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Identification of Genes Involved in Hematopoietic Stem Cell Differentiation and Leukemia Differentiation and Leukemia

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Identification of genes involved in hematopoietic stem cell differentiation and leukemia

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by

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ABSTRACT

Hematopoiesis is maintained by a proper balance between self renewal and multipotent differentiation of the hematopoietic stem cells (HSC). Acute myelogenous leukemia (AML) is characterized by the blockage in the differentiation of HSC, while self renewal and proliferation is preserved. It is important to understand the mechanisms involved in the inhibition of hematopoietic differentiation and maintenance of the HSC state in order to develop better therapies for AML. In these studies I have explored the role of Hsp90, omega-3 fatty acids and YB-1 in hematopoietic differentiation. EML, a hematopoietic precursor cell line, was used as a model for the hematopoietic system in these studies. My preliminary data showed the activation of Wnt signaling upon inhibition of Hsp90 in EML cells. This data suggested the involvement of Hsp90 in the regulation of Wnt signaling in EML cells. Moreover, my initial data with fatty acid studies indicated that omega-3 fatty acids could affect Wnt signaling in EML cells. Unfortunately, further progression of both these studies was marred by variability in my data. In my latest study, I have identified YB-1 as a marker involved in the maintenance of the hematopoietic stem cell state. YB-1 was found to be highly expressed in the EML cell line and in the mouse bone marrow-derived HSC and myeloid progenitor cells. In addition, YB-1 expression was downregulated during myeloid differentiation in retinoic acid (RA) and granulocyte macrophage colony stimulating factor (GM-CSF) treated EML cells, as well as in the granulocytes derived from mouse bone marrow. Further, abnormal YB-1 expression was observed in myeloid leukemic cell lines. Knockdown of YB-1 expression and arsenic trioxide treatment (As_2O_3) in erythroleukemic, K562 cell line resulted in apoptosis and inhibition of cell proliferation. Most importantly, these treatments led to the induction of megakaryocytic differentiation in these cells. Overall my data suggests that increased expression of YB-1 in the leukemic cells contributes to the leukemic cell properties by promoting cell proliferation, cell survival and blocking cell differentiation. Thus, YB-1 could be a potential target for therapy in myeloid leukemia.

DEDICATION

I would like to dedicate this work to my parents, Satpal and Jasmeet Bhullar. Without their knowledge, wisdom, and guidance, I would not have the goals I have; to strive and be the best to reach my dreams.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
17-AAG	17-N-Allylamino-17-demethoxygeldanamycin
ABC	ATP-binding cassette
ADP	Adenine diphosphate
AML	Acute myelogenous leukemia
ALA	α-linolenic acid
ALL	Acute lymphoblastic leukemia
APL	Acute promyelocytic leukemia
ATP	Adenine triphosphate
ATRA	All-trans retinoic acid
AS2O3	Arsenic trioxide
BCR	Breakpoint cluster region
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CML	Chronic myelogenous leukemia
CMP	Common myeloid progenitor
CSD	Cold Shock Domain
COX	Cyclooxygenase
DHA	Docosahexanoic acid
DMSO	Dimethylsulfoxide
EMP	Erythroid megakaryoctye progenitor
EML	Erythrocyte myeloid lymphocyte
EPA	Eicosapentanoic acid
EPO	Erythropoietin
ER	Endoplasmic reticulum
FA	Fatty acid
FLT3	FMS like kinase
GA	Geldanamycin

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA	Globin transcription factor
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte macrophage colony stimulating factor
GMP	Granulocyte macrophage progenitor
HDAC	Histone deacetylase
HOP	Hsp70-Hsp90 organizing protein
HSC	Hematopoietic stem cell
HSP	Heat shock proteins
IGF-1	Insulin like growth factor
IL-3	Interleukin 3
IMDM	Iscove's modified Dulbecco's medium
LEF	Lymphoid enhancer binding protein
LNA	Linoleic acid
M-CSF	Macrophage colony stimulating factor
MDR1	Multidrug resistant protein
MDS	Myelodysplastic syndrome
MM	Multiple myeloma
MMP-2	Matrix metalloproteinase
PCNA	Proliferating cell nuclear antigen
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
PUFA	Polyunsaturated fatty acid
Pgp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinases
PML	Promyelocytic leukemia gene
RA	Retinoic acid
RAR-α	Retinoic acid receptor

SCF	Stem cell factor
SFRP	Secreted frizzled related protein
TAG	Triacylglyceride
TCF	T-cell factor
TPO	Thrombopoietin
TPR	Tetratricopeptide repeat
Wg	Wingless
YB-1	Y box protein

CHAPTER One: INTRODUCTION

Hematopoiesis

Hematopoietic stem cells (HSC) are the stem cells that give rise to blood and immune cells. They are ultimately responsible for the constant renewal of blood as billions of new blood cells are produced every day. Hematopoietic stem cells have the capability of self renewal and multi-potent differentiation, and hematopoiesis is maintained by a proper balance between the two. Hematopoietic stem cells give rise to multipotent committed progenitors - common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) (Figure 1.1). These multipotential committed precursors are highly proliferative and express receptors for specific growth and survival factors- the colony stimulating factors (CSF). The CMPs further produce megakaryotic/erythroid progenitors (MEPs), which differentiate into erythrocytes and platelets; and granulocye-monocyte progenitors (GMPs), which then produce granulocytes and monocytes. The CLPs give rise to mature T lymphocytes, B lymphocytes and natural killer cells (Kaushansky *et al*., 2006; Tenen, 2003).

The production of hematopoietic cells is under the tight control by a group of hematopoietic cytokines. Among these, the most important regulators of HSC maintenance are stem cell factor (SCF), and thrombopoietin (TPO). Receptors for TPO and SCF, c-Mpl and c-Kit, respectively, are both expressed on the surface of HSCs. SCF is expressed constitutively in bone marrow (BM) stromal fibroblasts and endothelial cells and is essential to the survival, proliferation and differentiation of HSCs (Broudy, 1997; Hartman *et al*., 2001). Administration of a neutralizing antibody against c-Kit in mice led to the depletion of all progenitor cells in two days, indicating the role of SCF in HSC maintenance (Ogawa *et al*., 1991).
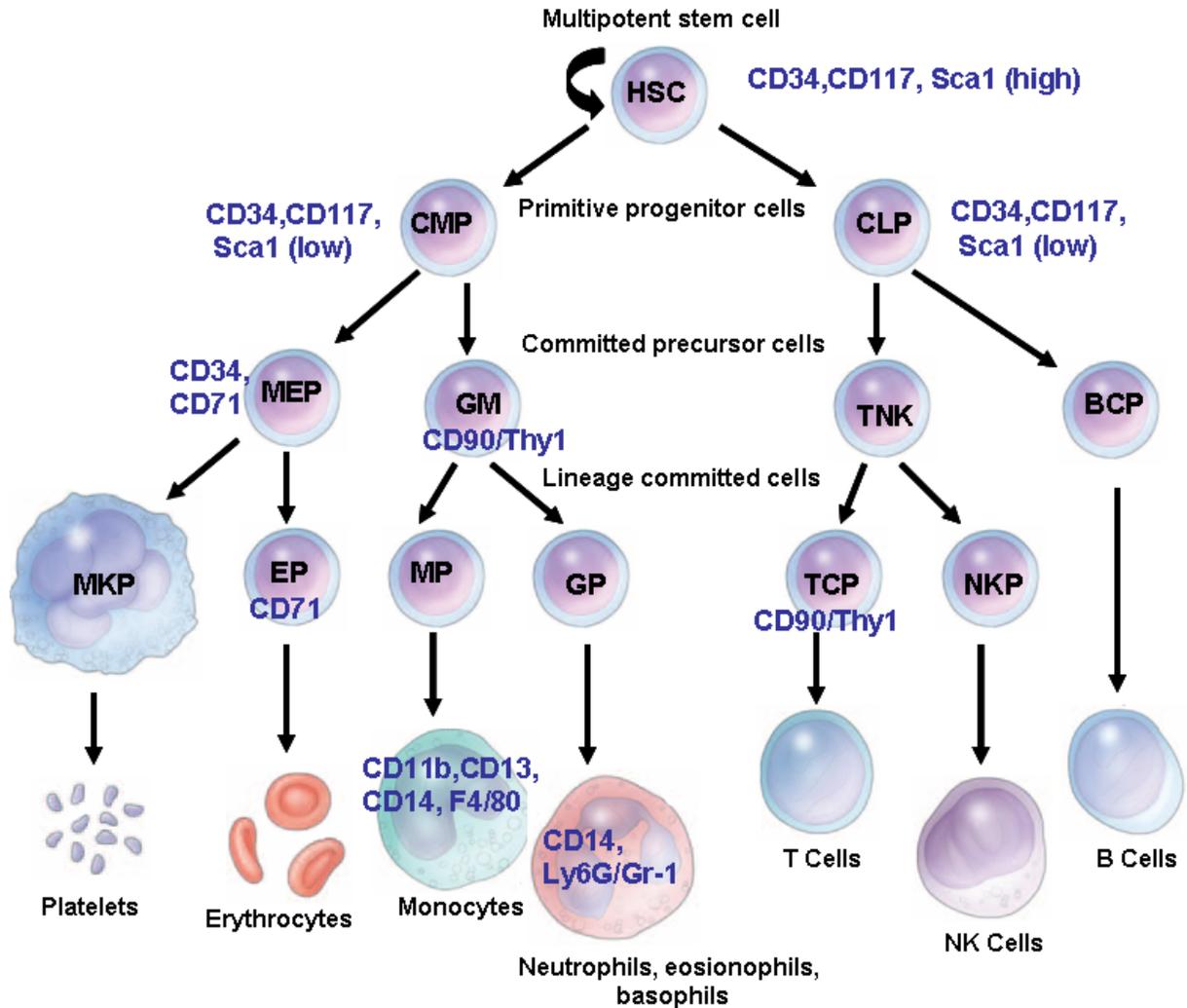
Figure 1.1. A general model of hematopoiesis. Blood-cells are produced from a hematopoietic stem cell (HSC), which can undergo either self-renewal or differentiation into a multilineage committed progenitor cells These include common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). CMP further divides to give rise to more differentiated progenitors, committed to granulocytes and macrophages (GMs), and megakaryocytes and erythroid cells (MEPs). CLP produces progenitors committed to T cells and natural killer cells (TNKs) and B cells (BCPs). Successive division and differentiation of these progenitors give rise to fully differentiated cells-B cells, NK cells, T cells, neutrophils, eosinophils, basophils, monocytes, platelets and erythrocytes. Different cluster of differentiation antigens (CD) and markers expressed on the surface of each cell type are shown in blue. NK cells (NKPs), T cells (TCPs), granulocytes (GPs), monocytes (MPs), erythrocytes (EPs), megakaryocytes (MkPs).

Mice with loss-of-function mutations in TPO or c-Mpl have a decrease in the number of HSC that can repopulate the bone marrow. Further, there is reduction in the expansion of HSCs that occurs after bone marrow transplantation. This suggests that TPO plays role in promoting the survival and the expansion of HSCs.

Myeloid cell development involves the interaction of various cytokines in the survival, proliferation and differentiation of myeloid progenitors (Figure 1.1). Interleukein-3 (IL-3), SCF and TPO are involved in the formation of MEP from CMP. The differentiation of MEP into megakaryocytes or erythrocytes depends on the presence of TPO or erythropoietin (EPO). CMP give rise to GMP in the presence of granulocyte-macrophage colony stimulating factor (GM-CSF). Differentiation of these committed progenitors into the granulocyte lineage or monocyte lineage requires granulocyte colony stimulating factor (G-CSF) or macrophage colony stimulating factor (M-CSF), respectively (Kaushansky *et al*., 2006).

Apart from cytokines, transcriptions factors are also thought to play critical role in regulating normal hematopoiesis. These include lineage-specific factors and non-specific transcription factors. The lineage-specific transcription factors are differentiating factors such as PU.1, CCAAT/enhancer binding protein α (C/EBP α) and GATA1, affecting only small number of related lineages. The non-lineage-specific transcription factors such as AML1 and SCL are involved in almost all lineages. Disruption of acute myeloid leukemia (AML1) or stem cell leukemia (SCL) affects formation of the entire blood cell lineage, as these transcription factors function at the pre-hematopoietic stem cell stage (Tenen, 2003; Rosenbauer and Tenen, 2007). Expression of PU.1 is considered one of the earliest events favoring HSCs to lineage commitment. PU.1 and GATA-1 are co-expressed in CMPs but their mutually exclusive expression coincides with further commitment to either granulocytic/monocytic or megakaryocytic/erythroid differentiation (Zhu and Emerson, 2002). In

erythroid/megakaryocytic leukemia cell line, K562 ectopic expression of PU.1 led to their differentiation into granulocytes and monocytes, instead of megakaryocytes upon Ras pathway activation. Further, overexpression of GATA-1 in granulocytic progenitor 32D cells redirects them to megakaryopoiesis (Matsumura *et al*., 2000).

Disruption of this transcription factor function can interfere with the normal cellular differentiation and lead to leukemia. Leukemia is characterized by a terminal differentiation block of hematopoietic cells while the self renewal and proliferation is preserved. Understanding the pathways by which transcription factors regulate differentiation is important to developing improved treatments for hematological malignancies including leukemia.

Acute myelogenous leukemia

Various hematological abnormalities including myelodysplastic syndrome arise from the dysregulation of the hematopoietic process. Myelodysplastic syndrome (MDS) is a preleukemic state which involves the ineffective production of the myeloid cells. In one third of patients with MDS, the disease transforms into acute myelogenous leukemia (AML), usually within months to a few years. Leukemia comprises 33% of childhood cancers and is the most prevalent cancer in the children. Among different types of leukemia, AML is the most common leukemia diagnosed in infants with a five-year survival rate of 22.6% (Surveillance Epidemiology and End Results, NCI). AML is characterized by bone marrow infiltration of abnormal hematopoietic precursors and resulting disruption of normal production of red blood cells, white blood cells, or platelets. It is diagnosed based on the presence of >30% immature and functionless cells called blasts in the bone marrow and peripheral blood. Symptoms in most patients arise from disruption of normal blood component production and include fatigue, anemia, bleeding and bruising (thrombocytopenia), and fever with or without infection.

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Transcription factor	Frequency in AML
RUNX1–ETO (t(8;21))	12–15%
CBFβ–MYH11 (inv16)	8–10%
PML–RARα (t(15;17))	6-7%
MLL fusions (t11q23)	4-7%
C/EBPα	7-9%
GATA1	Nearly 100% in AMKL associated with Down's syndrome
PU.1	<7%
RUNX1	9%

Table 1.1. Examples of transcription factor mutations in patients with AML. Abbreviations : AML, acute myeloid leukaemia; AMKL, acute megakaryoblastic leukaemia; CBFβ, core-binding factor-β; C/EBPα, CCAAT/enhancer binding protein-α GATA1, GATA-binding protein 1; MLL, mixed lineage leukaemia; PML, promyelocytic leukaemia; PU.1, transcription factor encoded by SPI1; RARα retinoic acid receptor-α; RUNX1, runt-related transcription factor 1.

Chromosome aberrations are detectable in the leukemic blasts of approximately 55% of adults with AML. Aberrant transcription factor activity in AML is associated with acquired chromosomal translocations that result in oncogenic fusion products such as RUNX1–ETO t(8;21) CBFβ–MYH11; Inv16, MLL (mixed lineage leukaemia; t(11q23) and PML–RARα (promyelocytic leukaemia–retinoic acid receptor-α; t(15;17) (Table1.1). These chromosomal changes have been recognized as the most important prognostic factor for achievement of

complete remission, risk of relapse, and overall survival (Döhner and Döhner, 2008). Several mutations that have been identified in genes such as C/EBPα GATA1, NPM1, FLT3 and c-Kit and are associated with poor prognosis in AML (Rosenbauer and Tenen, 2007). Mutations in the GATA1 gene that results in the formation of a truncated GATA1 protein with altered transcriptional function occurs in acute megakaryoblastic leukemia (AMKL) affecting patients with Down syndrome (Wechsler *et al*., 2002).

Stress response

Stress induced by change in temperature leads to a complex program of gene expression and adaptive response. These cell stress responses are of great interest both to basic biology and to medicine. The ability to survive and adapt to thermal stress appears to be a fundamental requirement of cellular life, as cell stress responses are ubiquitous among both eukaryotes and prokaryotes. Increase in temperature can result in a heat shock response and can lead to the production of heat shock proteins (Hsp), while a decrease in temperature induces a cold shock response resulting in cold shock proteins. Heat and cold shock responses are both involved in inhibition of cell growth and reduction in protein synthesis. Depending on the intensity of the exposure both heat and cold stress can lead to the activation of the apoptotic program and, in the extreme cases, necrosis (Sonna *et al*., 2002).

New approaches are emerging in order to find therapies for AML. Some of these approaches address the role of stress proteins in cancer. Cancer cells exist in a state of stress and rely upon stress related proteins for survival. Many malignant cells have constitutively high levels of stress proteins that correlate with aggressive and resistant tumors, resulting in poor prognosis. Thus, stress proteins have been identified as attractive targets for therapy in cancer.

Heat shock protein 90 (Hsp90)

In 1962, investigators reported that heat induced a characteristic pattern of puffing in the chromosomes of *Drosophila* (Ritossa, 1962). This discovery eventually led to the identification of the heat-shock proteins (Hsp) or stress proteins whose expression these puffs represented. It was found that brief exposure of cells to high temperature can provide protection from subsequent larger increases in temperature, even when this increase would normally be lethal. Increased synthesis of Hsp in *Drosophila* cells following stresses such as heat shock was first demonstrated in 1974 (Tissieres, 1974). Later it was shown that the heat shock response is ubiquitous and highly conserved in all organisms from bacteria to humans (Schlesinger *et al*., 1982).

Hsp act as molecular chaperone by enabling protein folding and assist in protein trafficking. Molecular chaperones are a ubiquitous class of proteins that play important roles in protein folding and in the protection of cells from several stresses associated with the disruption of protein structure including heat shock. Chaperones bind to and stabilize the unstable conformation of another protein therby helping in its folding and assembly. One major function of chaperones is to prevent both newly synthesized polypeptide chains and assembled subunits from aggregating into nonfunctional structures (Hartl, 1996).

Hsp90 is a specialized molecular chaperone required for the stability and function of numerous client proteins (substrate protein) that are mainly involved in signal transduction pathways. In unstressed cells, Hsp90 accounts for 1–2% of total protein, but in response to heat shock this increases to 4–6% of cellular protein. Hsp90 is distinct from other chaperone systems as it does not fold non-native proteins but rather binds to substrate proteins at a later stage of folding. It interacts with various protein substrates to assist in their folding and plays a critical role during cell stress by repairing damaged proteins (protein refolding), or by degrading them thus restoring protein homeostasis and promoting cell survival (Jolly *et al*., 2000). Moreover,

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Hsp90 is critical to the survival of the organism since the genetic knockout of Hsp90 is lethal in mice (Voss *et al*., 2000).

Hsp90 isoforms and structure

Hsp90 is highly conserved and expressed in a variety of different organisms from bacteria to mammals. There are five functional human genes which encode Hsp90 protein isoforms. In mammalian cells, there are two or more genes encoding cytosolic Hsp90 homologues Hsp90α (inducible form) and Hsp90β (constitutive form) which are the result of a gene duplication event (Csermely *et al*., 1998). Human Hsp90α shows 85% sequence identity to Hsp90β (Chen *et al*., 2005; Chen *et al*., 2006). An important difference is that the Hsp90α form readily dimerizes, whereas the β form does so with much less efficiency. Hsp90α is shown to play a regulatory role in muscle cell differentiation of zebrafish while Hsp90β is required for early embryonic development (Reviewed in Sreedhar *et al*., 2004). Recently another Hsp90 isoform has been identified, Hsp90N, which shares high sequence homology with the other two Hsp90 isoforms, but lacks the N-terminal domain (Figure 1.2). It has been shown to be associated with cellular transformation by activating Raf in rat F111 fibroblasts (Grammatikakis *et al*., 2002). However, a later study reported the existence of this isoform to be accidental and occurred due to chromosomal rearrangement in a single cell line (Zurawska *et al*., 2008).

Two other isoforms include GRP94 in the endoplasmic reticulum and TRAP1 in the mitochondrial matrix. GRP94 is glucose-regulated, induced by glucose starvation and is known to participate in protein folding and assembly (Gupta, 1995). TRAP1 acts as a molecular chaperone to retinoblastoma protein (Rb) during cellular stress. It possesses a unique LxCxE motif which is involved in binding to Rb. This motif is absent in all other Hsp90 family

members (Chen *et al*., 1996; Felts *et al*., 2000). There is still not much known about the function of these two isoforms of Hsp90.

Structure of Hsp90

Hsp90 consists of four structural domains (Figure 1.2). A highly conserved N terminal domain (NTD) which has an ATP binding pocket necessary for Hsp90 function. Various natural and synthetic inhibitors of Hsp90 bind at this site.
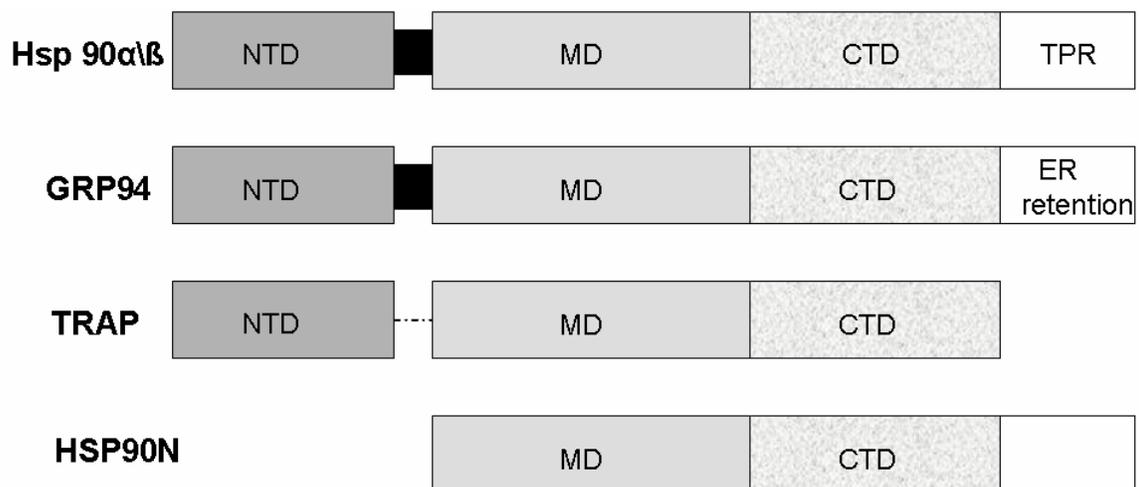
Figure 1.2. Structure of Hsp90 and its various isoforms. There are four structural domains of Hsp90. The N terminal domain (NTD) is highly conserved and has an ATP binding site. A charged linker region connects the NTD with the middle domain (MD). This linker region is absent in TRAP1. MD is involved in binding to client proteins. The C terminal domain (CTD) is the dimerization domain and has TPR binding site where cochaperones such as HOP bind. The TPR region is absent in TRAP1 and GRP94 has instead an endoplasmic retention site.

With the exception of TRAP1 there is a charged linker region that connects the NTD with the middle domain (MD). The MD is also involved in client protein binding. The C-terminal domain (CTD) is the dimerization domain. In eukaryotes, the CTD has the tetratricopeptide repeat (TPR) motif recognition site (consisting of amino acids MEEVD), which regulates ATPase activity (Wandinger *et al*., 2008). This region is responsible for the interaction with cochaperones such as HOP (Hsp 70-Hsp90 organizing protein). The TPR binding site is absent in TRAP1 while in GRP94 this is replaced with an endoplasmic retention site. While the C-terminal domain provides for the constitutive dimerization of Hsp90, the N-terminal domain undergoes transient dimerization driven by ATP binding (Prodromou and Pearl, 2003).

The protein folding activity of Hsp90 is regulated by the dynamic association of various co-chaperones (Figure 1.3). Hsp90 dimerizes at its CTD to form a U shaped structure such that the N terminal domain is available for protein interaction. Hsp 70 and Hsp 40 form a complex with the client protein for delivery to Hsp90 (Mahalingam *et al*., 2009). Hsp90 binds to the Hsp70 complex by the adapter protein HOP via the TPR domain which is present at the CTD of both Hsp90 and Hsp70. This is described as the "open conformation''. Recruitment of ATP to the NTD of Hsp90 results in dimerization and a conformational change leading to the formation of clamp around the client protein (Prodromou *et al*., 2003). This is referred as the "closed confirmation" and results in the dissociation of HSP70/HSP40/HOP complex. Aha is a cofactor that can bind and stimulate the activity of ATPase. Cdc37 and p23 are then recruited which help in the stabilization of the client protein and facilitates ATP hydrolysis (Pratt and Toft, 2003; Pearl *et al*., 2003). This complex assists in the conformational maturation of the client and maintains the protein in an active state capable of exerting its function.
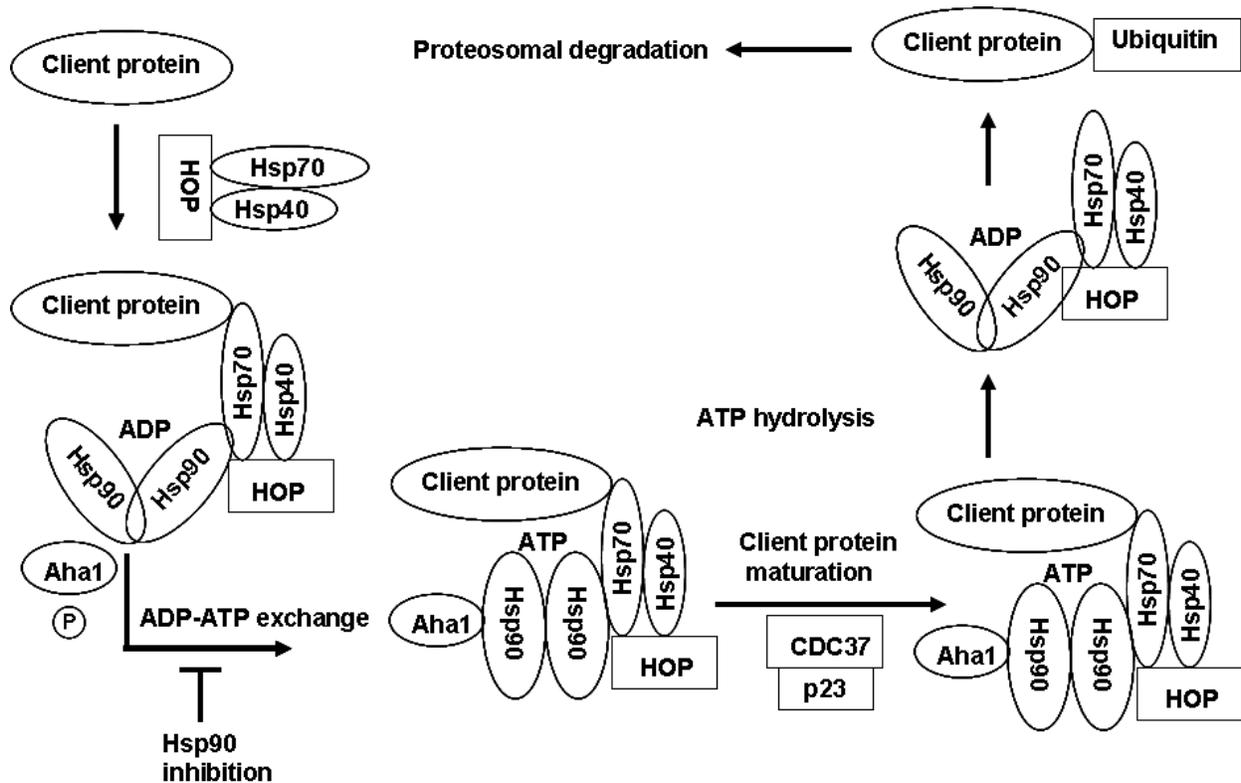
Figure1.3. **The Hsp90 superchaperone compex cycle.** The binding of a client protein to Hsp90 requires the co-operation of another chaperone, Hsp70 and its co-factor Hsp40. Hsp90 dimerizes at its C-terminal end. Hsp70 and Hsp 40 form a complex with client protein for delivery to Hsp90. Hsp70 complex binds to Hsp90 by an adapter protein (HOP), through the small helical TPR domains which is present at the C-terminal end of both Hsp90 and Hsp70. Initiation of ATPase activity by binding of cofactors like Aha results in a conformational change (clamp formation) in Hsp90 such that it dissociates from the Hsp70/Hsp40/HOP complex. Cdc37 and p23 then replace the original cochaperones to assist in conformational maturation of the client protein and its activation. Inhibition of ATP-binding through Hsp90 inhibitors prevents client protein maturation and results in degradation of these oncogenic proteins by the proteasome.

Role of Hsp90 in cancer

Hsp90 protein is found to be overexpressed constitutively in various types of cancer cells (Multhoff and Hightower, 1996). Hsp90 regulates the conformation, activity, function and stability of client proteins. There are over 100 known Hsp90 client proteins that include transcription factors and protein kinases involved in oncogenic signal transduction pathways. Hsp90 is overexpressed in breast, pancreatic, lung, ovarian cancers (Ochel and Gademan, 2002). Moreover, Hsp90 expression is high in various leukemic cell lines and human acute leukemia cells (Yu Fu *et al.*, 1992). Recently, Hsp90 was reported to be overexpressed in AML patients and was associated with poor prognosis (Flandrin *et al.*, 2008). Thus, Hsp90 is the principle molecular chaperone implicated in oncogenesis making it an attractive target for cancer therapy.

Cancer cells are particularly sensitive to Hsp90 inhibition and the therapeutic selectivity for cancer versus normal cells is based upon three main factors (reviewed in Pearl *et al.*, 2008). Firstly, according to the 'oncogene addiction model,' cancer cells exclusively depend on a sensitive Hsp90 client protein that drives malignancy. Degradation of a specific Hsp90 client has a greater impact on the cancer cells than in normal cells. For example, degradation of mutant BRAF in a melanoma cell or Bcr-Abl in chronic myeloid leukemia (CML) results in apoptosis and/or differentiation, whereas their degradation in normal cells leads to little or no effect. Secondly, many oncoproteins are expressed in mutated forms in cancer cells that are much more dependent on Hsp90 for their stability and activity than their normal counterparts, e.g. Bcr-Abl, EGFR and BRAF. Thirdly, cancer cells become dependent on Hsp90 to manage the cellular stress created by the oncogenic process and the hypoxia, acidosis and nutrient deprivation of the tumor microenvironment (reviewed in Solit and Chios, 2008). In support of this hypothesis, Kamal *et al.* have shown that Hsp90 in tumor cells is found entirely in an active complex with co-chaperones, whereas most Hsp90 in normal tissues

resides in a free, uncomplexed, or latent state. Since the binding affinity of Hsp90 inhibitors is higher for tumor derived Hsp90 than in normal cells it allows specific targeting of tumor cells (Kamal *et al*., 2003).

Hsp90 inhibition results in proteasomal degradation of large number of oncogenic client proteins which are involved in all the hallmark traits of cancer, including proliferation, evasion of apoptosis, immortalization, invasion, angiogenesis and metastasis. Targeting Hsp90 may be beneficial as it has a combinatorial impact on multiple oncogenic pathways. In addition, this combinatorial action should markedly reduce the opportunities for cancer cells to develop resistance to Hsp90 inhibition.

Hsp90 inhibitors

Targeting Hsp90 with drugs has shown promise in clinical trials. Hsp90 inhibitors cause the destabilization and eventual degradation of Hsp90 client proteins. Majority of Hsp90 inhibitors bind to the ATP pocket of Hsp90 and block the ATPase cycle that is essential for Hsp90 function (reviewed in Taldone *et al*., 2009). Hsp90 inhibitors Geldanamycin (GA) and Radicicol were discovered as natural products and originally isolated as antibiotics.

Geldanamycin (GA) is a benzoquinone ansamycin which binds to the ATP pocket of the N-terminal domain of Hsp90 and inhibits the binding of ATP. This inhibition targets bound client proteins for ubiquitination and proteasomal degradation leading to depletion of oncoproteins and consequent cell-cycle arrest and apoptosis (Hostein *et al*., 2001). Later it was found that GA is insoluble in water, unstable and exhibits severe hepatotoxicity. Therefore 17-allylamino-17-desmethoxygeldanamycin (17-AAG) was developed to enhance the solubility and therapeutic index of geldanamycin. 17-AAG is currently being used in animal models of cancer and has progressed to clinical trials (reviewed in Taldone *et al*., 2009).

Approximately one third of acute myeloid leukemias have activating mutations of FLT3, which are associated with adverse clinical outcome. 17-AAG exhibits a potent activity against leukemic cell lines, particularly those harboring a FLT3 mutation (Minami *et al.*, 2002). However a recent study reported the effect of 17AAG on a large scale of AML patients. In this study, overexpression of Hsp90 was associated with poor prognosis and resistance to chemotherapy in AML patients. Leukemic cells from the patients with higher Hsp90 expression showed spontaneous growth in liquid culture and colony formation. *In vitro* exposure of leukemic cells to 17-AAG resulted in inhibition of growth in liquid and clonogeneic cultures and induced apoptosis (Flandrin *et al.*, 2008).

Chronic myeloid leukemia (CML) is characterised by the BCR-ABL fusion gene, which is a constitutively active cytoplasmic tyrosine kinase. Imatinib mesylate targets the ATP-binding site of the kinase domain of ABL and is being used to treat CML patients (Druker *et al.*, 2001). However, patients can develop imatinib resistance (Gorre *et al.*, 2001). Heat-shock protein 90 maintains BCR-ABL stability and function. Treatment of BCR-ABL resistant cells with 17-AAG was shown to target BCR-ABL for degradation and to suppress cell proliferation (Nimmanapalli *et al.*, 2001). Furthermore, Hsp90 inhibitors have been used in combination with histone deacetylase (HDAC) inhibitors to induce apoptosis and inhibit cell growth in both imatinib-sensitive and resistant BCR-ABL cells (Rahmani *et al.*, 2005).

Stress induced by exposure to low temperature in cells can produce cold shock proteins which are involved in a cold shock response. The cold shock response is similar to the heat shock response in terms of inhibition of growth and reduction in protein synthesis in cells. However, the heat shock response is ubiquitous and highly conserved in all organisms from bacteria to humans and Hsp function as a molecular chaperone or protease. Moreover, cold shock proteins are found only in bacteria and function as an RNA chaperone (Matisumoto *et al.*, 1998). In eukaryotes, the homologous region to the bacterial cold shock proteins is found in

the nucleic acid binding region of the cold shock domain (CSD) protein family (Kohno *et al*., 2003).

Y Box protein (YB-1)

       Y-box proteins are members of the cold shock protein family that contain a CSD which is highly conserved during evolution. There are three Y-box proteins in human and mouse, two of which are expressed in both somatic and germ cells (Table 1.2). Contrin in human and MSY2 in mouse are germ cell-specific members of the Y-box protein family. The Y-box protein, YB-1 (p50 or dbpB) is the most extensively studied member which is ubiquitously expressed in various tissues (reviewed Matsumoto and Bay., 2005).

Human	Mouse	Expression
YB-1/DbpB	YB-1/MSY1	Ubiquitous
DbpA	MSY4	Ubiquitous (Abundant in heart, muscle and testis)
Contrin/DbpC	MSY2	Germ cells

Table 1.2. Y-box proteins in human and mouse

YB-1 expression, structure and function

YB-1 was originally identified as a transcription factor and was named because it binds to the Y-box (inverted CCAAT-box) sequence of the major histocompatibility complex class II gene (Didier *et al*., 1998). The CSD of the vertebrate protein is 40% homologous with the cold shock protein isolated from bacteria (Kohno *et al*., 2003).
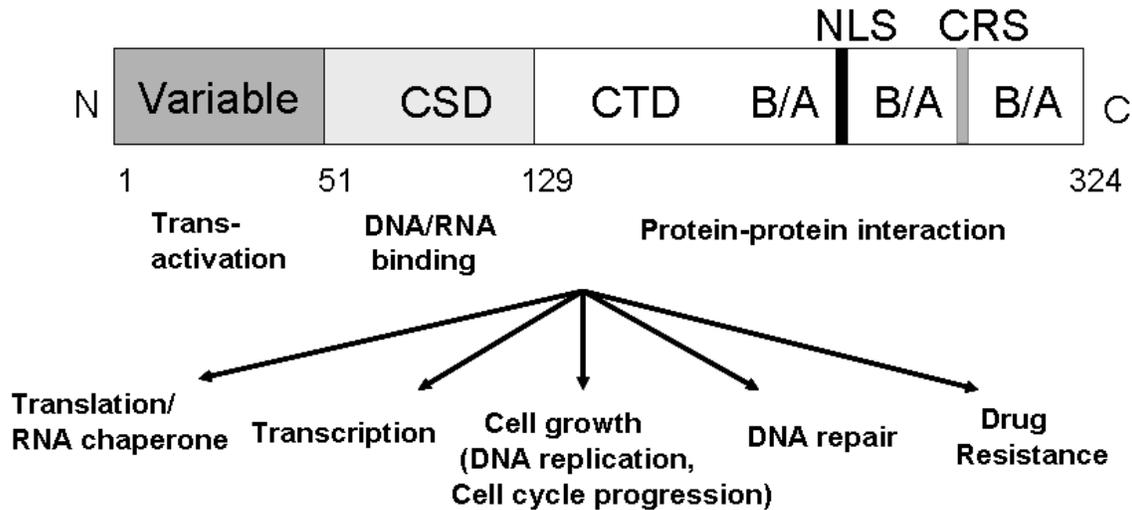
Figure 1.4 The Structure and Functions of YB-1. YB-1 protein has three domains: N-terminal, cold shock (CSD) and C-terminal domains (CTD). The N-terminal is involved in transactivation whereas the CSD is the most conserved domain and is important for RNA/DNA binding. The CTD has basic and acidic amino acids referred as B/A repeats and mediates protein: protein interactions. Cellular trafficking is regulated by the presence of a nuclear localization signal (NLS) and a cytoplasmic retention site (CRS) in the C-terminal domain.

The human YB-1 gene is contains 8 exons and is located on chromosome 1p34 (Toh *et al*., 1998).The mRNA is about 1.5 kb long and encodes a 43 kDa protein which has 324 amino acids (Didier *et al*., 1988). YB-1 is broadly expressed throughout development. High levels of YB-1 are present in human fetal tissues of heart, muscle, liver, lung, adrenal gland, bone marrow,

kidney and brain. On the other hand, YB-1 transcripts are not detected or are expressed at a very low level in many adult tissues (Spitkovsky *et al*., 1992). Expression level of YB-1 correlates with the cell proliferation state (reviewed in Lu *et al*., 2005). Also, high levels of YB-1 are detected in regenerating liver after tissue damage and the proliferating compartment of colorectal mucosa (Ladometry and Sommerville, 1995).

Structure of YB-1

The YB-1 protein consists of three domains: the variable N-terminal domain, a highly conserved cold shock domain (CSD) and the C terminal tail domain (CTD) (Figure 1.4). The N-terminal domain is rich in alanine and proline residues and is thought to be involved in trans-activation. The CSD is the most evolutionary conserved nucleic acid-binding domain that binds RNA, as well as single-stranded and double-stranded DNA. The CTD of YB-1 contains alternating regions of basic and acidic amino acids, called B/A repeats or charged zipper, and facilitates dimer formation. This region is suggested to mediate protein–protein interactions. YB-1 has been shown to interact with a number of cellular and viral proteins that are involved in various cellular processes. Cellular localization of YB-1 is controlled by the presence of a cytoplasmic retention site (CRS) and a nuclear localization signal (NLS) in the CTD (reviewed in Kohno *et al*., 2003; Wu *et al*., 2007).

Functions of YB-1

Transcription and Translation

As a transcription factor, YB-1 binds to the inverted CCAAT-box that is known as the Y-box in the promoter regions of several eukaryotic genes involved in cell growth including proliferating cell nuclear antigen (PCNA), epidermal growth factor receptor (EGFRα), DNA polymerase α

and DNA topoisomerase II α (Wolfe, 1994; Ladometry and Somerville, 1995; Kohno *et al*., 2003). In the cytoplasm, YB-1 acts as a RNA chaperone by associating with mRNAs to form messenger ribonucleoprotein particles. YB-1 regulates translation in a dose-dependent manner; low concentrations of YB-1 activate translation and high concentrations repress it (Evdokimova and Ovchinnikov, 1999). YB-1 can shuttle to the nucleus and back to the cytoplasm, which contributes to its function as a regulator of transcription and translation.

DNA Repair

YB-1 has been shown to be overexpressed in cisplastin-resistant cell lines and reduction of YB-1 leads to increased sensitivity to cisplatin and other DNA interacting drugs (Ohga *et al*., 1996). It is reported to interact with PCNA (Ise *et al*., 1999) and p53 (Okomoto *et al*., 2000), which suggests that YB-1 may be involved in DNA repair and the DNA damage response. Moreover, YB-1 is shown to possess 3'-5' exonuclease activity (Izumi *et al*., 2001). However, the regulation of this enzymatic activity *in vivo* is not known.

Drug resistance

The multidrug resistance gene (MDR-1) codes for P-glycoprotein (Pgp). Pgp is an ATP binding cassette (ABC) transporter that is responsible for the efflux of a variety of compounds out of the cell. These transporters act as a protective mechanism by reducing the toxins within a cell but they can also cause drug resistance by eliminating clinically useful drugs. Nuclear localization of YB-1 has been associated with the expression of Pgp and development of drug resistance in breast cancer, melanoma and multiple myeloma (Janz *et al*., 2002; Schittek *et al*., 2007; Chatterjee *et al*., 2007).

Biological role of YB-1

YB-1 plays a role in embryonic development. Homozygous deletion of YB-1 in mouse embryos results in late embryonic/perinatal lethality after embryonic day 13.5. This occurs due to major developmental defects such as neurological abnormalities, hemorrhage and respiratory failure. Moreover there is hypoplasia in multiple organ systems in late stage embryos that leads to growth retardation. In this study, mice heterozygous for loss of YB-1 did not develop any abnormalities in YB-1 function and were phenotypically indistinguishable from the wild type littermates (Lu *et al.*, 2005).
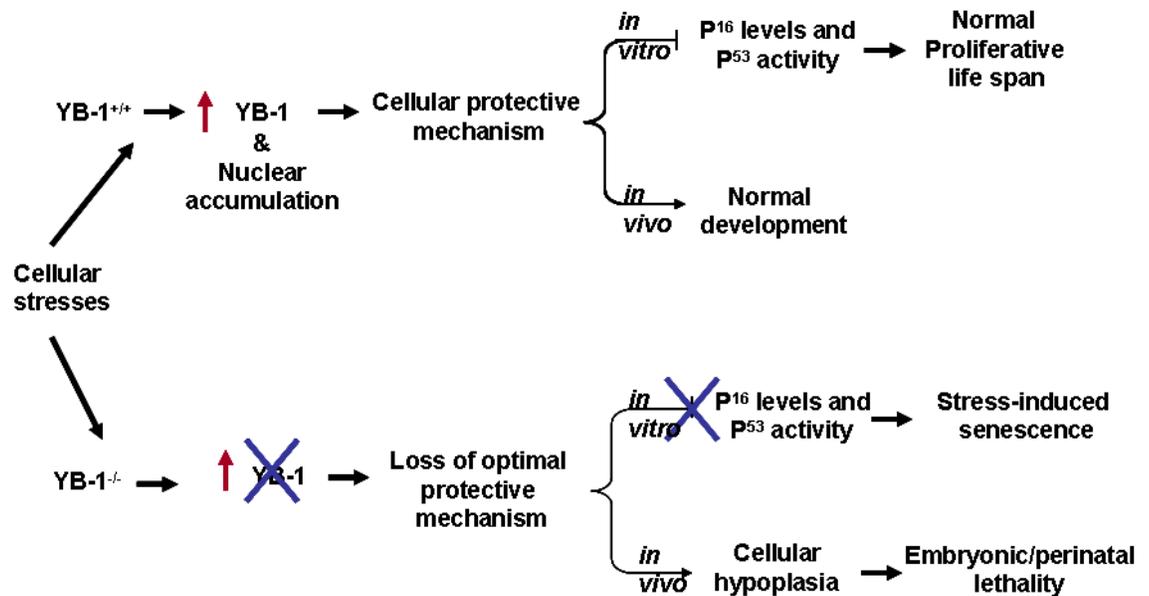
Figure 1.5. Model of YB-1 function. YB-1 is an important component of cellular stress response pathway which protects cells from a variety of stresses. In mammalian cells, YB-1 expression is induced in response to environmental stress leading to its accumulation in the nucleus. YB-1 deficiency results in the loss of this protective mechanism. Thus, cells deficient in YB-1 show an increased sensitivity to environmental stresses and undergo premature senescence.

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YB-1 is considered an important component of a cellular response pathway that is required to protect cells from a variety of stresses (Figure 1.5). Fibroblasts derived from YB-1$^{-/-}$ embryos demonstrated increased sensitivity to oxidative, genotoxic and oncogene induced stress. Under oxidative stress, populations of YB-1$^{-/-}$ mouse embryonic fibroblasts have a large percentage of cells in the G_0/G_1 phase of cell cycle. These cells accumulate negative cell cycle regulators such as p16 and p21 and senescence prematurely (Lu *et al*., 2005). Two previous studies reported that the loss of one functional allele of YB-1 resulted in a haplo-insufficient phenotype *in vitro*. Targeted YB-1 heterozygous mutations in the chicken lymphoid DT40 cell line showed major cellular defects such as aneuploidy and severe apoptosis (Swamynathan *et al*., 2002). Targeted disruption of one allele of YB-1 in mouse embryonic stem cells caused abnormal sensitivity to external cytotoxic stimuli (Shibhara *et al*., 2004). These studies suggest that YB-1 plays a crucial role in cell growth and stress response.

Role of YB-1 in cancer

Similar to Hsp90, YB-1 has been shown to be overexpressed in variety of human cancers including breast, thyroid, colorectal, prostate and melanoma (Kohno *et al*., 2003). Several clinical studies revealed that increased YB-1 expression is associated with poor prognosis in non small cell lung, prostate, ovarian and breast cancer (Kuwano *et al*., 2004). YB-1 has been shown to play role in cell proliferation and cell cycle progression. Increased YB-1 expression is correlated with PCNA and DNA topoisomerase IIα expression in colorectal cancer, human lung cancer and is linked to markers of cellular proliferation in osteosarcoma (reviewed in Matsumoto and Bay, 2005). Further, YB-1 acts as a cell cycle stage-specific transcription factor. In HeLa cells, nuclear accumulation of YB-1 transcriptionally activates cyclin A and B1 genes, which are crucial for cell cycle progression (Jurchott *et al*., 2003).
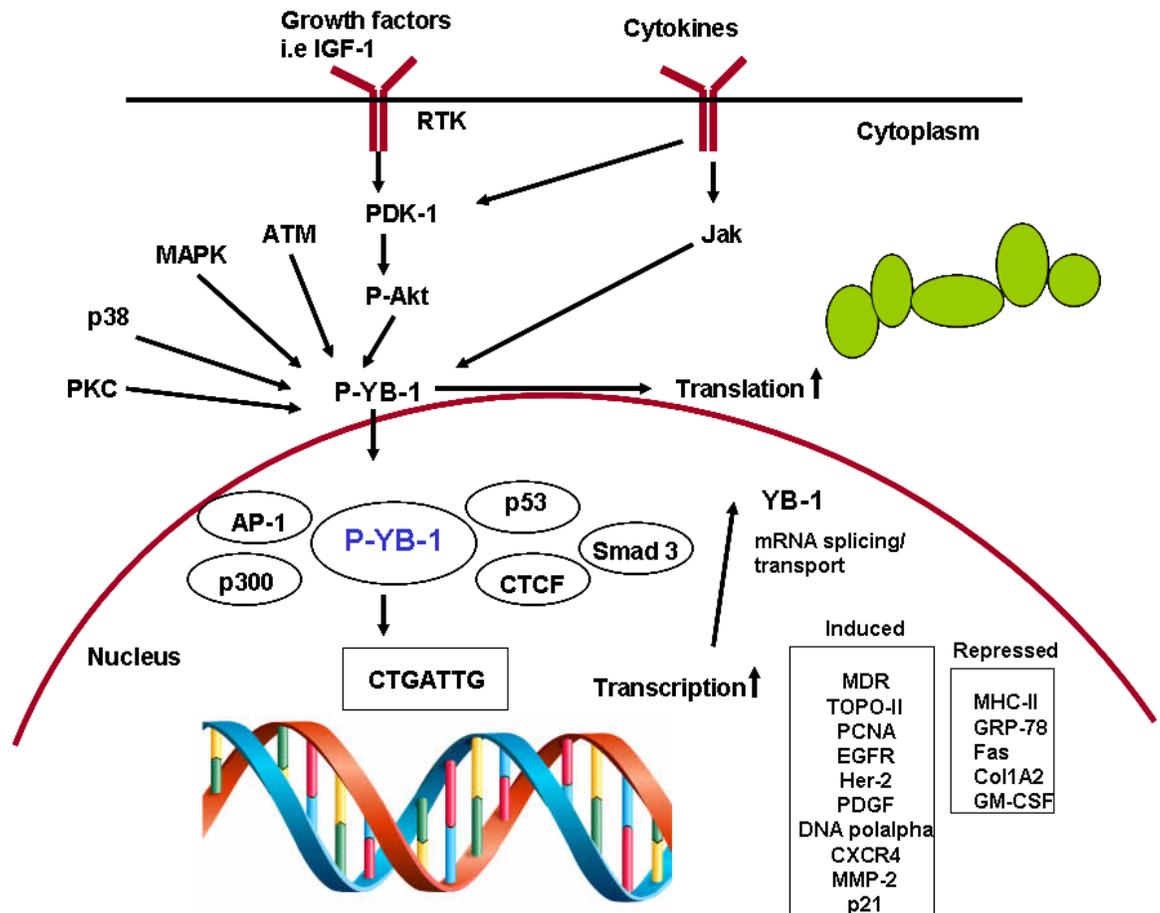
Figure 1.6. Schematic of the signal transduction pathways of YB-1 in cancer cells. Growth factors such as insulin like growth factor, IGF-1, and cytokines activate various kinases that can phosphorylate YB-1. It is generally thought that phosphorylation of YB-1 by kinases such as Akt in the cytoplasm leads to nuclear trafficking and DNA binding. The phosphorylation of YB-1 can also alter its role in translation initiation, mRNA splicing and/or transport. In the nucleus, YB-1 directly binds to the inverted CAAT boxes and activates multiple genes involved in cell growth. It can also indirectly induce the expression of oncogenes by binding to other transcription factors such as AP-1 and p53. Thus, oncogene expression can be induced by YB-1 through transcriptional as well as translational control.

In a transgenic mouse model, YB-1 has been shown to induce mammary tumor formation through chromosomal instability. This chromosomal instability occurred due to mitotic failure and centrosome amplification (Bergmann *et al*., 2005). In addition, YB-1 is believed to promote tumor invasion and metastasis by controlling the expression of a matrix metalloproteinase (MMP-2) (Cheng *et al*., 2002). Also, YB-1 has been reported to be involved in inducing drug resistance in cancer cells. YB-1 positively regulates the transcription of MDR1, and in a number of malignancies YB-1 levels are closely associated with the expression of Pgp (Janz *et al*., 2002; Schittek *et al*., 2007; Chatterjee *et al*., 2007; Bargou *et al*., 1997).

Many proteins that phosphorylate YB-1 have an important role in the signal transduction pathways associated with growth and survival of cancer (Figure 1.6). Various growth factors such as insulin like growth factor (IGF-1) and cytokines activate kinases that can phosphorylate YB-1. Phosphorylation by kinases such as Akt, MAPK, or Jak in the cytoplasm leads to the nuclear translocation of YB-1 where it transactivates genes involved in cell growth (EGFR, PCNA, Topoisomerase II α, DNA polymerase α), cell invasion (MMP-2) and represses genes that induce apotosis (Fas) and cell differentiation (GM-CSF). Phosphorylation of YB-1 can also alter its role in translation initiation, mRNA splicing and transport. In the nucleus, YB-1 directly binds to inverted CAAT boxes and activates multiple genes involved in cell growth. It can also indirectly induce the expression of oncogenes by binding to other transcription factors such as activator protein (AP-1) and p53. Thus, oncogene expression can be induced by YB-1 through transcriptional as well as translational control (Wu *et al*., 2007).

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Role of YB-1 in hematological malignancies

Evidence for the involvement of YB-1 in hematological disorders such as MDS comes from a gene array study where YB-1 expression was found to be higher in the bone marrow

samples of MDS patients (Lee *et al*., 2001). A later study identified YB-1 as a gene that is differentially expressed in the wild type and heterozygous GATA-1 knockdown mice. GATA-1 is a transcription factor essential for erythropoiesis, and heterozygous knockdown of GATA-1 in mice leads to maturation arrest and transformation of erythroblasts. GATA-1 knockdown mice exhibit increased expression of YB-1 in its spleen as compared to wild-type mice (Yokoyama *et al*., 2003a). GATA-1 mutant mice have a phenotype similar to humans with MDS in early stages of life which transforms into acute leukemia in later stages of life. Another study by the same group showed YB-1 mRNA to be highly expressed in the erythroblasts from the patients with myelodysplastic syndrome refractory anemia (MDS-RA) relative to normal patients (Yokoyama *et al*., 2003 b). These studies suggested a role for YB-1 in hematopoiesis, particularly erythroid development. Recently, YB-1 was demonstrated to be strongly expressed in immature and anaplastic multiple myeloma (MM) cells from the bone marrow of patients and in various MM cell lines indicating its potential involvement in leukemia (Chatterjee *et al*., 2007).

Though YB-1 has been studied extensively in solid tumors there is a paucity of information about its role and regulation of gene expression in hematopoiesis. Also, there are few studies demonstrating the association of YB-1 with MDS and leukemia. The objective of the study in chapter 2 was to determine the role of YB-1 in hematopoiesis and leukemia. In chapter 2, we show that YB-1 is highly expressed in stem/progenitor cells and is downregulated during myeloid differentiation. Moreover, abnormal expression of YB-1 in leukemic cells could contribute to their leukemic cell properties by blocking differentiation.

Wnt signaling

In previous studies, it was shown that mutations in the chromatin remodeling genes or Hsp90 in *Drosophila* resulted in the gain-of-function expression of Wg to produce an abnormal eye phenotype. This phenotype was epigenetically inherited (Sollars *et al*., 2003). The studies presented in chapter 3 were aimed to determine the effect of Hsp90 modulation on the Wnt pathway in a mammalian model system.

Wnt signaling is involved in embryogenesis and controls diverse cellular behaviors such as cell proliferation, stem cell maintenance and cell fate decisions. Dysregulated Wnt signalling has been shown to be associated with cancer. Therefore, researchers have focused on targeting this pathway for developing better therapies against cancer.

Investigators first identified Wnt genes independently in *Drosophila* and mouse. Wingless (Wg) was identified as a segment polarity gene in *Drosophila* (Sharma and Chopra, 1976), while int-1 was cloned as a proto-oncogene in mouse (Nusse, 1984). The name "Wnt" was derived from "wingless" and "int-1" after these two genes were shown to encode homologous proteins (Rijsewijk *et al*., 1987).

In humans, there are at least 19 members of the Wnt family and at least 10 members of its receptor family, FZ (frizzled) (Reya and Clevers, 2005; Mao *et al*., 2001). Wnt signals transduce two distinct pathways: the canonical pathway for cell fate determination and the non-canonical pathway for the control of cell movement and tissue polarity. Canonical Wnts include Wnt1, Wnt3A and Wnt 8 while non-canonical Wnts are Wnt4, Wnt5A and Wnt11.
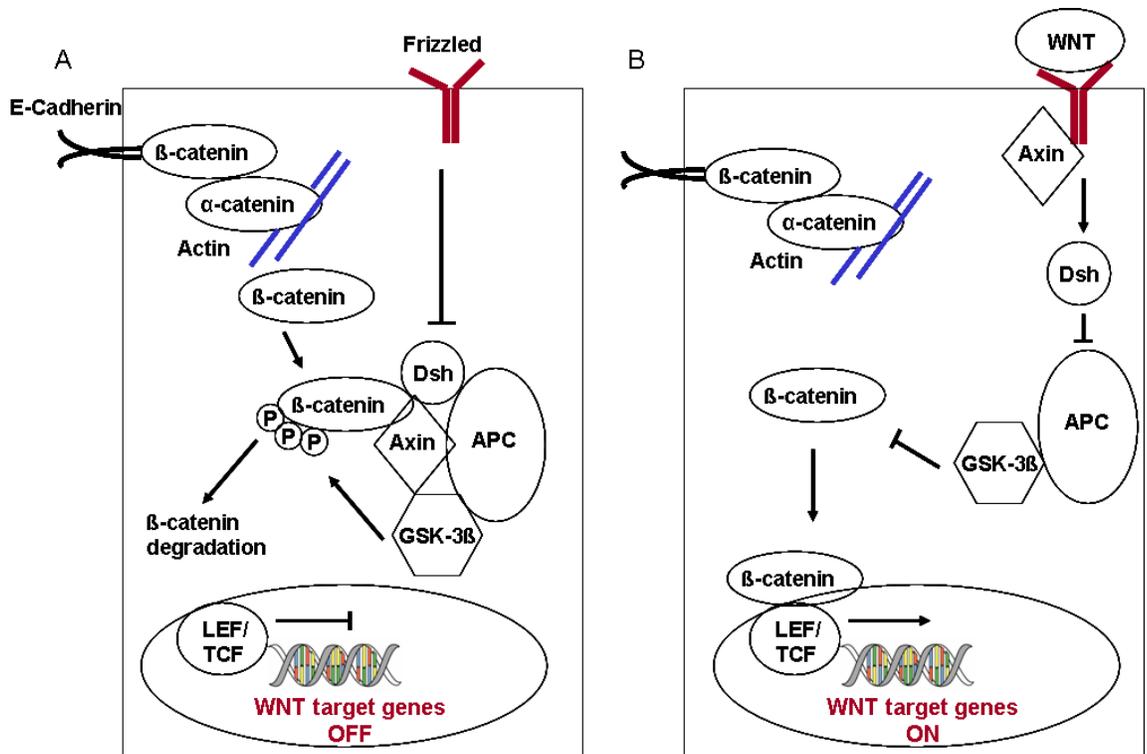
Figure 1.7. The canonical Wnt signaling pathway. (**A**) In the absence of Wnt binding to Fz, Dsh remains unactivated and ß-catenin is bound by the destruction complex composed of APC/ Axin/ GSK-3β. This complex phosphorylates β-catenin and is targeted for degradation. (**B**) When Wnt binds to Fz, Dsh is activated and uncouples β-catenin from the complex. β-catenin can then associate with transcription factors and mediate transcription of the target gene. Frizzled (Fz), Adenomatous polyposis coli (APC), dishevelled (Dsh), Glycogen synthase kinase 3β (GSK-3β), Lymphoid enhancer-binding factor (LEF), T-cell factor (TCF), Adenomatous polyposis coli (APC).

The most studied Wnt pathway is the canonical pathway (Figure 1.7), which is activated by the binding of a Wnt ligand to a frizzled receptor (Reya and Clevers, 2005). In the absence of Wnt, ß-catenin is bound by the multiprotein "destruction complex" composed of the tumor suppressors, APC (adenomatous polyposis coli) and Axin. These latter protein bind and present ß-catenin to the kinases, glycogen synthase kinase 3 ß (GSK3 ß) and casein kinase I (CKI). This results in phosphorylation of ß-catenin and thereby it is targeted for

polyubiqitination and proteasomal degradation. In the presence of Wnt, ß-catenin binds to FZ and inhibits the destruction complex so that ß-catenin remains unphosphorylated. This results in the translocation and accumulation of ß-catenin in the nucleus. In the nucleus it interacts with the TCF/LEF family of transcription factors to activate downstream target genes such as c-Myc, CyclinD1 and matrix metalloproteinase (MMP) that regulate cell proliferation, differentiation and survival (Mc Donald *et al*., 2006).

Canonical Wnt signaling is associated with the pathogenesis of several carcinomas. Dysregulation of Wnt/ ß-catenin signaling is involved in the initiation of colorectal carcinogenesis. APC is a tumor suppressor gene that can down-regulate the transcriptional activation mediated by Wnt/ß-catenin. APC mutations result in its inactivation and concominant loss of the inhibition of Wnt. Moreover, mutations of ß-catenin in the functionally significant phosphorylation sites have been detected in colorectal cancer (Morin *et al*., 1997). Furthermore mutations in members of the Wnt/ß-catenin pathway have been reported in hepatocellular and gastric cancers (Polakis *et al*., 2000). Aberrant activation of the Wnt canonical signaling pathway is involved in pathogenesis of many types of cancers, making this pathway an attractive therapeutic target.

Wnt signaling and hematopoietic stem cells

The Wnt/ ß-catenin signaling pathway has been shown to have an effect on controlling the proliferation, survival and differentiation of hematopoietic cells. It has been shown that expansion of HSC occurs in long-term cultures due to the overexpression of activated ß-catenin. These expanded HSCs retained the functional characteristics of HSCs and are able to reconstitute the hematopoietic system *in vivo* (Reya *et al*., 2003).

Activation of Wnt signaling pathway has been implicated in the pathogenesis of various

hematological malignancies including leukemia. ß-catenin activation coupled with GSK3 ß inactivation has been demonstrated in precursor B-cell acute lymphoblastic leukemia (ALL). The granulocyte macrophage progenitor (GMP) has been identified as a candidate leukemic stem cell in blast crisis chronic myelogenous leukemia (CML), and it has elevated levels of nuclear ß-catenin (Jamieson *et al*., 2004). Also, constitutive expression of active ß-catenin *in vivo* has been shown to result in loss of myeloid lineage commitment at the GMP stage, blocking erythrocyte differentiation and disrupting lymphoid development (Kirstetter *et al*., 2006). Recently, it has been found that the Wnt/ ß-catenin signaling pathway is required for self-renewal of leukemic stem cells (LSCs) that are derived from either HSC or more differentiated GMP (Wang *et al*., 2010). This suggests the involvement of active ß-catenin in leulemogenesis.

In the *Drosophila* model Wnt signaling was shown to be epigenetically regulated (Sollars *et al*., 2003). Epigenetics involves changes in gene expression that are propagatable through mitosis or meiosis, and are caused by mechanisms other than a change in the DNA sequence. These can occur through DNA methylation, histone modifications, or non-coding RNA. Secreted frizzled related proteins (SFRP), which act as Wnt antagonists, can inhibit Wnt-Frizzled interaction by sequestration of the ligand. SFRPs have been shown to be regulated by promoter hypermethylation and have been implicated in various cancers (Suzuki *et al*., 2004; Fukui *et al*., 2005; Marsit *et al*., 2006). Recently, SFRPs have also been found to be downregulated or inactivated by promoter hypermethylation in acute lymphocytic leukemia (ALL) and AML (Jost *et al*., 2008).

Inhibition of Hsp90 in *Drosophila* resulted in the up-regulation of Wg to produce an abnormal eye phenotype via epigenetically regulation (Sollars *et al*., 2003). In chapter 3 we wanted to determine the effect of Hsp90 modulation on the Wnt pathway in a mammalian model system. We obtained mixed results in this study since the *in vitro* model system does not

recapitulate the stem cell niche seen *in vivo*. Hsp90 interacts with numerous signal transduction proteins including chromatin remodeling complexes. Therefore, there is a possibility that Hsp90 has an effect on the Wnt pathway, and if this can be demonstrated it will have profound implications for the treatment of cancer as Wnt signaling is activated in various cancers.

Omega Fatty acids and cancer

In recent years, there has been increased focus on the role of specific dietary fatty acids and their effect on health and disease. Researchers have been studying the effects of poly unsaturated acids (PUFA) on cancer. This interaction will be important in devising new therapies for treatment and chemoprevention against cancer. AML is characterized by the inhibition of myeloid progenitor cell differentiation. Omega fatty acids have been shown to promote myeloid differentiation. Moreover, Wnt signaling has been demonstrated to be active in AML. Therefore, in the studies described in chapter 4 we have investigated the effect of omega fatty acids on Wnt signaling in the hematopoietic system.

Fatty acids (FA) are carboxylic acids with long hydrocarbon chains that can be saturated or unsaturated depending upon the presence of double bonds in the carbon chain. Fatty acids with multiple sites of unsaturation are termed as PUFAs. Omega-6 FAs are derived from linoleic acid (LA, 18:2) and the omega-3 FA are derived from α-linolenic acid (ALA, 18:3). LA is metabolized to arachidonic acid (AA, 20:4, omega-6), while ALA can be metabolized to eicosapentaenoic acid (EPA, 20:5, omega-3) and ultimately docosahexanoic acid (DHA, 22:6) (Anderson and Ma, 2009). Linoleic acid and α-linolenic acid are omega-3 and omega-6 PUFAs that are essential for survival and must be obtained from dietary source, since they cannot be synthesized in mammals. Omega-3 (EPA) and Omega-6 (AA) PUFA are classified depending on the location of the first double bond relative to the methyl terminus

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(Das, 2008) (Figure 1.8).
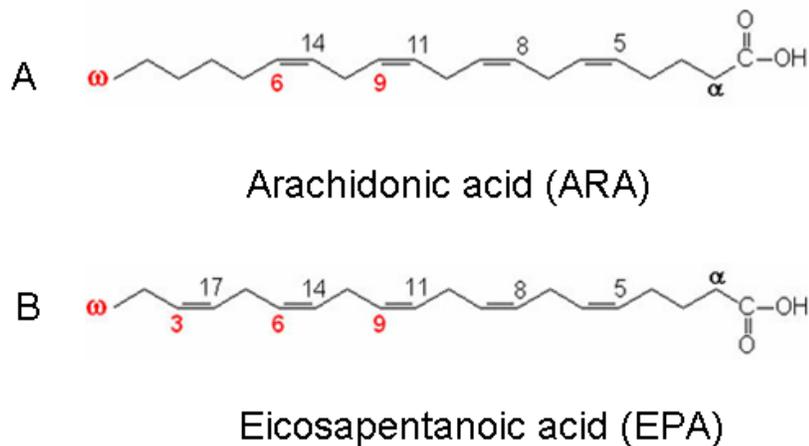
Figure 1.8. Omega-3 and omega-6 Polyunsaturated fatty acids (PUFA). (A) Arachidonic acid with first double bond at carbon 6 from the methyl terminus. **(B)** Eicosapentanoic acid with first double bond at carbon 3 from the methyl terminus.

Omega-6 and omega-3 FA are important structural components of the phospholipid cell membranes and are essential for the activity of membrane-bound enzymes and receptors as well as signal transduction. Polyunsaturated FAs are substrates for eicosanoid synthesis, with omega-6 FAs converted into pro-inflammatory eicosanoids and omega-3 FAs being converted into anti-inflammatory eicosanoids. High omega-6/omega-3 tissue ratios contribute to the development of chronic diseases in later life such as coronary heart disease and stroke, or diabetes (Institute of medicine, 2005). Several studies suggest that omega-6 FA promote cancer development (Williams *et al*., 2011) while omega-3 FA suppresses tumor carcinogenesis (Augustsson *et al*., 2003; DeDecker, 1999; Calviello *et al*., 2009).

Omega fatty acids have been found to have effects on the hematopoieis. Eicosanoids derived from AA metabolism (omega-6 FA) have been shown to play a role in myelopoiesis and erythropoiesis (Dupuis *et al*., 1997). High omega-3/omega-6 FA ratio in the diet has been

reported to promote differentiation and reduce the frequency of myeloid progenitor cells in the bone marrow of mice (Varney *et al*., 2009). Abnormal hematopoiesis results in excessive proliferation of immature blasts and inhibition of their differentiation into mature blood cells. This can lead to myeloproliferative disorders and leukemia. Since omega-3 FAs have been shown to promote differentiation they can be used as a therapeutic approach in leukemia.

Wnt signaling plays an important role in hematopoietic self renewal and differentiation (Reya *et al*., 2003). Prostaglandin (PGE2) derived from AA (Omega-6 FA) metabolism regulates vertebrate HSC induction and engraftment (North *et al*., 2007). Recently, it was reported that PGE2 interacts with Wnt and together they regulate murine stem and progenitor populations *in vitro* and *in vivo* (Goeslling *et al*., 2009). Inhibition of PGE2 synthesis blocked Wnt-induced alterations in HSC formation at the level of ß-catenin. This suggests that omega-6 FAs might play role in promoting HSC proliferation.

Aberrant Wnt signaling is associated with different cancers including AML and CML (Jamieson *et al*., 2004). Earlier studies with *Drsophila* demonstrated the up-regulation of Wnt signaling after inhibition of Hsp90 by an epigenetic mechanism. SFRP which act as Wnt antagonists can inhibit Wnt-frizzled interaction by sequestration of the ligand. SFRP are negatively regulated by epigenetics (DNA methylation) and this leads to up-regulation of Wnt signaling. Up-regulation of Wnt signaling through down-regulation of SFRP occurs in ALL and AML (Jost *et al*., 2008). Recently investigators reported that omega-3 fatty acids inhibited Wnt signaling in hepatocellular and cholangiocarcinoma (Lim *et al*., 2008; Lim *et al*., 2009). This suggests that omega-3 FA may be involved in the inhibition of Wnt signaling in leukemia by the down-regulation of SFRP.

Since Wnt signaling is important in hematopoietic stem cell maintenance and is involved in leukemia, I hypothesized that omega-3 FA might induce differentiation in leukemic cells by inhibiting the Wnt signaling via an epigenetic mechanism. To investigate the effects of

omega-3 and omega-6 FAs on the Wnt pathway I used a stem cell model, EML cells and leukemic cells. Wnt signaling activity was examined based upon the accumulation of ß-catenin in the nucleus. In chapter 4, our prelimiary data initially showed that Wnt signaling is down-regulated upon exposure to omega-3 FAs in EML cells as well as HL-60 leukemic cells.

CHAPTER TWO: YB-1 expression and function in early hematopoiesis and leukemic cells

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Abstract

Hematopoietic transcription factors play a critical role in directing the commitment and differentiation of hematopoietic stem cells along a particular lineage. Y box protein (YB-1) is a transcription factor which is widely expressed throughout development and is involved in erythroid cell development, however its role in early hematopoietic differentiation is not known. Our objective was to investigate the role of YB-1 expression in early hematopoietic differentiation and leukemia. Here, we show that YB-1 is highly expressed in mouse erythroid, myeloid lymphoid-clone1 (EML), a hematopoietic precursor cell line, but is downregulated in myeloid progenitors, and GM-CSF treated EML cells. Moreover, we found that lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^+$ (LKS; enriched fraction of hematopoietic stem cells) and lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^-$ myeloid progenitor cells showed a high level of YB-1 expression as compared to the differentiated cells like granulocytes in mouse bone marrow (BM). Also, YB-1 protein was expressed at high levels in myeloid leukemic cell lines blocked at different stages of myeloid development. We further investigated the role of YB-1 in leukemic cells by knockdown studies and observed that down-regulation of YB-1 expression in K562 leukemic cells inhibited their proliferation ability, induced apoptosis and differentiation towards megakaryocytic lineage. Overall, our data indicates that YB-1 is down-regulated during myeloid differentiation and the aberrant YB-1 expression in leukemic cells could be a contributing factor in the development of leukemia by blocking their differentiation. Thus, YB-1 protein could be an excellent molecular target for therapy in myeloproliferative disorders and leukemia.

Key words: YB-1, EML cells, K562 cells, differentiation, mouse stem and progenitor cells

Introduction

Hematopoiesis is the process by which all the different cell lineages that form the blood and immune system are generated from a common pluripotent stem cell. It is maintained by a proper balance between self renewal and multi-potent differentiation of the hematopoietic stem cells (HSC) (Huntley and Gilliland., 2005). Transcription factors play a major role in differentiation in a number of cell types, including the various hematopoietic lineages (Tenen *et al*., 1997). Among the best examples are PU.1, CCAT/enhancer binding protein α (C/EBPα), AML1, Globin Transcription Factor (GATA-1), c-myb, and SCL/Tal1 (Rosenbauer *et al*., 2005). Myeloid gene expression is controlled by the combinatorial effects of several key transcription factors. Alteration of myeloid transcription factors (changes in expression and structure) lead to abnormal myelopoiesis and dysplasia (Tenen *et al*., 1997). Consequently, a major focus of research in this area has been on the molecular mechanism controlling normal myeloid differentiation. To better understand the process of normal hematopoietic differentiation, it is important to identify and characterize the differential expression of transcription factors in HSCs and terminally differentiated cells.

It is difficult to study the early stages of hematopoietic differentiation because few experimental models are available to approach this question. The Erythroid Myeloid Lymphoid-clone 1 (EML) cell line, developed from murine bone marrow cells transfected with a vector expressing a dominant negative form of the retinoic acid receptor, provides a unique *in vitro* model to address this question. EML cells can be indefinitely propagated in medium containing stem cell factor (SCF) and can be differentiated into erythroid, myeloid, lymphoid lineages by the addition of appropriate cytokines (Tsai *et al*., 1994).

The Y-box protein (YB-1), also known as p^{50} or dbpB belongs to a superfamily of cold shock proteins that are highly conserved during evolution (Toh *et al*., 1998). Y-box protein is involved in a wide variety of cellular functions, such as regulation of DNA transcription and

translation (Evdokimova *et al*., 2006). YB-1 null mice can survive organogenesis and the majority of homozygous null embryos survive to day 18.5 of gestation (Lu *et al*., 2005).YB-1 is an integral part of the cellular stress response signaling pathway required for protecting cells from a variety of stresses and prevention of premature senescence in cultured primary cells (Lu *et al*., 2005). Y-box elements are present in the promoters of several genes associated with cell division, therefore it is suspected that YB-1 has a role in promoting cell proliferation (Wolfe 1994; Ladomery and Sommerville 1995). It has also been shown that down-regulation of YB-1 results in reduced proliferation and increased apoptotic cell death rates in multiple myeloma cells (Chatterjee *et al*., 2008). The YB-1 transcript and protein have also been detected in mouse embryonic stem cells (Shibahara *et al*., 2004). In summary, there is evidence for YB-1 having a role in cell proliferation, cell survival and protecting against apoptosis.

Increased nuclear and cytoplasmic expression of YB-1 has frequently been detected in a wide range of human cancers, including breast, thyroid, colorectal, osteosarcomas, and synovial sarcomas (Kohno *et al*., 2003). Clinical studies on YB-1 have shown close association of the cellular level of YB-1 with tumor growth and prognosis in ovarian, lung and breast cancers (Kuwano *et al*., 2004). YB-1 controls the expression of genes involved in tumor progression including matrix metalloproteinase-2 (MMP-2) and the multidrug resistance gene 1 (MDR-1) (Mertens *et al*., 1997; Ohga *et al*., 1998). Enhanced YB-1 expression is associated with tumor progression and drug resistance in melanoma and multiple myeloma (Schittek *et al*., 2007; Chatterjee *et al*., 2008).

Knockdown of GATA-1, a transcription factor essential for erythropoiesis in mice, leads to maturation arrest and transformation of erythroblasts. The GATA-1 mutant mouse (knockdown mouse) exhibits increased expression of YB-1 in its spleen as compared to the wild type mouse (Yokoyama *et al* 2003 a, b). Moreover, expression of YB-1 was found to be higher in the bone marrow samples of MDS patients (Lee *et al*., 2001). The expression of YB-1

is higher in erythroblasts in myelodysplastic syndrome-refractory anemia (MDS-RA) than in normal cells suggesting a role of YB-1 in erythropoiesis (Yokoyama *et al*., 2003 a, b).

Since the role of YB-1 in normal hematopoietic differentiation has not been elucidated, we examined the expression of YB-1 in the mouse hematopoietic EML cell line and *in vivo* during myelopoiesis. To further investigate its possible role in leukemogenesis, we have determined the expression and function of YB-1 protein in human leukemic cells.

Materials and Methods

Cell Culture

EML C1 cells were the kind gift of Dr. Schickwann Tsai and were maintained in Iscove's modified Dulbecco medium (IMDM,) supplemented with 20% horse serum (American Type culture collection, ATCC, Manassas, VA) and 10% BHK/MKL-conditioned medium (Tsai *et al*., 1994). For differentiation studies, EML cells were induced to differentiate into myeloid cells with 10 µM all-trans retinoic acid (ATRA (RA); Sigma, St. Louis, MO, USA), 10% BHK conditioned medium (source of stem cell factor) and 15% WEHI conditioned medium (source of interleukin-3) for three days. Cells were then cultured again in IMDM/20% horse serum with 20 ng/ml of murine granulocyte-monocyte colony stimulating factor (GM-CSF) (Stem cell tech. Vancouver, BC, Canada) for the next three days. HL-60 (human promyelocytic leukemia), K-562 (human myelogenous leukemia) (Lozzio and Lozzio 1975); U-937 (human promonocytic leukemia), WEHI-3 (murine monocytic leukemia) cells were purchased from the ATCC and cultured according to their guidelines.

Mouse strains

C57BL/6J mice were bred and maintained at the AAALAC accredited animal care facility at Marshall University in accordance with the university guidelines. Donor mice used

for bone marrow isolation were 8-10 months of age. Mice were sacrificed for these experiments according to institutional guidelines.

Flow cytometry studies

Stable clones with YB-1 shRNA in K562 cells: Sh6-, Sh7-, Sh8- were seeded at a density of $1x10^5$ cells/ml in a six-well plate and treated with 0.5μM $As_2O_{3\ for}$ 72 hours. Treated as well as untreated EML and K562 cells were washed twice with FACS buffer (Phosphate buffered saline, PBS supplemented with 3% BSA, 0.02% sodium azide and 1mM EDTA) and collected by centrifugation. Thereafter they were incubated with FcγRII/III antibody (553142, BD Pharmingen) to prevent nonspecific binding by blocking the Fc receptors for half an hour at 4°C. For EML cells the cells were washed again and labeled with PE conjugated anti-Sca-1 (clone D7, 553108), PE conjugated anti-c-Kit (clone 2B8, 553355), biotinylated anti-CD11b (clone M1/70, 553309, BD Biosciences) or PE conjugated anti-F4/80 (clone BM8, MF48004, Caltag) for 30 minutes on ice. After washing the EML cells biotinylated anti-CD11b antibody was labeled with strepavidin APC (SA1005, Molecular probes). K562 cells were washed and labeled with APC conjugated mouse anti-human CD41a antibody (clone HIP8, 559777, BD biosciences) for 30 minutes on ice. Data acquisition was performed using BD FACS Aria sorter and data analysis was done using Flow Jo software v.7.5.5 (Treestar, Ashland, OR).

Cell staining and sorting

Bone marrow (BM) was harvested from C57BL/6J donor mice by flushing both femur and tibia with PBS/2% FBS. Heart punctures were performed to isolate serum from the mice. Cells were washed with 1X PBS by centrifugation and resuspended in PBS/2%FBS buffer followed by the addition of 10% mouse serum at 4°C for half an hour to block the Fc receptors to prevent any nonspecific binding. For isolation of HSC and progenitor cells, $2.5X10^7$ bone marrow cells were incubated for half an hour on ice with 100 μl of each of the following

biotinylated lineage specific antibodies to identify terminally differentiated cell types: CD3e (clone 145-2C11), CD11b (clone M1/70), B220 (clone RA3-6B2), Gr-1 (clone RB6-8C5), TER-119 (anti-erythrocyte specific antigen), IL-7Rα chain (clone B12-1) (Lineage panel; 559971, Becton Dickenson-Pharmingen). Cells were labeled with PECy7 conjugated anti-Sca-1 (clone D7, 25-5981) and APCCy7 conjugated anti-c-Kit (clone 2B8, 25-1171; eBioscience, San Diego, CA) monoclonal antibodies. After washing with PBS/2% FBS the cell suspension was incubated with strepavidin pacific blue conjugate (Molecular probes, Invitrogen) for 30 minutes on ice. Murine hematopoietic stem cells (lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^+$) and myeloid progenitors (lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^-$) were sorted from bone marrow, as described previously (Akashi *et al*., 2000). For the isolation of granulocytes, bone marrow cells were stained with biotinylated Gr-1 and visualized by a strepavidin pacific blue conjugate. All cell populations were sorted using BD FACS Aria multicolor cell sorter and data analysis was done using DIVA and Flow Jo software (Treestar, Ashland, OR). A second round of sorting was performed to ensure pure populations. RNA was isolated from multiple independently isolated samples containing normal HSC, myeloid progenitors and granulocytes.

RNA extraction and quantitative RT-PCR

RNA was isolated from double sorted HSC & progenitor cells obtained from mouse bone marrow or from EML cells using an RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA). cDNA was synthesized by using the Advantage RT-for-PCR kit® according to the manufacturer's guidelines (Clontech, Mountain View, CA). Gene expression analysis designed against mouse YB-1 (catalog no. ABI Mm00850878) was performed using TaqMan Gene Expression Assays on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, California, USA). Expression of the β-actin gene (catalog no. ABI, 4352341E) was used to normalize the amount of the investigated transcript. Data were corrected

for efficiency and loading using the Pfaffel method (Tichopad *et al.*, 2003). Data shown are representative of four independent experiments.

Western blotting

Treated cells from different experimental conditions were rinsed once with ice-cold PBS and then separated into nuclear and cytoplasmic fractions using the NE-PER extraction kit as per the manufacturer's instructions (Pierce, Rockford, IL). Protein concentration was determined using bicinchoninic acid (BCA) protein assay reagents from Pierce according to the manufacturer's guidelines. Cell extracts were then denatured at 95°C for 5 min in 2x sample buffer (62.5 mM Tris HCl (pH 6.8), 25% glycerol, 0.01% bromophenol blue, 2% SDS, 10% β-mercaptoethanol). Equal amount of protein extracts were separated on an 8-16% gradient Tris-Glycine SDS–poly acrylamide gel (Bio-Rad, Hercules, CA) by electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Millipore) using the Bio-Rad MiniProtean3® system. The membranes were treated in blocking solution (5% non fat dry milk in TBS containing 0.1% Tween 20) and incubated with primary YB-1 rabbit polyclonal antibody (1µg/ml, 2749, Cell Signaling Technologies, Danvers, MA) overnight at 4°C, followed by incubation with HRP-conjugated monoclonal rabbit secondary IgG antibody (1: 3000, 7074, Cell Signaling). An anti-mouse GAPDH antibody (MAB374, GE Healthcare, CT) was used to assess equal loading. Proteins were visualized by enhanced chemiluminescence (ECL) (GE Healthcare, CT). Benchmark ^{TM} protein ladder was used to visualize the transfer of protein onto the membrane and MagicMark ^{TM} XP (Invitrogen) was used as a molecular size standard.

Wright-Giemsa staining

EML cells at different stages of myeloid cell differentiation were cytospun onto microscopic slides and stained with Wright-Giemsa stain (Accustain, sigma) and light microscopy images (400x) taken using an Olympus BX51 system microscope attached to a DP70

microscope digital camera. Captured images were analyzed for morphological changes during myeloid differentiation with DP70 software on a Dell optiplex GX280 computer.

Transfection of leukemia cells and generation of stable clones

The shRNA expression vector for YB-1 (YB-1-pSUPER with the target sequence GAAGGTCATCGCAACGAAG, shYB-1) for generating YB-1 specific shRNAs and control shRNAs has been described previously (Schittek *et al*., 2007; Huber *et al*., 2004). This vector does not have a selectable marker. To generate stable clones, K562 leukemia cells (Lozzio CB, Lozzio BB, 1975) were cotransfected with the YB-1 pSUPER vector and for neomycin selection ; pEGFPN3 vector (Clontech, Saint-Germainen- Laye, France) by electroporation using BTX electroporation system as per manufacturer's instructions (Genetronics, San Diego, CA). K562 cells or pEGFP transfected K562 leukemia (Empty vector, EV) cells served as controls. K562 cells were selected with 800 µg/ml G418, 48 hrs after transfection, for 2 weeks, thereafter GFP +ve cells were sorted and recultured in G418 media for another 2 weeks. When stable clones were generated the G418 dose was reduced to half. Three stable clones sh6-, sh7- and sh8- from the YB-1sh/EGFP were confirmed for YB-1 knockdown by western blotting and were further analyzed.

Cell Viability Assay

Stable clones with YB-1 shRNA in K562 cells: Sh6-, Sh7-, Sh8- were treated with 0.5 µM As_2O_3 (Alfa Aesar, Massachusetts, USA) and cell viability assay was performed at 24, 48, 72 hours using the trypan blue dye exclusion method.

Apoptosis assay

Stable clones with YB-1 shRNA in K562 cells: Sh6-, Sh7-, Sh8- were seeded at a

density of 1×10^5 cells/ml in a six-well plate and treated with 0.5 µM As_2O_3 for 72 hours. The cells were harvested and washed with cold PBS and resuspended in the binding buffer (100 µl of calcium buffer containing 10 mM HEPES/NaOH, ph7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) containing 5 µl annexin V-pacific blue and 5 µl 7 AAD (5 µg/ml). The samples were incubated for 30 minutes in the dark at room temperature and then subjected to flow cytometry.

Morphological evaluation of differentiated cells

Stable clones with YB-1 shRNA in K562 cells: Sh6-, Sh7-, Sh8- were treated with 0.5µM As_2O_3 for 72 hours and cell morphology was determined by examining light microscopy images (400x) taken using an Olympus BX51 system microscope.

Cell cycle analysis by flow cytometry

Stable clones with YB-1 shRNA in K562 cells: Sh6-, Sh7-, Sh8- were seeded at a density of 1×10^5 cells/ml in a six-well plate and treated with 0.5µM As_2O_3 for 72 hours. Cells were harvested and washed twice with PBS, fixed in 70% ethanol overnight at 4°C. Cells were then stained with 5 µl (1 mg/ml) propidium iodide (PI) containing 2.5 µl (10 mg/ml) RNase for 30 minutes at 37°C and analyzed by flow cytometry on an Accuri C6 flow cytometer.

Statistics

Statistical analysis of the data was performed using the Student t test or ANOVA followed by a Student t test with corrections for multiple comparisons using the Bonferroni method as appropriate. The statistical test used for each data set is stated in figure legends; $p < 0.05$ was considered to be significant. Data are presented as means plus or minus standard error of mean (SEM).

Results

EML cells as a model of myeloid differentiation

The EML cell line is a SCF dependent multipotent cell line with myeloid, erythroid and lymphoid potentials. It was established from mouse bone marrow infected with a retroviral vector (LRARα403SN) harboring a dominant negative retinoic acid receptor (RAR construct) (Tsai *et al.*, 1994).
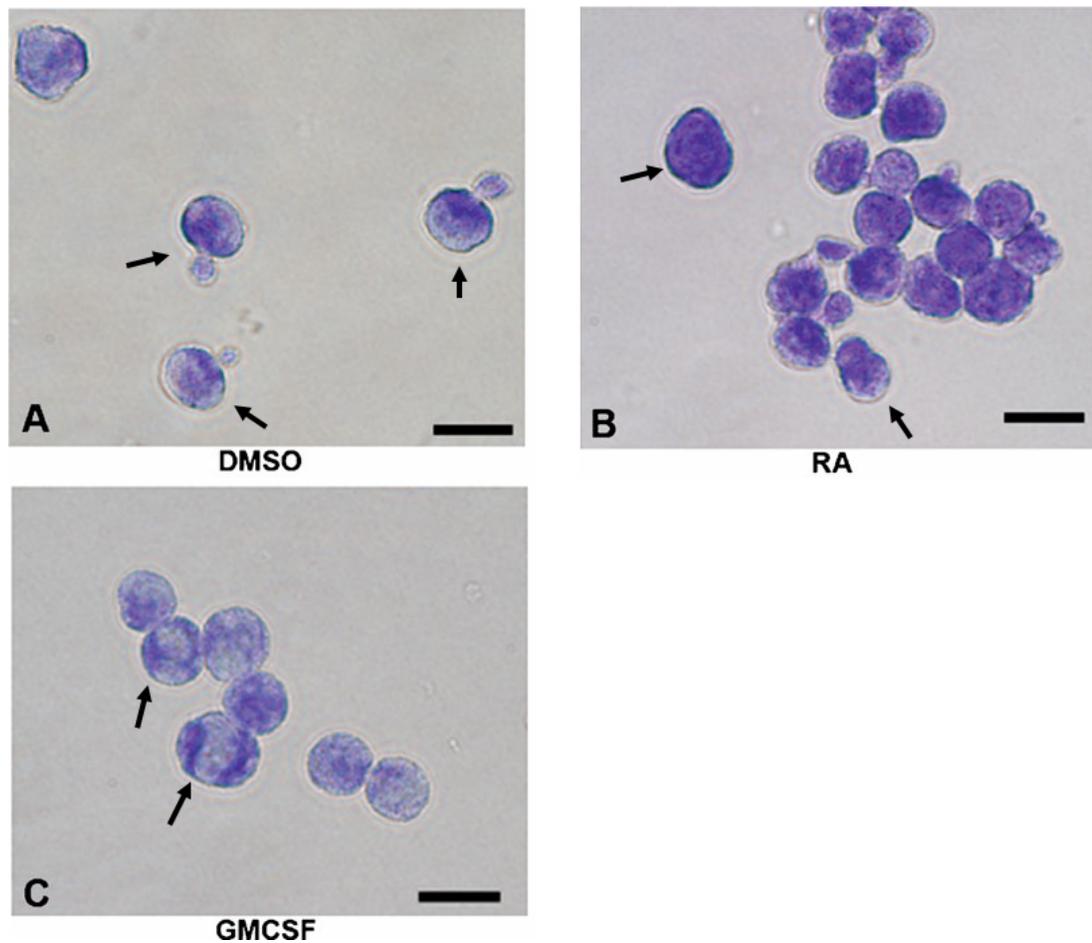
Figure 2.1. Morphology of EML cells during RA and GM-CSF induced differentiation. Cytospin preparations of the indicated cells were stained with Wright-Giemsa. (A) Native DMSO treated SCF dependent EML cells. Arrows indicate hand-mirror-shaped cells that are frequently seen in EML cell line. (B) EML cells treated with SCF/IL-3 and 10 µM RA for three days. Arrows show the granulocyte-monocyte progenitors (CFU-GM) indicated by the increase in nuclear to cytoplasmic ratio. (C) EML/RA+IL-3 cells treated with GM-CSF for another three days. Magnification is 400x. Arrows show the committed granulocytic/monocytic progenitors with bi-lobed nucleus. Scale bars are 50 µm.

It is a suspension cell line consisting of mostly blast like cells with 20-30% hand mirror shaped cells (Figure 2.1A). EML cells serve as an excellent model to study hematopoietic differentiation *in vitro* (Johnson *et al.*, 1999). It can be induced to differentiate towards granulocyte/monocyte progenitors (CFU-GM) by high concentration of RA in the presence of IL-3 (Figure 2.1B). These CFU-GMs can be further differentiated into more committed granulocyte/monocyte progenitors by GM-CSF treatment (Figure 2.1C).

To characterize the differentiation pattern of EML cells with our cytokine treatments, we analyzed cell markers associated with several stages of differentiation. We compared the cell surface marker profile of native, DMSO-treated EML cells, EML cells treated with RA+IL-3 (CFU-GM) for three days, and EML cells treated for an additional 3 days with GM-CSF after the RA+IL-3 treatment. Flow cytometry analysis was performed using the following panel of antibodies: (1) anti-Sca-1, which is specific for stem cells, (2) anti-c-kit specific for stem/progenitors, (3) anti-CD-11b specific for macrophage/neutrophil lineages, and (4) anti-F4/80 specific for macrophages (Figure 2.2). The parental EML cells appear relatively un-differentiated (Figure 2.2A) and display an immature surface antigen phenotype that is characterized by high Sca-1+ and c-kit+ (Figure 2.2 A, B) expression and absence of CD11b and F4/80 (Figure 2.2 C, D). In contrast, the EML/GM-CSF cells appear more differentiated (Figure 2.2 C) and display cell surface markers indicative of differentiated cells (CD11b+, F4/80+) with a loss of stem/progenitor markers such as Sca-1 and c-kit. Sca-1 expression in GMCSF treated EML cells was >90% reduced ($p<0.01$) while CD11b ($p<0.01$) and F4/80 ($p<0.01$) showed >95% & 59% increase respectively as compared to the untreated EML cells (Figure 2.2 E). This data indicates that EML cells express stem and primitive progenitor markers. When these cells are subjected to conditions that induced myeloid progenitors (CFU-GM), there is an increase in the expression of granulocyte/monocyte specific markers, which becomes more predominant with the appearance of more committed granulocyte/monocyte

progenitors.
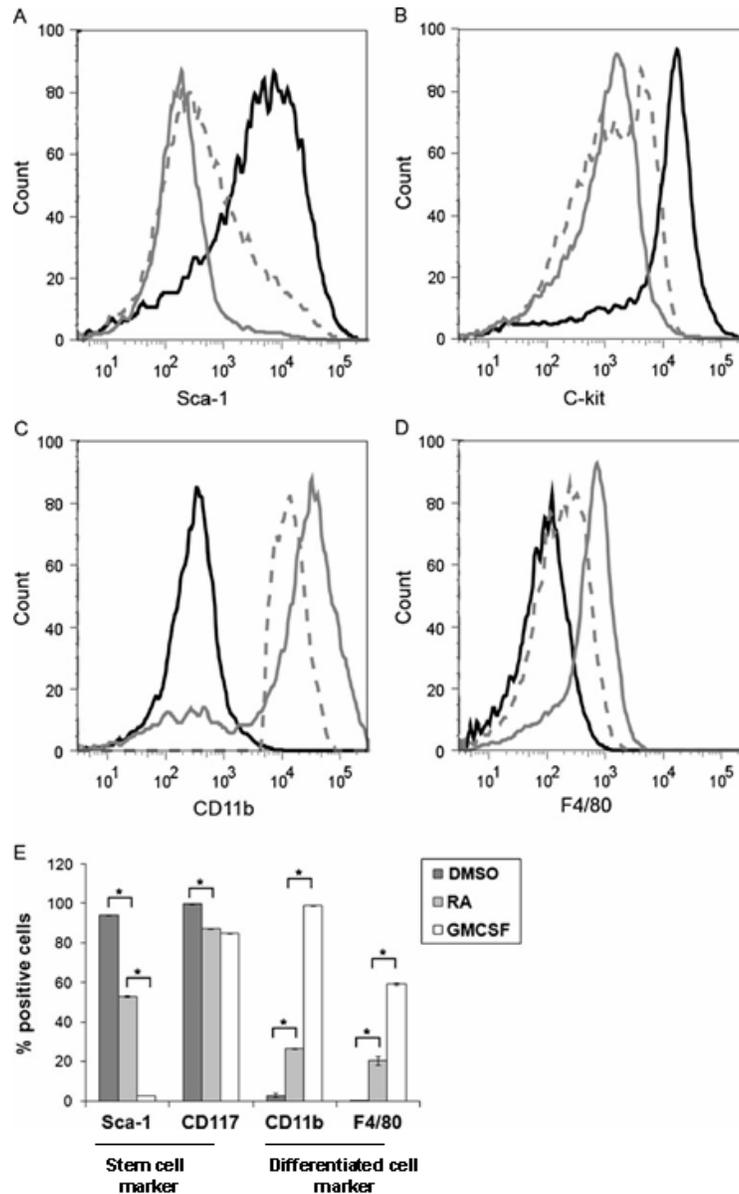
Figure 2.2. Cell surface marker profile of Untreated, RA and GM-CSF treated EML cells. EML cells were induced to undergo myeloid differentiation in the presence of SCF, IL-3 and RA for three days followed by GM-CSF for three days as described in materials and methods. Samples were collected at day 3 and day 6 for RA & GM-CSF treated EML cells respectively. Expression of (A) Sca-1, (B) c-kit which label primitive stem/progenitor cell types and (C) CD11b, (D) F4/80 specific for granulocytes and macrophages in DMSO treated (black line), RA (grey dashed line) and GM-CSF (solid grey line) treated EML cells. (E) Combined flow cytometric analysis of DMSO treated, RA+IL-3, and GM-CSF treated EML cells in terms of % positive cells for each surface antigen. Data is expressed as the mean ± SEM of triplicate values. Analysis of variance (ANOVA) for multiple pairwise comparisons was used to analyze the data along with Student t test comparing each to untreated EML cells. (* indicate $p \leq 0.01$).

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YB-1 expression in undifferentiated vs. differentiated EML cells

YB-1 functions in erythroid differentiation and aberrant expression of YB-1 leads to abnormal erythropoiesis but its role and regulation of gene expression in early hematopoiesis is still unknown (Yokoyama *et al*., 2003). In order to characterize its pattern of expression in early hematopoiesis, YB-1 protein expression was studied by western blot analysis in DMSO-treated, RA+ interleukin-3 (IL-3) treated and GM-CSF treated EML cells (Figure 2.3). YB-1 was detected as 49 kD protein in nuclear extracts. YB-1 protein was highly expressed in native EML cells relative to RA+IL-3 and GM-CSF treated cells (Figure 2.3A, B). RA+IL-3 treated EML cells (myeloid progenitors) showed a significant decrease (>60%; $p<0.05$) in YB-1 protein expression compared to the DMSO treated EML cells. This reduction was even more apparent in the GM-CSF treated (>80%; $p<0.05$) compared to the DMSO treated EML cells.

In order to determine if YB-1 protein levels correlate with the mRNA, we analyzed YB-1 mRNA expression in the above three cell populations by quantitative RT-PCR (Figure 2.3 C). YB-1 mRNA levels were down-regulated in RA+IL-3 treated relative to the DMSO treated EML cells and reduced further significantly with GM-CSF treatment ($p<0.05$). These data show that YB-1 mRNA and protein expression pattern correlate and are highly expressed in native undifferentiated EML cells but both levels are dramatically down-regulated during EML cell differentiation. Taken together, this data indicates that YB-1 is expressed in undifferentiated stem cells and reduces as these cells progress into early and later stage progenitors. The functional role of YB-1 expression is not known.
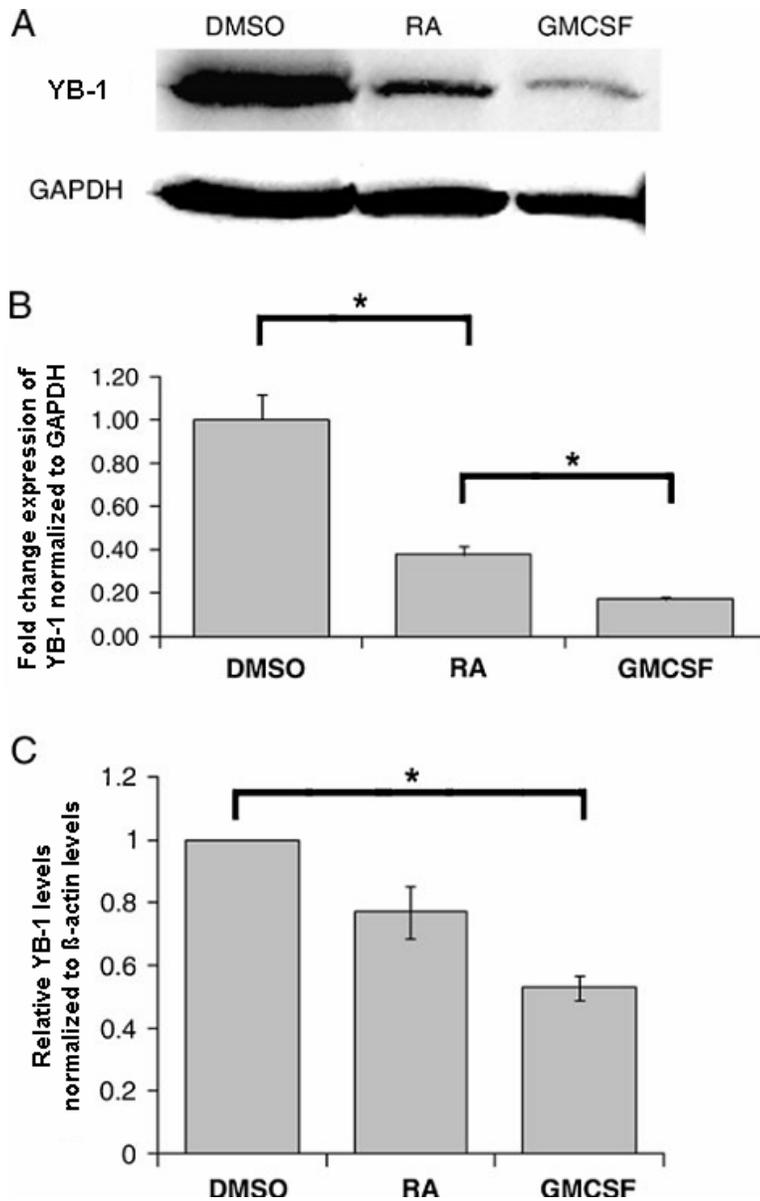
Figure 2.3. Expression of YB-1 mRNA and protein in EML cells. (A) Nuclear extracts from DMSO treated, RA and GM-CSF treated EML cells were subjected to SDS- PAGE and western blot analysis with an anti-YB-1 antibody (1µg/ml). GAPDH was used as a loading control. (B) The mean ± SEM of values obtained from densitometric analysis of three individual experiments. ANOVA for multiple pairwise comparisons was used to analyze the data along with Student's t-test comparing each to untreated EML cells (* indicate $p < 0.05$). This blot is representative of at least 3 different experiments, all of which gave similar results (N=9). (C) Total RNA was extracted from untreated, RA and GM-CSF treated EML cells by RNeasy kit and was reverse transcribed using Advantage RT-for-PCR kit®. Real-Time PCR analysis was performed using TaqMan probes directed at YB-1 and β-actin. Data was analyzed with ANOVA and is expressed as fold change, corrected for β-actin, relative to untreated EML cells (* indicate $p < 0.05$). Data is representative of experiments (N=6).

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Expression of YB-1 in mouse stem and progenitor cells

Though the EML cell culture system has proved useful in identifying changes in YB-1 gene expression during hematopoietic differentiation, it is an immortalized cell line and thus may not accurately represent normal hematopoietic cells (Ye *et al*., 2005). To compare changes in YB-1 gene expression observed in EML cells with normal hematopoiesis, we sorted stem cells, myeloid progenitors, and granulocytes from C57BL/6 mouse BM. In mice, multipotent hematopoietic activity resides in a small fraction of BM cells lacking the expression of lineage-associated surface markers but expressing high levels of Sca-1 and c-Kit (LKS fraction). Within the lineage⁻ BM cells myeloid progenitors can be isolated from the IL-7Rα⁻ fraction by the absence of Sca-1 expression, but the presence of c-Kit . Based on Sca-1 and c-kit profile of lineage⁻/IL-7R⁻bone marrow cells, a population enriched in HSC was sorted according to lineage⁻/IL-7R⁻/c-kit⁺/Sca1⁺ (LKS) marker profile while myeloid progenitors were separated based on lineage⁻/IL-7R⁻/c-kit⁺/Sca1⁻ profile (Figure 2.4A-C). Granulocytes were isolated from mouse BM by staining with anti Gr-1 antibody as shown in Figure 2.4D and E. In these three populations, YB-1 mRNA levels were measured by quantitative RT-PCR (Figure 2.4F). There was a significant two-fold increase in YB-1 mRNA levels in myeloid progenitors ($p<0.05$) as compared to the LKS fraction (enriched fraction for stem cells). More noteworthy is the statistically significant reduction in YB-1 mRNA levels in granulocytes which is reduced by more than 87% as compared to the stem/progenitor cells ($p<0.01$). These data indicate that YB-1 transcripts are highly expressed in stem/myeloid progenitors and down-regulated in more differentiated cell types like granulocytes. These results are consistent with the *in vitro* data, reinforcing the validity of the EML cell model and suggesting an *in vivo* role for YB-1 in hematopoietic stem and progenitor cells.
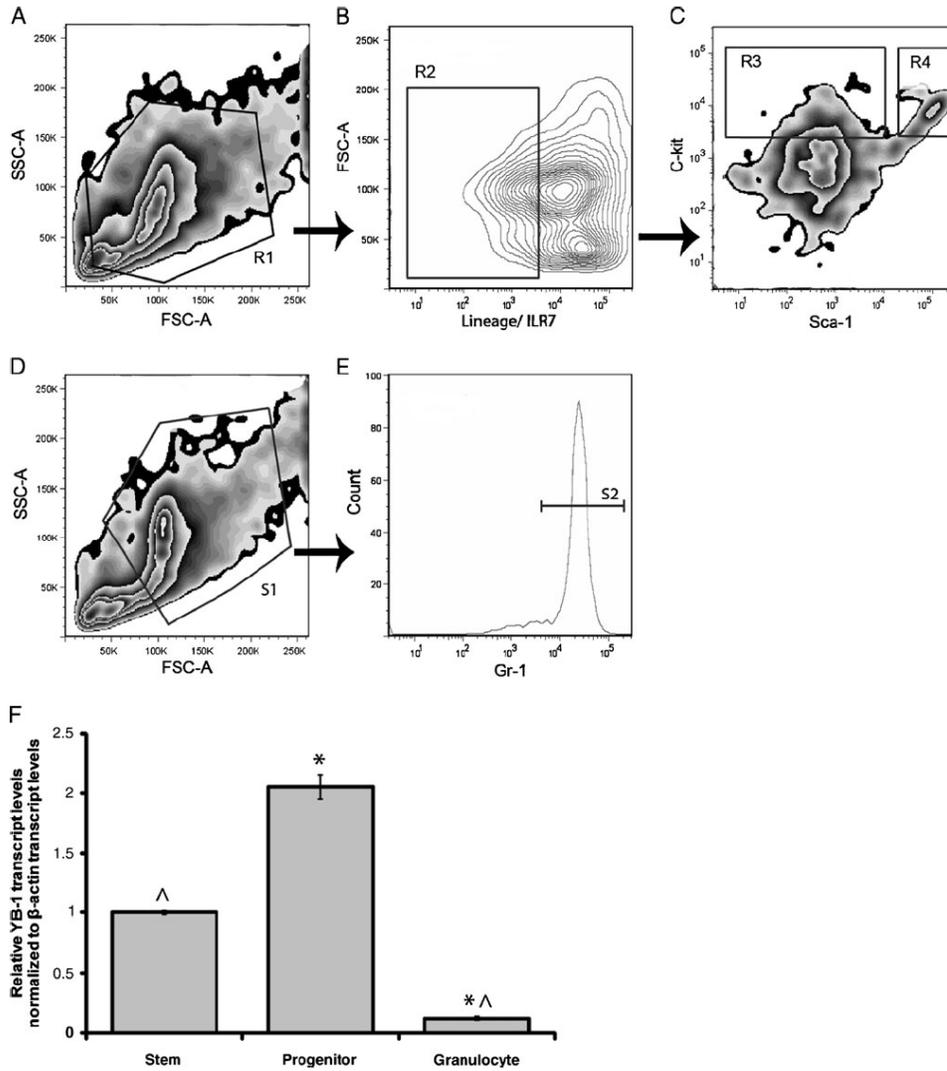
Figure 2.4. Expression of YB-1 in mouse stem and progenitor cells. (A) BM cells from adult mice were isolated by flow cytometry. Cells of the appropriate side scatter and forward scatter properties (R1) gate were further fractionated by (B) IL-7Rα and lineage panel depletion represented by gate R2. (C) Those remaining were selected based upon c-kit and Sca-1 expression as indicated. The lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^+$ (LKS, enriched fraction for stem cells) shown as R4 and lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^-$ (myeloid progenitors, R3) fractions were then sorted for quantitative RT-PCR. (D-E) Granulocytes were sorted from the mouse BM by forward/side scatter characteristics (S1) and labeling with anti-Gr-1 antibody (S2). (F) Total RNA was extracted from lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^+$ (LKS), lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^-$ (myeloid progenitors), and Gr-1$^+$ populations followed by real-time RT-PCR was performed using TaqMan probes directed at YB-1 and β-actin. The relative amount of mRNA in each cell population was normalized to an internal control gene β-actin and expressed as fold change relative to stem cells. The statistical significance of YB-1 expression was assessed by ANOVA combined with the student's t-test (* indicate comparisons to stem cell levels, ^ indicate comparisons to progenitor cell levels, $p \leq 0.01$). Data is representative of four independent experiments (N=12).

YB-1 expression in leukemic cells

YB-1 is involved in erythropoiesis and aberrant YB-1 expression may lead to abnormalities in erythroid differentiation (Yokoyama *et al*., 2003). MDS is characterized by ineffective production of mature blood cells due to increased proliferation and reduced differentiation of hematopoietic stem/progenitor cells, with a high predisposition to transform into acute leukemia (Greenberg *et al*., 1997).
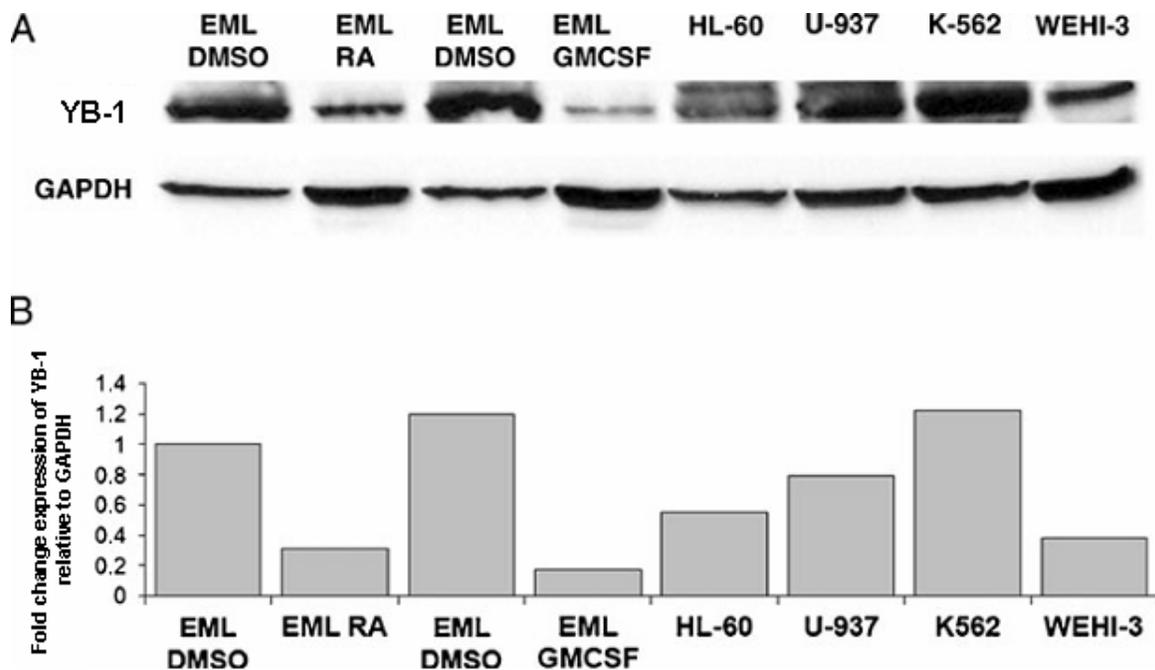
Figure 2.5. YB-1 expression in leukemia (A) Western blot analysis was performed on the nuclear extracts from HL-60 (human promyelocytic leukemia), U-937 (human promonocytic leukemia), K-562 (human chronic myelogenous leukemia), WEHI-3 (murine monocytic leukemia) cell lines using anti YB-1 antibody. Untreated, RA and GMCSF treated EML cells served as positive and negative controls. GAPDH was used as a loading control. (B) Densitometric analysis of the above western blot. This data is one representative of three independent experiments all giving similar results.

YB-1 has been shown to be up-regulated in the bone marrow of patients with MDS (Lee *et al*., 2001; Yokoyama *et al*., 2003). Since we saw a high expression of YB-1 in

stem/progenitor cells as compared to differentiated cells, we investigated whether higher expression of YB-1 is associated with leukemia. For this we performed western blot analysis on protein extracted from several human and mouse leukemic cell lines (Fig. 2.5 A, B). DMSO treated, RA+IL-3, and GMCSF treated EML cells served as references for these experiments. HL-60 (human promyelocytic leukemia), U-937 (human promonocytic leukemia), K-562 (human chronic myelogenous leukemia), WEHI-3 (murine monocytic leukemia) cell lines arrested at progenitor stage of myeloid differentiation expressed YB-1 at different levels. As native EML cells are more like stem/early progenitor cells, they express high levels of YB-1 protein as compared to the more committed myeloid progenitors. YB-1 expression levels in all the leukemic cell lines lie between stem and early myeloid progenitor state except the K-562 cell line, in which the expression is even higher than the EML cells. This data indicates that YB-1 is highly expressed in myelogenous leukemias.

Biological significance of down-regulation of YB-1 expression on cell proliferation apoptosis and differentiation in K562 leukemic cells

To determine the role of YB-1 in leukemic cells, we downregulated YB-1 in K562 cell line (Lozzio and Lozzio, 1975), using specific shRNA and studied the effects of low YB-1 levels on cell proliferation and apoptosis. Three stable clones sh6-, sh7-, sh8- were selected and down-regulation of YB-1 was confirmed by western blotting. Approximately a 40% reduction in YB-1 expression was achieved compared to EGFP transfected K562 cells, empty vector (EV) (Figure 2.6A).YB-1 down-regulation itself resulted in reduction in cell viability in sh6-, sh7-, and sh8- K562 relative to the normal K562 cells and EV at 72 hours. There was a statistically significant reduction in cell proliferation in sh6- (68%), sh7- (66%) & sh8- (66%) after treatment with 0.5 µM As_2O_3 relative to the untreated K562 cells ($p<0.01$) (Figure 2.6B).
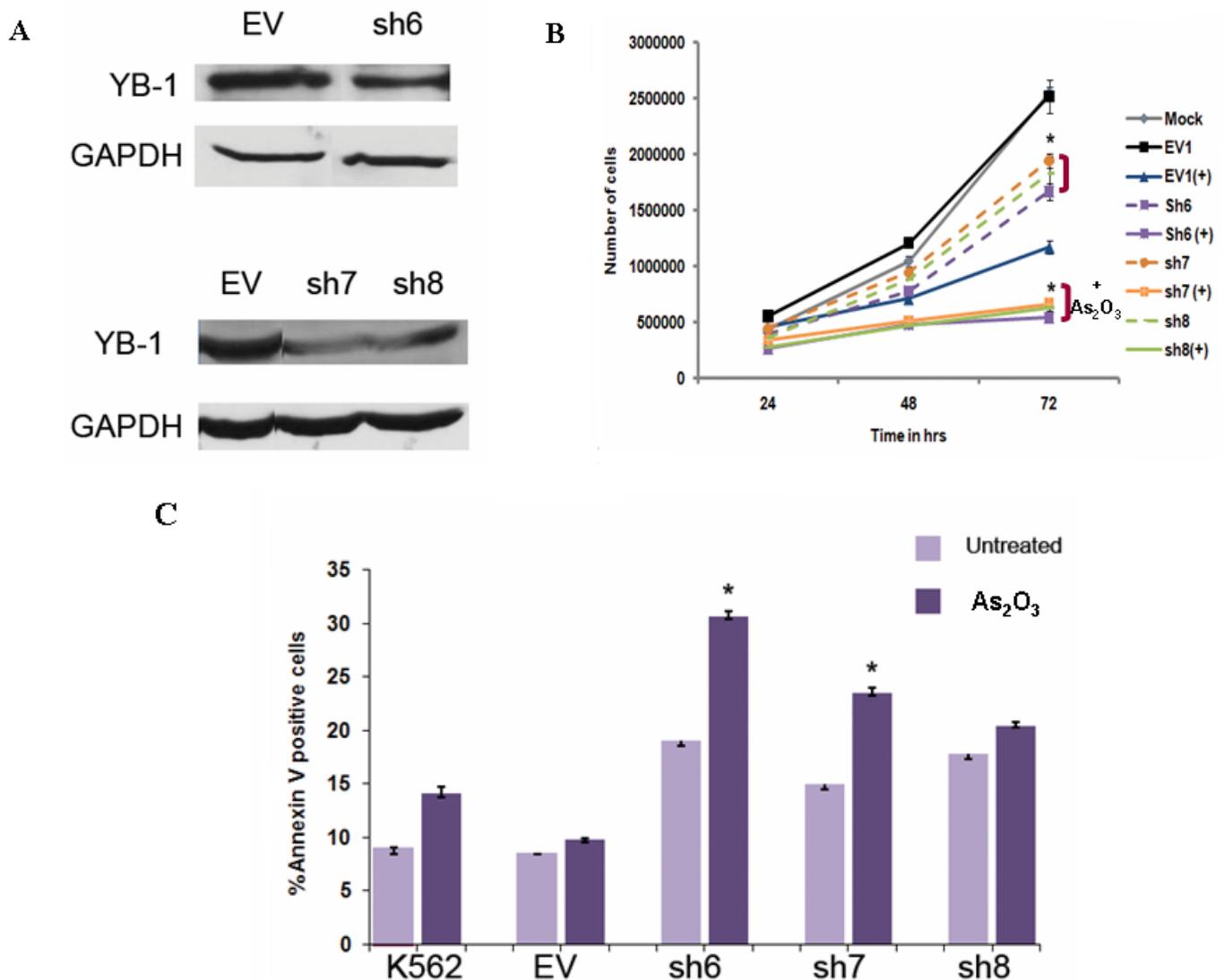
Figure 2.6. Effects of down-regulation of YB-1 expression on cell proliferation and apoptosis in K562 leukemic cells. K562 leukemic cells were co-transfected with a plasmid containing YB-1 shRNA and pEGFP. pEGFP was used as a selectable marker to establish stable clones. (A) Three clones sh6-, sh7- & sh8- were selected and down-regulation of YB-1 was confirmed by western blot using anti-YB-1 antibody (1µg/ml). GAPDH was used as loading control. Approximately 40% reduction in YB-1 expression was achieved in each of these clones. (N=3) (B) Sh6-, sh7-, & sh8- transfected K562 cells were treated with 0.5µM As_2O_3 for 72 hours and cell viability assay was done at 24, 48 &72 hours as described in methods (* indicate $p \leq 0.01$). (C) Apoptotic analysis was done in K562 cells after 72 hr of treatment with or without 0.5µM As_2O_3. These cells were labeled with annexin V pacific blue and 7-AAD and then subjected to flow cytometric analysis. Untransfected K562 cells and EV served as controls (* indicate $p \leq 0.01$). Data is expressed as the mean ± SEM of triplicate samples. Data is representative of at least two independent experiments. ANOVA for multiple pairwise comparisons was used to analyze the data along with Student t test comparing each to untreated cells.

A higher number of apoptotic cells were detected in sh6-, sh7-, sh8-K562 compared to the normal K562 cells and EV at 72 hours. A statistically significant increase in the number of annexin-V positive cells was seen in sh6- (61%), sh7- (57%) after treatment with 0.5 µM As_2O_3 for 72 hours ($p<0.01$) (Figure 2.6C). These data indicate that loss of YB-1 reduce cell viability in leukemic cells, and confers some protection from apoptosis in these cells.

YB-1 down-regulation leads to the induction of megakaryocytic differentiation in sh6-, sh7-, and sh8- in comparison to the normal K562 cells and EV at 72 hours after treatment with 0.5 µM As_2O_3. Phase contrast microscopic examination of As_2O_3 treated sh6- sh7-, and sh8-K562 cells revealed an increase in the nuclear to cytoplasm ratio, and an elongated shape characteristic of megakaryocytic morphology (Figure 2.7A). To confirm the megakaryocytic differentiation in sh6-, sh7-, and sh8- K562 cells, CD41a (GPIIb/IIIa) expression, a marker for platelet/megakaryocytes, was measured. Interestingly YB-1 down-regulation alone resulted in an increase in CD41a expression. Further increase in CD41a expression was observed after 72 hours of treatment with 0.5 µM As_2O_3 (Figure 2.7B). Polyploidization is a unique feature of megakaryocytes (Szalai *et al*., 2006). To further confirm megakaryocytic differentiation, DNA content was measured by PI staining. Ploidy analysis revealed a significant increase in the 4 and 8N population of cells 72 hours after treatment with 0.5 µM As_2O_3 in sh6-, sh7-, and sh8- in comparison to the normal K562 cells and EV (Figure 2.7C). From this data it is evident that down-regulation of YB-1 primes the sh6-, sh7-, and sh8-K562 cells for megakaryocytic differentiation after treatment with 0.5 µM As_2O_3.

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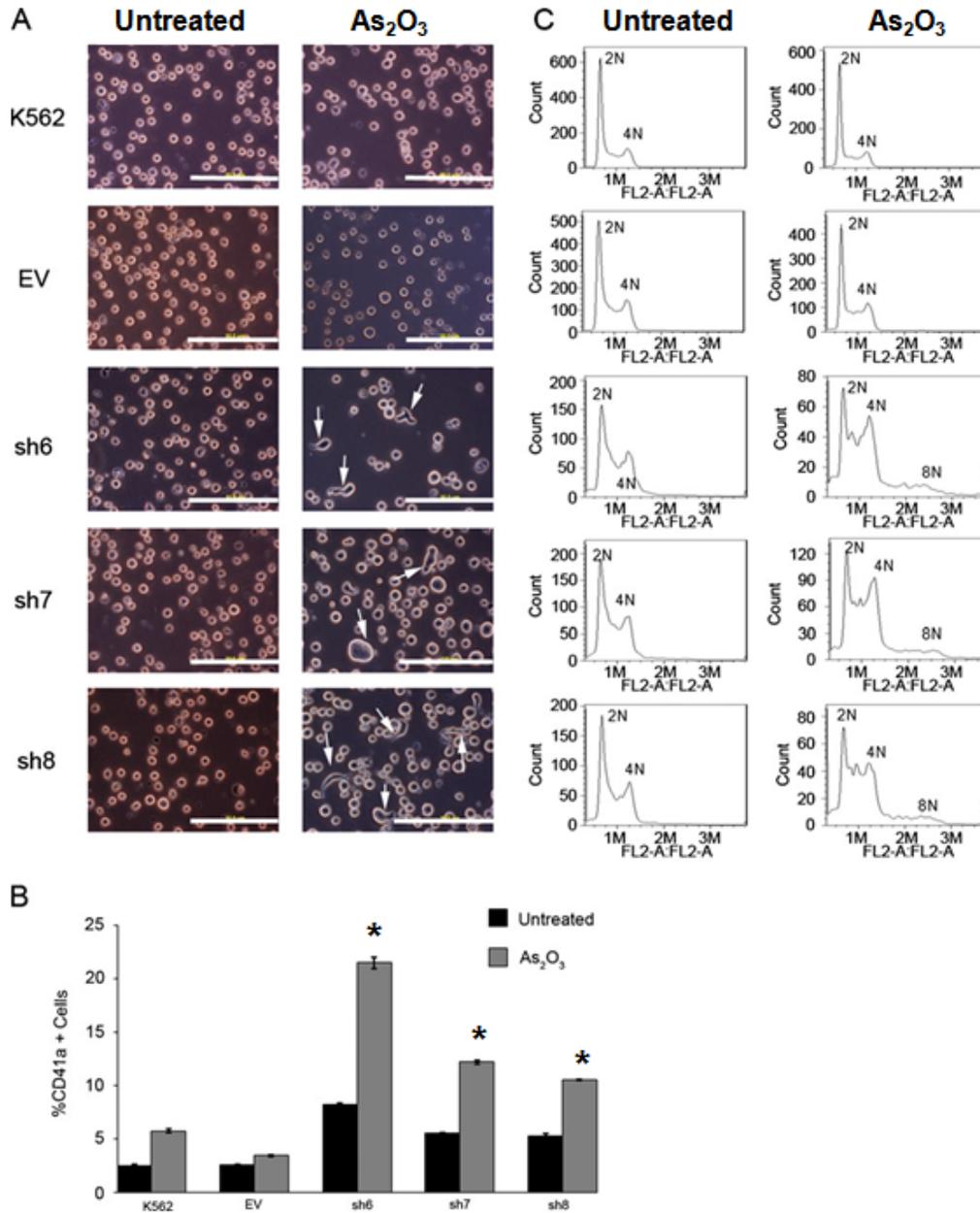
Figure 2.7. Effects of down-regulation of YB-1 expression on cell differentiation in K562 leukemic cells (A) Morphological changes in K562 cells at 72 hrs with or without treatment of 0.5μM As_2O_3. Arrows indicate megakaryocytic cells. Magnification is 400x. Scale bars are 50μm. (B) K562 cells were treated with or without 0.5μM As_2O_3 for 72 hrs and CD41a expression which labels platelets and megakaryocytes was measured (* indicate $p \leq 0.01$). Data are expressed as the mean ± SEM of triplicate samples and is representative of at least two independent experiments. ANOVA for multiple pairwise comparisons was used to analyze the data along with Student t test comparing each to untreated cells (C) K562 cells were treated with or without 0.5 μM As_2O_3 for 72 hrs and DNA content of cells was assayed by staining with propidium iodide (PI) and then analyzed by flow cytometry. Data is representative of at least two independent experiments. EV indicates empty vector.

Discussion

Y box binding protein is a multifunctional protein and is involved in regulating both transcription and translation. YB-1 promotes cell proliferation through transactivation of target genes such as proliferating cell nuclear antigen, epidermal growth factor receptor, DNA topoisomerase II, thymidine kinase and DNA polymerase α (Wolfe, 1994; Ladomery and Sommerville, 1995). It has also been implicated in cell cycle regulation, cell survival, stress response regulation, DNA repair, and drug resistance (Lu *et al*., 2005; Kohno *et al*., 2003).

YB-1 has been shown to be involved in erythroid development through interactions with GATA (Yokoyama *et al*., 2003); however the role of YB-1 in early hematopoietic differentiation has not been investigated. For the first time, we show in this study that YB-1 is expressed in early hematopoiesis and both YB-1 mRNA and protein levels are down-regulated during myeloid differentiation. EML cells undergoing myeloid differentiation down-regulate YB-1 expression after three days of culture in RA+IL-3. YB-1 expression is further down-regulated at six days when these myeloid progenitors are cultured in GM-CSF with the appearance of an increasing number of more committed granulocyte and monocyte progenitors. A previous cDNA microarray study that analyzed changes in gene expression during induced myeloid differentiation of EML cells showed similar results (Ma *et al*., 2002). This indicates that YB-1 is highly expressed during hematopoiesis in multipotent hematopoietic precursor EML cells but is down-regulated in more committed granulocyte/monocyte progenitors.

We have observed greater reduction in the YB-1 protein expression as compared to the YB-1 mRNA in both RA/IL-3 and GM-CSF treated EML cells relative to the DMSO treated EML cells. YB-1 has been shown to regulate gene expression not only at the level of transcription, but also at the level of mRNA translation (Fukuda *et al*., 2004). Therefore, lower levels of YB-1 protein expression as compared to YB-1 mRNA might be due to autoregulation of YB-1 translation by YB-1 mRNA. Another possibility could be that RA+IL-3 and GM-CSF

treatment might lead to post-translation regulation of YB-1 (Wu *et al*., 2007). YB-1 can shuttle between the cytosol and nucleus (Jurchott *et al*., 2003) and can also be localized to the mitochondria (de Souza-Pinto *et al*., 2009). However, we have examined only nuclear expression of the YB-1. Thus, a cytoplasmic fraction of YB-1 might account for the lack of correlation between theYB-1 protein and RNA levels.

Since we found a dramatic down-regulation of YB-1 expression in EML cells during myeloid differentiation, we investigated YB-1 expression *in vivo*. HSCs and progenitors were isolated based upon the parameters lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^+$ (which is an enriched fraction for HSC), lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca1$^-$ (myeloid progenitors). Like EML cells, we found high expression of YB-1 mRNA levels in the stem/progenitor cells of mouse BM as compared to the differentiated cells like granulocytes. YB-1 has a role in cell proliferation and is known to repress transcription of genes involved in differentiation (GM-CSF) and apoptosis (Fas) so one possible explanation for YB-1 to be down-regulated in granulocytes is to allow the expression of differentiation factors (Coles *et al*., 1996; Lasham *et al*., 2000). A gene expression study of mouse transcriptome agrees with this data as similar results have been shown in mouse BM for YB-1 expression (http://biogps.gnf.org). Taken together, the EML cell studies and primary bone marrow studies share consistent results and thus provide strong evidence that YB-1 is highly expressed in stem/ early progenitor cells and is down-regulated during granulocyte/monocyte lineage differentiation.

Our data revealed a significant reduction in the YB-1 expression in EML cells upon treatment with RA/IL-3, while we saw an increase in the YB-1 mRNA expression in myeloid progenitors as compared to the HSC in mouse BM. This difference could be attributed to the fact that the *in vitro* model involves using relatively high pharmacological concentrations of retinoic acid (10 µM) about 100–1000-fold higher than the endogenous physiological concentration of retinoids (1–10 nM) normally present in serum which could lead to off target

effects (Collins, 2002). Moreover, we have differentiated EML cells towards only CFU-GM, which generates granulocyte monocyte progenitors (GMP) while in the mouse BM we have assayed all the myeloid progenitors as one group including common myeloid progenitor (CMP), GMP and megakaryocyte erythroid progenitor (MEP). Possibly, YB-1 expression goes down in the GMPs, but not in the CMP and MEP.

YB-1 has been shown to be up-regulated in the bone marrow of patients with MDS (Lee *et al*., 2001; Yokoyama *et al*., 2003). Over-expression of YB-1 mRNA and protein is associated with several human cancers including breast, prostate, pancreas, colorectal and melanoma (Kohno *et al*., 2003), but its role in leukemia has not yet been investigated. Therefore, cell lines derived from patients with HL-60 (acute promyelocytic leukemia), U-937 (acute promonocytic leukemia), K562, chronic myelogenous leukemia (CML) and WEHI-3 (murine monocytic leukemia), which are arrested at various stages of myeloid differentiation, were used to analyze the expression of YB-1 protein. YB-1 protein was expressed by all the myeloid leukemic cell lines, indicating that YB-1 is associated with myelogenous leukemia. Additionally, it is intriguing that YB-1 was up-regulated in mouse progenitor cells as compared to mouse HSCs. This finding is consistent with the hypothesis of the progenitor being the cell of origin for some leukemias (Jamieson *et al*., 2004).

To determine the role of YB-1 in leukemia we investigated the biological consequences of YB-1 knockdown in K562 leukemia cells. This cell line was chosen due to high expression of YB-1. We treated cells with As_2O_3 because it is being used as a therapeutic agent for the treatment of acute promyelocytic leukemia. The concentration of As_2O_3 used was 0.5 μM, which is within the known plasma concentration in patients under treatment and does affect cell viability (Tang *et al*., 1997). YB-1 down-regulation resulted in growth arrest and induction of apoptosis. Upon treatment with As_2O_3 these effects were more pronounced. YB-1 promotes cell proliferation by transactivating various genes involved in cell division and also by controlling

cyclin A and B1 gene expression (Wolfe, 1994; Ladomery and Sommerville, 1995; Jurchott *et al*., 2003). A few studies have reported biological effects of YB-1 (Schittek *et al*., 2007; Chatterjee *et al*., 2008). In the multidrug resistant cell line, K562/A02 YB-1 down-regulation leads to decreased cell proliferation and induction of apoptosis. These results are consistent with our findings in untreated K562 cells (Xu *et al*., 2009).

K562 is an erythroleukemia cell line that is situated in the common progenitor stage of megakaryocytic and erythroid lineages of hematopoietic cell differentiation. K562 cells can be induced to erythroid or megakaryocytic differentiation by different agents (Baliga *et al*., 1993; Tabilio and Pelicei, 1983). As_2O_3 exerts double effects on acute promyelocytic cells, i.e., induction of apoptosis and partial differentiation (Tang *et al*., 1997). Interestingly, down-regulation of YB-1 and treatment with As_2O_3 led to megakaryocytic cell differentiation in K562 leukemia cells, further strengthenig our observation that YB-1 is associated with stem/progenitor cell maintenance. We saw a significant change in cellular morphology after 72 hr treatment of K562 leukemic cells with As_2O_3; evident by an increase in nuclear size and cytoplasmic mass. YB-1 knockdown alone resulted in an increase in CD41a, a cell surface marker associated with megakaryocytic differentiation expression. However, we did not see any large change in cellular morphology. After treatment with As_2O_3, there was a steep, statistically significant increase in the expression of CD41a when YB-1 was downregulated in sh6-, sh7- and sh8- K562 cells. Further confirmation of megakaryocytic differentiation was our detection of polyploidy in K562 cells when YB-1 was downregulated (Vitrat *et al*., 1998). The ability of K562 cells to partially differentiate upon YB-1 knockdown supplemented with As_2O_3 treatment is a novel finding.

In conclusion, our data reveals that YB-1 is highly expressed in the EML cell line but is down-regulated upon differentiation of these cells towards the myeloid lineage. Terminally differentiated cells, such as granulocytes, express low levels of YB-1 as compared to murine

BM stem and progenitor cells. Expression of YB-1 in several myeloid leukemia cell lines indicates its association with leukemia. Down-regulation of YB-1 expression reduced cell proliferation, induced apoptosis and cell differentiation. Further studies are needed to decipher the mechanism of how YB-1 contributes to the blockage of myeloid differentiation.

In leukemia there is uncontrolled proliferation of immature progenitors and blockage to their differentiation.Understanding the mechanisms involved in hematopoietic differentiation will help in developing therapies against leukemia. Our major focus of this work was to identify different genes that play role in hematopoietic differentiation. Hsp90 is a molecular chaperone that is involved with protein folding and various signal transduction cascades. Hsp90 has been suggested to regulate Wnt signaling in the *Drosophila* model. Wnt signaling is associated with hematopoietic stem cell maintainenace and is important for leukemia progression. In chapter 3, we investigated the role of Hsp90 in the regulation of Wnt signaling in mammalian hematopoietic system.

CHAPTER Three: Activation of the canonical Wnt pathway in the hematopoietic precursor EML cell line upon Hsp90 inhibition

Abstract

Acute myeloid leukemia (AML) in adults has a 20% 5-year disease-free survival, despite treatment with aggressive cytotoxic chemotherapy. Heat shock protein (Hsp90) is a chaperone for several client proteins involved in transcriptional regulation, signal transduction and cell cycle control. 17-AAG, the synthetic analogue of geldanamycin (GA), is an Hsp90 inhibitor that is presently in phase II clinical trials for the treatment of various leukemia and other cancers. In *Drosophila*, functional inactivation of Hsp90 resulted in a transdifferentiation event where the eye tissue becomes a limb-like outgrowth due to abnormal wingless expression (wg). Expression of this phenotype induced by Hsp90 inhibition becomes independent of chaperone after being inherited across successive generations, suggesting an epigenetic mechanism. Wnt signaling is associated with hematopoietic stem cell maintenance and is important for leukemia progression. The objective of the study in chapter 3 was to determine whether the Wnt pathway is regulated by Hsp90 in mammalian cells through epigenetic mechanisms as observed in flies. Our preliminary data showed the activation of the canonical Wnt pathway in a hematopoietic precursor cell line, EML, after inhibition of Hsp90. This effect seemed to be short lived and pharmacological inactivation of Hsp90 did not cause any changes in the Wnt pathway activity during myeloid differentiation of EML cells. However there was considerable variability in our results that prevented further progress in these studies. Further investigation into the molecular mechanism of Hsp90 inhibition and Wnt signaling in adult hematopoietic stem cells is needed to determine whether Hsp90 play a role in maintaining the leukemic state in AML.

Introduction

Hsp90 is a specialized molecular chaperone involved in the stress response and in normal homoeostatic control mechanisms. Several client proteins including transcription factors and protein kinases are Hsp90 substrates, where it facilitates their stabilization and activation. Inhibition of Hsp90 leads to disruption of various signal transduction pathways in the cell. Hsp90 inhibitors such as geldanamycin act by interacting specifically with a single molecular target, the Hsp90 chaperone, thereby destabilizing and degrading Hsp90 client proteins (Pearl *et al.*, 2008).

Although Hsp90 is expressed in normal cells, it is frequently overexpressed in cancer cells, suggesting a role in maintaining malignant transformation. Hsp90 has been implicated in oncogenesis by associating with multiple mutated, chimeric and over expressed signaling proteins that promote cancer cell growth and survival (Pearl *et al.*, 2008). Several studies have shown that Hsp90 inhibitors target tumor cells over normal cells because of higher binding affinity of the Hsp90 complexes present in these tumor cells (Chen *et al.*, 2010; Flandrin *et al.*, 2008). Thus targeting Hsp90 could result in simultaneous disruption of multiple oncogenic signal transduction pathways suggesting utility for treatment of advanced cancers.

According to Waddington's canalization model there are masked phenotypes in a wild-type population which can be expressed during environment stress. When these adaptive phenotypes are selected over subsequent generations they get fixed and can be expressed even in the absence of stress (Waddington, 1942). Under stressful conditions Hsp90 deviates from its usual role of activating signal transduction proteins and severe stress can temporarily overwhelm the Hsp90 chaperone system. This leads to generation of varied phenotypes, which when selected, can be fixed in the population and are significant in terms of evolution (Wong and Houry, 2006). Impairment of Hsp83, the *Drosophila* homolog of Hsp90 produced abnormal phenotypes which are inherited in subsequent generations (Rutherford *et al.*, 1998). Thus

Hsp90 can act as genetic capacitor by storing masked phenotypes for later release.

This impairment of Hsp90 either by genetic mutation or pharmacological inhibition in *Drosophila* led to phenotypic variation was also reported by another study. However, an epigenetic basis for the capacitor function of Hsp90 was revealed, as depletion of Hsp90 induced an altered chromatin state. Progeny of flies which were fed Hsp90 inhibitor, geldanamycin (GA) and a mutation in the trithorax group of genes (TrxG) showed an abnormal eye-bristle phenotype. Interestingly, phenotypic selection of this drug-induced phenotype resulted in an increase in the number of affected progeny in successive generations even in the absence of Hsp90 inhibition indicating, an epigenetic mechanism. Further evidence for an epigenetic phenomenon came from chromatin remodeling inhibitors, which were able to reverse this phenotype (Sollars *et al.*, 2003).

Moreover, it was found that this abnormal eye phenotype induced by Hsp 83 and TrxG mutations was due to ectopic expression of Wingless (wg) (Sollars *et al.*, 2003). Wingless is required for many developmental processes ranging from embryonic segmentation to limb development. Since mutations in TrxG genes induced up-regulation of the Wnt pathway, this indicates a role of epigenetic gene regulation in this pathway.

The Wnt signaling pathway is highly conserved during evolution and regulates hematopoietic stem cell maintenance and self renewal (Kirsttetter *et al.*, 2006). Its dysregulation is responsible for various hematological malignancies including leukemia. The canonical Wnt pathway regulates target gene expression via the stabilization and nuclear translocation of the cytoplasmic pool of ß-catenin (Brembeck *et al.*, 2003). The focus of this study was to find out whether Hsp90 modulation can regulate the Wnt pathway epigenetically in a mammalian model system.

Hematopoiesis is an excellent model to study differentiation due to the presence of different cell surface markers along the course of development. The Erythroid Myeloid Lymphoid clone 1 (EML) cell line was developed from murine bone marrow cells transfected with a vector expressing a dominant negative form of retinoic acid receptor. These cells are a unique *in vitro* model to study hematopoietic differentiation. EML cells can be indefinitely propagated in medium containing stem cell factor (SCF) and can be differentiated into erythoid, myeloid and lymphoid lineages by the addition of appropriate cytokines (Tsai *et al*., 1994).

Our hypothesis was that Hsp90 acts as an epigenetic modulator affecting the Wnt pathway in the EML cell model. To test this hypothesis, EML cells were treated with GA and Wnt pathway activation was examined based upon ß-catenin accumulation and translocation to the nucleus.

Materials and Methods

Materials

Geldanamycin, GA (G3381) and retinoic acid (R2625) were purchased from Sigma. FITC conjugated total ß-catenin (610155) and Fc Blocker (553142) antibodies were purchased from BD Biosciences while active ß-catenin (05-665) was purchased from Millipore. Paraformaldehyde (15014601) and Triton X-100 (02300221) were bought from MP Biomedicals. GM-CSF was purchased from Stem Cell technologies (Vancouver, BC).

Cell culture

EML C1 cells were the kind gift of Dr. Schickwann Tsai and were maintained in growth medium which is base medium (IMDM supplemented with 20% heat inactivated horse serum, American Type Culture Collection, ATCC, Manassas, VA) and 10% BHK/MKL-conditioned

medium, (Tsai *et al*., 1994). EML cells were maintained at a density below 5×10^5 cells/ml for these experiments. BHK cells were cultured in EMEM and WEHI-3 cells (TIB-68, ATCC) were cultured in IMDM supplemented with 10% FBS at 37º C and 5% CO_2. L Cells (immortalized mouse fibroblast) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (ATCC, CRL-2648). L-Wnt 3a cells, the Wnt 3a expressing clone of L cells and L-Wnt 5a, the Wnt 5a expressing clone of L cells (ATCC, CRL-2647 and 2814), were maintained in the same medium containing 0.5 mg/ml of G418.

EML cell differentiation

EML cells were seeded at a density of 1×10^5 cells/ml in base medium along with 10µM all-trans retinoic acid (ATRA; sigma, St. Louis, MO, USA), 10% BHK conditioned medium (source of stem cell factor) and 15% WEHI conditioned medium (source of interleukin-3) for three days. Cells were then washed twice with PBS and resuspended at 1×10^6 cells/ml in base medium with 20 ng/ml of murine granulocyte-monocyte colony stimulating factor (GM-CSF) (Stem Cell Technologies, Vancouver, BC, Canada) for the next three days.

Treatment with Geldanamycin (GA)

EML cells were seeded at 2×10^5 cells/ml in growth medium and treated with 5, 10 or 15 nM GA for 24 hours. DMSO was used as a vehicle. Cells were washed and reseeded at 2×10^5 cells/ml in growth medium after 24 hours of treatment followed by a 24 hour recovery period. All the experiments were performed after the 24 hour recovery period.

Flow Cytometry

Treated as well as untreated L, L-Wnt3a, L-Wnt 5a and EML cells were washed twice with FACS buffer (Phosphate buffered saline, (PBS) supplemented with 3% BSA, 0.02% sodium azide and 1 mM EDTA) and collected by centrifugation. Thereafter they were incubated with

FcγRII/III receptor (553142, BD Pharmingen) to prevent nonspecific binding by blocking the Fc receptors for half an hour at 4°C. Cells were then fixed with 4% paraformaldehyde /PBS for half an hour and permeabilized with wash buffer (PBS supplemented with 3% BSA, 0.1% Triton X-100). After washing the cells in buffer they were then labeled with FITC conjugated total ß-catenin antibody (610155, BD Transduction) for half an hour at 4º C. An isotype antibody labeled with FITC was used as a negative control for FITC. Data acquisition was performed using BD FACS Aria sorter and data analysis was done using Flow Jo software v.7.5.5 (Treestar, Ashland, OR).

Western blot

Treated cells from different experiments were rinsed once with ice-cold PBS and whole cell protein extracts were obtained by briefly sonicating the cell pellets and then boiling the lysates for 5 minutes in sample lysis buffer containing 50 mM Tris (tris hydroxymethyl aminomethane; pH 6.8), 1% sodium dodecyl sulfate (SDS), 10% glycerol and 1 mM dithiothreitol (DTT). Nuclear and cytoplasmic fractions were separated using the NE-PER extraction kit as per the manufacturer's instructions (Pierce, Rockford, IL). Protein concentration was determined using bicinchoninic acid (BCA) protein assay reagents from Pierce according to the manufacturer's guidelines. Cell extracts were then denatured at 95°C for 5 min in 2x sample buffer (62.5 mM Tris HCl, pH 6.8, 25% glycerol, 0.01% bromophenol blue, 2% SDS,10% β-mercaptoethanol). Equal amount of protein extracts were separated on an 8-16% gradient Tris-Glycine SDS–poly acrylamide gel (Bio-Rad, Hercules, CA) by electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Millipore) using Bio-Rad MiniProtean3® system. The membranes were treated in blocking solution (5% non fat dry milk in TBS containing 0.1% Tween 20) and incubated with primary active ß-catenin antibody (05-665, Millipore, 1 µg/ml) overnight at 4°C, followed by incubation with HRP-conjugated monoclonal mouse secondary IgG antibody (1: 3000, 7074, GE Healthcare, CT). An anti-mouse GAPDH antibody (MAB374,

GE Healthcare, CT) was used to assess equal loading. Proteins were visualized by ECL (GE Healthcare, CT). A Benchmark [TM] protein ladder was used to visualize the transfer of protein onto the membrane and MagicMark [TM] XP (Invitrogen) was used as a molecular size standard.

Results

EML cells have high total ß-catenin levels

Wnt signaling is important in embryogenesis and is required for the maintenance of the hematopoietic stem cell state (Reya *et al*., 2003). The canonical Wnt pathway involves the stabilization and nuclear accumulation of its downstream target, ß-catenin (Mc Donald *et al*., 2006). In order to determine the activity of Wnt signaling in the hematopoietic precursor cell line EML, we performed intracellular staining to assess the ß-catenin accumulation.

Cell line	FITC
L-Cells isotype	162.02
L-Cells +ve	676.29
L-Wnt 3a Cells	855.86
L-Wnt 5a Cells	542.99
EML Cells	1591.39
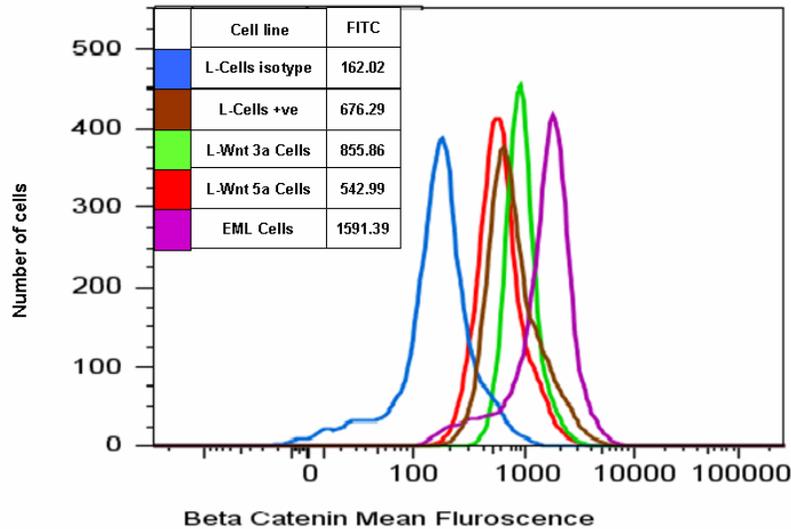
Figure 3.1 High β-catenin accumulation in EML cells. Intracellular staining was done to examine the level of total β-catenin protein in EML cells. Increased β- catenin accumulation is present in EML (purple) cells in comparison with other cell types normal L cells (brown), Wnt 3a (green) which have active canonical Wnt pathway due to secretion of Wnt in the medium. Noncanonical Wnt 5a (red) expressing cells do not appear to show any accumulation of β-catenin. FITC mean fluroscence values are listed in the figure. L-cells +ve is labeled with FITC conjugated β- catenin antibody.

EML cells showed higher ß-catenin accumulation compared to normal L cells, Wnt 3a L and Wnt 5a L cells (Figure 3.1). Wnt 3a transfected cells were used as a positive control for active canonical Wnt signaling and Wnt 5a as a positive control for non-canonical Wnt signaling. However, L cells represented basal levels of ß-catenin, which is expected because of its role in the cytoskeleton (Brembeck *et al*., 2006). The signal intensity of ß-catenin was two-fold higher in EML cells relative to Wnt 3a-L cells. The Wnt 5a expressing cells do not appear to show any accumulation of ß-catenin as the signal intensity of ß-catenin was similar in them relative to the L cells.

Hsp90 inhibition leads to reduction in total ß-catenin levels in EML cells

Functional inactivation of Hsp90 led to a transdifferentiation event in successive generations of *Drosophila* due to epigenetic regulation. This transdifferentiation event in *Drosophila* also led to abnormal activation of Wnt signaling (Sollars *et al*., 2003). My study was aimed at ascertaining whether in a mammalian model Hsp90 regulates the Wnt pathway through an epigenetic mechanism. A hematopoietic precursor cell line, EML, was used as a representative mammalian model system. Since the half life of Hsp90 dissociation and biological persistence is short (Supko *et al*., 1995), and EML cells undergo one cellular division during the 24 hour rest period, a protocol of pharmacological inhibition followed by a recovery period will help in analyzing epigenetic effects. Our goal is to achieve an epigenetic phenotype for the EML cells which will be analyzed in future experiments. Before testing the epigenetic mechanism we wanted to examine the effect of Hsp90 inhibition on the Wnt singaling. Thus, EML cells were treated with GA for 24 hours followed by a 24 hour rest period before determining the accumulation of ß-catenin. We observed a decrease in total ß-catenin levels after GA treatment, indicated by both the reduction in the percentage positive as well as the median fluorescence of total ß-catenin (Figure 3.2). There was more than a 40%, statistically significant reduction of

signal intensity for total ß-catenin in 10 nM GA treated EML cells relative to control.

A

B
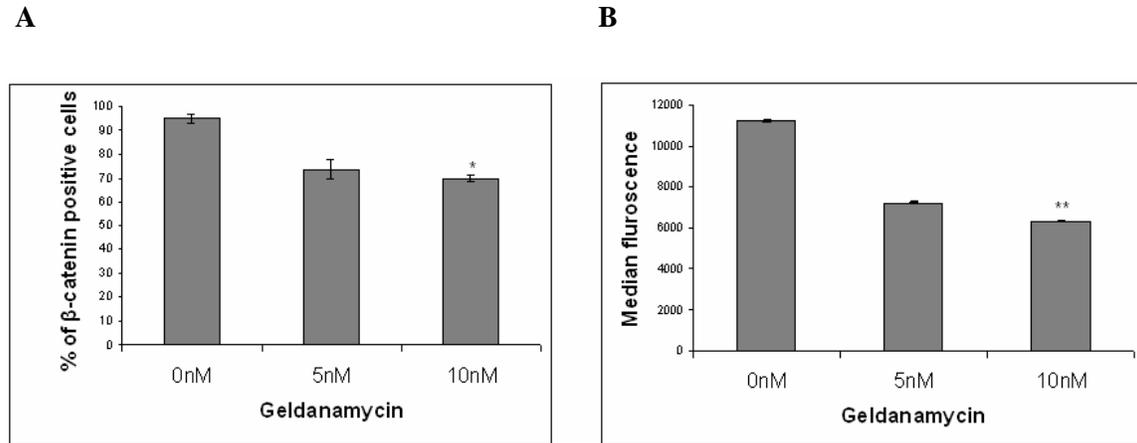
Figure 3.2 Decrease in total β-catenin protein in EML cells after Hsp90 inhibition. EML cells were exposed to vehicle (DMSO), 5 nM or 10 nM GA for 24 hours. The cells were then allowed to recover for 24 hours, and β-catenin levels were analysed using flow cytometry. Total volume of DMSO was constant in all trials. (A) shows the percentage of EML cells which were positive for β-catenin (*p value < 0.01). (B) shows the median fluorescence values for β-catenin protein (**p value < 0.05) (N=3).

Activation of the canonical Wnt pathway after inhibition of Hsp90 in EML cells

In the absence of phosphorylation by glycogen synthase kinase (GSK3 ß), ß-catenin is stabilized referred to as active ß-catenin, which translocates to the nucleus, an indication of active Wnt signaling (Van Noort M *et al*., 2002). Since total ß-catenin levels are not an accurate representation of the activation of Wnt pathway we wanted to determine the effect of Hsp90 inhibition on the active ß-catenin levels in EML cells by western blotting. When we treated EML cells with different concentrations of GA, we found that active ß-catenin protein was highly expressed in 10 nM GA treated EML cells relative to vehicle control and 15 nM GA treated EML cells (Figure 3.3 A).

To further determine the effect of 10 nM GA on ß-catenin levels and to determine how long the Wnt pathway remains activated, we did a time course study. It seemed that the up-regulation in the levels of active ß-catenin protein lasted for only 24 hours (Figure 3.3 B). In this figure, ponceau staining was used as a loading control. This data suggests that the Wnt pathway might be activated after the inhibition of Hsp90 in EML cells, but if this does occur it is of a short duration.

A

| GA | Control | 10 nM | 15 nM |

Active ß Catenin

B

| 24 hr | 48hr | 72 hr | 1 week |

Active ß Catenin

| GA | C | 10 nM | C | 10 nM | C | 10 nM | C | 10 nM | + ve Ctrl |
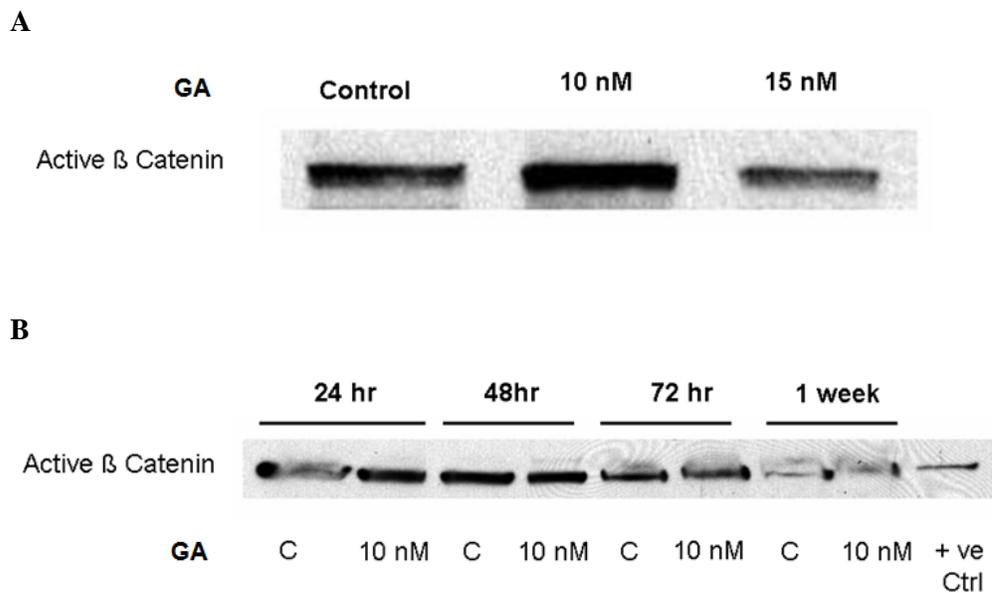
Figure 3.3 Activation of the Wnt pathway after inhibition of Hsp90 in EML cells. (A) EML cells were treated with vehicle, 10 and 15 nM GA for 24 hours and rested for another 24 hours. Whole cell lysates were extracted and analyzed by western blot analysis to detect active β-catenin protein levels. Ponceau staining was done to determine equal loading. This blot is representative of two independent experiments. **(B)** EML cells were treated with 10 nM GA for 24 hours and followed by a 24 hour rest period as before. Western blot was done on the whole cell extracts at 24, 48, 72 hours and 1 week time course. 'C' represents the vehicle control. This blot is representative of two independent experiments.

Loss of Wnt pathway activation in GA treated EML cells after differentiation

We wanted to observe whether our preliminary finding of the up-regulation in Wnt signaling due to Hsp90 inhibition (Fig. 3.3) results in changes in the expression of ß-catenin

after differentiation of EML cells. Therefore, we inhibited Hsp90 and then forced EML cells to differentiate toward the granulocyte macrophage pathway by treating them with RA and GMCSF.

A

B
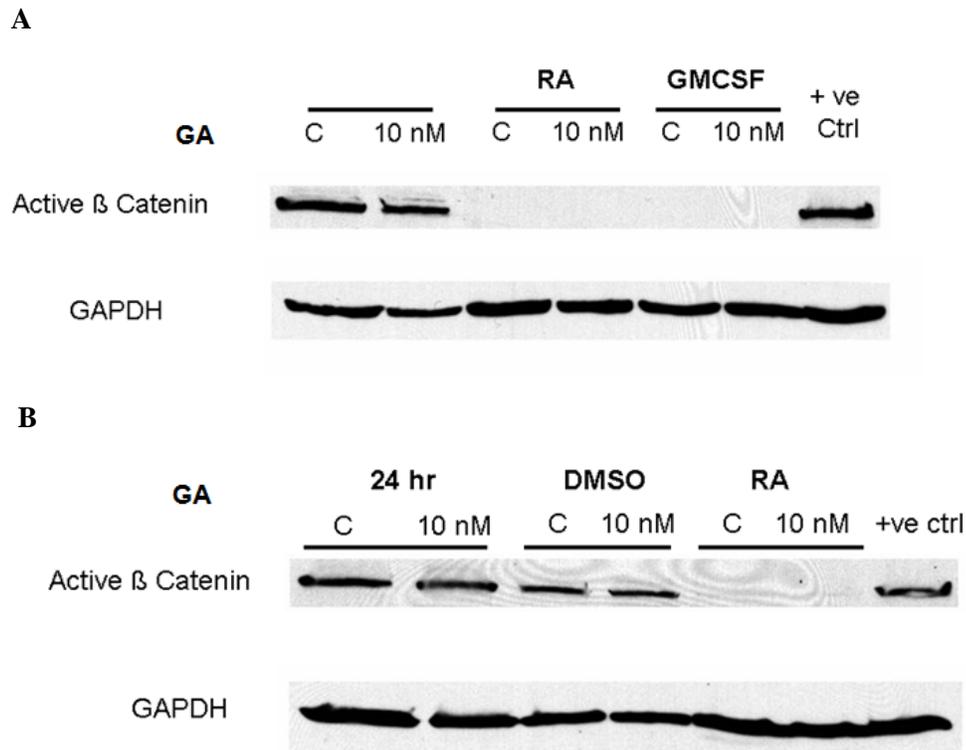
Figure 3.4 Wnt pathway activation in GA treated EML cells after differentiation. (A) EML cells were treated with 10 nM GA for 24 hours and rested for another 24 hours before proceeding with the differentiation protocol. Nuclear extracts were isolated and western blot analysis was done to determine the levels of active β-catenin after treatment with RA and GMCSF. This is a representative blot for two independent experiments. **(B)** EML cells were treated with 10 nM GA and then a second dose of 5 nM given during our differentiation protocol along with treatment with RA and IL-3. Western blot analysis was done after extraction of nuclear extracts to examine the levels of active β-catenin. GAPDH was used as a loading control. This is a representative blot for two independent experiments. 'C' represents the vehicle control.

In this experiment, Hsp90 inhibition did not show any effect on the active ß-catenin expression. Secondly, we found that upon differentiation with RA as well as GMCSF in the EML cells activity of ß-catenin was inhibited regardless of Hsp90 inhibition (Figure 3.4A). This data suggests that Hsp90 inhibition does not seem to play a role in Wnt pathway activation

with or without forced differentiation of EML cells. Next, we tried to modify our treatment protocol by giving a second dose of GA during differentiation but that also seem to have no effect on the levels of active ß-catenin (Figure 3.4B).

Wnt pathway activation through inhibition of Hsp90 in EML cells was not consistent

Next, we wanted to reproduce the earlier findings of Hsp90 inhibition on ß-catenin activity in EML cells. Therefore, EML cells were treated with various concentration of GA for 24 hours and active ß-catenin levels were measured by western blot.

A
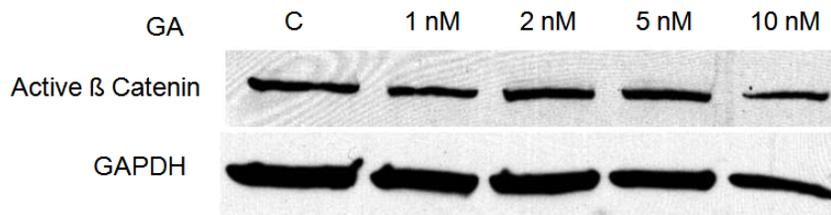
B
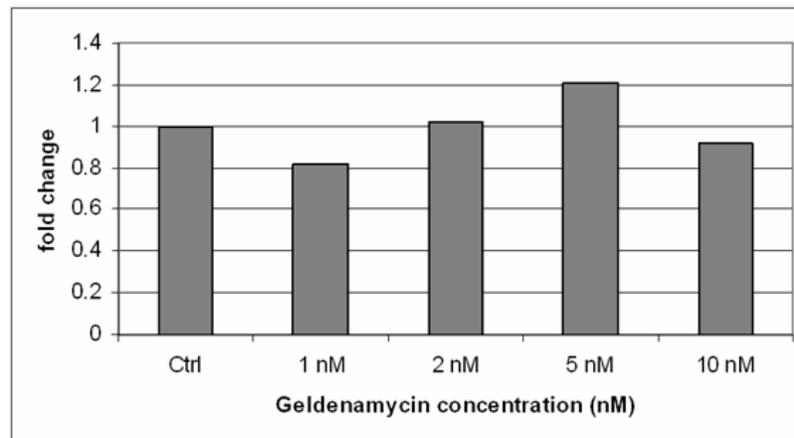
Figure 3.5 Up-regulation of Wnt signaling observed previously was not consistent. (A) EML cells were treated with vehicle, 1, 2, 5 or 10nM GA for 24 hours followed by a 24 hour rest period. Nuclear extracts were isolated and active ß-catenin levels were measured. GAPDH was used as a loading control. This blot is representative of at least three different experiments. 'C' represents the vehicle control. (B) The densitometric analysis for the above experiment.

We found that there was no significant difference in the active ß-catenin levels between the vehicle control relative to GA treated EML cells (Figure 3.5). This data indicates that the up-regulation of Wnt signaling upon Hsp90 inhibition in EML cells as observed in our preliminary studies is inconsistent.

Discussion

We observed an increased expression of ß-catenin in EML cells compared to the L-Wnt 3a cells, which have active canonical Wnt signaling. This is consistent with the fact that EML cells are stem cell in nature and the self renewal pathway such as canonical Wnt signaling is required for maintenance of a hematopoietic stem cell state. Deregulation of Wnt signaling, which is normally tightly regulated, has been identified as an important step in leukemic transformation. Common myeloid progenitor (CMP) gives rise to granulocyte macrophage progenitor (GMP) which can form either granulocytes or macrophages that have an active Wnt/ ß-catenin pathway. The GMP has elevated levels of nuclear ß-catenin and has been identified as candidate leukemic stem cell in blast crisis chronic myelogenous leukemia (Jamieson *et al*., 2004). Moreover mutations in members of the Wnt -ß- catenin pathway occur in 90% of the colorectal cancers and other cancers such as hepatocellular and gastric cancers (Brembeck *et al*., 2006).

In EML cells, after inhibiting Hsp90, we saw a down-regulation of total ß-catenin expression in contrast to what was observed in *Drosophila*. Hsp90 inhibitors have been shown to have higher binding affinity to tumor cells than normal cells leading, to proteosomal degradation of Hsp90 client proteins. However, several studies have also demonstrated the toxic effects of Hsp90 inhibitors on the normal cells. In two different studies, Hsp90 inhibitors were found to be extremely cytotoxic to the Oligodendrocyte precursor cells (OPCs) and human retinal pigment epithelial cells respectively *in vitro* (Alcazar and Cid, 2009; Wu *et al*., 2010). In another study, it has been shown that the Hsp90 inhibitor geldanamycin and its analogs have

cytotoxic effects on rat primary hepatocytes due to generation of reactive oxygen species (Samuni *et al*., 2010). Therfore Hsp90 inhibitors might be toxic to EML cells and this toxicity can result in a general down-regulation of protein expression and a lowering of ß-catenin protein levels. Moreover, given the role of Hsp90 in diverse biologic processes, drugs targeting Hsp90 will have side effects on normal cellular function.

Canonical Wnt pathway activation is achieved by stabilization of ß-catenin and translocation to the nucleus, activating the transcription of target genes responsible for cellular proliferation and differentiation (Brembeck *et al*., 2006). Therefore we measured the amount of active ß-catenin that is due to dephosphorylated on Ser 37 or Thr41 of the molecule (Fig. 3.3 and 3.4). Surprisingly, we saw an increase in active ß-catenin levels after inhibition of Hsp90 in EML cells, which is contradictory to what we previously found. This could be explained by the fact that ß-catenin is also involved in cell-cell adhesion at the plasma membrane by mediating association of integrins with intracellular actins in myeloid cells (Brembeck *et al*., 2006). Inhibition of Hsp90 affects both the transcriptional regulation and cellular adhesion functions of ß-catenin. Also, Hsp90 is required for constitutive and inducible activity of the IKB kinase (IKK) complex (Broemer *et al*., 2002). Therefore, inhibition of Hsp90 results in impairment of IKK activity. IKK regulates ß-catenin via phosphorylation and ubiquitin dependent degradation. It has been shown that IKK ß decreases ß –catenin dependent gene expression similar to the effects seen with GSK-3 ß (Lamberti *et al*., 2001). In addition, the up-regulation in Wnt signaling upon Hsp90 inhibition in EML cells is consistent with the previous finding of increased Wg expression in *Drosophila* upon Hsp90 inhibition (Sollars *et al*., 2003).

The up-regulation of Wnt signaling upon Hsp90 inhibiton in EML cells lasted only for short duration. The half life of the Hsp90- GA complex is short, indicating Hsp90 inactivation is short-lived once GA is no longer supplied to the EML cells. Also the biological half life of GA in mice is 77.7 minutes (Supko *et al*., 1995), which means it is rapidly degraded. Since the

biological persistence of Hsp90 is short, it is not surprising that the up-regulation in Wnt signalling was short-lived.

We did not observe any changes in the active ß-catenin levels upon Hsp90 inhibition in differentiating EML cells. This could be due to the fact that we did not see an effect of Hsp90 inhibition in the control and GA treated EML cells in this experiment. Interestingly, we found that active ß-catenin was not expressed in differentiating EML cells. This is consistent with the observation that Wnt signaling is involved in the maintenance and renewal of hematopoietic progenitors/stem cells and inhibition of Wnt pathway induces their differentiation (Kirstetter *et al*., 2006). Also, it has been shown that expression of a mutant active ß-catenin in normal progenitors impairs myelomonocytic differentiation (Simon *et al*., 2005).

Future experiments focused on attaining the inhibition of Hsp90 by treating EML cells with varying doses of GA. It was thought that the effect on the Wnt signaling might be seen at different concentration of GA. We also tried to use early passaged EML cells for our experiments. Moreover a longer rest period was given to EML cells after GA treatment. But our repeated attempts to revive the phenotype were unsuccessful and there was no significant difference in the active ß- catenin levels in the vehicle and GA treated EML cells. Another study conducted in our lab to look at the effect of Hsp90 inhibition on the myeloid cell differentiation also indicated variable results and no effect on the differentiation profile of EML cells (Napper, 2010). This could be attributed to the fact that inhibition of Hsp90 might not have any effect on the Wnt signaling at least in the *in vitro* EML cell model.

Two earlier studies have reported that EML cells are a heterogenous population. In one study the authors demonstrated that can be separated into two different populations based on CD34 expression. Levels of stem cell factor receptor (c-kit) are similar in both these populations but they differ in growth characteristics and response to cytokines. The CD34$^+$ population shows a growth response when treated with SCF while the CD34$^-$ population grew

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in the presence of IL-3.The CD34$^+$ population is able to regenerate the mixed population upon stimulation with SCF (Ye *et al*., 2005). Another study demonstrated the broad spectrum of Sca-1 expression in EML cells, which was enlisted as the cause of clonal subpopulations in these cells. Both high and low Sca-1 expression populations were able to reconstitute the parental distribution of Sca-1. Most strikingly, the cells at the extremes of the spectrum differed in their differentiation potential. Sca-1$^+$cells were strongly biased toward myeloid differentiation while Sca-1$^-$ cells toward erythroid differentiation (Chang *et al*., 2008). These studies indicate that EML cells are a heterogeneous population having different populations with phenotypic cell-to-cell variability. This is consistent with the notion that hematopoietic cells have an intrinsic ability that generates a spectrum of progeny with different differentiation biases, resulting in commitment either spontaneously or as a consequence of extracellular signals. This heterogeneity might be attributed to biological noise, which is the fluctuation of transcriptional regulators.

The Wnt signaling pathway controls hematopoietic stem cell (HSC) self-renewal and differentiation of hematopoietic progenitors. Aberrant Wnt signaling is associated with leukemia. Researchers have focused recently on the dietary fatty acids due to their beneficial effects on health and disease. Omega fatty acids have been shown to promote myeloid cell differentiation. Therefore, in the studies described in the next chapter, I have investigated the effect of omega fatty acids on Wnt signaling in the hematopoietic stem cell model.

Chapter Four: Effect of omega-3 and 6 fatty acids on the Wnt signaling in hematopoietic precursor, EML cell line

Abstract

The pro-inflammatory omega-6 fatty acids are metabolized through the cyclooxygenase (COX) pathway into inflammatory eicosanoids, including prostaglandin E2. In contrast, omega-3 fatty acids exhibit their anti-inflammatory properties by competitively inhibiting the arachidonic acid (AA) cascade, mainly at the COX pathway. The Wnt signalling pathway has a key function in stem cell maintenance and differentiation of haematopoietic progenitors and is involved in leukemia progression. Omega-3 FAs have been shown to promote differentiation and induce apoptosis in different cell lines. The objective of this study was to determine whether omega FA altered Wnt signaling in a hematopoietic stem cell model. Our preliminary data suggested that treatment of a lymphohematopoietic stem cell line, EML, with eicosapentanoic acid, EPA (omega-3 FA) might lead to the down-regulation of Wnt signaling compared to treatment with arachidonic acid, AA (omega-6 FA). Interestingly, similar results were seen when promonocytic leukemic HL-60 cells were treated with omega-3 FA. Unfortunately, these changes were not consistent and more investigation needs to be done to standardize the experimental conditions required to obtain reproducible changes in cells treated with omega-3 FA.

Introduction

Omega-6 fatty acids (FA) are derived from linolenic acid (LA, 18:2) and omegas 3 FA are derived from α-linolenic acids (ALA, 18:3). Linolenic acid and α-linolenic FA are essential FAs that cannot be synthesized in the body and have to be obtained from the diet (Das, 2008). Both omega-6 and omega-3 fatty acids (FA) are metabolized by the same set of enzymes to their respective long-chain metabolites. However, the metabolic products of each pathway are structurally and functionally distinct. LA is metabolized to arachidonic acid (AA, 20:4, omega-6), while ALA can be metabolized to eicosapentaenoic acid (EPA, 20:5, omega-3) and ultimately docosahexanoic acid (DHA, 22:6, omega-3). Alternatively, AA can be obtained from animal fat sources and EPA and DHA can be consumed directly from marine sources (Anderson and Ma., 2009).

EPA exhibits anti-inflammatory properties while AA has pro-inflammatory action. EPA and AA exert these effects as they are the substrates for the synthesis of a group of anti- and pro-inflammatory mediators respectively including thromboxanes, leukotrienes, and prostaglandins, collectively referred to as eicosanoids, via the cyclooxygenase (COX) and lipoxygenase pathway(Anderson and Ma, 2009).

The ratio of omega-6 to omega-3 FAs is believed to be of higher importance than the absolute levels of a particular fatty acid and is thought to play a role in inflammatory, heart, and metabolic diseases, as well as cancer (Gleissman *et al*., 2010). Impaired differentiation is the hallmark of many forms of cancer, but is particularly pronounced in myeloid leukemias. Omega-3 FA have been shown to promote differentiation by affecting myeloid progenitor cell frequency (Dupuis *et al*., 1997; Varney *et al*., 2009) and thus can be potentially used as a therapy in myeloid leukemias.

The Wnt signaling pathway controls hematopoietic stem cell (HSC) self-renewal and bone marrow repopulation; aberrant Wnt signaling is associated with carcinogenesis (Jamieson *et al*., 2004; Brembeck *et al*., 2006). It has been shown in previous studies using the *Drosophila* model system that the Wnt pathway can be epigenetically regulated (Sollars *et al*., 2003). In these studies mutations in trithorax group of genes or Hsp90 resulted in the gain of function expression of wingless, which was epigenetically inherited. It is thought that epigenetic silencing of secreted frizzled related proteins (SFRP) that have been implicated in colorectal cancer, results in the accumulation of the Wnt ligand and activated signaling (Suzuki *et al*., 2004). Also, Wnt activation in stem cells requires PGE2, a byproduct of AA metabolism (omega-6 FA). Inhibition of PGE2 synthesis blocks alterations in HSC formation at the level of ß-catenin Wnt-induced (Goeslling, 2009). Omega-3 FAs have been shown to inhibit COX-2 derived PGE2 and Wnt/ß-catenin signaling in hepatocellular and cholangiocarcinoma (Lim *et al*., 2008; Lim *et al*., 2009).

Thus, I hypothesize that high omega-3 FAs might inhibit leukemic progression by inducing differentiation via down-regulation of the Wnt pathway. We used EML cells as a model to study hematopoietic differentiation and determined the effect of exposure to various ratios of omega-3 and 6 FA on Wnt pathway activation. Wnt pathway activation was measured by the nuclear accumulation, ß-catenin.

Materials and Methods

Materials

Arachdonic acid, AA (A3555), Eicosapentanoic acid, EPA (E2011) and fatty acid free bovine serum albumin, BSA (A3782) were purchased from Sigma. Active ß-catenin antibody (05-665) was purchased from Millipore.

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Cell culture

EML C1 cells (American Type culture collection, ATCC, Manassas, VA) were maintained in growth medium which is base medium (IMDM supplemented with 20% heat inactivated horse serum and 10% BHK/MKL-conditioned medium, (Tsai S *et al*., 1994). HL-60 cells (ATCC, Manassas, VA) were cultured in IMDM and 20% FBS while U-937 cells were cultured in RPMI supplemented with 10% FBS at 37º C and 5% CO_2.

Fatty acid Treatments

EML cells were seeded at $2x10^5$ cells/ml in growth medium and treated with 60µM fatty acids including high AA (AA: EPA; 9:1), equal (AA: EPA; 1:1), high EPA (AA: EPA; 1:9) dissolved in vehicle (ethanol) for 96 hours. After 48 hours cells were washed with PBS and the same concentration of fatty acids was resupplemented along with the culture medium.

Immunoblotting

Nuclear and cytoplasmic fractions were separated using the NE-PER extraction kit as per the manufacturer's instructions (Pierce, Rockford, IL). Protein concentration was determined using bicinchoninic acid (BCA) protein assay reagents from Pierce according to the manufacturer's guidelines. Cell extracts were then denatured at 95°C for 5 min in 2x sample buffer (62.5 mM Tris HCl (pH 6.8), 25% glycerol, 0.01% bromophenol blue, 2% SDS,10% β-mercaptoethanol). Equal amount of protein extracts were separated on an 8-16% gradient Tris-Glycine SDS–poly acrylamide gel (Bio-Rad, Hercules, CA) by electrophoresis (PAGE) and transferred onto nitrocellulose membranes (Millipore) using Bio-Rad MiniProtean3® system. The membranes were treated in blocking solution (5% non fat dry milk in TBS containing 0.1%

Tween 20) and incubated with primary active ß-catenin antibody (05-665, Millipore, 1µg/ml) overnight at 4°C, followed by incubation with HRP-conjugated monoclonal mouse secondary IgG antibody (1: 3000, 7074, GE Healthcare,CT). An anti-mouse GAPDH antibody (MAB374, GE Healthcare, CT) was used to assess equal loading. Proteins were visualized by ECL (GE Healthcare, CT). Benchmark [TM] protein ladder was used to visualize the transfer of protein onto the membrane and MagicMark [TM] XP (Invitrogen) was used as a molecular size standard.

Results

Omega-3 fatty acids downregulate Wnt signaling in EML and HL- 60 cells

Wnt signaling is involved in maintaining the stem cell state in the hematopoietic system (Brembeck *et al*., 2006). Omega-3 FAs have been shown to promote myeloid differentiation (Dupuis *et al*., 1997, Varney *et al*., 2009). Also, omega-3 FAs have been reported to induce differentiation in breast cancer (Wang *et al*., 2000) and inhibit Wnt signaling in other cancers (Lim *et al*., 2008; Lim *et al*., 2009). To determine the effects of omega FAs on Wnt signaling in the hematopoietic system we used EML cells as a model. The EML cell line was established from DBA/2 mouse bone marrow infected with a retroviral vector (LRARα403SN) harboring a dominant negative retinoic acid receptor (RAR construct) (Tsai *et al*., 1994). It is a stem cell factor dependent multipotent cell line with myeloid, erythroid and lymphoid potential and serves as an excellent model to study the hematopoietic system. A concentration of 60 µM was chosen for all FAs (Finstad *et al*., 1998; Finstad *et al*., 2000). EML cells were treated with different ratios of omega-3 (EPA) and 6 (AA) FAs. The effect of omega FA on the Wnt signaling in EML cells was analyzed based on active ß-catenin accumulation in the nucleus. A 96 hour time point was chosen for our studies based on the preliminary data showing the effective loading of fatty acid on the cell membrane by gas chromatography. High omega-3 FA

treated EML cells showed reduction in active ß-catenin levels relative to high omega-6 treated
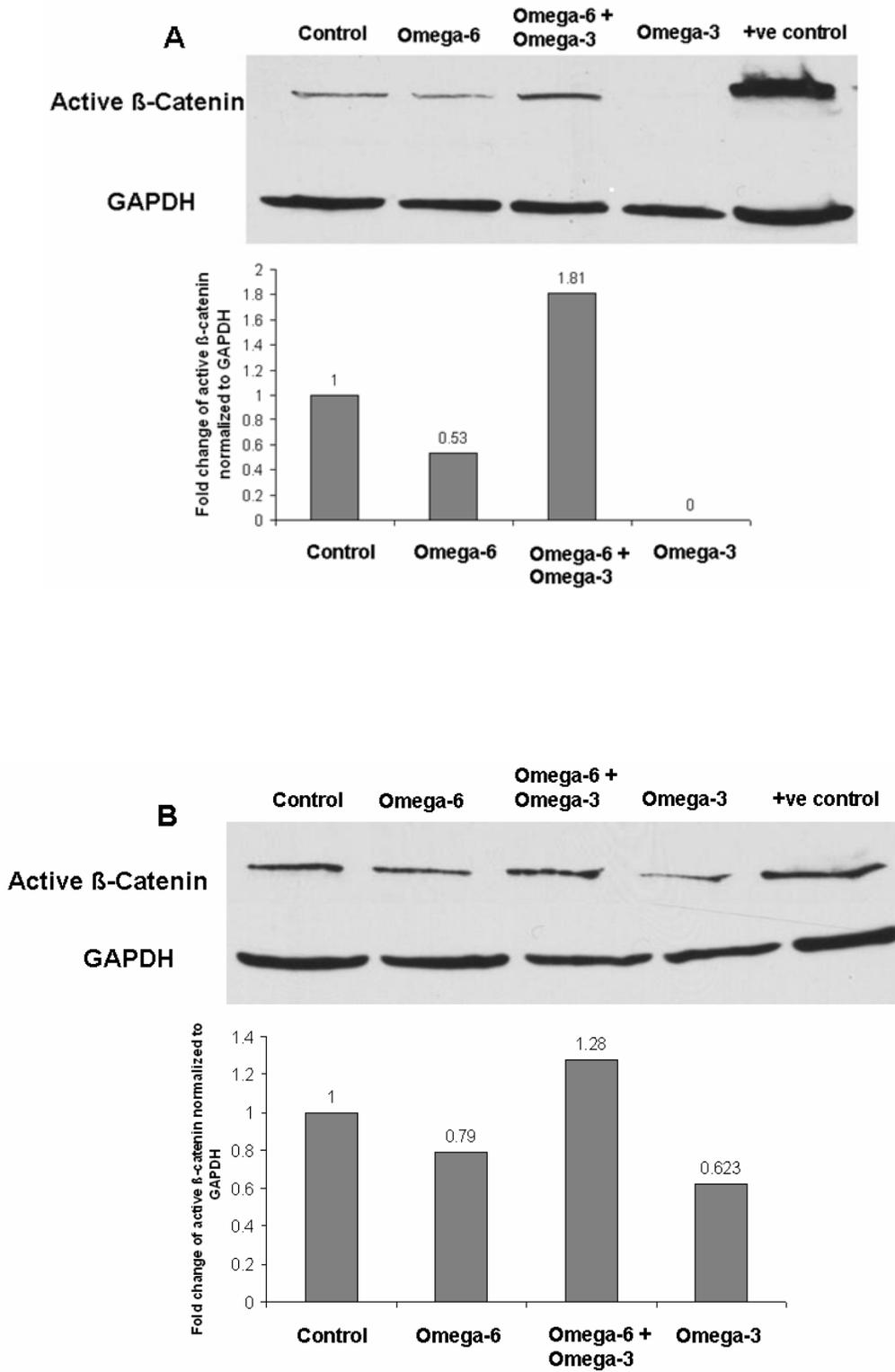
cells (Figure 4.1A).

Figure 4.1. Omega-3 fatty acids downregulate Wnt signaling in EML and HL- 60 cells.
Cells were exposed to vehicle, high AA (omega-6) (AA: EPA; 9:1), equal ratio of AA and EPA (omega-6+omega-3) (AA: EPA; 1:1), high EPA (omega-3) (AA: EPA; 1:9) for 96 hours. Thereafter, cells were washed with PBS and resupplemented with respective FA after 48 hours. Nuclear extracts were isolated and western blot was done to determine the levels of active β-catenin. Each panel shows a representative blot (A) EML cells (B) HL-60 cells. At the bottom of each blot is the densitometric analysis for that blot. Each blot is representative of two independent experiments. A549 cell lysate was used as a positive control for active ß-catenin and is represented as +ve control.

Activated Wnt signaling is involved in many forms of cancer including acute and chronic myeloid leukemia (Wang *et al*., 2010; Jamieson *et al*., 2004). Therefore, we examined the effect of omega fatty acids on Wnt signaling in HL-60, a human promyelocyte leukemic cell line. HL- 60 cells were treated with different ratios of EPA and AA as described above and ß-catenin accumulation was assayed by western blot analysis (Figure 4.1B). Our data suggested the down-regulation of active ß-catenin levels in high omega-3 FA treated HL-60 cells relative to high omega-6 treatment

This data indicates that the Wnt pathway is inhibited upon treatment with omega-3 fatty acids in hematopoietic stem cell culture as well as the leukemic HL-60 cell line.

Down-regulation of Wnt signaling by omega-3 fatty acids in EML, HL-60 and U-937 cells was inconsistent

Unfortunately when further experiments were done to understand the significance of the above data we found conflicting results. We did not see any significant difference between the levels of active ß-catenin in high omega-3 FA treated relative to high omega-6 FA treated EML cells (Figure 4.2 A).

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A

| | Control | Omega-6 | Omega-6 + Omega-3 | Omega-3 | +ve control |

Active ß-Catenin

GAPDH

Fold change of active ß-catenin normalized to GAPDH

Control	Omega-6	Omega-6 + Omega-3	Omega-3
1	1.33	0.82	0.973

B

| | Control | Omega-6 | Omega-6 + Omega-3 | Omega-3 |

Active ß-Catenin

GAPDH

Fold change of active ß-catenin normalized to GAPDH

Control	Omega-6	Omega-6 + Omega-3	Omega-3
1	0.91	1.42	0.88

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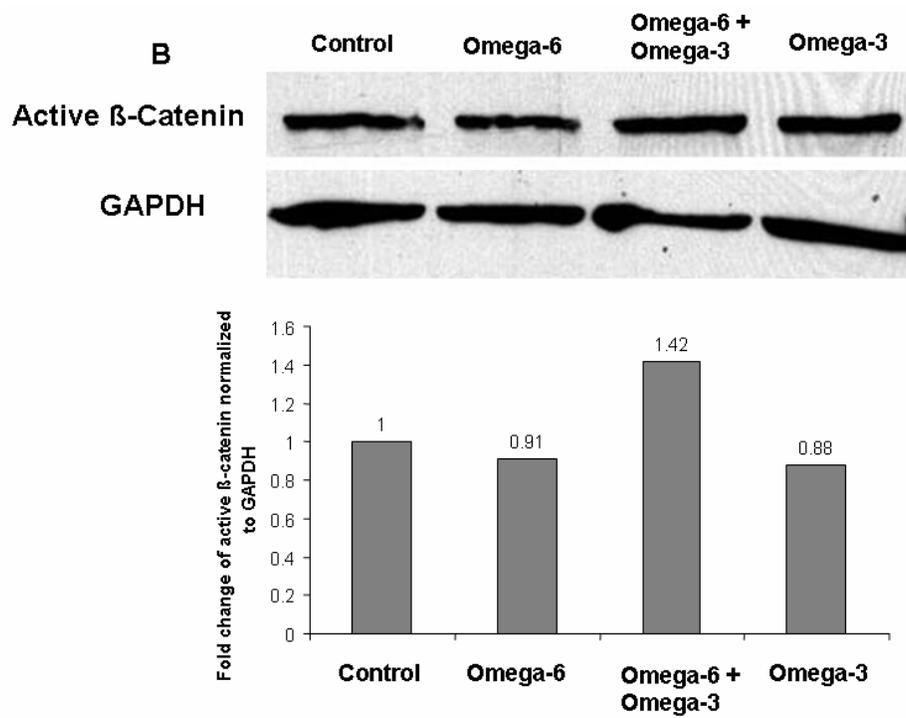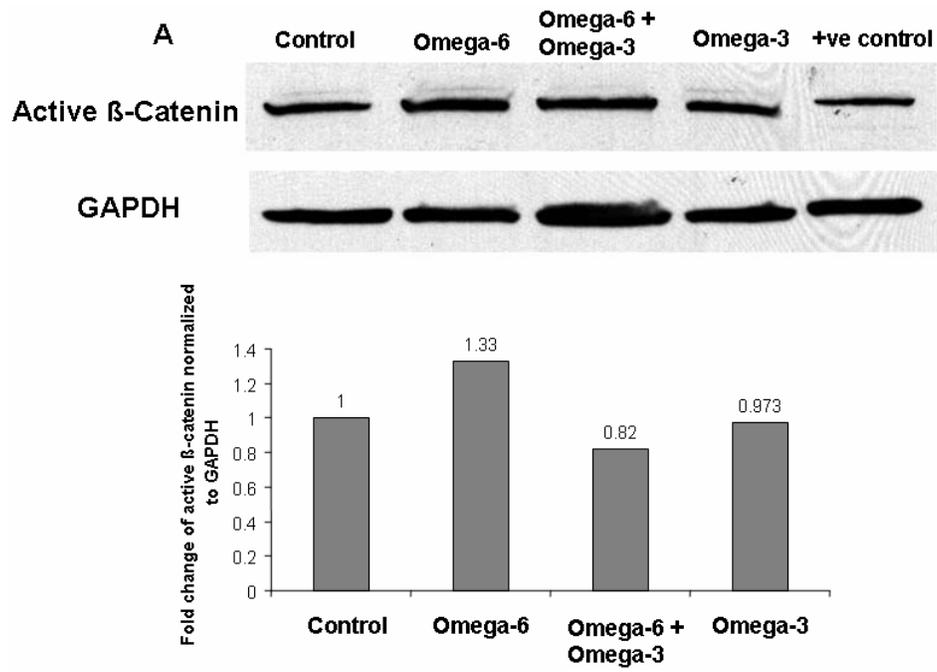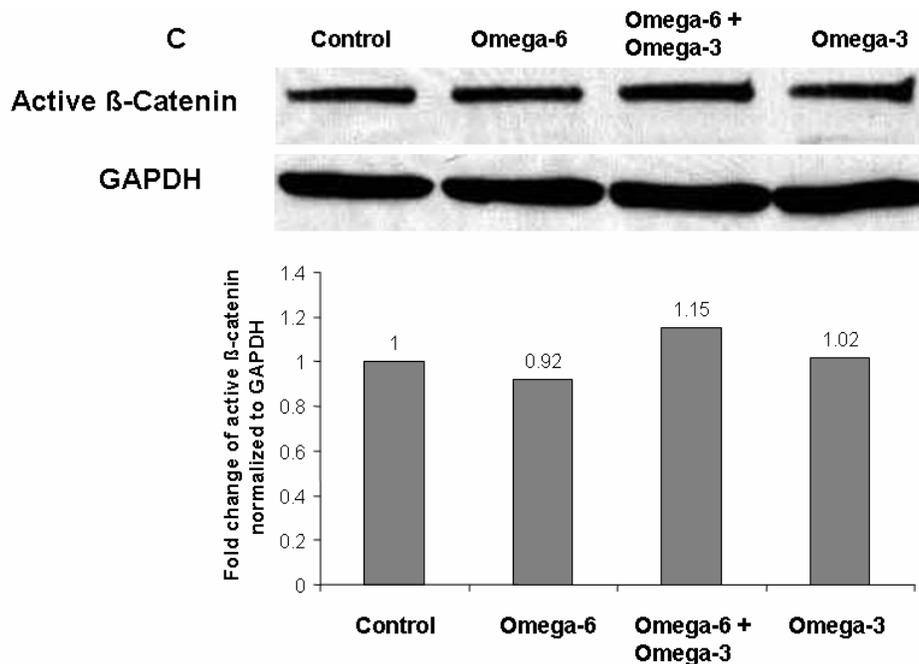
Figure 4.2. **Down-regulation of Wnt signaling by omega-3 fatty acids in EML, HL-60 and U-937 cells was inconsistent.** EML cells were exposed to vehicle, high AA (omega-6) (AA: EPA; 9:1), equal ratio of AA and EPA (omega-6+omega-3) (AA: EPA; 1:1), high EPA (omega-3) (AA: EPA; 1:9) for 96 hours. Cells were washed with PBS and resupplemented with respective FA after 48 hours. Nuclear extracts were isolated and western blot was done to determine the levels of active β-catenin. Each panel shows a representative blot (A) EML (B) HL-60 C) U-937. At the bottom of each blot is the densitometric analysis for that blot. Each blot is representative of two independent experiments. A549 cell lysate was used as a positive control for active ß-catenin and is represented as +ve control.

Similarly, the preliminary data showing that Wnt signaling might be downregulated in omega-3 FA treated relative to omega-6 FA treated HL-60 cells was not reproducible (Figure 4.2 B). We looked at the effects of omega fatty acid treatment in another monocyte like leukemia cell line, U-937, but we found no difference in the level of active ß-catenin expression in omega-3 FA treated versus high omega-6 FA treated U-937 cells (Figure 4.2 C)

Attempts to achieve the reduction in the Wnt signaling seen previously upon omega-3 fatty acid treatment were unsuccessful

We wanted to standardize our treatment protocol with omega FAs. Therefore, we used different methods to treat EML cells with omega FA to see if there was an effect on Wnt signaling. Some studies had demonstrated the use of EPA/AA with sodium salt as a base (Asano *et al*., 1997; Seung Kim *et al*., 2001; Edwards *et al*., 2004). Therefore, we treated EML cells with sodium salts of EPA and AA but did not observe any significant difference in the active ß-catenin levels in omega-3 and omega-6 FA treated EML cells (data not shown). Other studies reported the treatment of cells after serum starvation with FAs along with bovine serum albumin (BSA) (Melki and Abumrad, 1992; Ishola *et al*., 2006). To test this protocol, EML cells were serum starved for 24 hours and then treated with AA or EPA complexed to bovine serum albumin (BSA) at a molar ratio of 4:1 (FA/BSA) to a final concentration of 60µmol/L for 5 hours. Interestingly, treatment of FA itself led to massive mortality in EML cells. We also used variable molar ratios of FA/BSA; 2:1 and 1:1. Further, after serum starvation for 24 hours we supplemented EML cells with reduced concentration of serum (5, 10, and 15%) treated with AA and EPA and performed cell viability assay after 24 and 48 hours (Fig. 4.3). There was significant mortality in EML cells treated with FAs relative to vehicle control, but this effect was more pronounced in omega-6 compared to omega-3 FA treated EML cells. We speculated that omega FAs treated cells might be differentiating and may need a stimulant like IL-3 for survival. More investigation needs to be done to find a protocol for treating EML and leukemic cell lines with omega fatty acids that will yield consistent results.

A
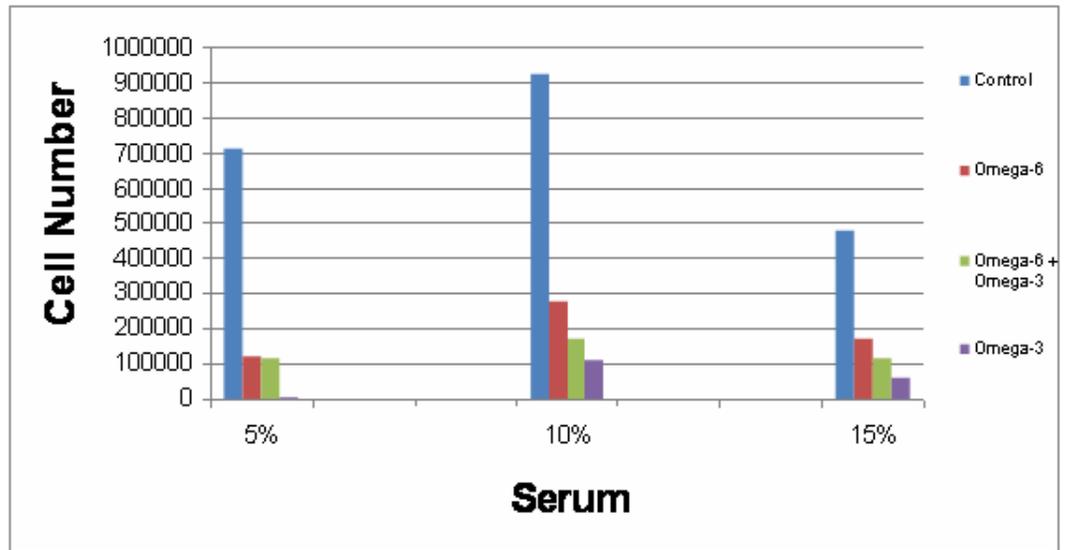
24 hour

B
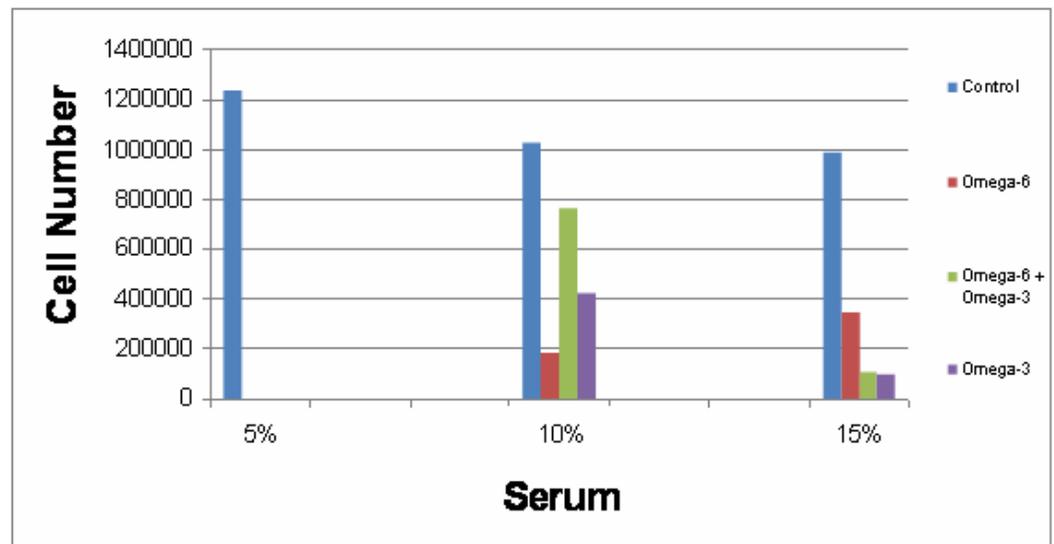
48 hour

Figure 4.3. Attempts to achieve the reduction in the Wnt signaling seen previously upon omega-3 fatty acid treatment were unsuccessful. EML cells were serum starved for 24 hours and then treated with vehicle, high AA (omega-6) (AA: EPA; 9:1), equal ratios of AA and EPA (omega-6+omega-3) (AA: EPA; 1:1), high EPA (omega-3) (AA: EPA; 1:9) in 5, 10 and 15% horse serum. Cell counts were performed by trypan blue dye exclusion method at 24 and 48 hours after the treatment.

Discussion

EML cells are stem cell in nature and have an active Wnt signaling pathway indicated by the nuclear translocation of active ß-catenin. Wnt signaling regulates stem cell fate in the hematopoietic system by self renewal of HSC. Deregulation of Wnt signaling, which is normally tightly regulated, has been identified as an important step in leukemic transformation. HSC differentiate into common myeloid progenitor cells (CMP) that gives rise to granulocyte macrophage progenitors (GMP). GMP has been identified as the candidate leukemic stem cell in the blast crisis stage of chronic myelogenous leukemia and has an active Wnt/ß-catenin pathway (Jamieson *et al*., 2004). We observed reduction in active ß-catenin expression in EML cells treated with high omega-3 FA relative to omega-6 FA, consistent with the fact that omega-3 FAs have been shown to promote myeloid differentiation (Dupuis *et al*., 1997; Varney *et al*., 2009).

We initially saw down-regulation of active ß-catenin protein in acute promyelocytic leukemia, HL-60 cells upon exposure to high omega-3 FAs. It has been shown previously that omega-3 FAs inhibit proliferation and induce differentiation in HL-60 cells which supports our data (Finstad, 1994). Also, Wnt signaling is active in various cancers including myeloid leukemia. Omega-3 FAs have been shown to inhibit omega-6 FA product, PGE2 and Wnt/ß-catenin signaling in hepatocellular and cholangiocarcinoma (Lim *et al*., 2008; Lim *et al*., 2009). Up-regulation of Wnt signaling through epigenetic gene silencing of SFRPs is involved in many forms of cancer. Wnt activation in stem cells requires PGE2 (product of omega-6 FAs), and it has been shown recently that inhibition of PGE2 synthesis blocked Wnt-induced alterations in HSC formation at the level of ß-catenin *in vivo* (Goeslling *et al*., 2009). These initial findings indicated that omega-3 FA therapies aimed at disruption of the Wnt/β- catenin pathways may be effective in the treatment and chemopreventive effects in leukemia.

Unfortunately, additional experiments aimed at understanding the mechanism and effects of down-regulation of Wnt signaling, gave variable results. There was no significant difference in the active ß-catenin expression in both EML and HL-60 omega-3 FA treated compared to the omega-6 FA treated cells. We also measured active ß-catenin levels in the pro-monocytic U-937 cell line but did not see any difference in the cells exposed to omega-3 versus omega-6 FAs. This inconsistency could be attributed to the fact that FAs exert their effects at several levels, both through signal transduction pathways and on gene transcription. Since the effect of FA on various cellular processes like differentiation and apoptosis depend upon the concentration of FA and serum, exposure time and the cell model used (Rudolph *et al*., 2001). It may be possible that inactivation of Wnt signaling by omega-3 FAs is a sensitive event which requires a specific set conditions to be met. Another possibility could be that omega FAs exerts their effects in the hematopoietic system through signal transduction pathways other than Wnt ß-catenin pathway.

We used different methods to treat EML and HL-60 cells with various ratios of FA to achieve our initial results but none of these were successful. Since, PUFA are not soluble in the aqueous medium we tried sodium salts of FAs which have increased water solubility. Moreover, FAs are carried within the body complexed to serum albumin and the FA: BSA complexes are the main factors controlling FA availability for uptake (Melki *et al*., 1992). Therefore, we serum starved EML cells to deplete any albumin present in the serum that might bind to the free fatty acid and then treated the cells with FAs complexed to BSA.

We observed massive cell mortality in EML cells after exposure to either omega-3 or 6 FAs complexed with BSA, relative to the vehicle control. Several studies have reported the cytotoxic effects of FA on different model systems. In one study, the authors demonstrated that FA sensitive Burkitt lymphoma cell lines, Raji and Ramos, die by necrosis and apoptosis than the FA resistant U-698 cell line upon incubation with EPA, respectively. These FA sensitive

cell lines exhibit a 2 to 3 fold higher uptake rate of EPA than the FA resistant cell line. Accumulation of TAG-rich lipid bodies was seen in Ramos cells incubated with 60 µm EPA indicating a role for lipid bodies in regulation of the cellular suicide program. A high number of TAG-rich bodies in Ramos cells, containing 2–3 molecules of EPA or DHA per glycerol backbone, may cause marked changes in intracellular signal transduction and thereby initiate apoptosis (Finstad *et al*., 2000).This response to *in vitro* fatty acid supplementation has previously been reported for U937-1 cells (Finstad *et al*., 1998). In another study AA and EPA were shown to induce apotosis and necrosis in a dose dependent manner in a murine macrophage cell line, J774. When these cells were treated with FAs, they exhibited higher granularity, suggesting accumulation of lipid droplets. The cytotoxic effects of the FAs were suggested to be related to their ability to be incorporated into TAG (Martins *et al*., 2006).

Moreover, the variability in our data could be due to the heterogenous nature of EML cells. It has been shown that EML cells can be separated into two different populations based on CD34 expression. Levels of stem cell factor receptor (c-kit) are similar in both these populations but they differ in growth characteristics and response to cytokines (Ye *et al*., 2005). Another study demonstrated a broad spectrum of Sca-1 expression in EML cells which was suggested to be the cause of clonal subpopulations in these cells. Most strikingly, the cells at the extremes of the spectrum differed in their differentiation potential (Chang *et al*., 2008). These studies indicate that EML cells are a heterogeneous population having phenotypic cell-to-cell variability.

Our preliminary results suggested that the down-regulation of Wnt signaling upon exposure to omega-3 FAs may occur in the hematopoietic precursor cell line EML and also leukemic HL-60 cells. Unfortunately we could not reproduce these results. This non-reproducibility might be due to the fact that EML cells take into account only cell autonomous factors. The stem cell niche, which is an important part of stem and progenitor cell biology, is

not recapitulated in this model. Also, EML cells in culture are a heterogenous mixture of cells with populations having different self renewal and differentiation potential. This heterogeneity might have contributed to the variable responses seen in our studies.

CHAPTER Five: Discussion and Conclusions

Hematopoiesis is maintained by a proper balance between self renewal and multipotent differentiation of the HSC. Acute myelogenous leukemia (AML) is characterized by the blockage in the differentiation of HSC while self renewal and proliferation is preserved. Understanding the mechanisms involved in hematopoietic differentiation will help in developing therapies against various hematological disorders including leukemia. To address this question I investigated: (1) the involvement of Hsp90 in the regulation of Wnt signaling in HSCs cell line, (2) the effect of omega-3 and 6 fatty acids treatments on the Wnt signaling in HSC cell line, (3) YB-1 expression and function in early hematopoiesis and leukemic cells.

(1) The involvement of Hsp90 in the regulation of Wnt signaling in HSCs cell line

Hsp90 acts as a molecular chaperone to ensure the proper folding and refolding of client proteins. Hsp90 clients are implicated in various signal transduction pathways and are involved in the pathogenesis of cancer making it an attractive therapeutic target for cancer. Previous studies in *Drosophila* demonstrated that inhibition of Hsp90 resulted in a transdifferention event where the eye tissue became limb like outgrowth and was demonstrated to occur through epigenetic mechanisms. This abnormal eye phenotype was associated with the upregulated expression of wingless (Wg), a *Drosophila* homolog of Wnt, in the eye imaginal discs. This study indicates that Hsp90 plays a critical role in determining the proper direction of normal stem cell differentiation and might have implications in haematopoesis. In addition, this study is particularly relevant in stem and progenitor cell biology as Wnt signaling regulates the hematopoietic stem cell maintenance and self renewal (Kirstetter *et al*., 2006). Thus, the aim of the part of my thesis was to determine whether Hsp90 inhibition would block the normal HSC differentiation via up-regulating Wnt signaling.

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EML, a hematopoietic stem/progenitor cell line, was used as a model for my studies (Tsai *et al*., 1994). In chapter 3, our preliminary data showed the transient up-regulation of Wnt signaling upon Hsp90 inhibition in EML cells. This was an important result since it was the first evidence of this interaction in a mammalian system and suggested the possibility of phenotypic plasticity as seen in the *Drosophila* model upon Hsp90 inhibition. Phenotypic plasticity is the ability to adapt to an unfavorable environment and this is particularly important for evolution. Cancer cells show this plasticity, which provides them the ability to survive under adverse conditions. Therefore, this increased adaptability could contribute to cancer progression. However, we had reproducibility issues with these experiments.

Two separate studies have demonstrated the heterogeneity of the EML cell line. This characteristic could be the reason behind the variability in our data. The published study separated EML cells into two separate populations based on the CD34 expression. The CD34 $^+$ population showed a growth response upon treatment with stem cell factor (SCF) while the CD34$^-$ cells did not grow in SCF but responded to IL-3 treatment. This shift from the SCF dependent growth to the requirement of IL-3 suggests an early differentiation event in the CD34$^-$ population. Moreover, CD34 $^+$ cells were Sca-1^{hi} while CD34$^-$ cells were Sca-1^{lo} indicating that CD34 $^+$ population possessed more stem cell characteristics (Ye *et al*., 2005). The second study reported the broad spectrum of Sca-1 expression in EML cells that was correlated with changes in differentiation potential. It is possible that these subpopulations exhibit different responses upon Hsp90 inhibition (Chang *et al*., 2008). Therefore, we might sort EML cells into different subpopulations based on the CD34 and Sca-1 expression and then inhibit Hsp90 in the different populations. This will allow the analysis of the specific response of Wnt signaling of each population upon Hsp90 inhibition.

In our initial experiments, we saw a down-regulation of total ß-catenin levels in EML cells after inhibiting Hsp90. Geldanamycin, a Hsp90 inhibitor, and its analogs have cytotoxic effects on normal cells (Alcazar and Cid., 2009; Wu *et al*., 2010). Therefore, down-regulation of total ß-catenin levels via geldanamycin treatment of EML cells might be toxic. Since the effect of Hsp90 inhibition on Wnt signaling is based on the changes observed in the ß-catenin expression, the overall down-regulation of ß-catenin expression due to toxic effects of geldanamycin would adversely affect the interpretation of our data.

When we differentiated EML cells, active ß-catenin was inhibited in RA and GMCSF treated cells but the undifferentiated EML cells pretreated with Hsp90 inhibitor did not show any effect on the active ß-catenin levels. There was no significant difference in the level of active ß-catenin in differentiated and undifferentiated EML cells upon Hsp90 inhibition.

It is possible that Hsp90 might not be involved in regulating Wnt signaling in the mammalian hematopoietic system. In the *Drosophila* model, researchers reported that inhibition of Hsp90 led to the up-regulation in Wg expression resulting in an abnormal eye phenotype (Sollars *et al*., 2003). However, later analysis of this finding suggested the ectopic adhesion of hemocytes under the eye imaginal disc to be a possible cause for the ectopic outgrowth rather than up-regulated Wg expression in the peripodial membrane. This could be attributed to the fact that hemocytes are involved in tissue remodeling (Ruden *et al*., 2003). Another possibility is that the EML cell model takes into account only cell autonomous factors. The Wnt axis might not be completely modeled in this system since Wnt is released from the niche cells and taken up by the stem cells. Further, the EML cell line has been derived from mouse bone marrow by the transfection of a dominant negative retinoic acid receptor. This blockage to hematopoietic differentiation in the EML cells could be disrupting the normal Wnt signaling process.

Thus, in this study we show the up-regulation of Wnt signaling in EML cells upon Hsp90 inhibition. Unfortunately, this data was not reproducible. One contributing factor to this lack of reproducibility could be that EML cells are known to have heterogenous populations. Also, this *in vitro* model does not recapitulate the effects of the stem cell niche.

In the future, the role of Hsp90 regulation of Wnt signaling could be investigated in an *in vivo* mouse model. For this study, mice could be treated with Hsp90 inhibitors such as geldanamycin or 17-AAG and the effect of Hsp90 inhibition on normal hematopoesis measured by colony formation assays and various differentiation markers. If Hsp90 inhibition successfully blocks the normal hematopoietic differentiation, then the expression of active ß-catenin in HSC or progenitor cell populations would be determined using flow cytometry. Thus, this study would determine the role of Hsp90 in regulating Wnt signaling during HSC differentiation.

(2) The effect of omega-3 and 6 fatty acids on Wnt signaling in a HSC cell line

AML is characterized by problematic differentiation of HSC while self-renewal and proliferation are preserved. Wnt signaling controls HSC self renewal and is active in various cancers including acute and chronic myeloid leukemia (Wang *et al*., 2010; Jamieson *et al*., 2004). Omega-3 FAs have been reported to promote myeloid differentiation by affecting myeloid progenitor cell frequency (Dupuis *et al*., 1997; Varney *et al*., 2009). Also, omega-3 FAs have been demonstrated to induce differentiation in breast cancer (Wang *et al*., 2000) and inhibit Wnt signaling in other cancers (Lim *et al*., 2008; Lim *et al*., 2009). Therefore, this section of my thesis research analysed the effect of omega FA on Wnt signaling in the hematopoietic system.

In chapter 4, I showed that treatment of EML and HL-60 cells representing HSC and myeloid leukemic cells respectively, with omega-3 FAs led to to the down-regulation of active ß-catenin. In the hematopoietic precursor cell line, EML we found that high eicosapentanoic acid (EPA) (omega-3) treated cells showed reduction in active ß-catenin levels relative to high arachidonic acid (AA) (omega-6) treated cells. Similar results were observed in the human promyelocyte leukemia cell line, HL-60. These results were exciting because they suggested that omega-3 FA could have therapeutic potential in the treatment of leukemia by disrupting the Wnt/β- catenin pathway.

Unfortunately, the data obtained in these initial experiments was not reproducible. One possibility that I cited earlier in discussing the Hsp90 studies is the heterogeneous nature of the EML cell culture. The presence of different subpopulations in EML cells which differ in their growth characteristics and differentiation potential could lead to variable results (Ye *et al*., 2005; Chang *et al*., 2008).

In addition, a report has shown that omega-3 (EPA) or omega-6 (AA) FAs treatment of EML cells do not seem to have a significant difference on their differentiation profile (Varney *et al*., 2011). In this study EML cells were differentiated into macrophage/granulocytic lineage after exposure to omega-3 and omega-6 FAs. There was no effect of EPA or AA on the early stem cell markers Sca-1 and CD117 upon differentiation of EML cells, though there was a small increase in F4/80 (macrophage marker) after AA treatment. Further, there was no difference in the viability or proliferation of cells treated with either EPA or AA. This suggests that omega FAs does not have any effect on the proliferation and differentiation of early differentiating EML cells. This could be the one of the reasons we did not observe any changes in the Wnt signaling with omega FA treatment of these cells. In the same study, when effects of omega FA were studied in an *in vivo* mouse model it was found that omega FA treatment affected later stages of differentiation and not the stem cell stage. This suggests that to see the

desired effects of omega-3 to omega-6 treatments in EML cells they might need to differentiate for a longer duration.

Stem cell characteristics are known to be regulated by several factors which include cell-cell interactions, growth factors, cytokines, and the physiochemical nature of the environment *in vivo*. These components may only be replicated to a fairly limited extent in an *in vitro* cell model; therefore the EML model gives a limited idea as to what effects FAs have *in vivo*. Thus, the potential effect of omega FAs on hematopoiesis requires the investigation of a mouse model.

Omega FAs can be fed to the mice in the diet as corn (omega-6) or canola oil (omega-3) and its effects on ß-catenin accumulation in HSC and myeloid progenitors could be quantified by flow cytometry. In addition, to further determine the effects of FA treatment on Wnt signaling in hematopoietic stem and progenitor cells, these cells can be sorted by flow cytometry using specific cell surface markers and the sorted cells cultured. Unphosphorylated or active ß-catenin interacts with TCF/LEF transcription factors to activate the downstream target genes (Mc Donald *et al*., 2006). Therefore, a reporter assay for the TCF/LEF can be performed in these sorted HSC/progenitor cells as a measure of ß-catenin mediated transcriptional activation. Thus, this study would confirm the effect of FA treatments on Wnt signaling by functional activation of ß-catenin in addition to its accumulation in HSC/Progenitor cells.

(3) YB-1 expression and function in early hematopoiesis and leukemic cells

YB-1 promotes cell proliferation through transcriptional regulation of various genes involved in cell division. It also enhances cell growth by promoting both cell cycle progression and DNA replication (Kohno *et al*., 2003). In addition it induces the expression of genes such

as MMP-2 which are associated with invasion and metastasis (Cheng *et al*., 2002). Thus, YB-1 is implicated in cancer pathogenesis. YB-1 is reported to be involved in erythroid cell development as it was shown to be highly expressed when erythroid cell maturation was inhibited (Yokoyama *et al*., 2003a, b). Moreover, YB-1 expression was found to be higher in the bone marrow samples of patients with MDS. Further, YB-1 expression was showed to be higher specifically in the erythroid progenitors of MDS patients. Although YB-1 is reported to be involved in erythropoesis its role and regulation in normal hematopoiesis is not known. Thus part of my thesis research was to investigate the role and function of YB-1 expression in the early hematopoiesis.

YB-1 mRNA and protein expression was high in the hematopoietic stem cell line, EML, but was down-regulated during myeloid differentiation. Similar results were observed in a previous cDNA microarray study that analyzed changes in gene expression during induced myeloid differentiation of EML cells (Ma *et al*., 2002). Moreover, we found YB-1 to be highly expressed in the lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca-1$^+$ (LKS, enriched fraction for HSC) and lineage$^-$/IL-7R$^-$/c-kit$^+$/Sca-1$^-$ (myeloid progenitors) compared to *in vivo* differentiated granulocytes. This finding is consistent with a previous study where YB-1 is highly expressed when erythroid cell maturation was inhibited upon knockdown of GATA-1, an essential factor for erythroid cell development. (Yokoyama *et al*., 2003b). YB-1 plays a role in embryogenesis and its expression level correlates with the cell proliferation state (Lu *et al*., 2005). High levels of YB-1 are present in human fetal tissues that represent more a stem cell stage of development, while YB-1 transcript is not detected or is expressed at very low level in many adult tissues (Spitkovsky *et al*., 1992). These findings are consistent with our data that YB-1 is highly expressed in HSC/progenitor cells while down-regulated in differentiating granulocytes. Our results with a more well-defined cell population further suggest that YB-1 has a role in maintaining the stem cell state.

We also found that YB-1 was highly expressed in various myeloid leukemia cell lines, supporting our hypothesis that YB-1 is involved in cancer progression. This finding is consistent with a previous study which reported increased expression of YB-1 in the bone marrow of patients suffering from MDS (Lee *et al*., 2001). MDS is considered to be the preleukemic state characterized by the ineffective production of mature blood cells due to increased proliferation and reduced differentiation of HSC. MDS has a high predisposition to transform into acute myelogenous leukemia. Knockdown of GATA-1, a transcription factor required for erythropoieis in mice, leads to inhibition of erythroid differentiation. YB-1 is highly expressed in the GATA-1 knockdown mouse. The mechanism behind YB-1 up-regulation after GATA-1 knockdown is not known. However, these mice have a phenotype similar to human MDS during early stages of life, and in later stages of life they develop acute leukemia (Yokoyama *et al*., 2003 b). This suggests that YB-1 might be playing a role in the development of MDS and leukemia.

To further investigate the biological effects of YB-1 expression in leukemia, we did loss of function studies. For this we chose the high YB-1 expressing cell line, K-562, and knocked down YB-1 using specific shRNA. Knock down of YB-1 resulted in a growth arrest and induction of apoptosis in K562 cells. This effect was much more pronounced when we used arsenic trioxide (As_2O_3). This compound has been shown to induce apoptosis in acute promyelocytic cells (APL) and is being used a therapeutic agent in leukemia (Tang *et al*., 1997). As_2O_3 treatment of K562 cells with YB-1 knockdown led to a statistically significant reduction in cell proliferation. There was also a significant increase in the apoptotic population of these cells. Down-regulation of YB-1 decreases cell viability and induces apoptosis in multiple myeloma and melanoma cells (Schittek *et al*., 2007; Chatterjee *et al*., 2008). Knockdown of YB-1 in the multi drug resistant K562/A02 cell line showed similar results and is consistent with our data (Xu *et al*., 2009).

Moreover, we found that treatment of As_2O_3 in K562 cells with down-regulation of YB-1 resulted in their differentiation toward the megakaryocytic lineage (Figure 5.1). Since impaired differentiation is the hallmark of myeloid leukemia, this finding has a significant impact on our understanding of myeloid differentiation and might be exploited for therapy. As_2O_3 exerts double effects on acute promyelocytic cells such as induction of apoptosis and partial differentiation (Tang *et al.*, 1997). We observed changes in the cellular morphology of the YB-1 knockdown cells treated with As_2O_3. Subsequently, we determined the expression of CD41a megakaryocytic/platelet marker. We found significant up-regulation of CD41a in YB-1 depleted K562 cells treated with As_2O_3. To confirm this data, we did ploidy analysis which showed an increase in polyploidy in the As_2O_3 treated K562 cells containing reduced YB-1 levels. There was a significant increase in CD41a in untreated K562 cells when YB-1 was reduced but we did not observe any significant change in cell morphology. This indicates that down-regulation of YB-1 expression could initiate the differentiation process.

In chapter 2, we show for the first time the expression of YB-1 in the hematopoietic stem/progenitor cells and its down-regulation during myeloid differentiation. Further, abnormal YB-1 expression in leukemic cells contributes to leukemic properties by enhancing cell survival and inhibiting cell differentiation (Figure 5.1).

We studied the expression and function of YB-1 *in vitro* using leukemia cell lines. Our findings can be further verified by measuring YB-1 levels in patient samples from acute and chronic myelogenous leukemia. This will help in translating our findings into humans and determine whether YB-1 might be a useful prognostic marker in leukemia.

An important experiment to study the mechanism for the down-regulation of YB-1 during myeloid differentiation would be knocking down YB-1 in EML cells. It would be interesting to see whether knockdown of YB-1 induces spontaneous differentiation in EML cells as seen in K562 leukemic cells. If EML cells differentiate it will further corroborate that

YB-1 plays role in early stage of hematopoietic differentiation at an early stage. It might be possible that EML cells need a stimulant such as IL-3 for differentiation, which would imply that YB-1 acts at a later stage of differentiation.

To investigate the potential effects of YB-1 gene knockdown in K562 cells a gene array study could be beneficial. This assay will help in finding the putative target genes for YB-1.

The PI3K/Akt signaling pathway plays an important role in the proliferation, differentiation and survival of hematopoietic cells. Constitutive activation of PI3K/Akt has been demonstrated in AML patients and is associated with poor prognosis (Min *et al*., 2003; Kubota *et al*., 2004). Akt has been shown to phosphorylate YB-1 leading to its nuclear translocation and activation in breast cancer. Inhibition of the PI3K/Akt pathway suppresses nuclear translocation of YB-1 in breast and ovarian cancer (Sutherland *et al*., 2005; Baski *et al*., 2006). Since there are no direct inhibitors available for YB-1 we can inhibit it indirectly by using Akt inhibitors in AML cell lines. Further, we could test whether these Akt inhibitors can reduce YB-1 expression in a CML mouse model.

Wnt and Notch pathways play an important role in promoting self renewal in hematopoietic stem and progenitor cells (Reya *et al*., 2003; Duncan *et al*., 2005). Examining whether YB-1 is involved in the regulation of Wnt and Notch pathways in the hematopoietic system would be of great interest. A recent study in breast cancer reported several members of Wnt and Notch signaling pathways to be the targets of YB-1. Further, c-Kit, which is a receptor expressed on the surface of HSC, was also identified as YB-1 target (Finkbeiner *et al*., 2009). Mutations in the c-kit gene have been documented in AML and are associated with a poor prognosis (Beghini *et al*., 2004). Thus, Wnt and Notch pathway elements and c-kit could be the putative targets of YB-1 in the HSC/progenitor cells and in leukemia. Our previous two projects focused on the regulation of Wnt signaling in the hematopoietic system by Hsp90 and omega FAs. If Wnt signaling elements are targets of YB-1 it will be interesting to determine the

association of YB-1 with Hsp90 or omega FAs.

In summary, my overall goal for these studies was to determine potential genes involved in the regulation of hematopoietic differentiation. The first study initially suggested Hsp90 might be involved in regulating Wnt signaling in the hematopoietic system. Our preliminary data showed the transient activation of the canonical Wnt pathway in a hematopoietic precursor cell line EML after the inhibition of Hsp90. Our second study initially indicated that omega-3 FAs might affect Wnt signaling in the hematopoietic system and leukemic cells. These results had great potential in increasing our understanding of how Hsp90 and omega FAs regulate Wnt signaling in the hematopoietic system. Unfortunately, further progress in both of these studies was marred by the variability in repeated experiments. However in our third study we have identified YB-1 as a marker in leukemia and that is responsible for HSC maintenance, making it an attractive therapeutic target. We show high YB-1 expression in the hematopoietic stem cell line EML, as well as in the HSC and myeloid progenitors from mouse bone marrow. We found down-regulation of YB-1 expression during myeloid differentiation in EML cells. Abnormal YB-1 expression was observed in myeloid leukemic cell lines. In addition, increased expression of YB-1 in leukemic cells contributes to the leukemic cell properties by stimulating proliferation, promoting cell survival and blocking differentiation. Since inhibition of YB-1 in leukemic cells can induce differentiation it is an attractive target for the development of new therapies for leukemia.
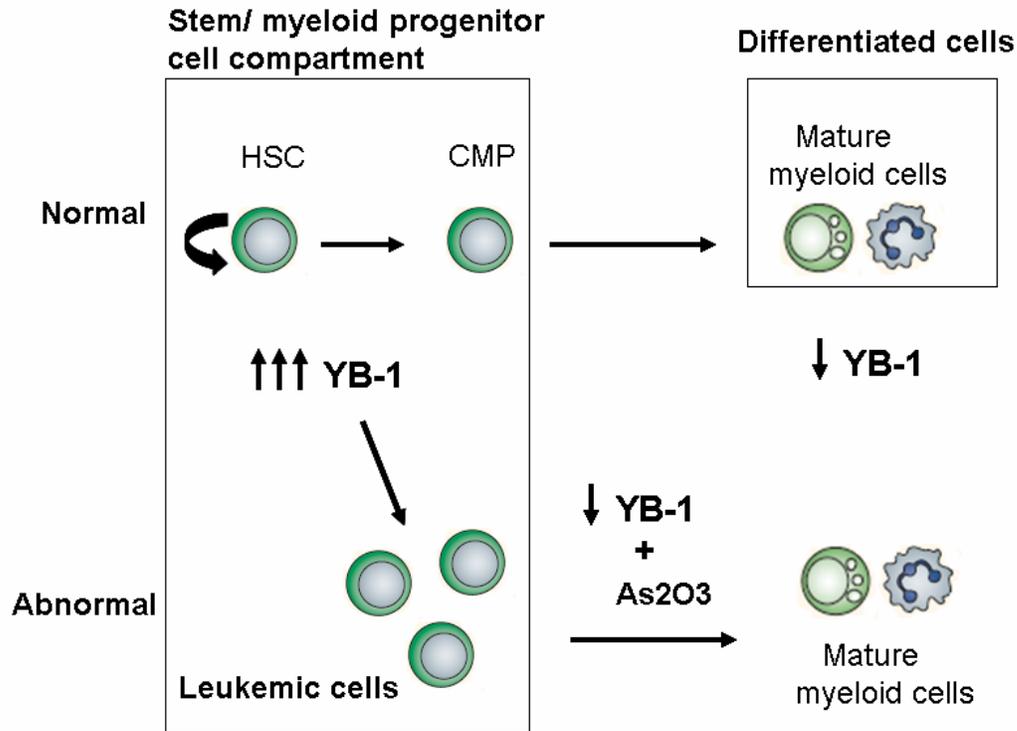
Figure 5.1 Model for the expression and function of YB-1 in normal and leukemic cells. Hematopoietic stem cells (HSCs) have the ability to self renew and give rise to common myeloid progenitors (CMPs). CMP further differentiates to produce mature myeloid cells. Blockage in the terminal differentiation of HSCs and CMPs can result in myeloid leukemia. YB-1 is highly expressed in the HSCs and early myeloid progenitors relative to the differentiated myeloid cells. Knockdown of YB-1 expression followed by treatment of arsenic trioxide (As_2O_3) in K562 cells resulted in their differentiation toward the megakaryocytic lineage. Thus, increased expression of YB-1 contributes to the leukemic cell properties by promoting cell survival and inhibition of differentiation.

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