem cells are resistant to cell cycle checkpoint-induced apoptosis. In stress conditions, such as during *in vitro* expansion cultures, apoptosis plays a major role in hematopoietic stem and progenitor cell loss. Intrinsic apoptotic and extrinsic apoptotic pathways may both be important for hematopoietic stem cell function.

The intrinsic apoptotic pathway is initiated from an intracellular signal, such as DNA damage, defective cell cycle, loss of cell survival factors, or other types of intracellular stress. This pathway involves the activation of pro-apoptotic Bcl-2 family members, Bax or Bak in the outer mitochondrial membrane, which eventually causes cytochrome c release from the inter-mitochondrial membrane space into cytosol. Cytochrome c in turn binds to the adaptor apoptotic protease activating factor-1, forming the apoptosome, which is able to activate the initiator caspase-9. Caspase-9 can activate the downstream effector caspases -3, -6 or -7 to cleave a variety of cellular substrates for executing apoptotic response. The anti-apoptotic Bcl-2 proteins, including Bcl-2, Bcl-xL and Mcl-1, act to prevent the mitochondrial outer membrane permeabilization (MOMP) through inhibiting the action of the pro-apoptotic Bcl-2 family proteins. The anti-apoptotic Bcl2 family members are involved in maintaining the function of hematopoietic stem cells. Mice lacking the anti-apoptotic *Bcl-2* gene show massive cell death of lymphoid cells.¹⁹⁹ The absence of Bcl-xL causes embryonic lethality; and homozygous null mutant mice die at E13.0 with massive cell death in neurons and immature hematopoietic cells.²⁰⁰ Bcl-xL is also indispensable for the late maturation of primitive and definitive erythroid progenitors.²⁰⁰ Inducible deletion of *Mcl-1* gene in mice results in the loss of early bone marrow progenitor populations including hematopoietic stem cells.⁷⁰ Overexpression of Bcl-2 in HSCs *in vivo* in a transgenic mouse model results in apoptotic protection, and a moderate two fold expansion of HSCs.^{22;201} Those hematopoietic stem cells overexpressing Bcl-2

can outcompete wild type cells following reconstitution.²² Based on this information, the intrinsic apoptotic pathway is important in hematopoietic stem and progenitor cell expansion.

On the other hand, the extrinsic apoptotic pathway also shows its role in hematopoietic cell expansion. The extrinsic apoptotic pathway starts with an extracellular signal, such as Fas ligand and TNF- α , which activates specific death receptors on the cell surface. In this case, the ligand binding induces receptor clustering and recruitment of the adaptor protein Fas-associated death domain (FADD) and the initiator caspases-8 or -10, forming a death-inducing signaling complex (DISC). The initiator caspases then can be processed into their active form and be released into the cytoplasm, where they activate effector caspases -3, -6, or -7. For example, TNF- α is able to inhibit SCF-induced expansion of hematopoietic progenitor cells *in vitro*.²⁰²

Our data proves that one mechanism of HOXB4 in promoting hematopoietic stem and progenitor cell self-renewal is through protecting cells from apoptosis by activating its direct downstream target gene-*Hemgn*. Human HEMGN was shown to act as a transcriptional regulator to upregulate the expression of Bcl-2 and Bcl-xL.¹⁷³ Due to their high homology, mouse Hemgn probably has the same ability to regulate the anti-apoptotic Bcl-2 family members to perturb the intrinsic apoptotic pathway. On the other hand, human HEMGN can activate NF- κ B, which in turn inhibits TNF- α receptor signaling induced apoptosis.¹⁷² It is possible that mouse Hemgn can activate NF- κ B to inhibit apoptosis. Intriguingly, HOXB4 was shown to inhibit TNF- α pathway and thereby to protect cells from TNF- α induced apoptosis.¹⁴⁴ Based on this information, the mechanism of Hemgn in protecting HOXB4-overexpressing cells from apoptosis could be that Hemgn can either inhibit intrinsic apoptotic pathways by upregulating of anti-apoptotic Bcl-2 family members, or inhibit extrinsic apoptotic pathways by interfering with TNF- α receptor signaling. To test these possibilities, examination of both intrinsic and extrinsic apoptotic pathways would be necessary in Hemgn-overexpressed cells. For instance, the expression level of Bcl-2, Bcl-xL and Mcl-1 in Hemgn-overexpressing or -deficient cells could be examined, and inhibition of the NF- κ B pathway could be used to discern whether the effects of Hemgn can be overcome.

5.4.1.2 Proliferation Signals

Apoptosis may not be the only mechanism of how HOXB4 functions in promoting hematopoietic stem and progenitor cell self-renewal. Indeed, the fact that HOXB4 can promote 40 fold expansion of hematopoietic stem cells *in vitro* is not likely to be explained merely by its anti-apoptotic effect. For instance, Bcl-2 overexpression adequately provides a signal for hematopoietic stem cell survival but merely causes a two fold expansion of hematopoietic stem cells because of the inability to maintain those cells in the undifferentiated status.²¹ Other signals,

presumably those regulating proliferation and differentiation, may also contribute to the dramatic effect mediated by HOXB4.

Cyclins and cyclin-dependent kinases (CDKs) are critical components controlling the cell cycle progression. When activated by a particular cyclin, CDK can activate or inactivate target proteins to orchestrate entry into the next phase of the cell cycle. Upon receiving mitotic signal, G₁ cyclin-CDK complexes become active to prepare the cell for S phase entry, through promoting the expression of transcription factors that in turn induce the expression of S cyclins and enzymes required for DNA replication, or through inducing the degradation of factors which function as S phase inhibitors. For example, cyclin A-CDK2 complex plays a key role in S phase entry through the phosphorylation of proteins including cdh1, Rb, p21 and p27. Accelerated S phase entry caused by HOXB4 may significantly contribute to the induced dramatic expansion of hematopoietic stem cells. The lack of alteration in cell cycle distribution in Hemgn-overexpressing bone marrow cells implies the existence of Hemgn-independent proliferative effects of HOXB4. Although it is unknown which other target genes of HOXB4 regulate proliferation, I hypothesize that cell cycle-related genes will be candidates. p21 contributes to the quiescent state maintenance of hematopoietic stem cells.⁶⁸ It has been demonstrated that hematopoietic stem cell pool size is regulated by p21, whereas proliferation of hematopoietic progenitor cells is regulated by p27.^{203;204} Deletion of *p18* results in enhanced self-renewal of the primitive hematopoietic cells and improved long-term engraftment.²⁰⁵ It is possible that HOXB4 can downregulate the expression of those CDK inhibitors to facilitate more rapid S phase entry. Correlatively, when HOXB4 was overexpressed in hematopoietic cells from *p21*^{-/-} mice, the primary CFU-C colony number and the repopulating ability were significantly increased compared to that seen in the wild type bone marrow cells.¹⁶⁷ These findings suggest that HOXB4 may affect the cell cycle process to promote hematopoietic stem cell self-renewal. However, the fact that we did not detect any differential expression change of cell cycle regulators including cyclins, CDKs and CDK inhibitors in our microarray data suggests that HOXB4 may indirectly regulate the activity of these cell cycle regulators.

5.4.1.3 Anti-Differentiation Signals

Differentiation is the transition process of a less specialized cell into a more specialized cell type. One cycling hematopoietic stem cell may generate two daughter cells with identical biological properties as itself in the process of self-renewal, or it may also divide to produce more differentiated progenies. During the differentiation, hematopoietic stem cell gradually loses its totipotency, but the generated hematopoietic progenitor cells may still retain a high degree of multipotency.

HOXB4-overexpressed bone marrow cells showed a more homogenous morphology and contained increased number of hematopoietic progenitor cells

capable of generating primitive colonies *in vitro*.¹⁵⁰ In contrast, control-transduced bone marrow cells in long-term cultures showed hallmarks of myeloid differentiation and reduced secondary colony forming activity.¹⁵⁰ It is reasonable to raise the possibility that HOXB4 has anti-differentiation function. Indeed, evidence shows that ectopic expression of HOXB4 can cause delay or impairment of myeloid differentiation.^{154;189} Similarly, there is also some evidence that Hemgn can interfere with differentiation. Human HEMGN blocks the differentiation of hematopoietic cell lines.¹⁷² Transgenic expression of human HEMGN under the cd11a promoter in mice causes massive myelopoiesis and repressed lymphopoiesis.¹⁷³ However, in contrast to these reports, we found that either HOXB4 overexpression or Hemgn overexpression in bone marrow cells did not cause blockage of myeloid differentiation based on the observation of the secondary CFU-C assays *in vitro*. Furthermore, after the HOXB4 or Hemgn-transduced donor bone marrow cells were transplanted into lethally irradiated recipient mice, peripheral blood from the reconstituted recipient mice were examined to confirm that no hematopoietic lineage was blocked due to the overexpression of HOXB4 or Hemgn. These evidences indicate that HOXB4 or Hemgn may not irreversibly block differentiation of hematopoietic stem and progenitor cells, and thus suggest that HOXB4 or Hemgn could be good candidates for therapeutic application since they are not potentially tumorigenic.

5.4.2 Cytokines, HOXB4 and Hemgn

It is known that in hematopoietic tissues, Hoxb4 expression can be upregulated by cytokine-stimulation. TPO, Flt-3 or SCF were reported to induce Hoxb4 expression up to 10 fold through activating its direct trimeric transcriptional activator complex USF1(upstream stimulating factor 1)/USF2/NF-Y(nuclear factor Y).²⁰⁶⁻²⁰⁸ Cytokine receptor signals, such as TPOR, Flt3R and c-KIT, are normally required for hematopoietic stem cell survival during *in vitro* expansion. Now we provide potentially new insights into the downstream pathways of these cytokines and HOXB4 through identification of a key regulator-Hemgn. It is possible that Hemgn can be directly induced by Hoxb4 following cytokine stimulation and thus provide a signal for hematopoietic stem and progenitor cell survival. Human HEMGN was shown to act as a transcriptional regulator to upregulate the expression of Bcl-2 and Bcl-xL.¹⁷³ Due to high homology, mouse Hemgn probably has the same ability to regulate the anti-apoptotic Bcl-2 family members to perturb the intrinsic apoptotic pathway. The fact that hematopoietic stem cells from Bcl-2 transgenic mice are hyper-responsive to normal level cytokines than wild type cells²¹ is correlated to the hypothesis that cytokine signaling may upregulate Hemgn which may further upregulate Bcl-2. Consistently, we have shown that Hemgn was able to protect bone marrow cells from cytokine withdrawal-induced apoptosis. This suggests that Hemgn might substitute for the function of those cytokines to support cell survival. To test this possibility, we can compare cultures with or without

hematopoietic cytokines IL-3, IL-6 and SCF to see whether Hemgn can be induced by these cytokines.

5.4.3 HOXB4 Functional Redundancy

Hoxb4 and its other three paralogs: Hoxa4, Hoxc4 and Hoxd4 were reported to have similar effect in promoting hematopoietic stem and progenitor cell expansion *in vitro*.¹⁶⁷ Each paralog group 4 member was shown to both promote proliferation and inhibit differentiation, enabling the exponential expansion of hematopoietic progenitors from the c-kit⁺CD41⁺ hematopoietic progenitor cell population of day 6 murine embryoid bodies.¹²⁰ Similar findings about the functions of HOX4 paralogs in promoting hematopoietic stem and progenitor cell expansion *in vivo* were also obtained in our lab (Brian P Sorrentino's lab, unpublished data). This redundancy may explain the mild phenotype in *Hoxb4*^{-/-} mice.¹⁵⁸ Quantitative analysis of mRNA from the fetal liver of *Hoxb4*^{-/-} mice revealed that a deficiency of Hoxb4 was associated with changes in the expression levels of several other Hox genes.¹⁵⁸ This result indicates that alternative genetic pathways initiated by other Hox4 paralog members could substitute for the function of Hoxb4. Since trans-paralog *Hox* genes share higher degree of homology in sequence than cis-paralog *Hox* genes,¹⁰⁴ it is suggested that the same trans-paralog genes could functionally compensate for each other. As Hox4 paralog genes lie on different chromosomes, it is feasible to examine the degree of redundancy among them by breeding the mice with individual disrupted gene. Although technically challenging, the analysis of double, triple and even quadruple mutants of Hox4 paralogy genes could address the question whether these Hox4 genes are functionally redundant in regulating hematopoiesis.

Overexpression of HOXB4 in ES cell-derived primitive progenitors induced a switch to the definitive hematopoietic stem cell phenotype and promoted rapid hematopoietic stem cell expansion *in vitro*.¹⁴⁶ Those HOXB4 induced progenitors from ES cells engraft lethally irradiated adults and contribute to long-term, multilineage hematopoiesis in primary and secondary recipients.¹⁴⁶ During embryogenesis, primitive hematopoiesis occurs in blood islands of the yolk sac at approximately E7.5, and generates mainly primitive erythrocytes. The definitive hematopoiesis occurs around E10.5 in aorta-gonadomesonephros (AGM) regions, and then shifts to the fetal liver, and eventually to the spleen and bone marrow in adult.²⁰⁹ The definitive hematopoiesis generates all hematopoietic lineages, including hematopoietic stem cells with multipotent long-term repopulating activity. The mechanisms regulating the emergence of the definitive hematopoietic stem cells at AGM region around E10.5 and the further rapid expansion is poorly understood. Evidence shows that the autonomous initiation and further expansion of definitive hematopoietic stem cells in the AGM region can be enhanced by application of exogenous interleukin-3 (IL-3) which acts as a proliferation and survival factor to the earliest hematopoietic stem cells

in the embryo.²¹⁰ The identified expression pattern of Hoxb4 and its other Hox4 paralogy members in definitive hematopoietic stem cells but not yolk sac suggests the potential role of Hoxb4 and other Hox4 paralogy members in promoting definitive hematopoiesis.¹⁰⁵ Since Hoxb4 can be regulated by some hematopoietic cytokines, it is possible that Hoxb4 acts downstream of IL-3 to convey its function in enhancing the development of definitive hematopoietic stem cells in embryo. Although the Hoxb4-deficient mice show essentially normal hematopoiesis, deletion of the other three functionally redundant Hox4 paralogy members could possibly lead to more severe phenotype in embryonic hematopoietic stem cell development.

In gene expression profiling assays with c-kit⁺Lin⁻ bone marrow cells that constitutively overexpress HOXB4 or HOXC4 by retroviral vectors, we found that many genes, including *Hemgn*, were commonly changed in HOXB4 and in HOXC4 overexpressed samples compared to the control vector-transduced samples (Brian P. Sorrentino's lab, unpublished data). It is possible that HOX4 paralogs share at least some common downstream targets, such as *Hemgn*, for enhancing expansion of hematopoietic stem and progenitor cells. Our real-time PCR analysis in *Hoxb4*^{-/-} c-kit⁺Scal-1⁺Lin⁻ cells showed a 5-fold decrease in *Hemgn* mRNA expression when compared to wild type cells. This result is not contradictory to the finding that *Hemgn* is a direct target of HOXB4 for conveying its function in promoting self-renewal, because the expression of *Hemgn* can still be detected in the absence of Hoxb4 possibly due to redundancy of other group 4 paralogs. Investigation of whether *Hemgn* is a common direct target gene of HOX4 paralog members is promising to unravel the mechanisms of HOX4 paralogs.

5.4.4 HOX Specificity

Functional specificity of Hox proteins depends on multiple mechanisms. Due to the high degree of homology within the homeodomain, a core DNA sequence "TAAT" is recognized and bound by the majority of Hox proteins.¹²⁸ Structural studies proved that DNA recognition by Hox proteins uses two sets of information that provide different degrees of specificity. The first one uses DNA-contacting residues that are common to all Hox proteins to promote Hox binding to AT-rich sequences, such as "TAAT". Some monomeric homeodomain-DNA structures have been solved, and they show a very similar mode of DNA recognition.²¹¹ The third recognition α -helix of homeodomain lies in the major groove of the DNA, where it makes direct water-mediated contacts with specific bases and the phosphate backbone. Ile47, Gln50, Asn51, and Met54 residues that are present in all Hox homeodomains are primarily responsible for contributing to these interactions.²¹¹ In addition, the N-terminal arm preceding the first α -helix typically lies in the minor groove of the DNA. Arg5, an N-terminal arm residue present in almost all homeodomains, is the most commonly observed residue in the minor groove. The second set of determination factor for specificity in addition to

common DNA-contacting residues is to use additional DNA-contacting residues from the N-terminal arm and linker regions of Hox proteins.²¹² These additional contacts are cofactor-dependent and paralog-specific. The N-terminal arm and linker residues of Hox family members are conserved in a paralog-specific manner. Thus, paralog-specific DNA sequences of Hox family members could be recognized of by specific Hox-cofactor complexes. The same Hox paralogy members probably share common targets for functional complement to initiate the similar downstream biological events.

As discussed previously, the DNA binding specificity, affinity and transregulating potential of a specific Hox protein can be modified by the flanking DNA sequence or interaction with cofactors.¹²⁹⁻¹³¹ A *in vitro* study showed that specific Hox protein is able to bind to DNA consensus sequence with preferred flanking sequence.¹²⁹ In the future, by applying ChIP-chip and ChIP-seq methodologies to each HOX protein, accurate DNA binding sequence of specific HOX protein could be identified. Hox family members may bind to their specific preferred DNA sequence as monomers or as heterodimers with other cofactors. However, when high affinity Hox monomer binding sites were applied *in vitro* to drive a reporter gene in *Drosophila* embryos, the generated expression patterns were not consistent with their identified ability to bind homeodomain proteins.²¹³ It is plausible to hypothesize that currently unidentified factors can bind with Hox proteins cooperatively or noncooperatively to these sites. As introduced in Chapter 1, interaction with the TALE family cofactors to form dimeric or trimeric Hox-Pbx/Meis complexes is hypothesized to increase the DNA binding ability of some Hox family members to their target genes.¹³⁶⁻¹³⁸

DNA binding affinity of Hox family members can also be regulated by posttranslational modification. For instance, phosphorylation of conserved tyrosine residues in the homeodomain can decrease or increase the DNA binding ability of Hox.^{133;134} In addition, the transcriptional specificity of Hox proteins also lies in their ability to act both as transcriptional activators and repressors in different contexts.

The fact that HOXB4 and other HOX4 paralog members but not other HOX family member show similar function in promoting hematopoietic stem and progenitor cell expansion indicates the specificity of HOX4 in regulating their target genes in hematopoietic cells. To my understanding, although it is known that PBX cofactor is not required for HOXB4-mediated expansion, it is possible that other unidentified cofactors or collaborators of HOXB4 exist. To screen the potential cofactor or collaborators of HOXB4, one could apply proteomics methods following immunoprecipitation of HOXB4-containing complex. After that, one could knockdown the identified interaction partner in HOXB4-overexpressed cells to validate its function. Except the conserved “YPWM” cofactor-binding domain, mutagenesis screen method could be applied in HOXB4 to identify other unknown protein-interaction domains which contribute to its specificity. On the other hand, phosphorylation could possibly increase the target binding affinity of

HOXB4. HOXB4 was tyrosine phosphorylated in the transduced mouse and dog cells, in which HOXB4 was introduced with high copy transcripts and thus high protein expression level.²¹⁴ Furthermore, the tyrosine phosphorylation of HOXB4 contributes to more profound expansion in the transduced cells.²¹⁴ Whether HOXB4 is phosphorylated in the transduced bone marrow cells can be determined by mass spectrometry following immunoprecipitation of HOXB4. If HOXB4 is phosphorylated, one could make point mutation (Ser/Thr/Tyr/ to Ala) in phosphorylation sites and determine whether this modification abrogates the ability of HOXB4 to activate target gene expression and thereby blunts its ability to increase self-renewal.

5.4.5 HOXB4 and Leukemia

Hematopoietic stem cells may fail to fully regenerate the stem cell compartment since their self-renewal ability is usually intrinsically limited and subjected to exhaustion under the transplantation stress. This defect is possibly a result of negative feedback mechanisms initiated when progenitors and mature blood cells have been regenerated to their normal levels, which prematurely inhibit further hematopoietic stem cell expansion.^{215;216} In contrast, HOXB4-transduced hematopoietic stem cells are capable of fully reconstituting the stem cell compartment but do not expand above levels normally observed in unmanipulated mice.⁸⁹ This indicates that HOXB4 overexpression does not override the intrinsic regulatory mechanisms *in vivo* that maintain the HSC pool size within normal limits. Several studies have indicated that, in steady-state hematopoiesis, the cell cycle entry of hematopoietic stem cells is tightly controlled. In mice, hematopoietic stem cell numbers remain relatively constant throughout their adult life time. Hematopoietic stem cells may transit between quiescent state in niche and undergoing cycling at non-niche sites *in vivo*. Genetic studies show that increasing the number of osteoblastic cells can cause parallel increases in the hematopoietic stem cell population, in contrast, osteoblastic cell depletion can reduce BM cellularity.^{217;218} The finding that HOXB4 overexpression did not lead to hematopoietic stem cell expansion beyond the normal level is thus not surprising, since the limited size of hematopoietic stem cell niche could prevent homing and residence of more hematopoietic stem cells.

Despite a profound and consistent effect on expanding hematopoietic stem and progenitor cells, overexpression of HOXB4 did not promote preferential expansion along any hematopoietic lineage or lead to leukemia in mice after persistent expression. In sharp contrast, retroviral overexpression of other HOX family members such as HOXB3 or HOXA10 in a similar transplantation model did lead to leukemia.^{118;219} The different outcomes suggest that these different HOX proteins regulate different sets of target genes in hematopoietic cells. A minimum of two events, one leading to enhanced proliferation with disabled apoptosis and the other a block in differentiation, may be sufficient for leukemia initiation.^{220;221} Of the known genetic events leading to leukemia, translocations

leading to fusion transcription factors or activated signaling kinases are among the most common events, and deletions in cell cycle checkpoint genes or mutation in tumor suppressor genes could act as a secondary hit to initiate leukemic transformation.²²² One possible reason HOXB4 overexpression did not cause leukemia in mice is that the intrinsic negative feedback regulatory signaling pathways were activated to limit the accumulation of leukemic transformation hits.

In contrast to the situation in mouse models as we discussed, myeloid leukemia was reported in dogs two years after they were transplanted with HOXB4-overexpressing CD34⁺ cells, but not in control retroviral vector transplanted animals.¹⁵⁷ shRNA-mediated knockdown of HOXB4 in the leukemic cell line derived from one animal caused profound growth inhibition and cell death, suggesting the involvement of HOXB4 in leukemogenesis.¹⁵⁷ Those HOXB4-overexpressing leukemic cells rapidly engrafted and expanded in immune-suppressed recipient dogs to cause leukemia transmission.²²³ This, although surprising, is not contradictory to the previous findings in mouse models, where HOXB4 is not leukemogenic. Instead, in this case, HOXB4 overexpression was demonstrated not to be the only contributor to the observed leukemic phenotype in dogs.^{157;224} Retroviruses have long been known to cause leukemia through insertional mutagenesis. In those two transplanted dogs, HOXB4-containing retroviral vectors were shown to integrate into genomic locus of several known proto-oncogenes, including *c-myb* and *PRDM16*, to elevate their expression, thus favoring the engraftment and expansion of those clones.^{157;224} HOXB4 overexpression is thus probably not the single trigger to induce leukemia; instead, it may function as an additional contributor for leukemogenesis following retroviral vector integration-caused insertional mutagenesis. One reason that HOXB4-overexpression leads to leukemia in dogs but not in mice possibly lies in the fact that a relatively small number of transduced cells and relatively less retroviral vector integration sites in transplanted mice lower the frequency of insertional mutagenesis.^{157;224} On the other hand, perhaps a latency period longer than the lifespan of a mouse is required for detection of leukemic transformation.^{157;224} In summary, the observed myeloid leukemia is probably a combined consequence of the deregulated proliferation caused by the activated proto-oncogenes and the expansion advantage mediated by HOXB4.^{157;224}

Based on the information aforementioned, one could speculate that as a potential application in gene therapy, retrovirus-mediated overexpression of HOXB4 in large animals may pose a significant risk of leukemia. To circumvent this drawback, new vectors based on lentivirus are in development, which may decrease the frequency of insertional mutagenesis.¹⁵⁸

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VITA

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