HOXA9 as a Risk Factor in Acute Myeloid Leukaemia

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Declaration of originality

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Summary

Acute myeloid leukaemia (AML), also known as acute myelogenous leukaemia is a cancer of the myeloid line of blood cells, characterized by the rapid proliferation of abnormal myeloid cells accumulating in the bone marrow that interfere with normal haematopoiesis. Although AML is a relatively rare disease, which accounts for almost 1% of cancer deaths in the United Kingdom and 1.2% of cancer deaths in the United States, it is the most common acute leukaemia; primarily affecting adults, and its incidence is known to increase with age.

To date, there is still very little understood about the risk factors for the development of AML beyond the onset of old age. However, some key pre-existing haematological disorders such as myelodysplastic syndrome (MDS) and myeloproliferative neoplasms (MPN) are now known to have a relatively high incidence of developing AML (15-52% and 7% respectively). In recent years, there have been great strides made towards understanding some of the major genetic changes that occur in AML. Normal haematopoiesis is strictly regulated by the *HOX* family of transcription factors which have been shown to play a key role in the development of cancer when elevated in the normal peripheral blood mononuclear cells (PBMCs) of the elderly. Together with its known role as an oncogene in AML, and a predictor of survival in this disease, it is suggested that *HOXA9* expression in PBMCs could be a predictive marker of non-therapy related AML.

This study suggests that elevated *HOXA9* expression is indeed a predictive marker for AML development and attempts to shine a light on the progression of AML from pre-leukemic diseases such as MDS and MPN as well as offering a possible solution which may be used as a therapeutic drug either alone or in combination with current commonly used therapeutics.

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List of Abbreviations

7-Amino-actinomycin (7-AAD)

Abdominal-B genes (Abd-B)

Activator protein 1 (AP-1)

Acute myeloid leukaemia (AML)

Acute promyelocytic leukaemia (APML)

ALL1-fused gene from chromosome 9 protein (AF-9)

Three Amino acid loop extension (TALE)

Antennapedia (Ant-C)

Bithorax complex (BX-C)

Bone marrow (BM)

Caudal-type homeobox transcription factors (CDX's)

Chronic eosinophilic leukaemia (CEL)

Chronic myeloid leukaemia (CML)

Chronic neutrophilic leukaemia (CNL)

Class I homeobox genes (HOX)

Complementary DNA (cDNA)

Complete remission (CR)

Cyclic AMP-dependent transcription factor (c-Atf3)

Cysteine-aspartic protease (Caspase)

Daunorubicin (DNR)

Deoxyribonucleic acid (DNA)

Diethylpyrocarbonate (DEPC)

Early growth response protein 1 (EGR1)

Enzyme-Linked Immunosorbent Assay (ELISA)

Essential thrombocythemia (ET)

Extracellular Matrix (ECM)

Foetal bovine serum (FBS)

Fluorescence-activated cell sorting (FACS)

Genomic DNA (gDNA)

Haematopoietic stem cells (HSC's)

Haematopoietic progenitor cells (HPC's)

Homeotic genes (HOM-C)

Janus Kinase (JAK)

Messenger RNA (mRNA)

Mitogen-activated protein (MAP) kinases (MAPK)

Mixed lineage, lymphoid, leukaemia (MLL)

Myelodysplastic syndrome (MDS)

Myeloid ectopic insertion site (MEIS)

Myeloproliferative neoplasms (MPN)

Normal karyotype-Acute myeloid leukaemia (NC-AML)

Nuclephosmin 1 (NPM1)

Nucleoporin 98 (NUP98)

Overall survival (OS)

Phosphate buffer saline (PBS)

Poly (ADP-ribose) polymerase (PARP)

Polycythemia vera (PV)

Polymerase chain reaction (PCR)

Pre B cell leukaemia (PBX)

Primary myelofibrosis (PMF)

Protein kinase-C (PK-C)

Relative quantitative-polymerase chain reaction (RQ-PCR)

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Ribonucleic acid (RNA)

Signal Transducer and Activator of Transcription (STAT)

Standard error of the mean (SEM)

Chapter 1: General Introduction

1.1 The evolution of cancer

Cancer is a family of genetic diseases resulting from uncontrolled cell division and tissue invasion (metastasis). The inability to regulate cell proliferation and an increase in metastasis is caused by alterations in the genome and/or epigenome of cancer cells. Over time, somatically acquired abnormalities in DNA sequences can lead to alterations in the cancer cell genome. Somatic mutations according to their contribution to cancer development can be divided into driver or passenger mutations. Driver mutations possess growth advantages to cancer cells and are often positively selected during the evolution of the cancer. These mutations tend to cause clonal expansion of cancer cells. However, passenger mutations are present in the cancer genome as a by-product of cancer cell development and therefore do not often contribute to the development of cancer, although may be associated with a clonal expansion, usually caused by driver mutations (Alberts *et al*., 2002, Hanahan and Weinberg, 2000, Futreal *et al*., 2001, Stratton *et al*., 2009, Berdasco and Esteller, 2010).

There is a growing body of evidence from recent studies of epigenetic mechanisms in cancer that highlights evidence supporting the idea that cancer is not solely a consequence of genetic alteration of the cancer-critical genes. Cancer cells have a different epigenome compared to their normal cell counterparts. For example, hypo-methylation of cancer cells has been one of the first epigenetic alterations found in human cancers (Berdasco and Esteller, 2010). The development of cancer is a multi-step process that requires the accumulation of different mutations during the lifetime of any cancer patient. During cancer, cells acquire genetic alterations that progressively transform previously normal cells into more malignant ones (Figure 1.1) (Alberts *et al*., 2002, Hanahan and Weinberg, 2000, Stratton *et al*., 2009). Cancers however, can be classified into various types according to their original cell: carcinoma (epithelial cell), glioma (glial brain cell), sarcoma (mesenchymal cell), lymphoma and leukaemia (bone marrow and blood cells), mesothelioma (mesothelial cells that cover the peritoneal and pleural cavities), choriocarcinoma (placenta), and germinoma (germ cell of the testes or ovary)(Alberts *et al*., 2002).

1.2 Cancer critical genes

These include all genes whose mutations eventually contribute to the tumorigenicity of the cell. Mutated genes however are divided into two categories according to their function. Cancer risks arise either from the activation or the inactivation of cancer-critical genes. Genes of the first category, where a gain-of-function mutation may drive a cell towards cancer are also called proto-oncogenes; where their mutant forms are known as oncogenes. Genes of the second category, where a loss-of-function mutation impacts normal cellular mechanisms, are defined as tumour suppressor genes (Alberts *et al*., 2002, Frei and Antman, 2003).

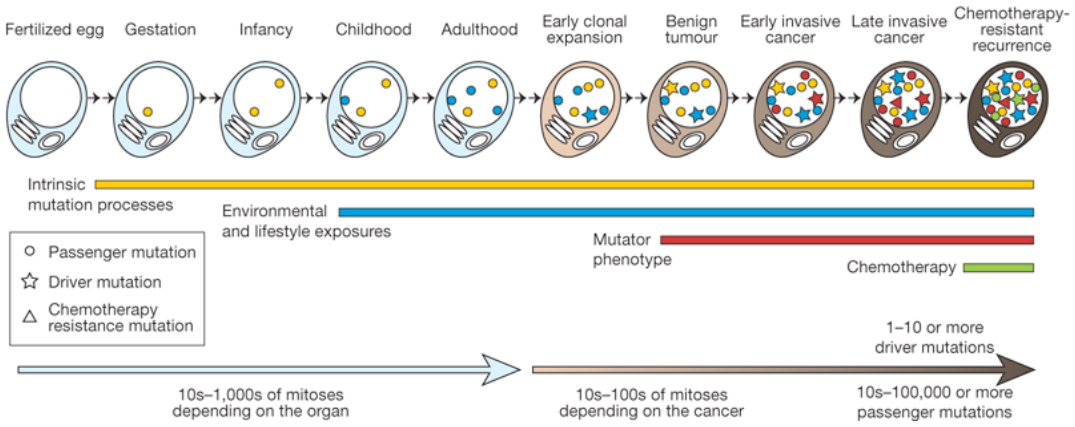

Figure 1.1 *A schematic diagram depicting somatic mutations that occur from a fertilized egg to a single cell within a cancer. Mutations accumulate due to the intrinsic mutation rate during normal cell division and the formation of mutations due to exogenous mutagens. Passenger mutations may be acquired as the cell lineage is phenotypically normal, however, driver mutations cause a clonal expansion and resistance to chemotherapy (Stratton et al., 2009).*

1.3 Discovery of oncogenes

In the 1960s research had discovered that some animal cancers were caused by viruses. This led to the discovery of the very first oncogene from the Rous Sarcoma Virus (RSV) called *v-src* (the viral oncogenes are called v-oncogenes) in 1970. The studies around the RSV revealed that RSV did not require *v-src* gene for its replication. After this however, studies began to show that *v-src* was

homologous to a host cellular gene (*c-src*) widely conserved in eukaryotic species. Studies from other transforming retroviruses from other species have since then led to the discovery of different retroviral oncogenes. These retroviral oncogenes are copies of normal cellular genes, the proto-oncogenes, that are captured from the genome of the host through a process known as retroviral transduction (Frei and Antman, 2003, Duesberg and Canaani, 1970, Martin, 1970).

1.4 Function of oncogenes

Proto-oncogenes encode proteins that control cell proliferation or apoptosis. They are activated to become oncogenes by alteration of their structure or amplification of genes. The activated oncogenes, capable of inducing neoplastic phenotypes, can be divided into six groups based on function and biochemical characteristics of the protein products of their normal counterparts (proto-oncogene): chromatin remodelers, growth factors, transcription factors, growth factor receptors, signal transducers and apoptosis regulators (Frei and Antman, 2003, Croce, 2008).

1.5 Activation of oncogenes

Oncogenes are activated by specific genetic alterations that generally involve a gain of function. This is often mediated through three main genetic mechanisms in human neoplasms: (1) mutation, (2) gene amplification, and (3) chromosome rearrangements. Some of these changes may lead to changes in the structure of the proto-oncogene or lead to the deregulation of expression (Holland-Frei 2003). Various mutations occur, base substitutions, deletions, and insertions for example, activate proto-oncogenes through alterations in the structure of their encoded proteins. Alterations, usually impact critical regulatory regions of a specific protein and may often enhance the transforming activity of the mutated protein (Bishop, 1991). Point mutations in key codons, are frequently detected in the RAS family of proto-oncogenes. RAS encodes a specific protein that remains in the active state, leading to a continuous signal induction. The signal transduction however, is responsible for inducing continuous cell growth

(Rodenhuis and Slebos, 1992). Cancer cells contain several copies of structurally normal oncogenes. An increased copy of any of these specific genes due to some genomic changes is called gene amplification, initially discovered in tumour cells, which acquires a resistance to anti-growth treatments. The process of gene amplification often creates chromosomal abnormalities, double-minute chromosomes (DMs) and homogeneously staining regions (HSRs) (Frei and Antman, 2003, Keung *et al*., 1997). High frequency of DMs and HSRs in human tumours suggests the amplification of specific proto-oncogenes may be a common occurrence in cancer malignancies. Studies have recently shown that three proto-oncogene families including MYC, ERBB, and RAS are often found amplified in many human tumours (Keung *et al*., 1997).

Chromosomal rearrangements are a lot more common in haematological malignancies than solid tumours. Cytogenetic abnormalities consist mainly of chromosomal translocations. Chromosomal rearrangements have been shown to increase or deregulate transcription of oncogenes or proto-oncogenes by transcriptional activation of proto-oncogenes or the creation of fusion genes. Transcriptional activation for example, occurs when a proto-oncogene is moved adjacent to an immunoglobulin or T-cell receptor gene. In doing so, the regulatory elements of the immunoglobulin or T-cell receptor locus may control the transcription of the proto-oncogene (Frei and Antman, 2003). For example, the t(8;14)(q24;q32) translocation found in Burkitt lymphoma is to date one of the most studied examples of proto-oncogene activation. This chromosomal rearrangement occurs when the c-Myc gene controls regulatory elements of the immunoglobulin heavy chain locus (Siebert *et al*., 1998, Aspland *et al*., 2001). Gene fusions may often form chimeric transcription factors, like the E2A/PBX1 fusion protein found in childhood pre-B-cell ALL is a t(1;19)(q23;p13) translocation (Aspland *et al*., 2001).

1.6 Tumour-suppressor genes

The discovery of many oncogenes has fuelled the idea that different classes of genes must exist to elicit tumour-preventing functions. Somatic cell fusion and chromosomal segregation experiments have

confirmed the existence of genes involved in tumour suppression (Stanbridge EJ, 1976). Over the years many tumour suppressor genes have been identified. Interestingly, these prototypic tumour suppressor genes were thought to be recessive and follow the "two-hit hypothesis" proposed by A.G. Knudson (Knudson, 1971). This then, well-respected hypothesis that implies that biallelic gene inactivation is in fact required before an effect is observed in cancer cells (Knudson, 1971) has been a little outdated. Further studies have shown however, that not all tumour suppressor genes follow this well-known hypothesis. Other tumour suppressors are haplo-insufficient for tumour progression; meaning that the inactivation of a single tumour suppressor gene by mutation, deletion or methylation-mediated transcriptional silencing may give a selective advantage during tumorigenesis. For example: inactivation of single alleles of genes encoding PREP1, p53, TGF, 27KIP1 and DMP1 can be sufficient to predispose mice to tumour development (Krimpenfort *et al*., 2001, Balmain *et al*., 2003). Over 30 tumour suppressors have been identified all of which appear to control a broad range of critical yet highly conserved normal cellular functions, including: signal transduction, cell differentiation, cell cycle checkpoint control, apoptosis, control of genomic integrity, repair of DNA damage and adhesion, and angiogenesis. These functions can be deregulated in cancer cells (Balmain *et al*., 2003). For this reason, tumour suppressor genes are generally divided into gatekeepers, caretakers and landscapers based on primary functions (Macleod, 2000). This "Gatekeeper" term was first proposed in an attempt to explain the role of adenomatous polyposis coli tumour suppressor gene, consistently found mutated in colorectal tumorigenesis. Gatekeeper genes encode proteins that act directly to inhibit tumour growth by either suppressing proliferation, inducing apoptosis or by promoting differentiation. Since the loss of function of these genes is the rate-limiting event in tumorigenesis, restoration of their function suppresses neoplasia. Individuals who may have a hereditary mutation in one of the two alleles of a gatekeeper gene are predisposed to neoplasia (Macleod, 2000).

"Caretaker" genes have been shown to help maintain genomic stability by encoding proteins in DNA repair and mitotic checkpoint pathways like *MLH1, BRCA1, MYH, and XPA*. These genes indirectly suppress cell proliferation ensuring the integrity of DNA. Caretaker genes do not directly contribute to the development of cancer; however, their loss of function increases the DNA mutation rate, raising the

probability that gatekeeper gene function will be altered. Cancer risk is not easily quantified although research into overall survival (OS) with drug treatments are currently one of the best ways to quantify this. These alterations increase cancer development risk from 5 to 50-fold (Macleod, 2000). The products of the third class of tumour suppressor genes named the "landscapers", act by modulating their microenvironment and the microenvironment of the tumour cells. These genes are known to regulate extracellular matrix proteins, cell surface receptors, adhesion proteins and secreted growth factors. Loss of function mutations of landscapers can generate an abnormal microenvironment prompting the neoplastic transformation of the adjacent epithelia (Macleod, 2000).

1.7 The hallmarks of cancer

18 years ago, Hanahan and Weinberg (2000) described the six "Hallmarks of Cancer" which at that time provided a logical framework summarizing and understanding several decades of research dedicated to cancer. These six common traits or "Hallmarks" that are thought to govern the transformation of normal cells to cancer cells, are essential for cells to acquire a cancer phenotype in a multi-step process (Hanahan and Weinberg, 2011). In 2011, two emerging hallmarks were added to this list due to the conceptual progress in research in the last decade. The complexity of upwards of 100 different types of human cancers often arises from disruption of the distinct regulatory circuits of cells that govern normal cell proliferation and homeostasis. Hanahan and Weinberg proposed that this complexity can often be explained by a small number of common traits between most, if not perhaps all types of human tumours (Figure 1.2)(Hanahan and Weinberg, 2011).

1.7.1 Sustaining proliferative signals

Tumours arise from unconstrained proliferation of cells that harbour either oncogenic activating or tumour suppressor inactivating mutations. Therefore, uncontrolled proliferation is the most fundamental feature of cancer development (Cheng *et al.*, 2005). Normal cells require mitogenic growth

signals to evolve from a quiescent state and enter a proliferation state. Growth-promoting signals that are released from cells are transmitted through transmembrane receptors to their neighbouring cells. Cell proliferation relies on the important availability of growth promoting signals with normal cells stopping proliferation in the absence of these signals. Cancer cells may develop a few ways when growing and proliferating independent of the absence of exogenous mitogenic signals (Hanahan and Weinberg, 2011) as seen in Figure: 1.2. Some cancer cells, however, produce their own growth factors. The production of tumour growth factor and platelet-derived growth factor (PDGF) by sarcomas and glioblastomas, are two very important examples (Frei and Antman, 2003). They also stimulate normal cells that are present in the tumour microenvironment and produce growth factors (Cheng *et al*., 2005). Cell surface receptors that bind to the growth factors and transmit the growth signals inside cells are overexpressed in many cancers. The increase in the number of these receptors makes cancer cells hypersensitive to minimal amounts of the growth factor which would normally be unable to trigger proliferation (Frei and Antman, 2003). For example, *HER2/NEU* is overexpressed in stomach and mammary carcinomas. Structural alterations in the receptor may also lead to ligand-independent signalling and growth factor autonomy can be achieved by activation of components of signalling pathways that operate downstream of these receptors (Medema *et al*., 1993).

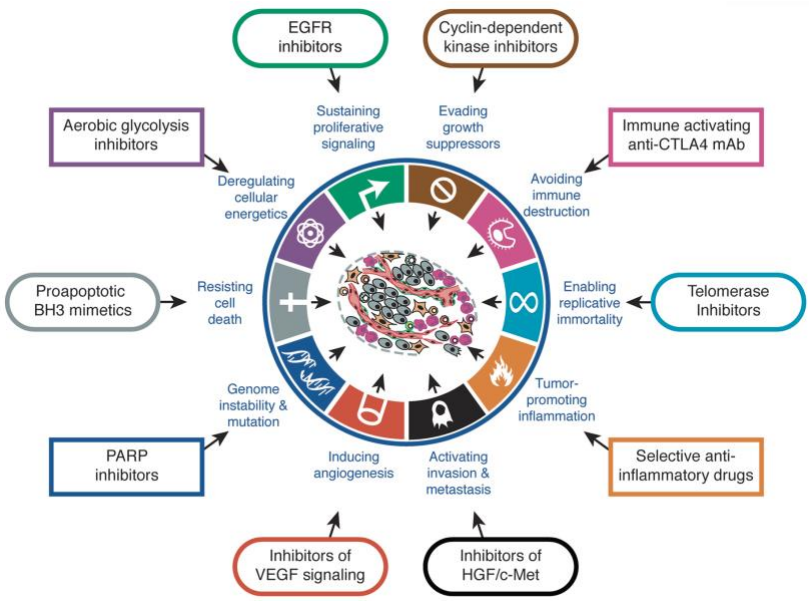

Figure 1.2 *A schematic diagram depicting the newest and updated version of "The Hallmarks of Cancer" (Hanahan and Weinberg, 2011).*

1.7.2 The evasion of growth suppressors

Cells have evolved mechanisms to control proliferation and tissue homeostasis by manipulating positively and negatively acting growth signals. Like the growth-promoting signals mentioned previously, the growth-inhibitory signals may be transmitted through transmembrane cell surface receptors. These signals act by blocking proliferation by either transiently forcing cells out of the proliferative state into the quiescent state or by preventing proliferative ability, which drives cells into a post-mitotic state like quiescence. Inactivation of tumour suppressors often conveys various capabilities to cancer cells to evade the anti-proliferative signals (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). RB (retinoblastoma-associated) and p53 proteins are typical examples of key tumour suppressors that control cell proliferation or activation of senescence and apoptosis programs. These tumour suppressor genes are frequently inactivated in cancer by loss of function mutations (Burkhart and Sage, 2008, Hupp *et al*., 1992, Sherr and McCormick, 2002).

1.7.3 Resistance to cell death

Normal tissues can maintain their homeostasis by balancing rates of cell proliferation and cell death. Programmed cell death or apoptosis, plays a major role in maintaining cell population in different tissues. Apoptosis may be triggered by either an extrinsic pathway mediated by cell surface death receptors bound by extracellular ligands, or by an intrinsic pathway mediated by the mitochondria. The second, is triggered in response to different extracellular and intracellular stresses, like: growth factor depletion, hypoxia, DNA damage and oncogene induction. The ability of transformed cancer cells to bypass this apoptotic barrier is widely known in the pathogenesis of cancer. Tumour cells may develop different strategies to escape apoptosis the most common of these is the loss of the p53 tumour suppressor, which increases expression of anti-apoptotic regulators and survival signals or down regulating the pro-apoptotic signals (Hanahan and Weinberg, 2011, Adams and Cory, 2007).

1.7.4 Enabling replicative immortality

Normal cells have a finite capability of replication and therefore may be able to pass through a limited number of cell growth and division cycles. Mammalian cells whilst in culture, stop growing and go into senescence after around 60-70 doublings (Burkhart and Sage, 2008, Hupp *et al*., 1992, Sherr and McCormick, 2002). Some cells succeed and bypass this barrier, going into a crisis phase, involving apoptosis and karyotypic abnormalities. Rarely however, cells from a specific population in crisis acquire indefinite replicative potential. This immortalization is one of the characteristics of the tumour cells (Wright *et al*., 1989). In non-immortalized cells, telomeres responsible for protecting the ends of chromosomes progressively shorten with each cell division, often leading to the end-to-end fusions of chromosomes, karyotypic disarray, crisis and eventually cell death. Telomerase is responsible for maintaining telomeric DNA and is mainly absent in non-immortalized cells, however it is expressed at high level in a large proportion of immortalized cells including human cancer cells (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

1.7.5 Induction of angiogenesis

The induction and sustaining of angiogenesis are crucial for the growth of all tumours. In the adult, angiogenesis is only transiently turned on due to physiological processes such as wound healing. However, in neoplastic growth an "angiogenic switch" can be activated and may remain permanently on, forcing the normal quiescent vasculature to generate new vessels and help expand tumour growth by supplying oxygen and nutrients. The activation of this angiogenic switch is mediated when changing the ratio of angiogenic inducers versus inhibitors. Vascular endothelial growth factor (VEGF) induces angiogenesis and can be over expressed in tumours compared to their normal tissue counterparts. Alternatively, the angiogenic inhibitor thrombospondin-1, can be positively regulated by p53 tumour suppressor protein, and is down regulated in tumours (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

1.7.6 Invasion and metastasis

Metastasis, the dissemination of cancer cells from a primary site to an adjacent or distant organ, which is the leading cause of death in patients with solid cancers. Invasion and metastasis are both multistep processes beginning with local invasion followed by intravasation of cancer cells in nearby vessels and circulation of these cells through the lymphatic and haematic systems (Figure 1.3). This often results in the extravasation of cancer cells to distant tissues and eventually with the formation of micro-metastatic lesions. At a molecular level, proteins involved in cell-to-cell and cell-to-matrix adhesion are important. E-cadherin is an important Ca(2+)-dependent cell-to-cell adhesion molecule and plays an important role as a key suppressor of metastasis. This protein along with various other adhesion molecules involved in cell-to-matrix adhesions is often down regulated in cells possessing invasive or metastatic capabilities (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

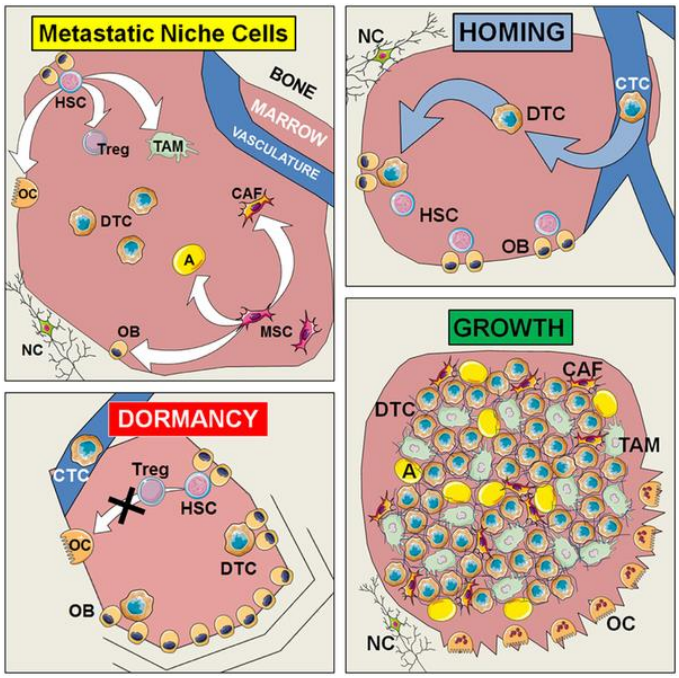

Figure 1.3 *A schematic diagram depicting development of a 'metastatic niche' due to the interaction of some disseminated tumour cells with that of the bone marrow micro-environment. Evidence supports the hypothesis that tumour cell behaviour is often dependent on the surrounding micro-environment, suggesting that the micro-environment in distant tissues, like bone, may be essential for disseminated tumour cell (DTC) survival and metastatic growth (Shiozawa et al., 2011).*

1.7.7 Reprogramming of energy metabolism

During normoxic conditions, cells process glucose through glycolysis followed by oxidation of pyruvate in the mitochondria of the cells. In anaerobic conditions however, glycolysis occurs in the cytosol of the cell and is shortly followed by lactic acid fermentation. Malignant cells usually limit their energy metabolism to glycolysis, even when under normoxic conditions. This phenomenon is known as the Warburg-effect (Liberti and Locasale, 2016). To date there are several hypotheses to explain the Warburg-effect, for example mitochondrial damage, adaptation to hypoxic environments, and the shutdown of mitochondria due to their involvement in apoptosis. Glycolysis can provide most of the intermediates that are necessary to produce nucleosides and amino acids, facilitating biosynthesis of the macromolecules and organelles that are required for active cell proliferation. Cancer cells often switch metabolic pathways to anaerobic glycolysis and support the uncontrolled, continuous cell proliferation (Hanahan and Weinberg, 2011).

1.7.8 Evading the immune system

There is currently a lot of evidence suggesting that the immune system plays an important role in the recognition and eradication of malignant cells. The cancer immunosurveillance theory suggests that immune cells constantly monitor cells and tissues recognizing and eliminating continuously arising nascent transformed cells by immunoediting. Immunoediting is an important process, responsible for protecting the individual from cancer growth and development of tumour immunogenicity. Immunoediting is composed of three main phases: elimination, equilibrium, and escape. Although it has been observed that both innate and adaptive immune systems contribute to immunosurveillance, many tumours escape the immune barrier and drive immunological tolerances. This has the potential to lead to tumour progression by mimicking immune signalling pathways that impact the tumour microenvironment and activate immunosuppressive cells such as regulatory T (Treg) and myeloid-derived suppressor cells (MDSCs) (Hanahan and Weinberg, 2011).

1.8 Transcription factors

Transcription factors are sequence-specific DNA binding factors responsible for the regulation of the transcription of target genes in regulatory regions such as promoters or enhancers (Mitchell and Tjian, 1989). Most of the 2600 proteins in our human genome containing DNA-binding domains are thought to act as transcription factors. Almost 10% of the protein-coding genes in our genome encode proteins which are known to be responsible for the regulation of transcription, making this family the single largest family of human proteins (Babu *et al*., 2004, Levine and Tjian, 2003). Multigene families of some transcription factors share common DNA-binding domains, for example: zinc finger, leucine-zipper, helix-loop-helix and homeodomain motifs (Pabo and Sauer, 1992). Transcription factors often act in a complex binding to other proteins and operate as the final link in a signal transduction pathway that translates cellular signals by altering gene expression (Alberts 2002). However, proto-oncogene transcription factors were initially discovered from their retroviral homologs. Chromosomal translocations are often responsible for the activation of transcription factors in haematological and solid malignancies, for example proto-oncogenic transcription factors include HOXA9, MEIS1, PBX1, Erb, Ets, Fos, Jun, Myb, and c-Myc. Relevant to this work is the HOXA9 transcription factor known to cooperate with the MEIS1 transcription factor in the induction of AML (Kroon *et al*., 1998, Mamo *et al*., 2006, Thorsteinsdottir *et al*., 2001). Equally, E2A-PBX1 chimeric protein resulting from chromosomal translocation of the basic helix-loop-helix transcription factor E2A with the gene encoding the homeodomain protein PBX1 has been shown to cause pre-B cell acute lymphoblastic leukaemia (ALL) (Aspland *et al*., 2001).

1.9 AML

In the western world, acute myeloid leukaemia (AML) accounts for approximately 25% of all leukaemias in adults. According to the World Health Organisation (WHO) there is an incidence of 3.7 per 100,000 people with a mortality rate of 2.7, rising to almost 18 per 100,000 people within the older age groups. Rarely diagnosed before 40 years of age, with newly diagnosed cases having a median age

of 65 (<65 years incidence 1.8 per 100.00, >65 years incidence 17 per 100.000). Complete remission can be achieved in 60%-80% of younger adults, however, treatment related mortality (TRM) can vary considerably, between 15% to 50%, and maintaining a balance between remission and TRM is vital. This is particularly relevant for those patients ages >60, who are more likely to show increased incidence of resistance to therapy and TRM.

	Acute	Chronic
Myeloid origin	Acute myeloid leukaemia	Chronic myeloid leukaemia
Lymphoid origin	Acute lymphoid leukaemia	Chronic lymphoid leukaemia

Table 1.1 *A table depicting the current and simple classification of leukaemia.*

Acute myeloid leukaemia is a heterogeneous group of genetically and phenotypically aggressive disorders in which several acquired somatic genetic changes accumulate and result in differentiation blockage of human progenitor cells, increasing the self-renewal ability of cells and disturbing the normal regulation of proliferation (Fröhling *et al*., 2005). Non-random chromosomal alterations, such as balanced translocations, monosomies, trisomies, inversions and deletions have been found in the leukemic blasts in approximately 55% of AML patients. These chromosomal aberrations are essential in the classification of AML and in the past they have been considered to be the most crucial prognostic factor for cure rates, possibility of relapse, and overall survival (Estey and Döhner, 2006, Mrózek *et al*., 2007). Recently, due to advances in molecular diagnosis, several gene alterations and specific dysregulation of gene expression has also been recognized. This has helped to clarify the numerous heterogeneities of AML subsets, particularly AML (Döhner and Döhner, 2008a) and furthered the understanding of the molecular mechanisms of leukaemogenesis. However, to date there is no current evidence for a direct molecular link between a person's age and their risk of developing AML, although several age associated potential haematological factors have been identified. Interestingly, some of these include myelodysplastic syndromes (MDS) and myeloproliferative neoplasms (MPN), which like AML, are also classified by the WHO and are an extremely heterogeneous group of haematological

disorders. MDS and MPN are clonal haematological disorders characterized by ineffective haematopoiesis, bone marrow dysplasia in MDS or hyperplasia in MPN, frequently progressing to AML 15-52% and 7% respectively, incurring a poor prognosis. In MPN, Hematopoietic Stem Cell (HSC) dysregulation occurs due to failure of normal cytokine feedback or hypersensitivity. This often results in the production of excessive blood cells and involves the three main myeloid lineages; erythroid, megakaryocytic/platelet and granulocytic, predominantly affecting only one lineage (Döhner and Döhner, 2008a). In MDS however, several factors are believed to contribute to the molecular pathology of this disorder, including increased HSC apoptosis, defective stroma, altered response to cytokines and chromosomal abnormalities including deletions of chromosome 5q31–q32. *HOXA9* and *HOXA7* are both often linked to the status of HSC underlying MDS and AML, either through overexpression or reciprocal translocations (e.g. NUP98-*HOXA9*) although very little, if any profile of *HOX* expression has been explored for MPN (Estey and Döhner, 2006, Mrózek *et al*., 2007).

1.10 Subtypes for AML

The diagnosis and classification of AML since its discovery has proven challenging. In most cases, the presence of specific morphologic features such as auer rods or specific markers can distinguish AML from other leukaemias. In the absence of these features, diagnosis may be a little more difficult. The two most commonly used classifications to date are the French-American-British (FAB) system and the newer WHO system.

The French American–British (FAB) classification system is divided into eight AML subtypes, M0 through to M7, based on the type of cell from which the leukaemia has developed and the degree of maturity (Table 1.2). This is analysed by examining the appearance of the malignant cells via light microscopy and by using cytogenetics to then characterize any underlying chromosomal abnormalities. The subtypes have varying prognoses and responses to therapy. These eight FAB subtypes were proposed in 1976 (Bennett *et al*., 1976). FAB classification requires a blast percentage of at least 30%

in the bone marrow (BM) or the peripheral blood (PB) for diagnosis of AML (Amin *et al*., 2005). It is important that AML is differentiated from "pre-leukemic" conditions such as myelodysplastic syndrome or myeloproliferative neoplasm, that have very different treatment options.

Type	***Name***	***Cytogenetics***	***Percentage***
M0	Acute myeloblastic leukaemia, minimally differentiated		5%
M1	Acute myeloblastic leukaemia without granulocytic maturation		15%
M2	Acute myeloblastic leukaemia, with granulocytic maturation	T(8;21)(q22;q22), t(6;9)	25%
M3	Promyelocytic, or acute promyelocytic leukaemia (APL)	t(15;17)	10%
M4	Acute myelomonocytic leukaemia	Inv(16)(p13q22) del(16q)	20%
M4eo	Myelomonocytic with bone marrow eosinophilia	inv(16), t(16;16)	5%
M5	Acute monoblastic leukaemia (M5a) or acute monocytic leukaemia (M5b)	Del(11q),t(9;11), t(11;19)	10%
M6	Acute erythroid leukaemia (M6a) (M6b)		5%
M7	Acute megakaryoblastic leukaemia	T(1;22)	5%

Table 1.2 *A table depicting the acute myeloid leukaemia classification according to the French-American-British classification scheme*.

World Health Organisation (WHO): classification of acute myeloid leukaemia that was published in 2008, aims to be more clinically useful and aims to produce more meaningful prognostic information than the FAB criteria. The WHO classification has various descriptive subcategories however, most of the clinically significant information of this WHO schema can be organised via categorization into one of the subtypes listed below (Table1.3). The WHO diagnosis of AML can be established by

demonstrating involvement in more than 20% of the blood and/or bone marrow by leukemic myeloblasts, except three best prognosis forms of AML that have recurrent genetic abnormalities (t(8;21), inv(16), and t(15;17)) of which the presence of genetic abnormality is now diagnostic irrespective of blast percentage (Harris *et al.*, 1999, Foucar *et al.*, 1982). The WHO

Categories
AML with recurrent genetic abnormalities
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); *CBFB-MYH11*
APL with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML (megakaryoblastic) with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated NPM1
Provisional entity: AML with mutated CEBPA
AML with myelodysplasia-related changes
Therapy-related AML
AML, not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Acute erythroid leukemia
Pure erythroid leukemia
Erythroleukemia, erythroid/myeloid
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Down syndrome related AML
Transient abnormal myelopoiesis
Myeloid leukemia associated with Down syndrome

Table 1.3 *A table depicting the current acute myeloid leukaemia classification according to the WHO classification scheme (WHO 2008)*

1.11 Pathophysiology of AML

The myeloblast is the malignant cell in AML which in haematopoiesis, is an immature precursor of myeloid white blood cells. Normally a myeloblast gradually matures into a mature white blood cell.

However, in AML, a single myeloblast accumulates genetic changes halting the cell in an immature state that prevents differentiation (Fialkow, 1976). This mutation alone does not cause leukaemia, however when a "differentiation arrest" happens in conjunction with other mutations that disrupts proliferation control, the result is an uncontrolled growth of immature cells leading to AML (Fialkow, 1976). There has been a large diversity and heterogeneity of AML identified since leukemic transformation may occur at several different steps along the differentiation pathway (Bonnet and Dick, 1997). Classification schemes for AML recognize the characteristics and behaviour of leukemic cells and may depend on the stage at which differentiation was halted (Figure 1.5, Figure 1.6).

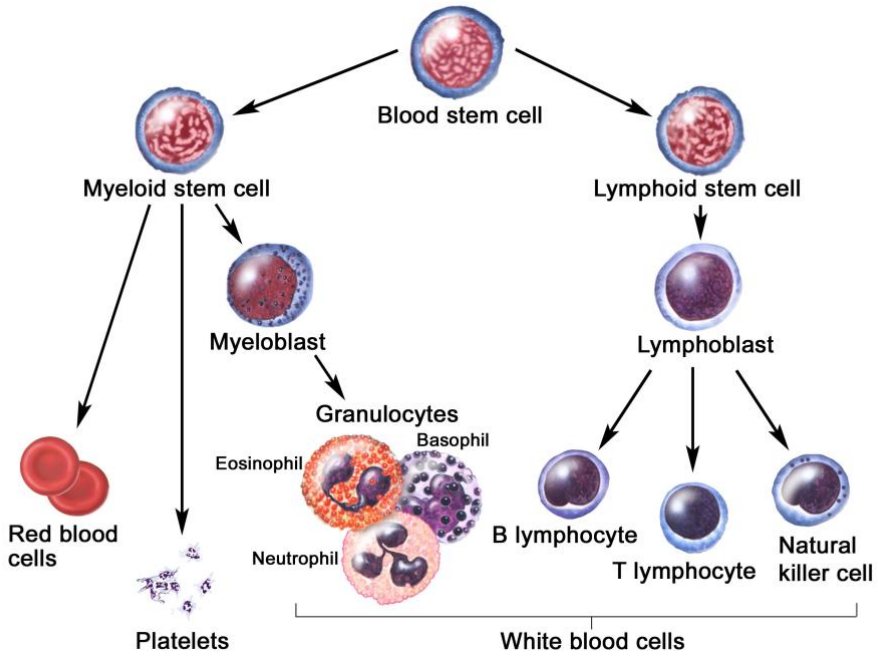

Figure 1.4 *A schematic diagram depicting blood cell development, showing the steps a blood stem cell goes through to become either a red blood cell, platelet, or white blood cell. This also shows a lymphoid stem cell becoming a lymphoblast and then one of several different types of white blood cells (Winslow 2007).*

Cytogenetic abnormalities may be found in people with AML, with the types of chromosomal abnormalities usually have a prognostic significance (Abeloff and Martin 2004). Chromosomal translocations encode abnormal fusion proteins that are usually transcription factors whose altered properties may lead to "differentiation arrest" (Greer *et al*., 2004). For example, in acute promyelocytic leukaemia, the t(15;17) translocation mutation is responsible for producing a PML-RARα fusion protein known to bind to the retinoic acid receptor element in the promoters of several myeloid-specific genes inhibiting myeloid differentiation (Melnick and Licht, 1999). Clinical signs and symptoms of AML result from growth of leukemic clone cells, that tends to displace and interfere with the development of normal blood cells in the bone marrow (Martin 2004). Disturbances like these often lead to neutropenia, anaemia, and thrombocytopenia. Symptoms of AML are often due to low numbers of these normal blood elements. In some rare cases however, people with AML may develop a chloroma, or solid tumour made up of leukemic cells that form outside the bone marrow. This can cause symptoms depending on location (Dally *et al*., 2005). An extremely important pathophysiological mechanism of leukaemogenesis in AML is the epigenetic induction of dedifferentiation by some genetic mutations that may alter the function of epigenetic enzymes. Some examples of these include the DNA demethylase TET2 and the metabolic enzymes IDH1 and IDH2 (Molenaar *et al*., 2015), which may lead to the generation of a novel oncometabolite, *D*-2-hydroxyglutarate and inhibits activity of some epigenetic enzymes such as TET2 (Molenaar *et al*., 2014). The general hypothesis is that such epigenetic mutations often lead to the silencing of tumour suppressor genes and/or the activation of proto-oncogenes.

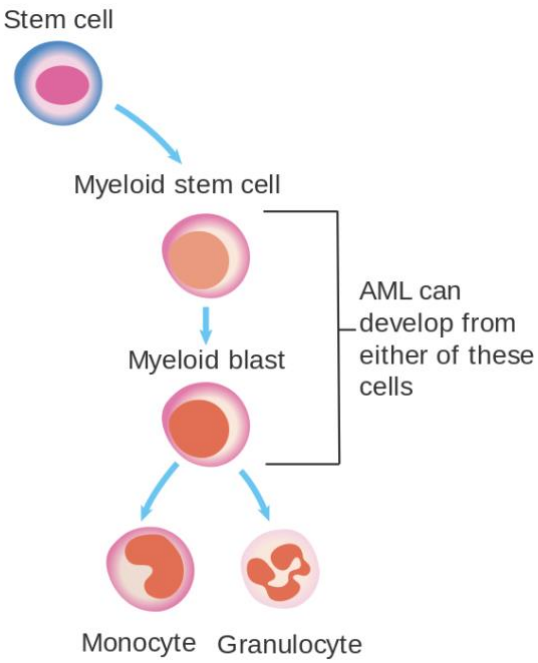

Figure 1.5 *A schematic diagram depicting the development of acute myeloid leukaemia both from either an abundance of myeloid stem cells and/or myeloid blast cells (Winslow 2007).*

1.12 AML and mutations

There has been a widely accepted hypothesis based on data obtained from animal models and human diseases over recent years termed, "the two-hit model hypothesis". The idea behind this hypothesis is that there are two types of genetic changes that may occur and are known to be involved in AML emergence. Molecular mutations in AML are divided into classes. The first class, Class I consists of mutations that enhance proliferation signal transduction pathways and induce the proliferation of HSC's or HPC's and usually affect kinase signalling pathways, such as FLT3, KIT, NRAS/KRAS, RAS/MAPK and JAK/STAT mutations. These Class I mutations take place later on and cause disease progression. Class II mutations on the other hand, consist of target hematopoietic transcription factor genes that may lead to a block in myeloid differentiation and give the self-renewal ability of HPC's. These mutations take place early on and initiate the AML disease (Döhner and Döhner, 2008b, Renneville et al., 2009, Betz and Hess, 2010). In fact, one set of the most affected transcription factors

are the *HOX* genes. Consequently, over-expression of *HOX* genes, which play significant roles in development of embryogenesis, organogenesis as well as normal differentiation of HPC's, is a frequent characteristic of AML (Ferrando *et al*., 2003). Class I mutation mainly cause changes in kinase signalling pathways and this occurs in the later stage of AML, leading to disease progression. The class II mutations take place in the early stages causing initiation of AML disease. *HOX* genes are the main type of transcriptional factors mutated in AML (Figure 1.7) (Bloomfeild, Small, & Stock, 2006).

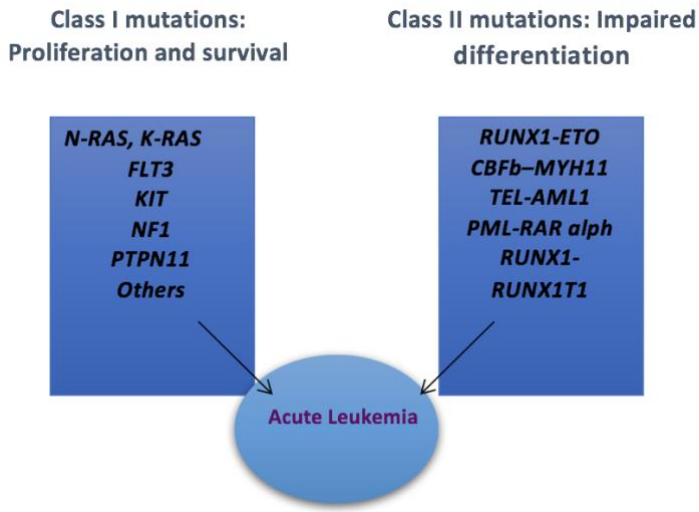

Figure 1.6 *A schematic diagram depicting both Class I (proliferation and survival) mutations and Class II (impaired differentiation) mutations that may lead to acute myeloid leukaemia.*

1.13 Current treatment

The current curative treatment for AML starts with chemotherapy. The aim of the first cycle, induction chemotherapy, is to achieve complete remission (CR), defined as microscopical disappearance of cancer cells from blood and bone marrow (Dahlstrom *et al*., 2011). Following induction therapy, consolidation therapy is administered the aim of which is to eliminate any remaining leukemic cells (Dahlstrom *et al*., 2011). Approximately 71% of patients can achieve CR. Unfortunately however, many patients relapse, explaining why long-term survival remains as poor as 22–32% despite the high rate of CR (Vardiman *et al*., 2009). In addition to chemotherapy, some patients receive allogenic stem cell

transplantation (Dahlstrom *et al*., 2011). Associated with this treatment is significant mortality and morbidity (Dahlstrom *et al*., 2011), making the choice of whether to proceed with a transplantation something to carefully consider. The risk of relapse, the availability of a matching donor and the overall general condition of the patient are considered (Dahlstrom *et al*., 2011). Knowledge of the genetic changes and the mechanisms behind AML has increased significantly (Vardiman *et al*., 2009), which is an important step towards the identification of new and more specific targets for therapy. However, the prognosis remains poor and there is still need for a better understanding of the mechanisms behind the disease and how these can be exploited to improve patient outcome.

1.14 Myelodysplastic syndromes

MDS or the myelodysplastic syndromes are a large group of clonal hematopoietic stem cell disorders previously classified as "pre-leukaemia" often characterized by ineffective haematopoiesis, peripheral blood cytopenias, dysplasia, and a propensity for the transformation to acute myeloid leukaemia. Patients who develop MDS often go on to develop severe anaemia and require blood transfusions. In some cases, the disease progresses and worsens and the patient may go on to develop cytopenias or a low blood count caused primarily by progressive bone marrow failure (Mufti *et al*., 2010). The overall outlook for patients with MDS often depends on the severity and type of the disease. Very often however, many live very normal life spans with the disease, and many are often asymptomatic for MDS until detected in a routine blood test. These MDS are all disorders of the HSCs in the bone marrow. Notably, in MDS, haematopoiesis is ineffective and disorderly with the number and quality of blood-forming cells declining irreversibly, further impairing blood production.

1.15 Myeloproliferative neoplasms

Myeloproliferative neoplasms (MPN), previously myeloproliferative disorders, are diseases of the bone marrow from which excess cells are produced. In the more recent WHO classification of hematologic

malignancies, these diseases have been renamed from "myeloproliferative diseases" to "myeloproliferative neoplasms" (Tefferi *et al*., 2011, Vainchenker *et al*., 2011), better reflecting the underlying clonal genetic changes that are a very salient feature of this group of diseases giving a far more accurate description of the disease itself. These malignancies are often characterized by a clonal proliferation of one or more of the hematopoietic cell lineages, often in the bone marrow as well as the liver and spleen. The discovery of myeloproliferative disease/neoplasms came when they were first proposed in the 1950's by the eminent haematologist William Dameshek (Dameshek, 1951). In contrast to myelodysplastic syndromes however, MPNs demonstrate a terminal myeloid cell expansion into the peripheral blood. In 2008, a revision of the WHO classification of MPNs. MPNs included: chronic myelogenous leukaemia (CML), chronic neutrophilic leukaemia, polycythemia vera (PV), primary myelofibrosis (PMF), essential thrombocythemia (ET), chronic eosinophilic leukaemia, mastocytosis, and unclassifiable MPNs. Overlap disorders between MDSs and MPNs are those chronic myeloid disorders unable to be classified as "classic" MPN or MDS. These include: chronic myelomonocytic leukaemia (CMML), atypical CML, juvenile myelomonocytic leukaemia, and unclassifiable MDS/MPN. Over the last few years work has been done to highlight a link between myeloproliferative neoplasms and their ability to evolve into MDS and most significantly for the work presented here into AML. Although this is true, the MPDs overall have a much better prognosis than both MDS and acute myeloid leukaemia. MDS and other "pre-leukaemic" diseases will be discussed.

1.16 *HOX* genes

Homeobox-containing proteins are a highly expressed family of proteins, the genes of which encode DNA-binding transcription factors (Garcia-Fernandez, 2004). These genes have been extensively studied since they were first discovered in Drosophila as master regulators of the trunk and the tail during embryogenesis (Shah and Sukumar, 2010). Genetic duplication of *HOX* genes in Drosophila results in eight genes in a single cluster, while in mammals the genetic duplication produces 39 genes divided into four different chromosomes, *HOXA, HOXB HOXC and HOXD* (Rice and Licht, 2007).

The four paralogs or four clusters are produced because of the original gene in the first cluster. Within these clusters, the same paralogous group will show genetic homology and therefore has functional redundancy and high sequence similarity as seen in a more recent review, He *et al*., 2011a. *HOX* clusters are believed to have been derived from a single gene that produced tandem duplications (Ruddle *et al*., 1994). Each cluster is approximately 100 Kb in length, found on different chromosomes, with *HOXA at 7p15.3, HOXB at 17p21.3, HOXC at 12q13.3 and HOXD at 2q31* (Akam, 1989). HOX gene products, HOX proteins, primarily function as transcription factors that are essential in the patterning of the anterior and posterior axis during embryonic development but also help determine tissue fate (Garcia-Fernandez, 2004).

Mammalian genes have a notable homology with Drosophila melanogaster *HOX-C genes*. These genes are distributed between two gene complexes termed the *Ant-C and BX-C.* Many mammalian HOX genes (Paralogous groups 1-8) are like *Ant-C* genes. The others (9-13) are known as *Abd-B Hox* genes because of their similarity to the *Abd-B* genes of the *BX-C* complex (Phelan *et al*., 1995). The anterior-posterior expression of these *HOX* genes is a unique feature among developmental regulators, termed "HOX code" (Knittel *et al*., 1995). *HOX* code in mammals has four distinctive criteria: (1) Temporal distribution: which is that the expression of *HOX* genes is initiated from the 5' to 3' ends of the DNA. (2) spatial distribution: so that in terms of a 5'-3' co-linear sequence of each single cluster, the expression of 5'end genes are caudal, whereas the expression of the 3' end genes is labial. (3) posterior prevalence: that in the individual cluster itself, the function of these posterior gene products is dominant over the more anterior genes. (4) Overlapping expression: *HOX* genes form a nested expression pattern (He *et al*., 2011b). Typically, 3' prime *HOX* genes are expressed in anterior tissue whilst 5' prime tissue will be expressed in posterior tissue (Shah and Sukumar, 2010). *HOX* proteins play several other roles, including: cellular proliferation, differentiation as well as apoptosis and the ability to modulate cell-cell and cell-extracellular matrix interactions (Cillo *et al*., 2001). These functions highlight the importance of understanding the disruption of activated *HOX* genes implemented in the initiation, promotion and progression of several cancers, however this presents a great treatment opportunity (Figure 1.7).

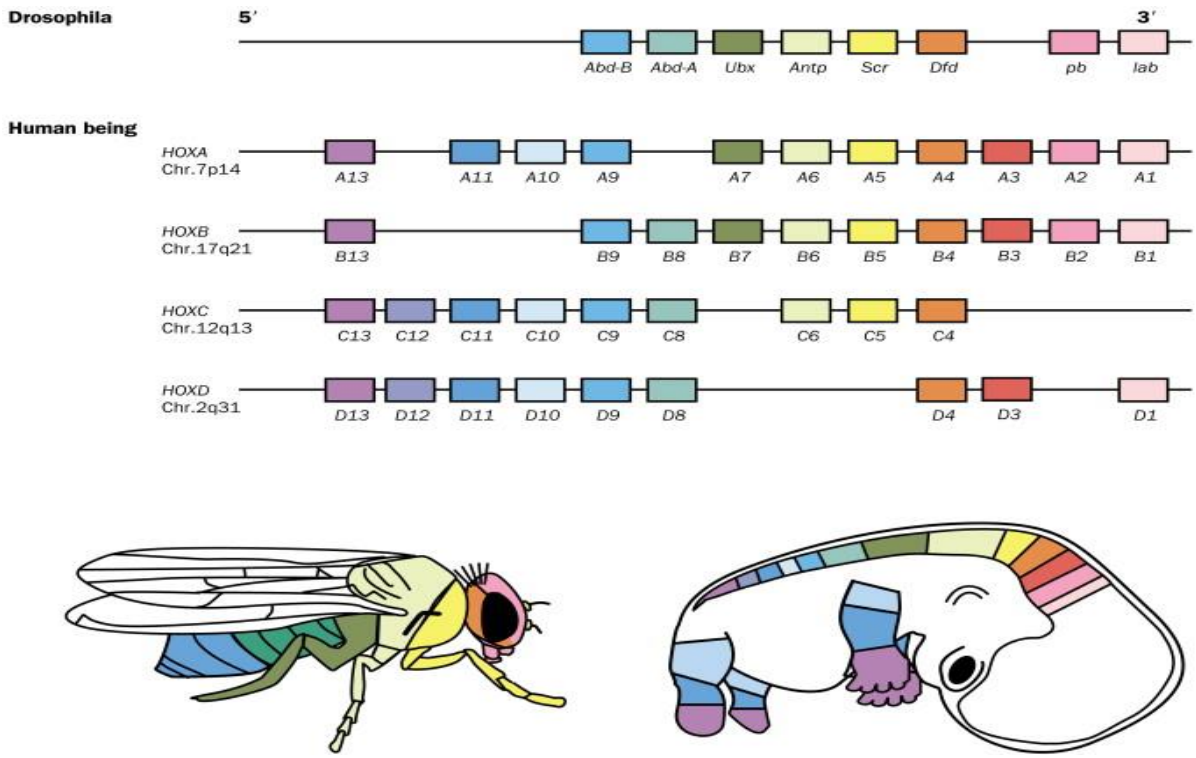

Figure 1.7 *A schematic diagram depicting the HOX gene layout in Drosophila with only a single HOX cluster, containing 8 HOX genes, compared to the 39 seen in Humans. Separated into 4 clusters, it is believed that the additional HOX clusters arose from a single ancestral cluster like the one seen in Drosophila. Vertebrate HOX groups 1, 2, 4, 5, 6, 7, and 8 are very like drosophila labial (lab), proboscipedia (pb), deformed (Dfd), sex combs reduced (Scr), antennapedia (Antp), ultrabithorax (Ubx), and abdominal-A (Abd-A), respectively. The vertebrate HOX group 9-13 are strongly related to the Drosophila abdominal-A (Abd-A). The Drosophila however, is not seen to have a group 3 corresponding HOX gene (Goodman, 2003).*

1.17 *HOX* genes and haematopoiesis

HOX genes are key regulators of trunk and tail development during normal embryogenesis, as an important family of homeodomain-containing transcription factors as already discussed. However, DNA-binding site studies highlight the fact that *HOX* proteins have relatively limited selectivity and specificity, with co-factor interactions needed to increase both. The co-factors associated with *HOX* genes also have crucial roles in development and haematopoiesis. For example, due to severe

hematopoietic defects, PBX1 null mice die during the embryonic stage and MEIS1-deficient mice are known to fail to generate megakaryocytes, resulting in severe haemorrhaging and fatality during the embryonic stage. *HOX* proteins 1–10 bind to PBX1, whereas *HOX* proteins 9–13 bind to MEIS1 (Thorsteinsdottir *et al*., 2001) both key co-factors in this study.

HOX genes are highly expressed in HSCs and progenitors with lineage and differentiation stage-restricted patterns. In uncommitted hematopoietic cells HOXB3, HOXB4 and HOXA9 are highly expressed, on the other hand, *HOXB8* and *HOXA10* are expressed in myeloid committed cells. The different *HOX* clusters also have specific patterns of lineage-restricted expression, whereby *HOXA* genes are expressed in myeloid cells, *HOXB* genes in erythroid cells and *HOXC* genes in lymphoid cells. Interestingly, *HOXD* genes are not expressed in haematopoiesis despite having similar regulatory regions to the other clusters. The function of *HOX* genes in normal haematopoiesis has been widely studied using gene expression analysis and knock-in or knockout studies in HSCs and early hematopoietic progenitors. In general, overexpression of *HOX* genes leads to expansion of stem and progenitor cell populations together, through an increased proliferation with a block on differentiation. Overexpression of murine *HOXB6*, results in the expansion of murine HSCs and myeloid precursors, together with the inhibition of erythropoiesis and lymphopoiesis. Overexpression of murine *HOXB3* results in several haematological abnormalities, such as a block of B- and T-cell differentiation along with a delay in myeloid precursor proliferation. Other *HOX* genes are required for the maintenance of progenitor or stem cell status and promote self-renewal, especially *HOXA9* and *HOXB4*. The former is the most preferentially expressed *HOX* gene in early hematopoietic progenitors and *CD34þ* HSCs and is downregulated during differentiation (Thorsteinsdottir *et al*., 2001).

Interestingly however, the exception to the general prevalence of functional redundancy amongst the *HOX* gene family is *HOXA9*. *HOXA9* is the most highly expressed member of the *HOX* family in HSCs. Although the mechanism by which *HOX* genes regulate haematopoiesis is not yet fully understood, genome-wide analyses after experimentally induced changes in *HOX* gene expression have identified some potential downstream targets. Amongst these are the *HOX* genes themselves, some of which have been shown to auto-activate their own expression or to cross regulate their neighbours, or their co-factors. *HOXA9, HOXA10* and *HOXB4* are the most comprehensively studied genes in this respect

because of their key roles in normal haematopoiesis and leukaemia. It is particularly noteworthy that *HOXA9* positively regulates the transcription of other *HOX* genes including *HOXA7* and *HOXA10* and its cofactors *PBX3* and *MEIS1* (Thorsteinsdottir *et al*., 2001).

1.18 *HOX* over-expression in AML

HOX genes have also been shown over the last decade to be indirectly involved in AML through chromosomal rearrangements that may involve some of their upstream regulators, such as MLL. MLL fusion proteins are known to constitute about 10% of therapy-related AML and just over 3% of *de novo* AML (Slany, 2009). There are almost 65 known translocation partner genes that are thought to fuse with the MLL N- terminus (Meyer *et al*., 2009). MLL positively regulates the transcription of *HOX* genes, maintaining their expression by directly binding to a proximal promoter region (Milne, Briggs *et al*. 2002). However, MLL fusion proteins activate *HOX* gene transcription far more efficiently than MLL alone (Liu *et al*., 2009, Slany, 2009), in particular the 5' end members of the *HOXA* cluster with their co-activator MEIS1. Due to this, myeloid differentiation is blocked and proliferation is vastly enhanced (Marschalek, 2011). As well as this current proposed mechanism, it has been shown that MLL-AF9, just like NUP98-*HOXA9*, often leads to a block in erythroid/myeloid cell maturation and erythroid hyperplasia (Abdul-Nabi *et al*., 2010). Interestingly, studies have suggested that the *MLL- Enl* fusion protein seems to require *HPXA7* and *HOXA9* for efficient immortalization of haematopoietic stem cells (HPSCs) (Ayton and Cleary, 2003). Conversely, some studies have shown that the expression of *HOXA* genes is not necessary for MLL leukaemogenesis, but their expression may affect disease phenotype. For example, *HOXA7* and *HOXA9* have been shown to influence AML latency and phenotype; but are not essential to initiate MLL-Gas7-mediated leukaemogenesis (So *et al*., 2004). Suppression of *HOXA9* expression in specific cells with a chimeric MLL-AF9 gene has been shown to reduce the survival of leukaemic cells and possibly changes the disease phenotype, without affecting AML initiation (Faber *et al*., 2009).

The dysregulation of the CDZ gene family (another regulator of *HOX* genes), has also been shown to

progress the development of AML. The CDX2 protein is expressed in around 90% of AML cases but not in normal adult haematopoiesis, with CDX2-elevated expression often leading to AML with a very short latency period (Scholl *et al*., 2007). The CDX4 gene is expressed in just over 25% of AML cases, as well as being expressed in normal adult haematopoiesis. CDX4 being over-expressed in some murine BM can result in AML, with a long latency period (Bansal *et al*., 2006). Latency in relation to the CDX4 gene is accelerated in mice through co-operation of MEIS1 resulting in the over-expression of several *HOX* genes *including HOXA6, HOXA7, HOXA9, HOXB4, HOXB8* and *HOXC6* (Bansal *et al*., 2006). *CDX2* expression alone has been shown to be sufficient in driving the up-regulation of a set of *HOX* genes (Thoene *et al*., 2008), which demonstrates the importance of this CDX family in dysregulation of *HOX* genes during AML.

Dysregulation of HOX gene expression is strongly associated with the nucleophosmin 1 *(NPM1)* mutation. This NPM1 protein is a chaperone protein that the cell uses to translocate between the nucleus and cytoplasm, even though its predominant localization is the nucleus (Rau and Brown, 2009). *NPM1* plays a critical role in biological processes, such as genomic stability, ribosome biogenesis, and cell cycle progression. In adult AML patients however, the *NPM1* mutation appears to be one of the most common genetic mutations, which has been reported in just over 33% of all adult AML and in 45-55% of *NK-AML* cases (Luzi Falini, Mecucci *et al*., 2005). However, in paediatric AML, *NPM1* mutations are far less common occurring in just less than 10% of cases, and in less than a quarter of the normal karyotype cases (Brown *et al*., 2007, Hollink *et al*., 2009). *NPM1* relocation into the cytoplasm (NPMc+) occurs only in AML (Falini *et al*., 2009). This cellular relocation facilitates the up regulation of several *HOX* genes, of which some are very like those seen in AML initiated by an MLL chimeric gene fusion, although some are distinct. *HOXA4, HOXA6, HOXA7, HOXA9, HOXB9* and *MEIS1* are all over-expressed in both contexts, whereas *HOXB2, HOXB3, HOXB5, HOXB6* and *HOXD4* are up-regulated in NPMc+ AML only (Mullighan *et al*., 2007). This will be discussed in a lot more detail throughout this study, however recent studies report activation of a humanized NPM1 allele leading to over-expression of *HOXA5, HOXA7, HOXA9* and *HOXA10,* induction of HSC self-renewal and an increase in myelopoiesis (Vassiliou *et al*., 2011). The mechanism of the relationship between the *NPM1*

mutation and the up regulation of *HOX* genes remains relatively unclear. A logical explanation for this may be that *NPM1* is directly affecting the expression of *HOX* genes, or potentially, that mutations in *NPM1* arrest the differentiation of early stage HPs in which *HOX* expression is up-regulated (Rau and Brown, 2009).

HOX protein	***Targets of transcriptional activation***	***Targets of transcriptional repression***	***Species***
HOXA9/ Hoxa9	Pim1 (Hu, Passegué *et al*. 2007), ID2 (Nagel, Venturini *et al*. 2010), CYBB (Bei, Lu *et al*. 2005), HOXA7, HOXA10, PBX3, MEIS1 (Faber, Krivtsov *et al*. 2009). Bcl-2 (Brumatti, Salmanidis *et al*. 2013), Flt3 (Gwin, Frank *et al*. 2010), Cerb1 and Pknox1 (Bei, Lu *et al*. 2005), Camk2d, Cdk6, Erg, Etv6, Foxp1, Gfi1, Kit, Lck, Lmo2, Myb and Sox4 (Huang, Sitwala *et al*. 2012).	BIM (Nagel, Venturini *et al*. 2010)/ Itfi1, Tlr4, Ccl3, Ccl4, Csf2rb, Ifngr1, Runx1, Cd28, Cd33 (Huang, Sitwala *et al*. 2012).	Human/mouse
Hoxa9-Meis1	c-Myb (Hess, Bittner *et al*. 2006).		Mouse
NUP98-HOXA9	HOXA7, HOXA9, MEIS1, PBX3, EVI1, MEF2C, FLT3 and KIT (Takeda, Goolsby *et al*. 2006).		Human
HOXA10/ Hoxa10	P21 (Bromleigh and Freedman 2000), DUSP4 (Wang, Lu *et al*. 2007), Itgb3	CYBB (Eklund, Jalava *et al*. 2000)/ Gata1 (Magnusson,	Human/mouse

	(Bei, Lu *et al*. 2007), Hlf (Magnusson, Brun *et al*. 2007), Tgfβ2 (Shah, Wang *et al*. 2011), Fgf2 (Shah, Bei *et al*. 2012), Dusp4 (Wang, Lu *et al*. 2007), Hoxa5 (Magnusson, Brun *et al*. 2007), Cdx4 (Bei, Huang *et al*. 2011).	Brun *et al*. 2007)	
Nup-Hoxa10	Flt3, Prnp, Hlf and Jag2 (Palmqvist, Pineault *et al*. 2007).		Mouse
HOXB4/ Hoxb4	MEIS1, DNTT, HLF, SOX4, RUNX2 (Lee, Zhang *et al*. 2010), c-MYC (Pan and Simpson 2001), Hoxb2, Hoxb3 (Satoh, Matsumura *et al*. 2004), Fra-1, JunB (Krosl and Sauvageau 2000).	B220 and HMBS (Lee, Zhang *et al*. 2010), c-Myc (Satoh, Matsumura *et al*. 2004).	Human/mouse

Table 1.4 *A table listing the important mammalian HOX target genes (Alharbi et al., 2013).*

1.19 *HOX* gene upstream regulation

Knock out models of *HOX* gene upstream regulators over the last decade have helped to define the role of *HOX* genes in normal haematopoiesis. Upstream regulators of *HOX* genes are thought to function as activators for *HOX* genes, such as MLL, and a family of CDX's (CDX1, CDX2, CDX4), or potentially act as repressors, for example the PcG genes. These regulators play some crucial roles in development and haematopoiesis by regulating *HOX* genes (Schuettengruber *et al.*, 2007, Jude *et al.*, 2007, Liu *et al*., 2009, Ernst *et al*., 2004, Bansal *et al*., 2006). Studies have shown that MLL-deficient mice express a dramatic reduction in HSC's and hematopoietic progenitors. Additionally, mice deficient in MLL exhibit a remarkable reduction in certain *HOX* genes, such as *HOXA7/HOXA9, HOXA10*, and other *HOXB* and *HOXC* genes (Ernst *et al*., 2004, McMahon *et al*., 2007, Jude *et al*., 2007, Gan *et al*., 2010).

Similarly, compound *CDX*-deficient mice have shown a dysregulation of the embryonic hematopoietic progenitors as well as impaired expression of certain *HOX* genes (Davidson and Zon, 2006, Wang *et al*., 2010). Most interestingly, *CDX's* may act in a redundant manner and therefore many of their functions are conserved in embryonic haematopoiesis but may not always influence adult haematopoiesis (Wang *et al*., 2010, Lengerke *et al*., 2009, Koo *et al*., 2010). On the contrary however, the PcG genes (PRC1 and PRC2) are known to effectively suppress the expression of *HOX* genes (Beuchle *et al*., 2001, Schuettengruber *et al*., 2007, Simon, 2010).

1.20 *HOX* gene dysregulation in ALL

The dysregulation of *HOX* genes has also been identified in ALL including B- and T-progenitor ALL (B-ALL and T-ALL), especially when MLL translocations are involved. Human HOXA9, HOXA10 and *HOXC6*, and their co-factor *MEIS1* are up-regulated in both *MLL-ENL T-ALL* and *MLL B-ALL* (Ferrando *et al*., 2003). The human *HOXA9* and *MEIS1*, and the murine *HOXA5*, *HOXA9* and *MEIS1* were also shown to be up-regulated in *MLL-AF4 B-ALL* (Rozovskaia *et al*., 2001, Krivtsov *et al*., 2008). A common signature of *MLL* rearrangements in both AML and ALL has been the over-expression of *HOXA* genes including *HOXA3, HOXA5, HOXA7, HOXA9* and *HOXA10* (Zangenberg *et al*., 2009). Additionally, *HOX* genes are involved in translocations such as *CALM-AF10 T-ALL*, where human *HOXA5, HOXA9, HOXA10* and *MEIS1* are also over-expressed (Dik *et al*., 2005). HOXA proteins have been identified and shown to form chimeric fusion proteins with T-cell receptors in T-ALL, resulting in the global over-expression of *HOXA* genes (Soulier *et al*., 2005, Speleman *et al*., 2005). However, the aberrant expression of the *CDX2* protein in ALL, which is an upstream regulator of *HOX* genes, is not correlated with *HOX* gene dysregulation (Thoene *et al*., 2009).

1.21 Role of *HOX* genes in AML

There are two primary mechanisms by which *HOX* genes are hypothesized to promote the transformation of myeloid progenitor cells in *vivo*. *HOX* genes are known to cause AML by forming

parts of new fusion proteins such as *NUP98-HOX* have been recently confirmed (Nakamura *et al*., 1996, Borrow *et al*., 1996). In addition, to this *HOX* genes may also indirectly lead to AML through their aberrant expression due to chromosomal rearrangements involving upstream regulators or co-factors, such as *MEIS1* (Zangenberg *et al*., 2009). *HOX* genes are known to facilitate the initial transformation of fusion proteins, most notably with NUP98. NUP98 is a known member of the nuclear pore family. It is localized in the nuclear membrane and functions as a selective transporter for RNA and proteins between the nucleus and the cytoplasm. *NUP98-HOX* fusion proteins have been reported in AML and other leukaemias (Slape and Aplan, 2004). In AML, *NUP98-HOXA9* is found to be a t(7;11)(p15;p15) positive translocation (Nakamura *et al*., 1996, Borrow *et al*., 1996). Eight other *HOX* genes have the ability to be fused with *NUP98,* including *HOXA11* and *HOXA13* (Fujino *et al*., 2002, Suzuki and Kuroiwa, 2002), *HOXD11* and *HOXD13* (Raza-Egilmez *et al*., 1998, Taketani *et al*., 2002b), and *HOXC13* (Taketani *et al*., 2002a). To date, only *abd-B* clustered *HOX* genes have been shown to fuse with *NUP98* in *AML* (Palmqvist *et al*., 2007). *HOXB3* under an experimental expression has been reported to be a leukemogenic partner with *NUP98* (Pineault *et al*., 2004), supporting its ability to be a fusion *NUP98* partner although is not limited to *abd-B* clustered *HOX* genes. The potential explanation for the *Abd-B* clustered genes' leukemogenic ability is that they have the ability to disturb hematopoietic differentiation (Pineault *et al*., 2004). *HOXA9 i*s a key regulator of haematopoiesis and has been shown to behave as an oncogene in many types of leukaemia. It is unsurprising therefore that it activates the transcription genes known to regulate cell proliferation and survival. *HOXA9* is known to directly activate the *PIM1* gene (Hu *et al*. 2014). The product of this activation can then enhance proliferation by activating *c-Myb* and exerts anti-apoptotic effects by phosphorylating and inactivating the *BAD* protein (Figure 1.8).

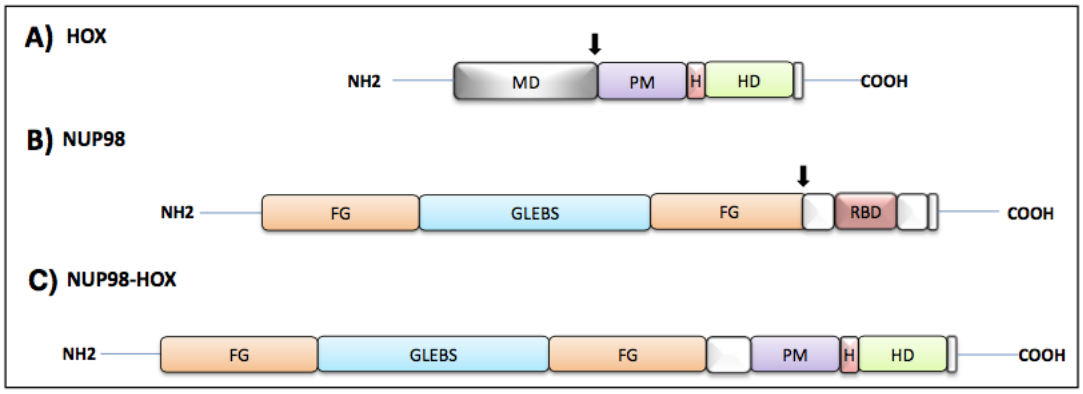

Figure 1.8 *A schematic diagram depicting the structures of AbdB HOX, NUP98 and the predictive fusion protein NUP98-HOX. (a) A general structure of AbdB HOX (9-13) proteins that have been reported to fuse with NUP98. (b) Structure of normal NUP98 protein. (c) Structure of predictive NUP98-HOX fusion protein. This fusion eliminates MD and RBD from AbdB and NUP98, respectively. Arrows represent breakpoints, MD: MEIS domain, PM: PBX motif, H: hexapeptide, HD: homeodomain, FG: phenylalanine-glycine, GLEBS: GLE2P-binding like motif, RBD: RNA-binding domain (Alharbi et al., 2013).*

HOXA9 directly activates the *PIM1* gene, the product of which enhances *c-Myb* which has recently been identified as an indirect transcriptional target of *HOXA9-MEIS1* mediating the transformation in *Mll-Enl* Leukaemia (Hu *et al*. 2014). *HOXA9* has several other targets associated with tumorigenesis including the oncogene *ID2*, seen to be upregulated. *BIM* that encodes an apoptotic factor is downregulated in response to *HOXA9. HOXA9* also activates the *CYBB* gene; encoding *Gp91phox*, a phagocyte respiratory burst oxidase protein expressed in differentiated myeloid cells (Thorsteinsdottir *et al*., 2001). Interestingly however, *HOXA9* in mice has been shown to directly activates the transcription of the *Flt3* gene, associated with the unfavourable prognosis of AML, and has also shown to regulate its own co-factor, *MEIS1*, by binding to both *MEIS1*'s upstream regulatory genes *CERB1* and *PKNOX1* (Morgado *et al*., 2007). There is a list of proliferative genes that have been recently linked and identified as downstream targets for *HOXA9* including; *CAMK2D, CDK6, ERG, ETV6, FLT3, FOXP1, GFI1, KIT, LCK, LMO2, MYB* and *SOX66.* Over 50% of AML cases over express *HOXA9* along with the co-factor already mentioned, *MEIS1* (Morgado *et al*., 2007). This is thought to be a result

of some highly conserved evolutionary enhancers that function to promote self-renewal. These binding sites are also enriched for transcription factors such as *PU1*, *C/EBPa* and *STAT5* that are mutated or otherwise activated in acute leukaemia. *HOXA9* binding enhancers promotes CBP/P300 recruitment, histone acetylation and transcriptional activation of pro-proliferative genes while at other sites *HOXA9* binding is associated with repression (Rajala *et al*. 2013). A key area of study therefore is to try and validate how *HOXA9* over-expression or mutation of associated transcription factors deregulates transcription and on identifying these, what might be the crucial targets for leukaemogenesis (Figure 1.10).

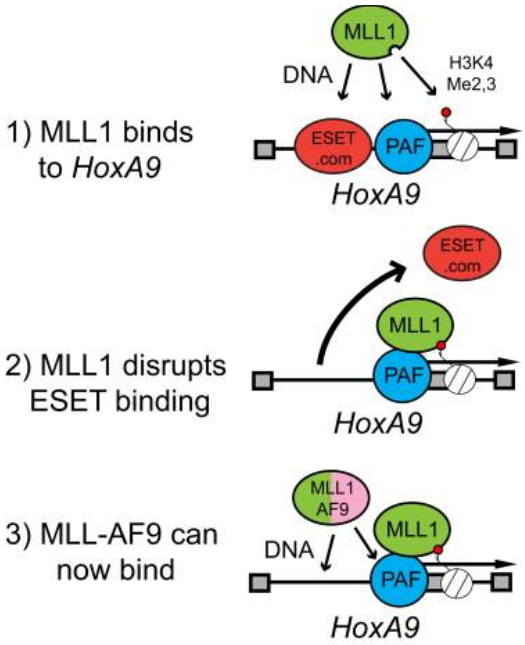

Figure 1.9 *A schematic diagram depicting cell specific transcriptional programs involved in AML progression via MLL fusions and MLL PTD near HOXA9 and the ability for differentiation genes and pro-proliferation genes to be manipulated (Allis et al., 2010).*

1.22 *HOX* genes as prognostic markers

The expression of *HOX* genes is an important prognostic factor in AML. Over-expression of *HOX* genes is now widely accepted as being closely associated with an unfavourable cytogenetic subset of AML. Among all 6817 genes investigated in AML patients, the single gene linked to the worst outcome and

relapse of disease, including short survival was *HOXA9* (Golub *et al*., 1999). Equally, high expression of *HOXA9* is associated with low CR rate (Li *et al*., 2013). Low *HOXA9* expression on the other hand was found to be strongly correlated with the best outcome and response to therapy (Andreeff *et al*., 2008). It is important to note at this point that low expression of *HOXA4* and *MEIS1* are also shown to be predictors of more favourable AML patient outcome (Zangenberg *et al*., 2009). High expression of *HOXA7, HOXA9* and *HOXA11* as well as the co-factor *PBX3* are independent prognosis markers of adverse overall survival (OS) in abnormal karyotype AML (Li *et al*., 2012). The global increase in *HOX* expression appears to strongly reflect the outcome of disease, supporting the theory of functional redundancy between many *HOX* proteins. The highest levels of *HOX* can be seen in cases where an *FLT3* mutation is present, and has unfavourable outcomes, in general *HOX* genes are expressed at very low levels only in the favourable subsets of AML.

Studies have established the importance of *HOX* genes in the progression of AML, however, it is still unclear exactly what the functions of these genes are other than inhibiting differentiation and increasing cell proliferation. The inability to identify a precise mechanism for individual *HOX* genes may be due to the co-operative functional redundancy expressed by members of this family, meaning the knockdown of any single *HOX* gene is extremely difficult to interpret, but may also help to explain the contrast in gene knock-in and knockout results seen in some myeloma and solid malignancies by targeting the *HOX* proteins and antagonizing their interactions with the *PBX* co-factor using *HXR9* (a peptide inhibitor) (Morgan *et al*., 2007, Shears *et al*., 2008, Plowright *et al*., 2009, Daniels *et al*., 2010, Morgan *et al*., 2010). *PBX* is known to bind to the *PBX* motif in *HOX* paralogous group 1-10. *HOX-PBX* dimers have been shown to have an increased binding affinity and specificity for their target DNA sequences compared to isolated *HOX* monomers alone. *HOX-PBX* dimers are involved in the interaction of PBX to the conserved hexapeptide sequence WYPWMK found in the N terminal region to the homeodomain of *HOX* proteins from paralogous groups 1-10 responsible for the cooperative binding to DNA by PBX and *HOX* partners (Chang *et al*., 1995, Shen *et al*., 1997, Medina-Martínez and Ramírez-Solis, 2003). A novel peptide inhibitor, HXR9, expresses a sequence which mimics the *HOX* protein hexapeptide sequence responsible for PBX binding and therefore interferes with the DNA

binding of *HOX-PBX*. *HXR9* has been shown to be extremely cytotoxic to cells, due to an increase in expression of c-Fos (Morgan *et al*., 2007, Shears *et al*., 2008, Plowright *et al*., 2009, Daniels *et al*., 2010). Targeting this could be a useful approach in AML for addressing some of the redundant functions of *HOX* genes. Dysregulation of *HOX* genes is very common in many types of haematological malignancies, including AML (Ferrando *et al*., 2003), but also in some solid malignancies including lung (Abe *et al*., 2006, Plowright *et al*., 2009), renal (Shears *et al*., 2008), ovarian (Cheng *et al*., 2005, Morgan *et al*., 2010), and other cancers. Although it is well documented that over-expression of HOX*A9* in AML is a poor prognostic factor (Golub *et al*., 1999), *HOX* genes have a very clear oncogenic function in leukaemia (Eklund, 2007) with their over-expression in melanoma maintaining cell survival, by likely preventing c-Fos transcription (Morgan *et al*., 2007).

1.23 *HOXA9*

As mentioned already, out of all 6817 genes investigated in AML patients by a larger study, the single gene linked to the worst outcome and relapse of disease, including poor survival was *HOXA9* (Golub *et al*., 1999), with high expression of *HOXA9* associated with a low CR rate (Li *et al*., 2013) and low *HOXA9* expression strongly correlated with the best outcome and response to therapy (Andreeff, Ruvolo *et al*. 2008). *HOXA9* is necessary for maintaining leukemic transformation; however, the molecular mechanisms through which it promotes leukaemogenesis remain extremely elusive. Studies have helped establish that *HOXA9* regulates downstream gene expression through binding at promoter distal enhancers along with a subset of cell-specific cofactor and collaborator proteins. Efforts are being made to identify the critical co-factors and target genes required for maintaining transformation in *HOXA9*-overexpressing leukaemia. With continued advances in understanding *HOXA9*-mediated transformation, and opportunity for developing novel therapeutics that would be applicable for greater than 50% of AML cases with overexpression of *HOXA9* (Figure 1.11).

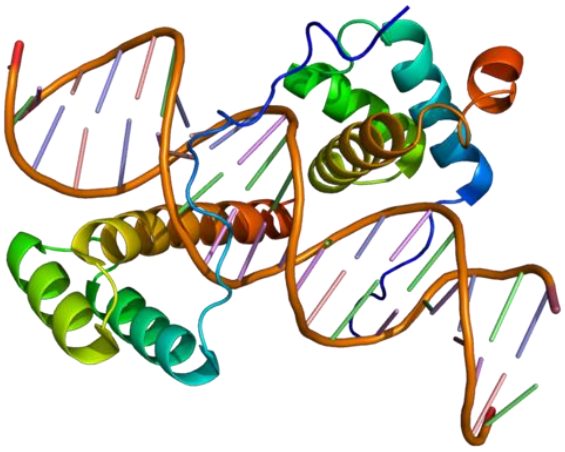

Figure 1.10 *A schematic diagram depicting tertiary structure of HOXA9 binding to DNA under x-ray (LaRonde-LeBlanc and Wolberger, 2003).*

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Human HOXA9 Interacting Partners	*Mouse HOXA9 Target Genes*
CEBPA	Bcl-2
CEBPB	c-Myb
CEBPE	Erg
CREB1	Fgf-2
CREBBP	Flt3
CUL4A	Fos
G9A	Igf1
JUN	Igf1R
MEIS1	Ink4a/ARF/Ink4b
MEIS2	Lmo2
NFKBIA	Pim1
PBX1	Sox4
PBX2	Vav2
PBX3	
PRMT5	
SMAD4	
STAT1	
STAT5	

Table 1.5 *A table listing all HOXA9 interacting proteins and all HOXA9 target genes (LaRonde-LeBlanc and Wolberger, 2003).*

1.24 *HOX-PBX* targeting

Over the last 20 years there has been a definitive change in the approach to cancer therapy from the use of untargeted chemotherapeutic agents to utilizing cancer-specific targeting drugs. As previously mentioned *HOX* proteins can act as dimers or trimers, bound to different co-factors in many different spatial arrangements. The co-factor of most interest here is pre-B-cell leukaemia homeobox (PBX). PBX is a family of transcriptions co-factors, which are members of the three-amino acid loop extension superclass of Homeobox proteins, known to bind with a large variety of *HOX* proteins. Exploiting the interaction between *HOX* and PBX binding which is mediated by a small tryptophan-containing motif which incorporates a highly conserved hexapeptide region, which can be located within the *HOX* protein itself, a hydrophobic pocket positioned in the PBX tertiary structure is responsible for this binding and became an area of keen interest. HXR9 is a synthetic peptide, designed by Professor Richard Morgan at The University of Bradford, formerly of The University of Surrey, which consists of a hexapeptide region and a small polyarginine sequence that can mimic this hexapeptide motif inhibiting *HXR9's* ability to successfully bind to *HOX* proteins, therefore down-regulating some of their target genes. This 18-amino-acid peptide consisting of a previously identified hexapeptide sequence binds to PBX and nine C-terminal arginine residues (R9) facilitating cell entry into the cytoplasm and nucleus by facilitating binding to the extra cellular proteins heparan sulphate (Morgan *et al*., 2007). The N-terminal and C-terminal amino bonds are in a D-isomer conformation which has already been shown to extend the half-life of the peptide to 12h in human serum (Morgan *et al*, 2007). CXR9 is used as a control peptide which lacks the functional hexapeptide sequence including the R9 sequence.

Since the discovery of HXR9, it has been shown to increase apoptosis in several cancers including myeloma, kidney, melanoma, non-small cell lung and ovarian cancer (Phelan *et al*., 1995, Chang *et al*., 1995, Shanmugam *et al*., 1997, Piper *et al*., 1999, Morgan *et al*., 2000, Medina-Martínez and Ramírez-Solis, 2003). HXR9 treatment causes a dramatic reduction in proliferation in tumour cells from multiple myeloma, ovarian, and melanoma both in *vivo* and in *vitro* (Phelan *et al*., 1995, Chang *et al*., 1995, Shanmugam *et al*., 1997, Piper *et al*., 1999, Morgan *et al*., 2000, Medina-Martínez and Ramírez-Solis, 2003). It is important however, to note that as HXR9 exerts its ability by specifically binding to the

PBX family, only *HOX* proteins 1-10 use PBX as a co-factor. *HOXA11,* which is a tumour suppressor, is not affected. E4 is a 300 Dalton small molecule which is a mimic of HXR9 that could exert its cytotoxic effects on the cell, binding to PBX and blocking its ability to interact with *HOX* proteins. E4 is lipophilic and passes through the phospholipid bilayer in a passive manner. HXR9 is a peptide and therefore it cannot be taken orally and must be administered intravenously or intratumorally for effective delivery to the target tumour (Figure 1.11).

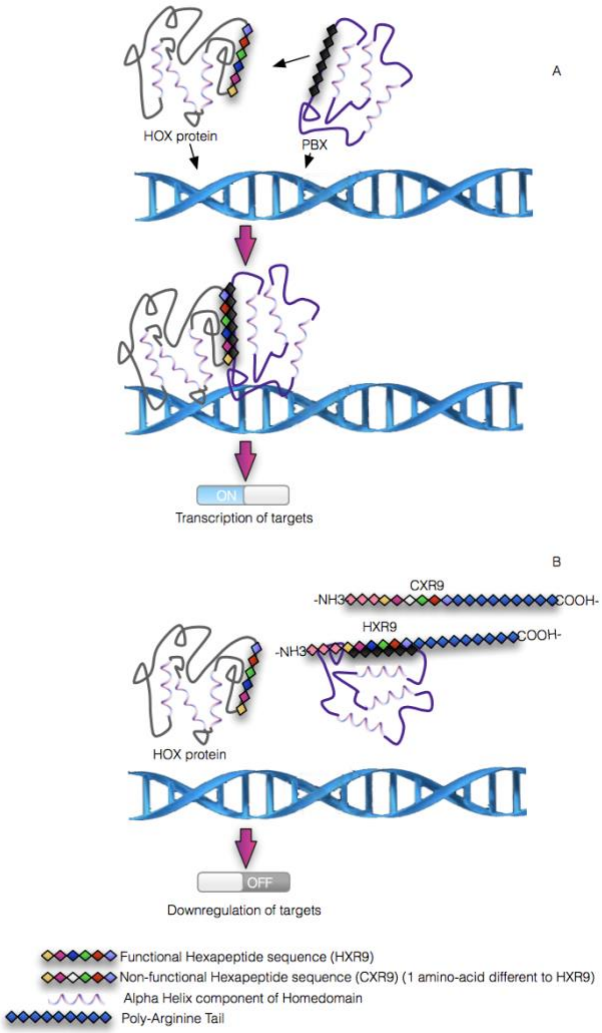

Figure 1.11 *A schematic diagram depicting the competitive binding of HXR9, where HXR9 mimics the hexapeptide motif of HOX proteins binding to PBX, inhibiting its own ability to successfully bind to HOX proteins and down regulating some of their target genes (Morgan and Grey 2016).*

1.25 Targeting HOX-PBX Dimers

The widespread aberrant HOX protein expression in cancer, and the key pathways they exert influence over has provided much interest as potential therapeutic targets. Various independent groups have targeted individual HOX genes with siRNA and miRNA, to varying degrees of success. This therapeutic approach is neither seen as especial effective, mainly due to functional redundancy of HOX proteins, and the limited application of siRNAs and miRNAs in the clinical setting. An alternative approach is to target and inhibit the interaction between HOX and PBX proteins, disrupting HOX paralog groups' 1-10 functionality. This interaction is mediated by a small tryptophan-containing motif, which incorporates a highly conserved hexapeptide region located within the HOX protein. This hexapeptide motif binds to a hydrophobic pocket positioned in the PBX's tertiary structure known as the PID (Laurent, 2008; Phelan, 1994; Morgan, 2000). A previous attempt at targeting this interaction with a small molecular inhibitor has been published, but with its dissociation constants (Kd) in the micromolar range, the experimental drug was abandoned (Jl, 2004). Independently an alternative approach was developed utilising a peptide- based drug named HXR9.

1.26 Peptide therapies: HXR9, CXR9 and HTL00-1

HXR9 is an 18 amino acid synthetic peptide consisting of a hexapeptide sequence connected to a short polyarginine sequence (Li, 2003; Errico, 2013). The hexapeptide motif mimics the same amino acid sequence found in HOX proteins, thus allowing it to bind to PBX1-4 and inhibiting its dimerization to HOX (Morgan, 2010). Figure 1.32 shows computer modelling of HOXA9-PBX1 inhibition by HXR9.

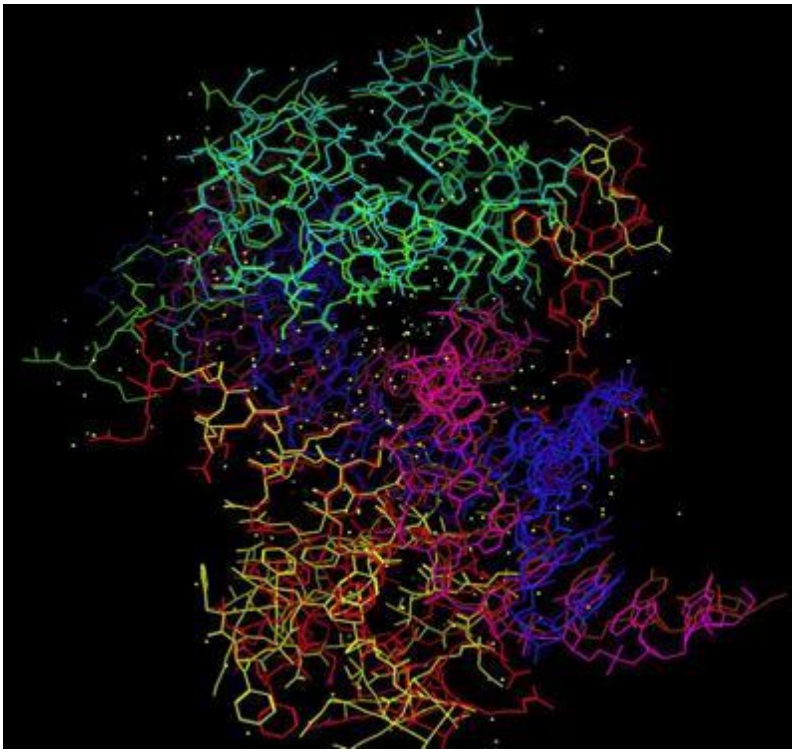

Figure 1.32 *Computer modelling based on X-ray crystallography of HXR9 binding to PBX1 PID, inhibiting the dimerization with HOXA9 and HOXB1 (LaRonde-LeBlanc and Wolberger, 2003).*

The polyarginine repeat promotes the cellular uptake of HXR9, via endocytosis, by binding to the extra cellular protein heparin sulphate. HXR9 was initially proved to be cytotoxic on melanoma cell lines and primary melanoma tumour cells and was shown to reduce growth of B16F10 murine melanoma cells in an orthotropic model. Subsequent experiments have shown HXR9 to be cytotoxic in numerous cancers including myeloma, mesothelioma, meningioma, melanoma, prostate cancer, breast cancer, non-small cell lung cancer and ovarian cancer both in vitro and in vivo (Li, 2003; Errico, 2013; Morgan, 2010; *Morgan and Grey 2016).*

1.28 Apoptosis resistance mechanisms

Apoptosis can be triggered by intrinsic and extrinsic mechanisms, with cross-talk common, both leading to loss of mitochondrial membrane potential and the activation of the cysteine-aspartic acid proteases (caspase) cascade. The extrinsic apoptotic pathway is mediated by membrane death receptors such as, Fas receptor or tumour necrosis factor (TNF) receptor. Once a death factor has bound to its' respective receptor, it causes a cascade reaction in which procaspases are cleaved to form their activated forms. These activated caspases cleave specific protein targets within the cell causing apoptosis. The intrinsic pathway is not dependent on external stimuli but rather stimuli from inside the cell. Cellular stress causes BH3 interacting-domain death agonist (BID) protein to translocate and bind to B-cell lymphoma 2 associated X protein (Bax). This binding event causes a conformational change in Bax, which allows it to insert itself into the outer mitochondria membrane. Bax oligomerises (6-8 molecules) to produce its final form. This final form of Bax allows cytochrome C and procaspase 9 to be released from the mitochondria into the cytosol (Pastorino, 1999; Eskes, 1998). In the cytosol molecules of cytochrome C, procaspase 9 and apoptotic protease activating factor 1 (Apaf-1) bind together to form the wheel-like complex known as apoptosome. The newly formed apoptosome triggers a caspase cascade reaction which ends with cell apoptosis. Members of the IAP family inhibit apoptosis by binding, and inactivating, caspases 3, 7 and 9 (Deveraux, 1999) During the release of cytochrome C from the mitochondria, two other proteins known as Smac and DIABLO are also liberated. Smac and DIABLO act as inhibitors to members of the inhibitor of apoptosis (IAP) family. B-cell lymphoma 2 (Bcl-2) and B-cell lymphoma-extra-large (Bcl-XL) can inhibit Bax and thus stop it from being inserted into the mitochondrial membrane. However, Bcl-2 and Bcl-XL can both are inhibited themselves by the inhibitor Bad. Figure 1.12 (de Vries, 2006) illustrates key proteins needed in the intrinsic and extrinsic apoptosis pathways. GBM tumours have been shown to have aberrant expression of key pro and anti-apoptotic proteins either by direct activation/suppression or by inactivation of p53. Inactivation of P53 has shown associated with an up-regulation of Bcl-2 and Bcl-XL leading to increased resistance to chemo and radiotherapy.

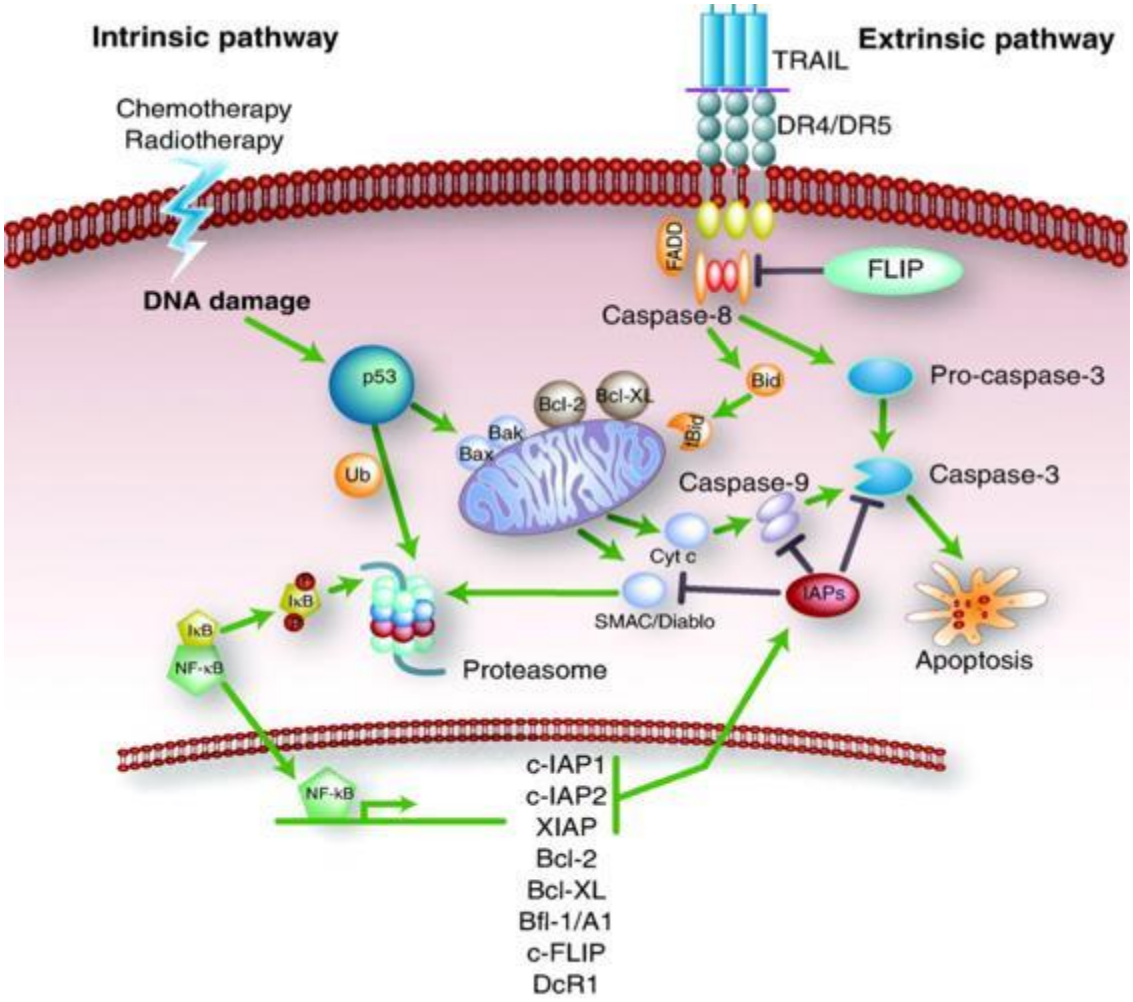

Figure 1.12 *A schematic illustration of the intrinsic and extrinsic apoptosis pathway. Though apoptotic stimuli are a key difference, both pathways converge at the mitochondria leading to the release of cytochrome C and caspase cascade (de Vries, 2006).*

Chapter 2: General Materials & Methods

2.1 Leukemic cell lines

The human leukaemic cell lines have been key over the last decade in attempting to understand what is going on at a cytogenetic level with patients with AML and other leukaemia's. These cell lines provide useful model systems to study various aspects of the disease including control of differentiation in human myelogenous leukaemia and, in a broader framework, the control of normal myeloid development. The AML cell lines used in this study represented various AML subtypes with these cell lines being both primary or secondary cell lines. Some of these primary AML cell lines derived from patients that had *de novo* mutations like KG-1, a cell line which is derived from patients with erythroleukaemia (Koeffler and Golde, 1978b, Koeffler and Golde, 1978a), HEL 92.1.7, an erythroleukaemia cell line (Martin and Papayannopoulou, 1982) and importantly, HL-60, a cell line that was initially obtained from acute promyelocytic leukaemia (APML) (Collins *et al*., 1978). Secondary AML cell lines are just as important as these cell lines are derived from patients that developed AML from pre-leukemic diseases such as MDS, chronic myeloid leukaemia (CML) or following previous treatment with chemotherapy, including K562, a CML cell in blast crisis (Lozzio and Lozzio, 1975) and KU812F, also a CML cell in blast crisis indicative of a poor prognosis (Lozzio and Lozzio, 1975, Kishi, 1985, Roche *et al*., 2004, Rücker *et al*., 2006, Weisberg *et al*., 2008).

- **HL-60:** cells also known as human promyelocytic leukaemic cells are widely used for laboratory research on how specific kinds of blood cells are formed. HL-60 cells proliferate continuously in a suspension culture of nutrient and antibiotic chemicals. The doubling time of these cells is about 36–48 hours. This cell line was derived from a 36-year-old woman with acute promyelocytic leukaemia at MD Anderson Cancer Centre (Gallagher *et al*., 1979). HL-60 cells are predominantly neutrophilic promyelocytic (Gallagher *et al*., 1979), with proliferation occurring through transferrin and insulin receptors, expressed on the cell surface. The requirement for insulin and transferrin is absolute, as HL-60 proliferation ceases if either of these compounds is removed from serum-free culture media (Breitman *et al*., 1980). With this cell line, differentiation to mature granulocytes is highly induced by compounds like dimethyl sulfoxide (DMSO), or RA. Other compounds like 1,25-dihydroxyvitamin D_3 and GM-CSF can also induce HL-60 cells to differentiate to monocytic,

macrophage-like and eosinophil phenotypes, respectively. The HL-60 cultured cell line provides a continuous source of human cells to study the molecular events of myeloid differentiation and the effects of physiologic, pharmacologic, and virologic elements of this process.

- **KG1:** cells are derived from the bone marrow aspirate of a 59-year-old male Caucasian who was diagnosed with erythroleukaemia that happened to transform into acute myelogenous leukaemia. These cells form colonies in soft-agar in the presence of colony stimulating factor (CSF). KG-1 cells appear to resemble acute myelogenous leukaemia and show considerable pleomorphism with a large predominance of myeloblasts and promyelocytes. However, a small percentage of these cells are mature granulocytes that have the occasional presence of macrophages and eosinophils. It is important to note, however, that they do not express surface IG or EBV antigens and lack specific markers for lymphocytic cells (Figure 3.1).

- **K562:** cells are erythroleukaemia-like cells derived from a 53-year-old female chronic myelogenous leukaemia patient in blast crisis (Lozzio and Lozzio, 1975, Britten *et al*., 2002). This cell line, like most leukaemic cell lines is non-adherent and rounded. They are positive for the BCR-ABL fusion gene, and bear proteomic resemblance to both undifferentiated granulocytes (Klein *et al*., 1976) and erythrocytes (Andersson *et al*., 1979). In culture these cells exhibit a lot less clumping than many other suspension cell lines, which is thought to be due to downregulation of cell surface adhesion molecules by the BCR-ABL gene (Jongen-Lavrencic *et al*., 2008). On the other hand, another study suggests that BCR-ABL over-expression could in fact increase cell adherence to cell culture plastic (Karimiani *et al*., 2014). K562 cells spontaneously develop characteristics that resemble early stage erythrocytes, granulocytes and monocytes (Lozzio, 1981) however are easily killed by natural killer cells (Lozzio, 1979) that lack the MHC complex required to inhibit NK activity (Figure 3.1). These cells also lack any trace of Esptein-Barr virus and other herpes viruses. In addition to the Philadelphia chromosome though, K562 cells exhibit a second reciprocal translocation between the long arm of chromosome 15 with chromosome 17 (Lozzio and Lozzio, 1975).

- **HEL92.1.7:** cells are derived from a 30-year-old male Caucasian and express ß-2 microglobulin and Ia antigen often used in the study of erythroid cell differentiation and globin gene expression. They are nalogous to friend cell leukaemia in mice and have similar characteristics to K562 cells. These cells differentiate spontaneously into erythroblast-like cells. Macrophage-like differentiation may be induced with phorbol esters like TPA (12-O-tetradecanoyl-phorbol-13-acetate) and PMA (phorbol myristic acid).

- **KU-812:** cells are a chronic myelogenous leukaemia cell line in which the cells are morphologically characteristic of basophils (Fc receptors, basophilic granules, histamine production), and are negative for lymphoid markers. These cells contain at least one Ph1 (Philadelphia) chromosome and have some characteristics of basophilic leukocytes (Fc receptors, basophilic granules, histamine production), along with being negative for lymphoid markers.

2.2 Instruments and materials

Instruments and materials that were used in this study were: Beckman coullier, a plate reader (Miltenyibiotec), a fluorescence-activated cell sorting (FACS) machine (Lebtech), a Nanodrop spectrophotometer ND-1000, Stratagene Mx 3005P, cytospin 4, a fluorescent microscope, and an eppendorf centrifuge 5415R. Cloned AMV first-strand synthesis kit, glutamine, foetal bovine serum and IMDM medium were obtained from Invitrogen, Paisley. SYBR green jumpstat Taq ready mix, penicillin-streptomycin solution and RPMI-1640 were all supplied by Sigma, Dorset. RNeasy mini kit was obtained from Qiagen, (West Sussex). Primers were supplied by Eurgentec (Belgium).

2.3 Cell culture

All cell lines were cultured in their appropriate media Table 2.2, which were made using the relevant reagents from Table 2.3. All cells were incubated in a Forma Steri-Cycle CO_2 incubator (Thermo

Scientific, UK) at 37°C with 5% CO_2 and 25% O_2. Cells were regularly passaged and weren't allowed to pass 90% confluency. Media was changed every 3-5 days or as required. When cells were passaged, cells were centrifuged at 690g for 3 minutes, the media was removed and the cell pellet was then re-suspended and washed with Hanks to remove any FBS. The cell solution was then re- centrifuged again at 690g for 3 minutes and the supernatant was removed. Once cells had been centrifuged down, fresh media containing foetal calf serum was added and the cell pellet was then re-suspended in the appropriate media and then split into the required dilutions and required flasks. All work was carried out in a sterile environment provided by a laminar flow hood with a double filter.

Cell line	***Source***	***Disease***	***Culure media***	***References***
KG-1	Human	Erythroleukaemia	IMDM, 20% FBS, P/S	(Koeffler and Golde 1978)
HEL92.1.7	Human	Erythroleukaemia	RPMI, 10% FBS, P/S and L-glutamine 5%	(Martin and Papayannopoulou 1982)
HL-60	Human	APML	IMDM, 20% FBS, P/S	(Collins, Ruscetti *et al*. 1978)
KU812F	Human	CML in blast crisis	RPMI, 10% FBS, P/S and L-glutamine	(Kishi 1985)
K562	Human	CML in blast crisis	RPMI, 10% FBS, P/S and L-glutamine	(Lozzio and Lozzio 1975)

Table 2.1 *A table listing cell lines used in this study. This table shows sources and diseases that cell lines were derived from. All cell lines were cultured at 37°C, 5% CO_2 and split 3 times a week or as required.*

2.4 Patients, controls and ethics

Blood samples obtained from normal healthy donors at the Royal Surrey County Hospital (Guildford, UK). Ethics for this work covered by a pre-existing multi-project approval. Of these, 143 healthy donor samples were analyzed in this study, between the ages of 18 and 86. These samples were collected in November 2011, where peripheral blood mononuclear cell isolation was undertaken. Cells were transferred into a 25ml universal tube and centrifuged at 690g for 3 minutes. The supernatant was removed and cell pellets were re-suspended in 1ml RPMI, supplemented with 10% (v/v) dimethyl sulphoxide DMSO (Sigma-Aldrich, UK) and 10% (v/v) FBS. The cell suspension was then transferred into 1.8ml cryovials. Cells were stored in cryovials and initially frozen overnight using a Mr Frosty Freezing Container (Thermo Scientific, UK) which has a temperature drop of 1°C/minute overnight before being transferred to the -80°C for storage. The collection of tissue and blood from AML patients and patients with other hematological disorders was approved by the local research ethics committee (REC no: 15/EM/0537). Suitable patients were identified at St. George's Hospital Haematology and Oncology. Patients were and asked to sign a written consent form. In total, ethics requested 100 patients, 73 of which were used in this study from patients with AML or other various haematological malignancies.

2.5 Cryopreservation of cell stocks

After calculating the cell density, a volume of media containing $5x10^6$ cells were transferred into a 25ml universal tube and centrifuged at 690g for 3 minutes. Then, supernatant was removed and cell pellets were re-suspended in 1ml storage medium, which was full growth medium supplemented with 10% (v/v) dimethyl sulphoxide (DMSO). The cell suspension was then transferred into 1.8ml cryovials. Cryovials were initially frozen overnight using a Mr. Frosty Freezing Container (Thermo Scientific, UK) which has a temperature drop of 1°C/minute. The following day the cells were transferred to liquid nitrogen storage

2.6 Revitalisation of cryopreserved cells

Cryopreserved cells were thawed rapidly by incubating in a 37°C water bath for 2 minutes. Then, cells were transferred to 10ml of pre-warmed FBS and centrifuged at 690g for 3 minutes. The supernatant was discarded to remove DMSO traces and cell pellets were re-suspended in 10ml full growth medium in a T-25 sterile cell culture flask and cultured at 37°C in a humidified environment containing 5% CO_2. After cell growth had resumed, cells were transferred to a T-75 flask and used for experiments. To maintain stock of all cell lines used, and provide varying passage numbers, cells were regularly cryopreserved. Cells in log phase of growth were centrifuged at 690g for 3 minutes to form a cell pellet. The pellet was then re-suspended at 10_6-10_7 cell/ml in appropriate complete media, with the addition of 10% DMSO (Sigma-Aldrich, UK). Cells were then transferred into 1ml aliquots, under sterile conditions, into labelled cryovials. Cryovials were frozen overnight. Rapid thawing at 37°C was used to revive cells from liquid nitrogen storage.

2.7 PBMC isolation

PBMCs were isolated from 10-15ml of human blood collected in EDTA tubes from normal healthy donors and patients. Blood was diluted in 10ml PBS and layered carefully onto 15ml of Ficoll before being centrifuged at room temperature for 25 minutes at 690g. PBMC's were harvested from the interface (buffy coat/ring) and placed in separate tubes, washed with 50ml of ice cold PBS before being centrifuged at 4°C for 5 minutes at 690g. The supernatant was aspirated without disturbing the cells and incubated for 10 minutes with 20ml ice-cold Red Blood Cell lysis buffer and centrifuged down at 4°C for 5 minutes at 690g. Isolated PBMCs were then re-suspended in 1ml of storage media [RPMI-1640 supplied by Sigma, 10% foetal calf Serum (FCS), 10% DMSO] placed in a cryotube and frozen down at -80°C.

2.8 RNA extraction

Cells were centrifuged down at 690g for 3 minutes, the media was removed, and the cell pellet was then re-suspended and washed with HBSS to remove any FBS. The cell solution was then re- centrifuged again at 1500rpm for 3 minutes and the supernatant was removed. The cell pellet was then lysed using 600µl of Lysis Buffer RLT (Qiagen, UK) with 6µl (10%) of β-mercaptoethanol (Sigma-Aldrich, UK). RLT and β-mercaptoethanol amounts used varied upon the size of the cell pellet. Complete RLT mix was then transferred to a gDNA eliminator spin column (Qiagen, UK) placed in a 2ml collecting tube and centrifuged for 30 seconds at 10,000rpm. The spin column was discarded 70% ethanol was added at a 1:1 ratio and mixed. The 1:1 mixture was then transferred to an RNeasy MinElute spin column (Qiagen, UK) placed in a 2ml collecting tube and centrifuged for 30 seconds at 10,000rpm. The collecting tube containing the flow through was discarded and replaced with a fresh 2ml collecting tube. 700µl of Buffer RW1 (Qiagen, UK) was added to the RNeasy MinElute spin column and centrifuged at 10,000rpm for 30 seconds. The collecting tube with flow through was discarded and again replaced with a new 2ml collecting tube. Then 500µl of complete Buffer RPE (Qiagen, UK), with ethanol added, was added to the RNeasy MinElute spin column and centrifuged at 10,000rpm for 30 seconds. The collecting tube was discarded and replaced before an additional 500µl of complete Buffer RPE was added to the RNeasy MinElute spin column and centrifuged at 10,000rpm for 2 minutes. Again, the collecting tube was discarded and replaced before the RNeasy MinElute spin column was centrifuged at 10,000rpm for 1 minute. The RNeasy MinElute spin column was then placed in a new 1.5ml collecting eppendorf with 30µl of RNase-free water (Qiagen, UK) added to the RNeasy MinElute spin column. This was left for 1 minute at room temperature before being centrifuged at 10,000rpm for 1 minute. The RNeasy MinElute spin column was discarded, and the collecting Eppendorf kept. RNA was quantified using a NanoDrop spectrophotometer ND-1000 (NanoDrop Technologies, USA). Samples were stored at -80°C for later use. All products used during the RNA extraction from Qiagen were supplied in the RNeasy Plus Mini Kit (Qiagen, UK).

2.9 Bioanalyzer

During gel preparation 550µL of RNA gel matrix was pipetted into a spin filter and spun at 1500g for 10 minutes at room temperature. 65µl filtered gel was aliquoted into 0.5 mL RNase-free micro centrifuge tubes. Preparing the Gel-Dye Mix: The RNA dye concentrate was left to equilibrate to room temperature for 30 minutes. RNA dye concentrate was then spun for 10 seconds. 1µl of dye was added into a 65µl aliquot of filtered gel. This solution was vortexed well and centrifuged at 13000g for 10 minutes at room temperature. During loading, put a new RNA chip on the chip priming station, 9µl of gel-dye mix was pipetted into the appropriate wells, waiting for exactly 30 seconds the clip was released before waiting a further 5 seconds. The plunger was slowly pulled back to the 1mL position. The chip priming station was opened and 9µl of gel-dye mix was added to the wells marked before discarding the remaining gel- dye mix. Loading the Marker 1: 5µl of RNA marker was loaded into all sample wells and in the wells of interest, accordingly marked. When loading the ladder and samples 1µl of prepared ladder was added to the wells marked. 1µl of sample was pipetted into each of the sample wells. 1µl of RNA Marker was added into each unused sample well. The chip was put horizontally into the IKA vortexer and vortexed for 1 min at 2400rpm before being analysed in the Agilent 2100 Bioanalyzer instrument within 5 minutes of preparation.

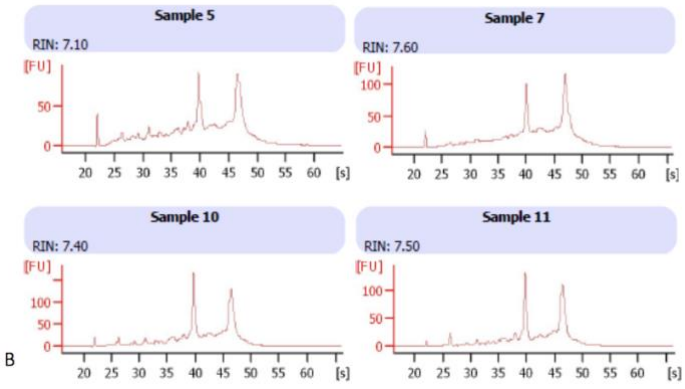

Figure 2.1 *The quality control run on RNA extracted from peripheral mononuclear blood cells that was used throughout the study. RNA was run on 2% agarose Egel when determining RNA integrity with RIN*

scores being obtained from running each sample on an agilent 2100 bioanalyser. Any RNA with a RIN score <7 was discarded.

2.10 Complementary DNA synthesis

RNA was reverse transcribed by mixing 1µg of RNA in a total volume of 20µl with final concentrations of 0.1M DTT (Invitrogen, UK), 10mM of deoxynucleotide triphosphate mix (dNTP) (Invitrogen, UK), 50µM Oligo $(dT)_{20}$ primers (Invitrogen, UK), 15 U/µl of Cloned AMV reverse transcriptase (Invitrogen, UK) and 40 U/µl of RNaseOut (Invitrogen, UK). RNase-free water was added to make up the volume to 20µl. This mixture was incubated at 50°C for 45 minutes. The cDNA synthesis reaction was terminated by incubating the sample at 85°C for 10 minutes. The cDNA samples were stored at a total volume of 500µl (2ng/µl) at 20°C. Total volume was made up by adding Nuclease-free water. All products used during the cDNA synthesis from Invitrogen were supplied in the Cloned AMV First-Strand Synthesis Kit (Invitrogen, UK). One sample of total RNA from normal human adult brain (Clontech Laboratories, UK) purchased for cDNA synthesis was stored at -80°C.

Well Reagent	***Well Reaction Volume***
cDNA	2µl (4ng/µl)
Primers (Forward and Reverse)	5µl
SYBR Green JumpStart Taq ReadyMix	12.5µl
Reference Dye	0.25µl
Nuclease-free water	5.25µl

Table 2.2 *A table of the reaction volumes for an individual well for qPCR*

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2.11 Quantitative reverse transcription polymerase chain reaction

A Quantitative Reverse Transcription Polymerase Chain Reaction (QRT-PCR) was performed using the Stratagene Mx3005P (Agilent Technologies, UK) real time PCR machine. The Stratagene Mx3005P machine measures PCR product accumulation during the exponential phase of the reaction by SYBR green fluorescence (Sigma-Aldrich, UK). The qRT-PCR master mix was prepared using 625µl SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich, UK), 12.5µl Reference Dye (ROX) (Sigma-Aldrich, UK), 262.5µl nuclease-free water and 100µl of 2ng cDNA. A primer master mix was also prepared by mixing 20µl each of relevant forward and reverse primers with 160µl of RNase-free water. Table 2.4 shows a breakdown of reagents and reaction volumes of an individual well. β-actin and GAPDH were used as housekeeper genes and thus the internal controls. Details of primers can be seen in the Appendix. Each experimental well was loaded with 20µl of qRT-PCR master mix and 5µl of appropriate primer master mix. Reaction conditions used were 1 cycle at 95°C for 10 minutes, followed by 40 cycles of 30 seconds at 95°C, 1 minute at 60°C and 1 minute at 72°C. The final cycle was 1 cycle of 1 minute at 95°C, 30 seconds at 55°C and 30 seconds at 95°C. The thermal profile can be seen in Figure 2.3. Results were analysed using the calculation derived from Livak's comparative Ct calculation method (Livak and Schmittgen, 2001). Cycle threshold (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold to be detected. Below is the calculation used:

Relative Expression = $2^{(-\mathbf{\Delta\Delta CT})}$ x 10,000

2.12 Calculations (qPCR)

There are two common methods used to analyse real-time PCR data: absolute quantification and relative quantification. Absolute quantification is known as the input copy number, which relates the PCR signal to a standard curve. Relative quantification on the other hand relates the PCR signal of a target transcript in a treatment group to the PCR signal of another sample just like an untreated control. The $2^{(-\mathbf{\Delta\Delta CT})}$ method is an extremely useful way of analysing relative changes in gene expression from real-time

quantitative PCR experiments. Normalizing to an endogenous reference allows us to use a method for correcting results with differing quantities of input RNA. The $2^{(-\Delta\Delta CT)}$ method is useful in that it uses data, which is already generated as part of the real-time PCR experiment that can perform this normalization function. This is a huge positive when it is not practical to measure the exact amount of input RNA. As mentioned, results were calculated by first normalizing internally to B-actin or GAPDH before dividing against the gene of interest *(HOXA9*) for a relative comparison. This calculation is derived from Livak's PCR calculation method (Livak and Schmittgen, 2001). Cycle threshold (CT) is defined as the number of cycles required for the fluorescent signal to cross the threshold and be detected. Calculations for the quantification of gene expression was done using the Livak formula. It is important that we are able to amulate where threshold is consistent or where the gradient is similar if we are to compare and contrast there values.

2.13 ΔCT Values for qPCR:

	HL60	***K562***	***KG-1***
HOXA9	177.09	359.57	906.66
PBX1	0.42	21.57	3.96
PBX2	3.93	32.33	2.28
PBX3	179.82	20.30	160.63
PBX4	2.16	4.09	1.83
MEIS1	214.73	41.91	186.51
MEIS2	11.98	291.37	21.48
MEIS3	0.76	15.96	1.59

Table 2.3 *A table of the ΔCT values used for qPCR. Values were calculated by first normalizing internally to B-actin before dividing against the gene of interest for a relative comparison. This*

calculation is derived from Livak's PCR calculation method: Relative Expression = $2^{(-\Delta\Delta CT)}$ *(Livak and Schmittgen, 2001).*

2.14 Preparation of cell lysate for western blotting

Western blotting is a very common technique in cell and molecular biology that is used to separate and identify proteins. A mixture of proteins is separated based on molecular weight and type, as the antibodies only bind to the protein of interest, through gel electrophoresis where the thickness of the band corresponds to the amount of protein present only one band should be visible (Mahmood and Yang, 2012). Cells were harvested then centrifuged at 720g for 3 minutes to aspirate the supernatant. After adding 600µl of RLT buffer (Qiagen) to disrupt the cells, the lysate was pipetted into QIAshredder spin columns placed in 2ml collection tubes and centrifuged for 2 minutes at full speed. This step was followed by transferring the homogenized lysate to AllPrep DNA spin columns that were placed in 2ml collection tubes and centrifuge for 30 seconds at full speed. The flow-through was then used for RNA purification by adding 400µl of 100% ethanol. The samples including any precipitate were then transferred to an RNeasy spin column placed in a 2ml collection tube and centrifuged for 15 seconds at full speed. The resulting flow-through was mixed vigorously with 1ml of buffer APP (Qiagen) and incubated for 10 minutes at room temperature to precipitate the protein. Afterwards this was centrifuged at full speed for 10 minutes and then 500µl of 70% of ethanol was added to the protein pellet and centrifuged at full speed for 1 minute. The supernatant was removed and the protein pellet was dried for 5-10 minutes at room temperature as incomplete drying may lead to problems when loading the protein onto the gel due to residual ethanol. 100µl of buffer ALO (Qiagen) was added and mixed vigorously to dissolve the protein pellet and then incubated for 5 minutes at 95°C to completely dissolve and denature the protein. The sample was cooled at room temperature and centrifuged for 1 minute at full speed. The resulted supernatant was used later in western blotting. The protein concentration in cell lysates was determined using a bicinchonic acid (BCA) assay.

2.15 Measuring protein concentrations by BCA

This assay was used to measure protein concentrations in cell lysates. It is based on reduction of copper Cu^{2+} to cuprous ion Cu^{+} by proteins. Cu^{+} then chelates with two molecules of BCA to form a purple-coloured product that can be detected by a 96-well plate reader at 562nm. The intensity of the formed colour increases linearly with increasing protein concentrations allowing protein concentrations to be calculated using a standard curve made of bovine serum albumin (BSA). This assay was performed as follows: 25µl of samples were added in duplicate in 96-well flat-bottom plates. Additionally, each plate contained a BSA series dilution added in duplicate. To each sample and standard well, 200µl of BCA working reagent, a combination of reagents A (1000mL (containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and B (25mL, containing 4% cupric sulfate) in a ratio 50:1, was added. The plates were gently agitated on a plate shaker and incubated for 30 minutes at 37°C. After the incubation, the plates were left at room temperature for 5 minutes in the dark before reading the absorbance on a plate reader. The absorbance means for the duplicate repeats of each sample and standard were calculated. The standard curve was then generated by the absorbance means of BSA series dilutions and their corresponding concentrations using Microsoft Excel. Finally, the protein concentrations of the samples were calculated using the standard curve linear regression equation.

2.16 Western Blot

After preparing, the resolving gel solution was poured between the glass plates and left for 20 minutes until the gel was solidified. 10% stacking gel solution was prepared and the gel plate filled to the edge of the glass plate. The comb was inserted to ensure no air bubbles. Butanol was added on top of the resolving gel and washed with ddH2O. The running buffer was poured into the electrophorator to cover the gel completely. The ladder (Bio-Rad protein ladder) (1µl) and protein sample (15µl) was loaded

into each well of the gel carefully and the gel was run for 1 hour at 200V until the dye front reached the end of the gel. A transfer sandwich was created with 3 pieces of filter paper, nitrocellulose membrane and then the gel, which had been removed from the glass plates followed by 3 pieces of the filter paper. These were all immersed in transfer buffer before creating the sandwich. The sandwich was placed on the transfer apparatus and run at 45V for 1 hour 30 minutes. After transfer, the membrane was coated with Ponceau S stain to visualize transfer efficiency and then washed thoroughly.

Running Buffer:	***20x***
Tricine (free base)	71.7g
Tris (free base)	72.6g
SDS	10g
Sodium Bisulphite	2.5g

Table 2.4 *A table showing the preparation of 20x running buffer.*

Transfer Buffer:	***10x***
Tricine (free base)	15.2g
Glycine	72.1g
SDS	5g

Table 2.5 *A table showing the preparation of 10x transfer buffer.*

2.17 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation

SDS-Page resolving gel:	***10%***	***Stacking gel***
H2O	4.00ml	3.4ml
1.5M Tris PH 8.8	2.50ml	-
1M Tris PH 6.8	-	630µl
30% Protogel acrylimide	3.34ml	750µl
10% SDS	100µl	50µl
10% APS	80µl	50µl
TEMED	8µl	8µl

Table 2.6 *A table showing the preparation of 10% resolving gel and stacking gel*

2.18 Electrophoresis

10% SDS PAGE Running buffer	
Tris base	30.3g
Glycine	144.1g
10% SDS	100mL
DdH2O	1L

Table 2.7 *A table showing the preparation of running buffer solution.*

2.19 Transferring proteins to poly-vinylidene fluoride (PVDF) membranes

Transfer buffer	
Tris	3g
Glycine	15g
Methanol	200mL
DdH2O	1L

Table 2.8 *A table showing the preparation of transfer buffer solution.*

2.20 Detection of proteins by antibodies

After transferring proteins, PVDF membranes were blocked in 5% blocking buffer (5% (w/v) dried non-fat milk powder, 0.1% (v/v) Tween-20) with gentle rocking for 2 hours at room temperature. Then, the membranes were incubated with primary antibodies, 10µl of primary antibody (Rabbit polyclonal to *HOXA9,* Goat polyclonal to MEIS1) (Abcam, UK) was appropriately diluted in 10ml of TBST and added to the membrane with gentle rocking overnight at 4°C. After overnight incubation, the membranes were washed 4 times with 0.1% Tween PBS solution for 10 minutes at room temperature with gentle rocking to remove unbound antibodies. Then, the membranes were incubated with secondary antibodies (anti-Rabbit for *HOXA9* and anti-Goat for MEIS1) and (Abcam, UK) diluted for 2 hours at room temperature with gentle rocking; the antibodies used in western blots (Table 2.9). After 2 hours incubation, membranes were washed 3 times, as above. The membranes were exposed to superSignal® West Pico Chemiluminescent substrate according to the manufacturer's recommendation. Membranes were exposed to carestream® Kodak BioMax® light film for 3 minutes to visualise the chemiluminescence. Films were developed and then fixed using developer and fixer, respectively.

Antibody	***Type***	***Isotype***	***Molecular weight (KDa)***	***Working concentration***	***Cat #***	***Supplier***
Primary anti-HOXA9	Polyclonal	Rabbit IgG	30	1/100	ab83480	Abcam
Primary anti-PBX1	Polyclonal	Rabbit IgG	47	1/100	ab97994	
Secondary for anti-Rabbit	Polyclonal	Goat IgG	32	1/2000	ab205718	

Table 2.9 *A table listing the antibodies used in western blot and their working dilutions.*

2.21 HXR9 and CXR9 peptide synthesis

The synthesis of the HXR9 and CXR9 peptides have been previously described (Morgan, Pirard *et al*. 2007). HXR9 is a short peptide of 18 amino acids, that is comprised of a hexapeptide sequence and nine C-terminal arginine residues (R9) enabling cell penetration. The HXR9 hexapeptide mimics the hexapeptide sequence of *HOX* proteins of paralogs 1-10, this sequence functions as a competitive inhibitor of *HOX/PBX*. CXR9 is the control peptide that does not have the functional domain but has the R9 region. The stability of the peptides was increased using D-isomers NH_2- and COOH- terminals. The half-life of these peptides is approximately 12 hours in the serum (Morgan, Pirard *et al*. 2007). A conventional column-based technique was used to synthesise HXR9 and CXR9 which were purified to at least 80% (Biosynthesis Inc., Lewisville, TX, USA). Sequences for both peptides are as follows:

HXR9: WYPWMKKHHRRRRRRRRR (2700.06 Da)

CXR9: WYPAMKKHHRRRRRRRRR (2604.14 Da)

2.22 Statistical analysis

Student t-tests or one-way anovas were used for data analysis on all assays using GraphPad Prism software. Apart from the age related analysis where healthy volunteer mRNA expression levels were calculated using Livak's PCR calculation method (Livak and Schmittgen, 2001) and then grouped into decades of age, as in Morgan; 2005, where the graph depicts decades from 20's to 80's as a graph constructed using GraphPad accounted for and represented on the GraphPad prism package. This was used in statistical calculations where data was further analysed with t-test using GraphPad statistical software (GraphPad software Inc., San Diego, CA USA) and by Mann-Whitney-Test using unpaired Wilcoxon rank sum test from R. All results are expressed as the mean of three separate experiments performed in duplicate with error bars to show the SEM. Statistical significance was also determined by comparing the different conditions in each assay with each other [$p < 0.05$ (*), $p < 0.01$ (**), or $p <$

0.001 (***) or $p < 0.0001$ (****)]. Calcusyn software was used to determine the IC_{50} of the HXR9 treatment for the different cell lines.

Chapter 3: The role of HOXA9 and its co-factors in-*vitro*

3.1.1 The importance of *HOXA9*

The *HOX* genes are a family of transcription factors responsible for the identity of tissues in early embryonic development that subsequently play several roles in regulating adult processes involved in cell renewal. As highlighted, *HOXA9* is one of the most intensively studied examples of these genes, which is a key regulator of adult haematopoiesis. The dysregulation of *HOX* genes and *HOXA9* is common to many haematological malignancies, including AML (Ferrando *et al*., 2003), and solid malignancies including renal (Shears *et al*., 2008), ovarian (Cheng *et al*., 2005, Morgan *et al*., 2010), lung (Abe *et al*., 2006, Plowright *et al*., 2009) and other cancers. Over-expression of *HOXA9* in AML is well documented and has been shown as a poor prognostic factor (Golub *et al*., 1999), with down-regulation of *HOX* genes being a favourable prognostic factor (Andreeff *et al*., 2008, Zangenberg *et al*., 2009). *HOX* genes have been identified as playing an oncogenic role in leukaemia (Eklund, 2007) and their over-expression in some cancers, for example in melanoma, maintains cell survival, through preventing c-Fos transcription (Morgan *et al*., 2007), for this and many other reasons c-Fos becomes a gene of interest in the *HOXA9* death pathway.

3.1.2 Co-factors of *HOX*

DNA binding site studies over the last number of decades have suggested that *HOX* proteins have limited selectivity and specificity, and therefore need co-factor interactions to increase both (Phelan *et al*., 1995, Moens and Selleri, 2006, Mann *et al*., 2009). The most commonly studied *HOX* co-factors are the TALE family of proteins, which are compromised of the PBX and MEIS families (Moens and Selleri, 2006, Mann *et al*., 2009). These co-factors have been identified in playing crucial roles in development and haematopoiesis (Thorsteinsdottir *et al*., 2001). PBX1 null mice die during the embryonic stage due to severe hematopoietic defects and suffer severe anaemia (DiMartino *et al*., 2001). Mice that were deficient in MEIS1 failed to generate megakaryocytes, expressed severe haemorrhaging and also died during the embryonic stage (Figure 3.1) (Hisa *et al*., 2004). MEIS1 and PBX contribute to the inhibition of myelopoiesis and in the production of erythropoietic cells (Pillay *et*

al., 2010). MEIS1 has been shown over recent years to contribute to leukemic transformation (Kroon *et al*., 1998). Almost as a rule, *HOX* proteins (*HOX* 1-10) generally bind with PBX1 (Shen *et al*., 1996), whereas the paralog groups 9-13 are shown to bind with MEIS1 (Shen *et al*., 1997). This has been challenged in recent years although no definitive studies have managed to confirm this.

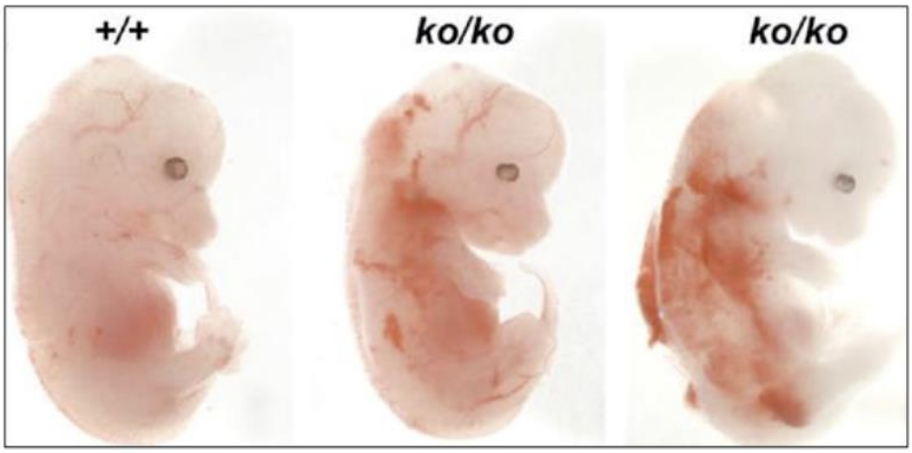


Figure 3.1 *A schematic diagram depicting the morphology of MEIS1-deficient embryos compared to the wild-type (Hisa T 2004).*

3.1.3 Translocation of *HOXA9*

Trying to underpin the mechanisms by which *HOXA9* and its co-factors interact, and function has been widely studied over the last decade. Research has tried to gather more information about the localization of *HOXA9* but also the localisation of its co-factors and use these findings for future studies. Significant advances over the last few years has been in nucleoporin *Nup98* associating with *HOXA9* binding and its localization not only to the NPC and intranuclear bodies that can be characterized by both deconvolution and electron microscopy (Griffis *et al*., 2004). The prevalence of these bodies can vary between cell lines and uncovered information on the whereabouts of *HOXA9* and its movements in and out of the nucleus. For example, in Xu *et al*, 2010, it was shown that in nearly all Nup98 leukemogenic translocations, an N-terminal half of the Nup98 protein containing the FG and GLFG repeats, is retained in the leukemic fusion protein suggesting that Nup98 leukemic fusion proteins may also be associated

with GLFG bodies as well as an endogenous Nup98 or with a distinct intranuclear compartment. Nup98 fusion proteins are valuable to the cell and are most frequently detected in AML patients that have been linked to *HOXA9*. Probably the most common of these is the *Nup98/HOXA9* fusion protein, which consists of the amino acids 1-469 of Nup98 joined to amino acids 163-271 of *HOXA9*. Other studies aimed at gathering more information about localization used GFP-tagged proteins visualized in HeLa cells after transient transfection (Griffis *et al*., 2004), with both the GFP-tagged Nup98 GLFG domain and the full-length protein present in intranuclear bodies.

Furthermore, the localization and regulation of transcription factor activity has in recent years been shown to play a pivotal role in various biological processes. Different mechanisms regulate transcription factor activity, as in post-translational modifications, expression level and protein stability. Since a transcription factor exerts transcriptional regulation in the nucleus, the control of nuclear localization plays a crucial role in this regulation (Stevens and Mann, 2007). For this reason, proteins like PBX1, MEIS1 and PREP1 (transcription factors) in the nucleus of cells is for the regulation of the appropriate target genes. Recent studies have shown that PBX1 (co-factor for *HOXA9)* nuclear/cytoplasmic distribution is very tightly regulated through other mechanisms (Laurent *et al*., 2009). As for the role of MEIS/PREP and PBX interactions and their nuclear translocation (Figure 3.3) for this interaction, the PBC-A domain of PBX1 (Chang *et al*., 1997, Knoepfler *et al*., 1997), and the HR1 and HR2 domains of the MEIS1/PREP1 are required. The LFPLL motif of HR1 is essential for PBX1 binding (Diaz-Blanco *et al*., 2007). It has been shown that PBX1 and MEIS1/PREP1 bind co-operatively to DNA, however they interact efficiently in the absence of DNA as well (Knoepfler *et al*., 1997, Berthelsen *et al*., 1998). These interactions in the absence of DNA can regulate the subcellular localization (Jaw *et al*., 2000, Berthelsen *et al*., 1998, Berthelsen *et al*., 1999) and the stability (Jaw *et al*., 2000, Longobardi and Blasi, 2003) of PBX proteins. PBX1 has a very dynamic subcellular localization. It contains two co-operative NLS (nuclear localization signal) (Saleh *et al*., 2000) and two independent NES (nuclear export signal) ((Kilstrup-Nielsen *et al*., 2003). The first NLS is situated in the N-terminal arm of the HD (amino acids 234-239) and contains the consensus RRKRR sequence. The second and less conserved domain (KRIRYKKNI), is situated within the helix 3 (amino acids: 285–294) (Saleh *et al*., 2000). The two NES are situated within the PBC- A domain spanning amino

acids 45-72 and 73-90 respectively ((Kilstrup-Nielsen *et al*., 2003). The two NES are able to mask and inhibit the NLS via an intramolecular interaction between the N-terminus and homeodomain of PBX1. This conformational change of PBX1 due to the interaction with MEIS/PREP exposes the PBX1 NLS, causing nuclear translocation (Saleh *et al*., 2000). The NES of PBX1 has been shown to mediate the interaction with the nuclear export receptor Crm1 responsible for exporting PBX from the nucleus. Importantly, two NES are located within the domains responsible for the interaction with MEIS/PREP and the deletion of either of the NES can impair this interaction. The contact domains for Crm1 and MEIS/PREP overlap, therefore the interaction of PBX1 with MEIS/PREP masks the NES and can facilitate PBX-MEIS/PREP to stay in the nucleus as heterodimers (Stevens and Mann, 2007, Kilstrup-Nielsen *et al*., 2003). Importantly, PBX1 nuclear localization is not only dependent on the MEIS/PREP interaction but various other mechanisms that regulate its subcellular distribution (Figure 3.2).

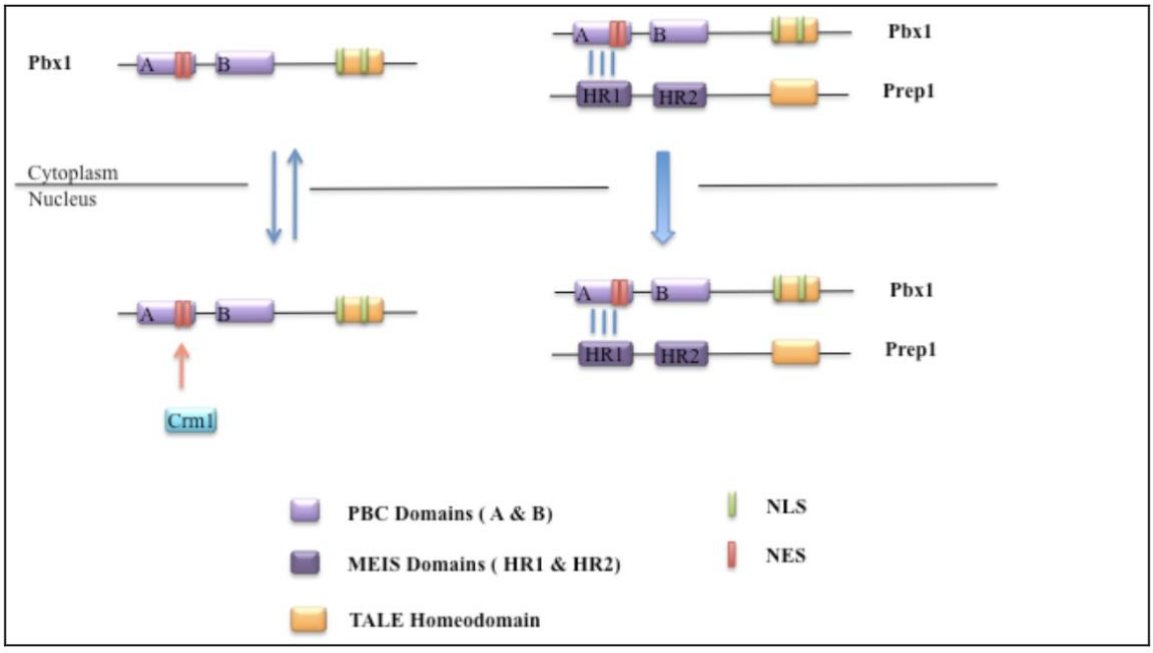


Figure 3.2 *A schematic diagram depicting PBX1 and PREP1 interaction and their subsequent translocation into the nucleus. PBX also interacts with MEIS through a similar domain and this complex then translocates into the nucleus as shown in this figure (Stevens and Mann, 2007, Kilstrup-Nielsen et al., 2003).*

Although a lot of this previous work gives some indication about the whereabouts of these proteins and their mechanisms of action very little to date has been done to try and analyse the localization of these proteins in AML cell lines. This poses the question whether *HOXA9* would have the same reaction with or without PBX1 and other co-factors implicated in *HOXA9* binding. Gathering a better understanding about these interactions and where in the cell these interactions occur along with the mechanism of action by which the cells transcribes these genes either in normal cells or in leukemic cells will outline the future of drug development (Figure 3.3).

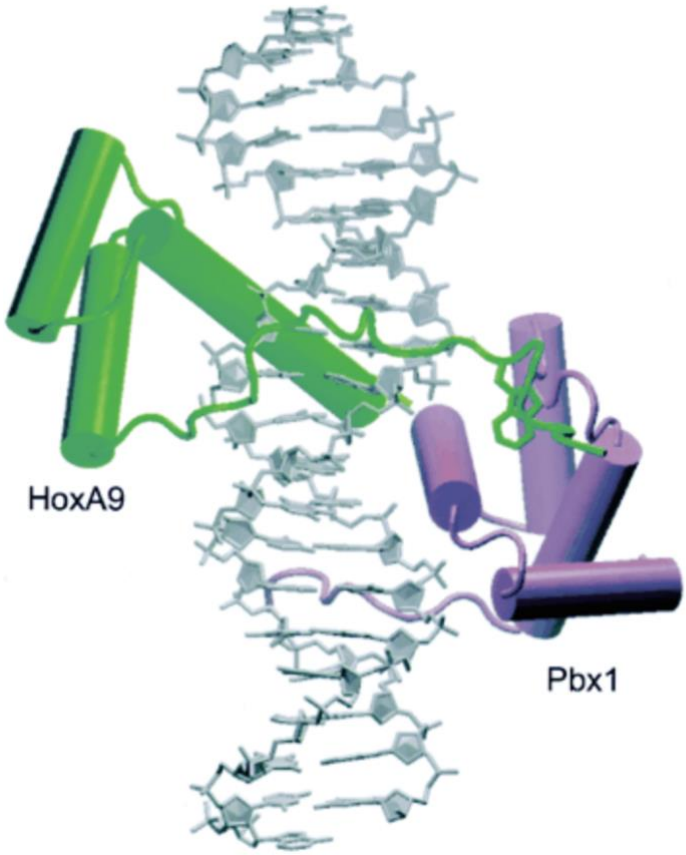


Figure 3.3 *A schematic depicting the VMD of the HOXA9-PBX DNA complex. HOXA9 is shown here in green and PBX1 is depicted in purple. The linker portion as well as the hexapeptide of HOXA9 spans the minor groove of the complex and the conserved tryptophan is inserted into the PBX1 binding pocket.*

Aims

To analyse the expression of *HOX* genes, mainly *HOXA9* and its co-factors in AML derived cell lines and asses their importance and the role they may play in the development of AML, related haematological malignancies and therefore the progression to AML.

Materials & Methods

3.2.1 Fixation and permeabilization of cell lines

A cell suspension of 10_6 cells was transferred from the flask into a micro-centrifuge tube and centrifuged at 690g for 3 minutes. These were then re-suspended in 1ml PBS and centrifuged at 690g for 3 minute. The supernatant was discarded and 160µl of PBS was added to 20µl cells to re-suspend them. Cells were fixed with 20µl 4% paraformaldehyde (PFA) solution at 37°C for 15 minutes then centrifuged at 690g for 3 minutes. PFA was freshly prepared in a fume hood due to its toxicity and was handled appropriately. A washing step was performed twice by using 1ml PBS and centrifuging at 690g for 3 minutes. This step was carried out after all the steps of this technique. Cells were then permeabilized to allow free access of the antibody to its antigen, since *HOXA9* and its co-factors (MEIS and PBX) are expressed inside the cells. 0.3% Triton X-100 in PBS was added to the cells before being centrifuged at 690g for 3 minutes. This step was also followed by washing with 1 ml PBS and centrifuging at 690g for 3 minutes.

3.2.2 Immunofluorescence labelling of *HOXA9* and *PBX1*

Before labelling the cells with antibodies, cells were blocked to minimize the adsorption of nonspecific antibody sites followed by incubation of cells with 5% PBSA, which was prepared by dissolving 5µg of bovine serum albumin (BSA) (Sigma-Aldrich, UK), in 1ml PBS, for 1 hour at room temperature. Cells were centrifuged at 690g for 3 minutes and the PBSA was aspirated. 1µl of the primary antibody (Rabbit polyclonal to *HOXA9* and Rabbit polyclonal to PBX1) (Abcam, UK) was appropriately diluted in 200µl of PBS; Afterwards cells were incubated with 200µl antibody at room temperature for 2 hours. This step was followed by two washing steps with 1ml PBS to remove unbound antibody and centrifuged at 690g for 3 minutes. 1µl of the secondary fluorochrome antibody, (Donkey polyclonal Secondary Antibody to Rabbit IgG - H&L, Alexa Fluor 488) (Abcam, UK), was diluted in 200µl, and added to the cells. Cells were incubated at room temperature for 1 hour followed by the centrifuging at 690g for 3 minutes and washing with 1ml PBS.

Antibody	*Type*	*Isotype*	*Molecular weight (KDa)*	*Working concentration*	*Cat #*	*Supplier*
Primary anti-*HOXA9*	Polyclonal	Rabbit IgG	30	.1/200	ab83480	Abcam
Primary anti-PBX1	Polyclonal	Rabbit IgG	47	1/200	ab97994	
Secondary for anti-Rabbit	Polyclonal	Goat IgG	/	1/2000	ab150077	

Table 3.1 *A table listing the antibodies used during immunofluorescence.*

3.2.3 Preparations for microscopy

Cells were counterstained with DAPI (4′,6-diamidino-2-phenylindole) by adding 2 drops to 100µl of PBS to the cells and then being left for 5 minutes. Afterwards cells were centrifuged at 690g for 3 minutes, washed with 1ml PBS and mounted on a glass microscope slide to be analysed using a Floyd microscope. A very small drop of clear acrylic nail varnish was applied to each of the 4 corners of an 18 x 18mm, 0 thickness (0.5um), borosilicate glass coverslip (BDH, Poole, UK) thereby preventing the cells from being damaged (squashed) when the slide is covered. The 0.5um thickness coverslip was used to maximize the depth of focus during confocal analysis. Vectashield anti fade reagent (Vector Laboratories Ltd, Peterborough, UK) 20ul, was applied to the centre of the coverslip and 40ul to the middle of the slide. The slide was inverted and lowered over the cover slip to rest onto the coverslip. It was carefully lowered allowing the two drops of antifade reagent to meet, the effect being to draw the cover slip rapidly up towards the slide. The slide was then rapidly turned over preventing movement of the cover slip (which would result in drawing cells from the slide) and placed to one side allowing the cover slip to settle on the nail varnish mounts for 1 minute. Using clear acrylic nail varnish, a line was painted along four edges of the cover slip, applying minimal pressure so as to not crush the cells and creating a sealed chamber. Slides were analysed within two days of staining and stored at 4°C in a blacked-out slide box.

3.2.4 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) provides a technique for optically sectioning fluorescent labelled whole cells or tissue, restricting out of focus light using a confocal imaging aperture (pinhole) within the optical system (Amos *et al*., 1987). The CLSM system also typically uses high numerical aperture objective lenses to focus the excitation laser light and reduce background fluorescence (Zemanová *et al*., 2003). Laser light is used, for excitation of the fluorescent probes or conjugated antibodies (Dunn *et al.*, 1994). The CLSM used throughout this project (Zeiss LSM 4.10) provided two different laser light sources for fluorescent excitation; low powered argon ion and helium neon producing two different wavelengths of laser light, 488nm and 543nm respectively, making it possible to undertake multi-fluorescent analysis. As for the EPF microscopy, this requires the use of specialized filters, ideally permitting fluorescent signals from only one fluorochrome to be detected when more than one fluorochrome is present within the specimen. The excitation light is reflected by a dichroic mirror and focused onto the specimen by the objective lens. The fluorescent and refracted light is then collected by the same objective lens. The dichroic mirror splits the fluorescent and refracted light, only allowing the fluorescent light to pass and removing the refracted light from the final image. Filters are also employed to ensure that reflected laser light is not allowed to reach the confocal aperture and contribute to the final image. The emitted fluorescent light is then focused onto the confocal imaging aperture. The focal point of the aperture and specimen are on the conjugated lanes restricting light from above and below the focal plane and resulting in high axial resolution free from stray light (Wilson and Pawley, 1995). The depth of focus is dependent on the size of the confocal aperture, with a smaller aperture producing a thinner focal plane, forming an image of an individual slice through a region of the cell.Additional advantages are obtained through the ability of the CLSM system to record only in focus light. It creates the possibility for the microscope to act as if there were an infinite depth of field compared to an extremely limited one of approximately 10im for ELM. By using an automated microscope stage, the depth of the focal plane can also be adjusted whilst undertaking simultaneous x, y scanning to create an axial z scan. A complete in focus representation of the specimen throughout the z-axis, z-scan slices can be achieved (Sheppard and Shotton, 1997). CLSM optics also enables the

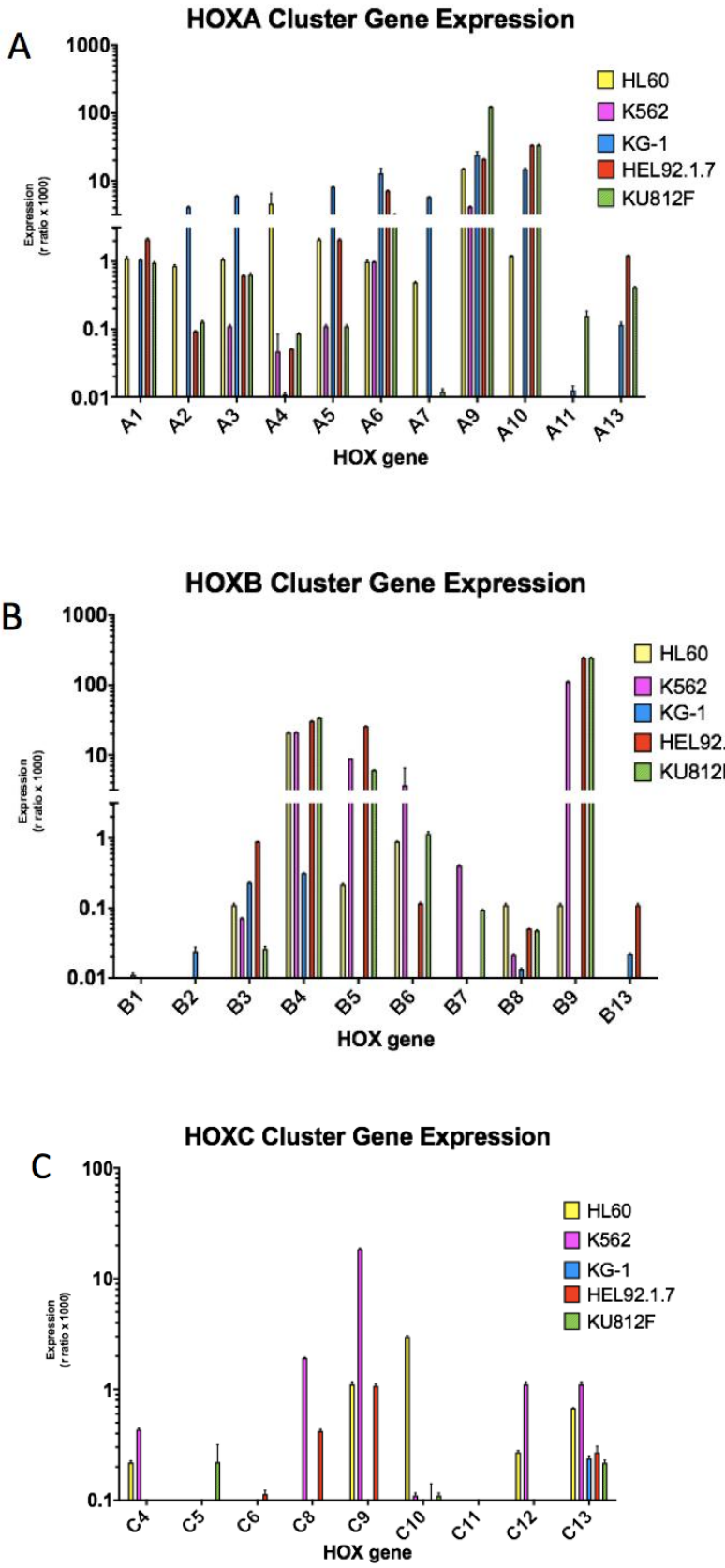
observation of cells that are adherent whilst minimising the interference from cells floating within the mounting medium and free fluorochromes or non-specific fluorescent debris also above the cell layer (Zemanová *et al.*, 2003).

Results

3.3.1 *HOX* gene expression in a whole panel of 39 *HOX* genes measured in leukemic cell lines

The expression of all *HOX* genes were assessed by qPCR in the five AML derived cell lines HL-60, KG-1, HEL92.1.7, KU812F and K562 (Figure R3.1). This analysis revealed substantial differences in *HOX* gene expression, whereby *HOXA* and *HOXB* cluster genes were far more highly expressed than those of *HOXC* and *HOXD* clusters. Importantly, many *HOXA* cluster were seen to be highly expressed in KG-1 cells including *HOXA5, HOXA6, HOXA9* and *HOXA10.* Noticeably, *HOXA9* and *HOXA10* were also highly expressed in KU812F cells, whereas *HOXB9* on the other hand was the most highly expressed gene in K562 and HEL92.1.7 cells. HL-60 cells show very little *HOX* expression.

Global *HOX* Gene Expression in AML-Derived Cell Lines



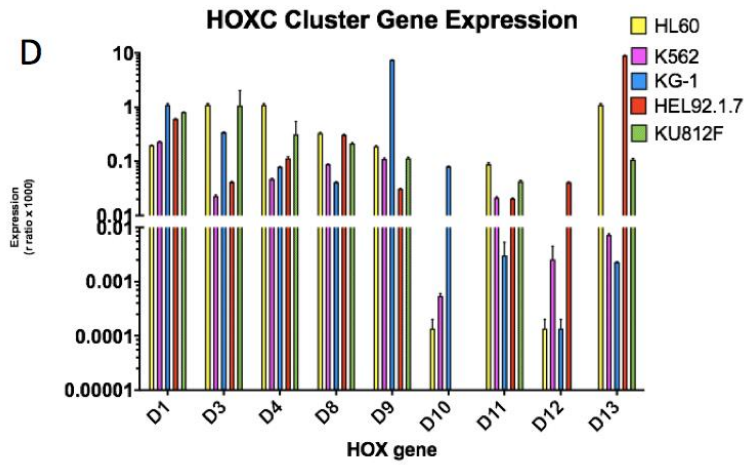


Figure R3.1 *HOX gene expression in KG-1, HEL92.1.7, KU812F, K562 and HL-60 cell lines. A) The most expressed HOXA genes in the KG-1 cell line appeared to be HOXA5, HOXA6, HOXA9 and HOXA10, with HOXA9 and HOXA10 being the most highly expressed in KU812F. B) HOXB9 was the most expressed HOX gene in HEL92.1.7 and K562. C) HOXC and D) HOXD genes showed very low expression in comparison in AML derived cell lines. The expression of these HOX genes are represented as the logarithm of the ratio of the expression of HOX genes to the housekeeping gene β-actin (×1000). The mean of three independent experiments are shown and error bars show the SEM in these graphs.*

3.3.2 Identifying *HOX* genes heavily linked to cancer in leukemic cell lines

To assess the variation in expression of *HOX* genes in leukemic cell lines, several HOX genes of most interest were selected based on previous literature: *HOXA5, HOXA6, HOXA7, HOXA9, HOXA10*, *HOXB4, HOXB9, HOXC4, HOXC9* and *HOXD9* (Figure R3.2). Mononuclear cells isolated from the peripheral blood of healthy volunteers were used as a control where n=3. As expected, expression varies between cell lines, qPCR was used to analyse 3 separate AML derived cell lines: HL-60, KG-1 and K562. *HOX* genes are generally of low expression in mononuclear cells isolated from the peripheral blood of healthy adults whereas gene expression varies significantly between cell lines. In terms of *HOXA9*, HL60 and KG-1 cells express this gene at a much higher level than K562, which is supported by previous studies as they are being both myeloid cell lines, with K562 being a CML-derived cell line. Other noticeable genes of higher expression include: *HOXA5, HOXA6, HOXA10, HOXB4* and HOXC9 in HL60*, HOXB9 HOXC4* and *HOXC9* in K562 cells and *HOXA5, HOXA6, HOXA7, HOXA10, HOXB4* and *HOXD9* in KG-1 cells.

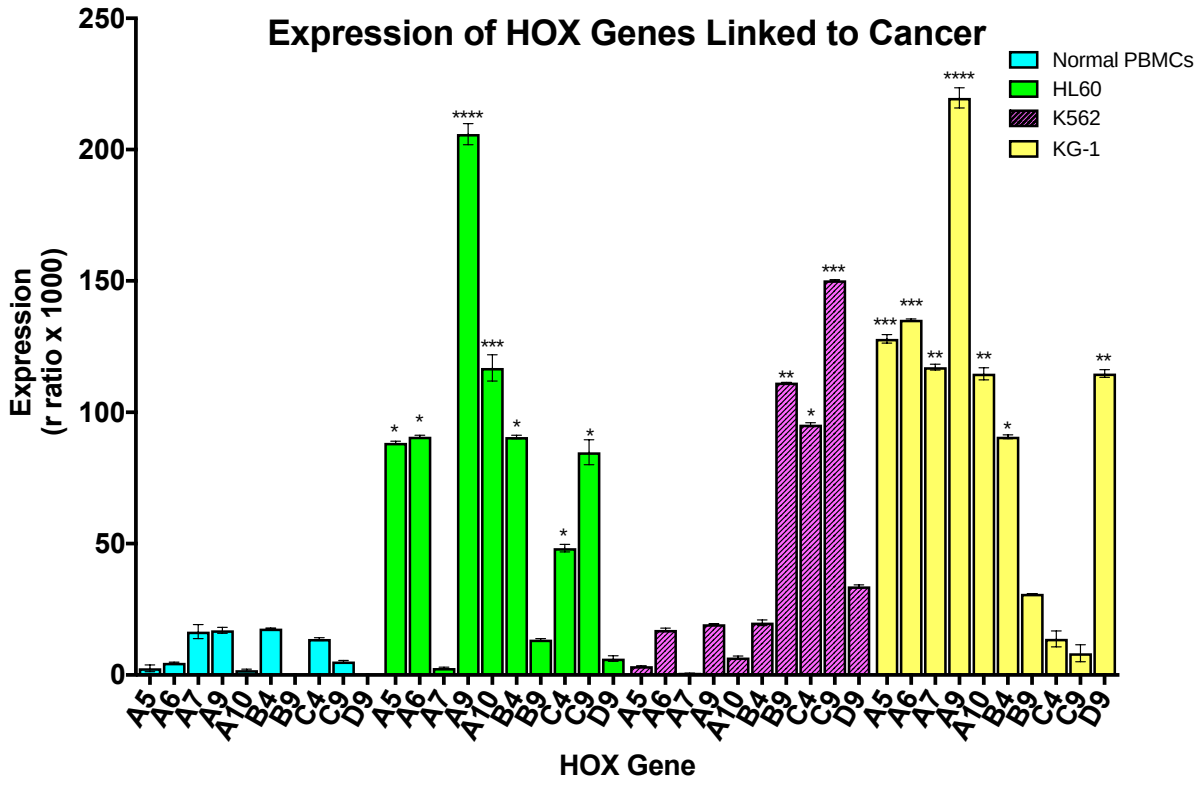


Figure R3.2 *Variation in expression of HOX genes heavily linked to cancer in mononuclear cells isolated from the peripheral blood of healthy adults against the HL60, K562 and KG-1 leukaemic cell lines. The expression of these HOX genes are represented as the logarithm of the ratio of the expression of HOX genes to the housekeeping gene, β-actin (×1000). The mean of three independent experiments are shown and error bars show the SEM in these graphs.* *p< .05, **p < .01, ***p <.001 and ****p <.0001.

3.3.3 PBX and MEIS are important co-factors of HOXA9 in leukemic cell lines

To determine the expression of *HOXA9* and its possible co-factor genes *(MEIS1, MEIS2, MEIS3, PBX1, PBX2* and *PBX3)* and how their expression may vary between cell lines, qPCR was used to analyse 3 separate AML derived cell lines: HL-60, KG-1 and K562 (Figure R3.3). As already seen, there are substantial differences in global *HOX* gene expression (Figure R3.1), whereby expression changed between cell lines, as expected. HL60 and KG-1 cell lines show similarity in most genes, both being primary derived AML cell lines. The only exception to this rule is in *HOXA9* expression with HL60 expressing much low levels. Both however express very low levels of *PBX1* with K562 cells showing a change in comparison exhibiting a lower *HOXA9* and a higher *PBX1* expression level. Much of this change is due to HL60 and KG-1 being myeloid cell lines with K562 being a secondary leukaemic CML derived cell line. Morphologically K562 cells differ to both HL60 and KG-1 cells, expressing positivity for the BCR-ABL gene. What was most interesting however, was that in almost every gene analysed K562 stood out and appeared to have an inverse relationship with the other two cell lines for each gene not just in HOXA9.

This analysis gives us a better understanding of the relative expression of *HOXA9* in healthy donors compared to that of leukemic cell lines, allowing us to quantify the variation in *HOXA9* expression as well as highlighting co-factor expression in the healthy volunteers. Most noticeable in this analysis was the variation in expression between the healthy volunteers and the leukemic cell lines in terms of *HOXA9* expression, however, it was observed in Figure R3.3, that *HOXA9* expression in each cell line as well as in the mononuclear cells isolated from peripheral blood differs to that of *PBX1*. This significance will be touched on in a little more detail when discussing *HXR9* and the strong interactions between *PBX1* and *HOXA9.*

Gene Expression in PBMCs and AML Derived Cell Lines

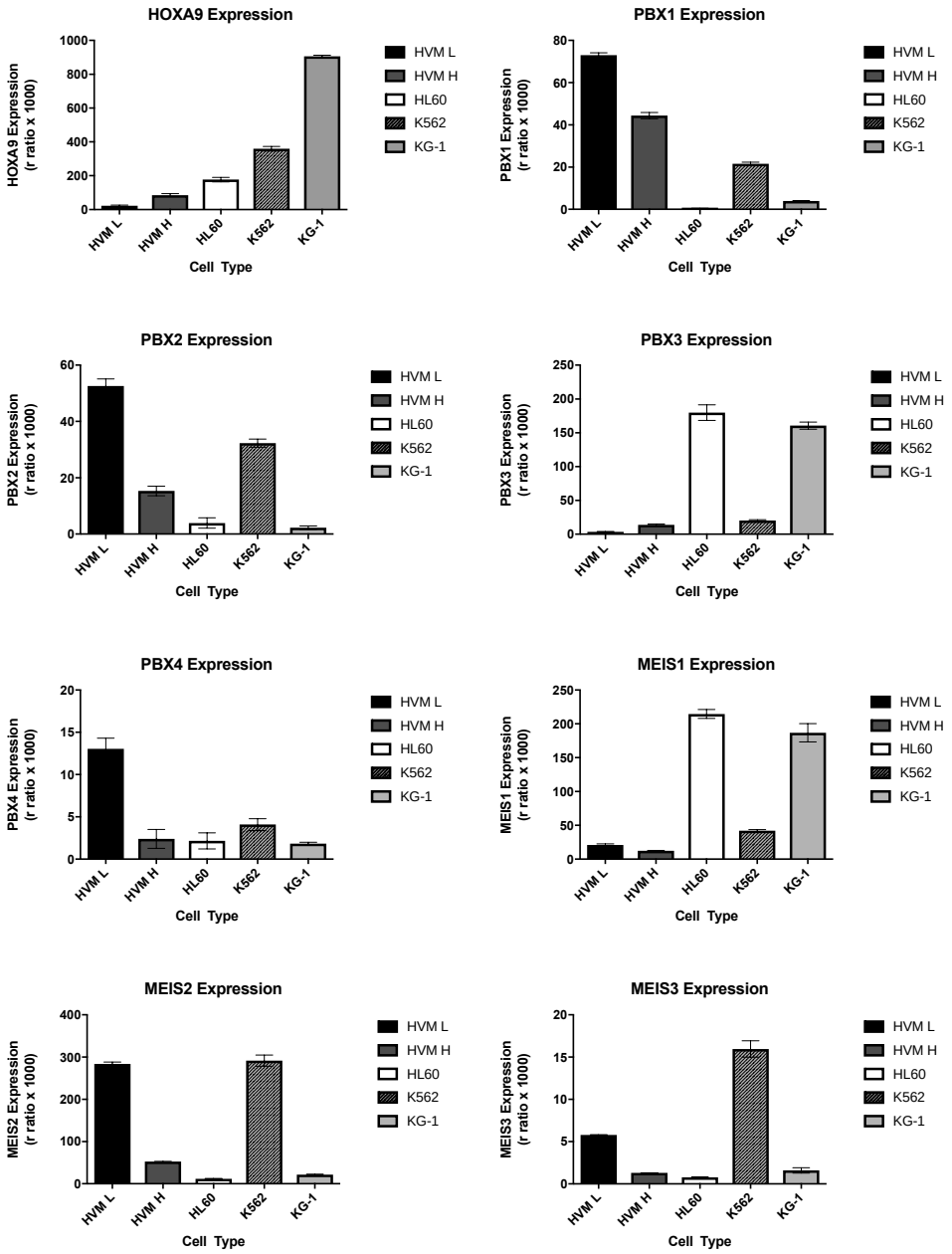


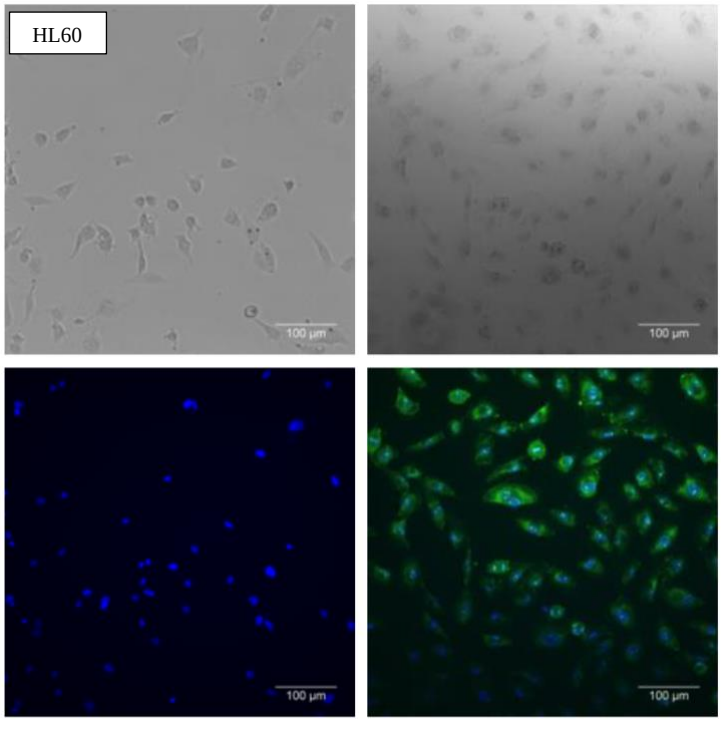
Figure R3.3 *Variation in gene expression of HOXA9 and its potential co-factors in mononuclear cells isolated from the peripheral blood of healthy adults (HVM Low HOXA9 expression, HVM High HOXA9 expression) against the HL60, K562 and KG-1 leukaemic cell lines (see appendix for ΔCT values). The expression of these genes are represented by the ratio of the expression of HOX genes to the housekeeping gene β-actin (×1000). The mean of three independent experiments are shown and error bars show the SEM in these graphs, normal PBMCs n=3.*

3.3.4 *HOXA9* is present in leukemic cell lines and appears to translocate to the nucleus

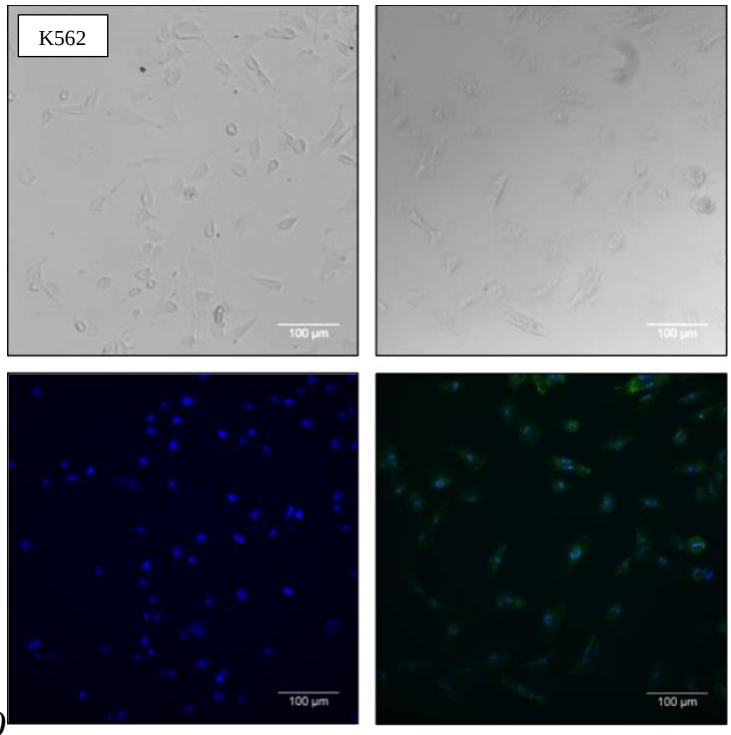
Immunofluorescence was used to validate *HOXA9* expression but also to observe localization in the cell. Immunofluorescent images of KG-1, HL60 and K562 and cells show a very similar pattern to that of the *HOXA9* gene expression analysed via qPCR in Figure R3.3 with the highest presence of positive antibody for *HOXA9* in KG-1, and K562 and HL60 showing a lower expression rate respectively. The is significance in variation of expression between the healthy volunteers and the leukemic cell lines in terms of *HOXA9* expression, however, it was observed in Figure R3.3, that *HOXA9* expression in each cell line as well as in the mononuclear cells isolated from peripheral blood. This is significant and will touch on this in a little more detail when discussing *HXR9* and the strong interactions between *PBX1* and *HOXA9.*

Localization of these proteins is important, with *HOXA9* appearing almost entirely within the nuclear envelope (Figure R3.4). The true mechanisms by which these proteins get chaperoned into the nucleus for transcription is currently unknown however our current hypothesis is that *HOX-PBX* binding facilitates the translocation of these proteins into the nucleus.

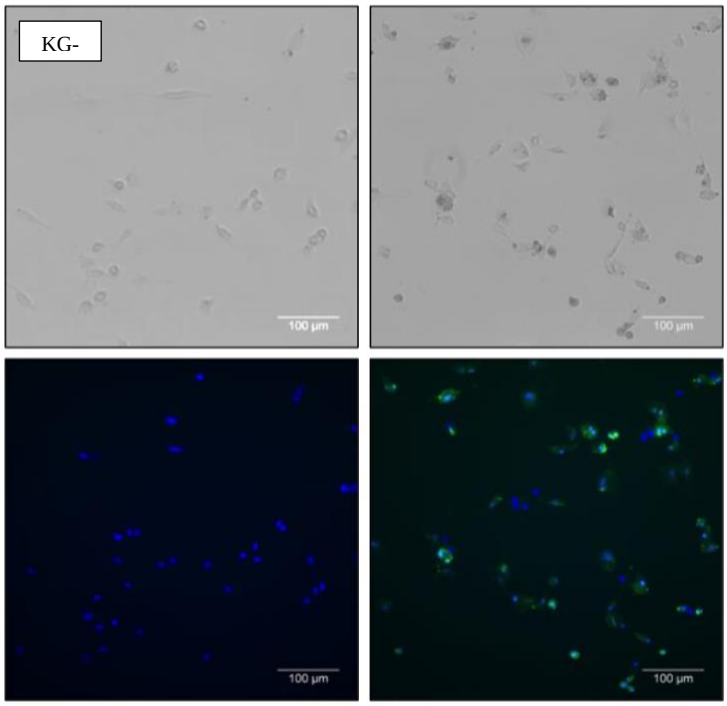
HOXA9 Localisation in AML-Derived Cell Lines



(A)



(B)



(C)

Figure R3.4 *Representative immunofluorescence images of (a) HL60 cells (b) K562 and (C) KG-1 cells stained with DAPI and HOXA9 respectively (scale bar shown, 100µm). All images were taken using an Olympus Fluoview FV1000.*

Discussion

In summary, the expression of all the 39 *HOX* genes were analysed in the five main AML-derived cell lines: *HOX* expression analysed concurred with previous studies (Figure R3.1), with the myeloid origin cell line KU812F highly expressing both *HOXA9* and *HOXA10* genes and the erythroid cell line HEL92.1.7 highly expressing the *HOXB9* gene, consistent with previous studies (Giampaolo *et al.*, 1994, Kawagoe *et al.*, 1999, Pineault *et al*., 2002). K562 as we know, originated from a CML patient in blast crisis, however, here we saw that the cell line highly expresses the *HOXB9* gene, supporting previous studies that have suggesting that K562 is in fact a human erythroleukaemia cell line (Andersson et al., 1979). Interestingly, KG-1 expresses high levels of *HOXA5, HOXA6, HOXA9* and *HOXA10* even though it is an erythroid cell line. Previously, studies have shown that KG-1 cells express high levels of *HOXA9* and *HOXA10* and it is hypothesized that this increased expression is due to the presence of a trisomy 8 mutation (Kok *et al.*, 2010). At present, the associating mechanism underpinning the up regulation of some of these *HOXA* genes and trisomy 8 is unclear. The APML cell line HL-60 expresses very little *HOX* which is seen across the board in all HOX families, concurrent with previous studies, APML was found to be characterized by a global down-regulation of *HOX* genes (Thompson *et al.*, 2003). It is worth noting at this point, however, that the most expressed subset of *HOX* genes in this cell line were posterior (6-13) *HOX* genes. Possibly most important of all is that the KG-1 cell line is CD34+, which is very often characterized by a poor response to most current conventional chemotherapies, and happens to strongly express *HOX* genes compared to the $CD34^-$ cell line HL60 that represents AML with a more favourable prognosis (Ahmed *et al.*, 1999, Marone, Scambia *et al.* 2002; Dores, Devesa *et al.*, 2012). This difference in prognosis suggests a link with the expression of *HOX* genes, as high and low expression of *HOX* genes has recently been shown to correspond with a poor or favourable prognosis, respectively (Golub *et al.*, 1999, Andreeff *et al.*, 2008, Zangenberg *et al.*, 2009). A good comparison between KG-1 and HL-60 in terms of *HOX* expression is as mentioned, consistent with previous studies showing that HOX genes are highly expressed in $CD34^+$ cells although they appear to show little or no expression in $CD34^-$ cells (Sauvageau *et al.*, 1994a, Pineault *et al.*, 2002). Although this is true, it is important to note here that the biggest limitation when

assessing the expression of *HOX* genes is to note the lack of normal $CD34^{+}$ cells which should be addressed in future studies. A potential explanation for the up-regulation in AML cell lines may be their ability to interrupt haematopoietic differentiation (Pineault *et al.*, 2004). The evaluation of the expression of *HOX* genes in all AML lines is important for us to get a basic understanding of the role *HOX* genes play in AML and other cancers and therefore identify the role *HOXA9* may play in these malignancies. These results, in general, are consistent with the already established hypothesis that HOX genes are expressed in a lineage-restricted manner with *HOXA* and *HOXB* genes being expressed in myeloid and erythroid cells, respectively (Giampaolo *et al.*, 1994, Kawagoe *et al.*, 1999, Pineault *et al.*, 2002).

The expression of *HOX* genes in leukemic cell lines were compared to that of healthy volunteer mononuclear cells isolated from the peripheral blood of adults. As we would expect, *HOX* genes are generally low expressed in mononuclear cells isolated from the peripheral blood of healthy adults. Gene expression varies between cell lines, although in terms *of HOXA9*, HL60 and KG-1 cells express this gene at a much higher level than in K562 cells which as we know are myeloid cell lines (Figure R.3.2). K562 on the other hand is a CML-derived cell line and differs from both HL60 and KG-1 cells (Figure 3.2). It is noteworthy that much like many *HOX* genes, *HOXA9* positively regulates the transcription of other *HOX* genes including *HOXA7* and *HOXA10* as well as its co-factors *PBX3* and *MEIS1* (Thorsteinsdottir *et al*., 2001). This was supported by our findings (Figure R3.2) with *HOXA7* and *HOXA10* resembling *HOXA9* expression to some degree and being noticeably expressed at high levels in both HL60 and KG-1 cells opposed to K562 cells, which exhibited a significantly lower level of *HOXA9*, which is in turn unable to positively upregulate *HOXA7* and *HOX10*. HOX gene expression may also contribute to tumorigenesis through activating anti-apoptotic pathways. This was first discovered in breast cancer with deficient p53 expression in breast cancer cell lines and primary tumours that has also been shown to correlate with the methylation of the *HOXA5* promoter and loss of *HOXA5* expression. *HOXA5* binding sites have been found in the promoter region of the TP53 tumour suppressor gene, and transient transfection of *HOXA5* activated the p53 expression in the breast cancer cell lines, MCF-7 and ZR75.1, which in turn led to an increased rate of apoptosis (Raman *et al.*, 2000).

HOXA5 is also known to induce apoptosis independently of p53 pathways, with activation of caspase-2 and caspase-8 mediated apoptosis (Chen *et al*., 2004) supported by our observations of high *HOXA5* expression levels in both HL60 and KG-1 cells (Figure R3.2). *HOXA10* is also increased in these cell lines with high *HOXA5* expression, which supports studies that have already shown *HOXA10* to play a similar role to *HOXA5* by activating p53 expression in breast cancer cells. The most logical explanation for this is through oestrogen-*HOX* signalling as oestrogen can upregulate the expression of *HOXA10* in these ER+ breast cancer cells (Chu *et al.,* 2004).

Analysing potential co-factors revealed substantial differences in gene expression between cell lines, whereby expression changed as would be expected when delving deeper into their morphology. HL60 and KG-1 cell lines are most similar to K562 cells showing a change in overall HOX gene profile expression. As mentioned previously, *HOXA9* positively regulates the transcription of its co-factors *PBX3* and *MEIS1* (Thorsteinsdottir *et al.,* 2001), in which cell lines expressing a higher level of HOXA9 also have a high level of *MEIS1* and *PBX3* in comparison to cell lines like K562, which has a lower level of all three genes. It is important to note at this point that low expression of *HOXA4* and *MEIS1* are also favourable predictors for AML patient outcome (Zangenberg *et al*., 2009) supporting our findings (Figures R3.2, Figure R3.3) with high expression levels of MEIS1 being demonstrated in HL60 and KG-1 cells, a lower expression level shown in K562 cells before observing an almost zero expression shown in healthy volunteer mononuclear cells isolated from the peripheral blood of adults with varying *HOXA9* expression (Figure R3.3). The signature up-regulation of *HOXA6, HOXA9* and PBX3 shown here has been demonstrated as a poor prognostic marker in some forms of AML over the last decade, with a simultaneous knockdown of both *HOXA6* and *HOXA9* increasing cell death and increased sensitivity of AML cell lines to cytarabine (Dickson *et al.,* 2013) with studies also showing that a high expression of *HOXA7, HOXA9* and *HOXA11* as well as *PBX3* (a co-factor) are independent prognostic markers of adverse overall survival in abnormal karyotype AML (Li *et al.,* 2012) (Figures R3.2, Figure R3.3).

Immunofluorescence helped validate the presence of *HOXA9* and *PBX1* (Figure R3.4). The presence of *HOXA9* and *PBX* reflects almost identically previous analysis and data obtained from the qPCR analysis of the genes at the mRNA level (Figure R3.2). This is not always the case for several reasons including

post-translational modifications being the most prevalent. Localization on the other hand, gives us a better indication of the mechanism by which *HOXA9* interacts with other proteins and its possible role in cell death and oncogenesis. Current understanding or hypotheses based on the literature and our findings, is that *HOX* proteins, *HOXA9* in our case, binds to its co-factors *(PBX1)* and is then transported into the nucleus where it functions as a transcription factor and facilitates the transcription of genes that are heavily implicated in haematopoiesis (Figure R3.4)

This *in vitro* data is a good foundation; however, it is also important to assess whether primary cells have or express similar characteristics to these cell lines, which is not always the case. For this, it is essential to analyse the above in primary cells, in aged matched healthy donors but also in patients with AML and other haematological disorders.

Chapter 4: *HOXA9* expression and aging

4.1.1 *HOXA9* and AML

Acute myeloid leukaemia is a heterogeneous group of genetically and phenotypically aggressive disorders whereby the differentiation of hematopoietic progenitor cells is increasingly blocked, which in turn increases their self-renewal ability and disturbing the normal regulation of proliferation (Fröhling *et al.*, 2005). In the UK, AML is the most frequent acute leukaemia in adults, accounting for just over 75% of cases. The median age at presentation of disease is 68-69 years and the male: female ratio is about 5:4. In this age group, AML has a very poor outcome with less than 5% of the patients surviving 5 years after the initial diagnosis of the disease, compared to 35-40% in the younger age groups.

Not only this, lower rates of complete remission (CR) in the elderly (40–50% vs 60–70% in the young) and a short duration seen in patients who are eligible for treatment strongly suggests there is a different disease biology in this age group. From a clinical standpoint, it is far more frequent that elderly patients have already received previous cytotoxic treatment or radiotherapy or perhaps they already have antecedent hematologic diseases, such as myelodysplastic (MDS) or myeloproliferative neoplasms, (see Chapter 5).

AML is commonly classified by either the French-American-British (FAB) system, or the World Health Organization (WHO). The former is based on morphology and the maturation stage that classifies AML into eight groups (M0-M7). The latter is also based on morphology, but includes immunophenotyping, genetics and clinical manifestations and classifies AML into four main groups: AML with recurrent genetic abnormalities, AML with myelodysplasia-related changes, therapy-related AML and MDS, or AML that does not fit into any of these groups. Non-random chromosomal alterations, such as balanced translocations, monosomies, trisomies, inversion and deletions have been found in the leukemic cells of almost 55% of AML patients, and until recently they were the most crucial prognostic factors for complete remission (CR) and higher probability of relapse and survival (Estey and Döhner, 2006, Mrózek *et al*., 2007). Recent advances in molecular diagnosis have resulted in both gene alterations and the dysregulation of specific genes becoming increasingly important as prognostic elements in AML.

This has helped to clarify the numerous heterogeneities of AML subsets, particularly AML subsets showing normal karyotype (NK-AML)(Bullinger *et al.*, 2008) and furthered understanding of the molecular mechanisms of leukaemogenesis.

4.1.2 *HOXA9* and aging

It has been suggested previously that *HOXA9* expression levels remain relatively constant throughout adult life until the age of about 50-55 (Morgan 2005). At this age, *HOXA9* begins to increase in many individuals averaging a 2.5-fold increase compared to normal *HOXA9*. The effect this change in gene expression may have on normal haematopoiesis is to date relatively unclear. However, age-related changes to *HOXA9* expression during later decades may support its association with increased incidence of AML. Until now, many studies carried out on the *HOX* cluster genes and aging has focused primarily on embryogenesis. There does appear to be a strong correlation. between *HOX* gene expression levels and the development of AML and an increased incidence of AML with age but very little to date has been done to investigate the relationship between *HOX* expression, AML and its subtypes and ageing together in the same study. In this chapter, we aim to examine this and analyse some outliers (high *HOX* expression) in depth, to assess if chronic inflammation is responsible in the elevated gene expression of *HOXA9* or if this elevated expression is increasing with age.

Younger age brackets also have a risk of developing AML, when breaking down the subtypes of AML. *HOXA9* expression has been detected in 63% of 52 bone marrow samples taken from 46 AL children (Jia *et al* 2013). The positive *HOXA9* expression rate in the AML group was significantly higher than in the ALL and control groups (86% vs 35% and 13%. Among these children with AML, those with M5 AML had the highest *HOXA9* mRNA levels, shortly followed by children with M4 AML and children with M1 and/or M2 AML. Similarly, when analysing *HOXA9* levels especially when looking at healthy volunteers, patients with AML but also those with pre-leukemic disorders that may potentially go on to develop AML to ascertain which of these subtypes may lead to a worse prognosis dependent on HOXA9 expression. However, *HOXA9* expression was not detected in children with

M3 AML at all. The treatment subgroup had significantly higher positivity for *HOXA9* expression and *HOXA9* mRNA levels than the remission subgroup and the control group, with no significant differences between the latter two groups. As expected, the non-remission subgroup had significantly higher *HOXA9* expression than the remission subgroup and control group and the high-risk subgroup of AML children had relatively high levels of *HOXA9* expression.

In summary, *HOXA9* is associated with the occurrence of AL, and its expression level is significantly higher in children who suffer from AML than in those who suffer from ALL. It has been widely suggested that there is a positive correlation between the expression level of *HOXA9* and the risk of childhood leukaemia, with a high expression of *HOXA9* suggesting a poor prognosis (Jia *et al*., 2013).

Despite previous studies into the changes in gene expression in an aged population, relatively little is known about the expression of *HOX* genes in the mature blood lineages and their activation efficiency. This becomes hugely important when dealing with the elderly or age-specific peripheral blood, whereby many these "healthy" donors may in fact have underlying abnormalities in relation to inflammation. Recent studies have purified different cell populations from human peripheral blood based on their expression of the surface markers and used this analysis to gather a better understanding of the activation of specific cell populations (Morgan and Whiting, 2008).

4.1.3 Important CD markers

Often distinguishing what type of tumour being looked at under the microscope is difficult. However, in this case with acute leukaemia, most cases have distinctive features, auer rods for example, which can tell what kind of leukaemia being looked at; other cases have no such clues and can be difficult to distinguish. In these cases where indications are minimal, in hematopoietic or lymphoid neoplasms, flow cytometry can give us a better understanding and observing what markers are on the surface of the cells is a great start. Flow cytometry involves fluorescent antibodies that tag molecules on the surface

of cells. The flow cytometer has a small tube that allows the cells to flow one at a time past a laser beam (Figure 4.1). In addition to showing what kinds of markers a cell has, we may also sort cells by other parameters like size and complexity. It's an incredibly useful technique that can be used for various purposes, one of the most common is to find out which markers are on the surface of specific cells. Important CD markers in AML are CD13, CD33 and CD117. The "CD" in the names of these markers, stands for "cluster differentiation." An important way of referring to different molecules on the surface of cells. There are over 350 CD markers. Sometimes, however, it's the absence of a marker that helps with diagnosis rather than the presence of one.

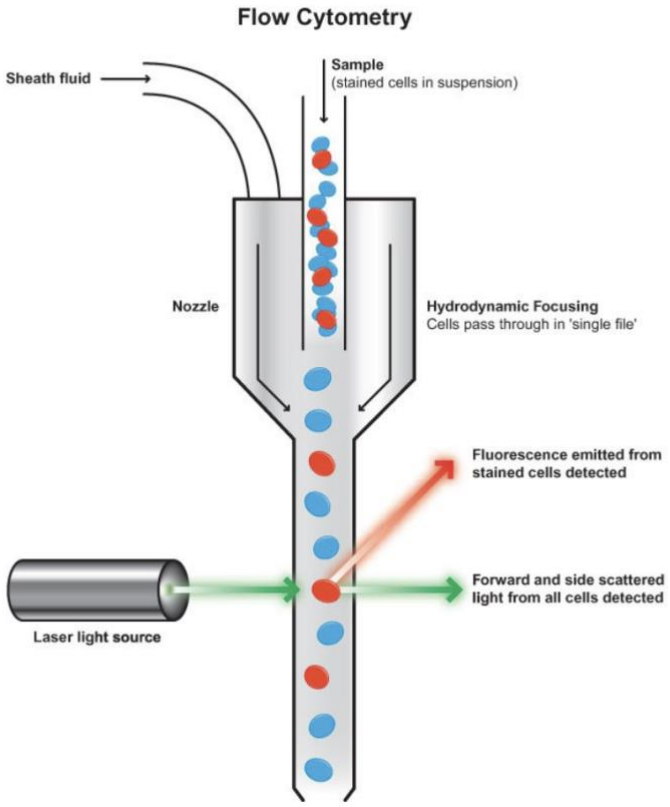


Figure 4.1 *A schematic diagram depicting how flow cytometry can be successfully used to identify cells, involving fluorescent antibodies that tag molecules on the surface of cells and the small tube that allows the cells to flow one at a time past a laser beam (Abcam).*

CD33: is a 67-kDa glycoprotein expressed on the surface. Of early multi-lineage hematopoietic progenitors, myelomonocytic precursors and generally to more mature myeloid cells, being absent from normal pluripotent hematopoietic stem cells. Almost 85–90% of AML cases have been shown to express the CD33 antigen. CD33 has also gained clinical importance as a suitable tumour-associated antigen and an important target for monoclonal antibody-based AML therapies (Harrington *et al*., 2014).

CD117: is a transmembrane protein receptor encoded by the c-kit proto-oncogene. The CD117 ligand is a stem cell factor and an important hematopoietic regulator. CD117 is present on just over 4-5% of normal bone marrow mononuclear cells and is extremely common in AML and CML in myeloid blast crisis but found rarely in ALL which makes CD117 an important marker when isolating AML and ALL. Initially CD117 was used as a primitive myeloid marker and has since been identified in all FAB subtypes of AML thought to predict a poor outcome. CD117 has been seen on most leukemic blasts of myeloid origin where 87% of AML, 80% of MPD-myeloid BC, and 75% of MDS. Although CD117 is a receptor for stem cell factor, its expression does not appear to correlate with CD34 positivity (Wells *et al*., 1996).

4.1.4 Cellular activation

Activation of specific cells is thought to affect the cells' probability of becoming cancerous. However, are genes expressed differently in cells based on activation or inflammation? Comprehensive analysis of HOX gene expression in activated leukocyte populations showed a unique pattern of HOX expression that may define cellular identity (Morgan and Whiting, 2008). The magnitude of these changes is striking, with activated T-cell subsets exhibiting virtually a complete loss of expression of many *HOX* genes. This is most prevalent in the more posterior members of the groups like *HOXA10*, *HOXC13,* and *HOXD12*. Conversely, activated monocytic and B-lymphocyte cells exhibit a large increase in expression of the same genes. Given the regulatory roles of *HOX* genes it is extremely likely that some of these changes in expression begin to facilitate the cellular changes associated with

activation. Of these the most noticeable seems to be increased proliferation, a response that is known to be mediated by *HOX* genes in several hematopoietic lineages.

CD14: is a myeloid differentiation marker primarily found on monocytes and macrophages, although low levels can also be found on neutrophils. In recent years, CD14 has been viewed simply as a useful marker molecule for both monocytes and macrophages. The function of CD14, its expression in different cell types, its regulation of expression have been described, and the diagnostic value of CD14 in various diseases was first discussed in detail in Ziegler-Heitbrock 1993. Upon cell activation, CD14 surface expression seems to decrease on monocytes and CD14 is released (Shive, 2015).

CD11b: is an integrin family member that pairs with CD18 to form the CR3 heterodimer. CD11b is expressed on the surface of leukocytes including monocytes, neutrophils, natural killer cells, granulocytes and macrophages, as well as on nearly 9% of spleen cells and almost 45% of bone marrow cells. Functionally, CD11b is known to regulate leukocyte adhesion and migration to mediate the inflammatory response. For this reason, it can be of benefit when assessing inflammation and/or activation of specific white blood cells.

The relevance of cellular activation in relation to gene expression was of great significance when looking at *HOXA9.* The hypothesis was that if these cells were chronically inflamed or activated, this may alter gene expression and therefore give a false positive which need to be addressed. Not only this but investigating the effects that activation/inflammation may have on the development of disease is of particular interest.

Aims

To observe how the expression of *HOXA9* changes with age in healthy donors and attempt to identify outliers in order to assess if chronic inflammation is responsible for the elevated gene expression of *HOXA9* or if this elevated expression is really increasing with age.

Observing if *HOXA9* is expressed highly in myeloid cells that were also double positive for the AML markers CD33 and CD117 in peripheral mononuclear blood also helps us to understand the relationship between *HOXA9* and disease progression. Analysis was also undertaken on *HOXA9* expression in cells positive for the activation markers CD14 and CD11b in peripheral mononuclear blood cells of healthy donors.

Materials & Methods

4.2.1 Patients, controls and ethics

Analysed blood samples for this section were obtained from 143 normal healthy donors (aged 18-86) collected from the Royal Surrey County Hospital (Guildford, UK).

4.2.2 Decade grouping

Healthy volunteer mRNA expression levels were calculated using Livak's PCR calculation method (Livak *et al*., 2001) and then grouped into decades of age. As previously reported in (Morgan; 2005).

4.2.3 LPS treatment

A vial of 10-15ml of human blood was collected in EDTA tubes from three healthy donors following informed consent and left at room temperature; all samples were used within 4 hours of collection. Each blood sample was treated for 3 hours with 1ug/ml LPS. The cells were placed into 6 well plates and suspended in RPMI with 5% FBS serum, penicillin and streptomycin. Cells were either: untreated, treated for 3 hours or treated for 3 hours and left to recover for 24 hours in fresh media (RPMI with 5% FBS serum, penicillin and streptomycin).

4.2.4 CRP ELISA

Samples of high *HOXA9* expression were selected and tested for chronic inflammation using CRP. Seven (7) outliers were selected and PBMCs were isolated as above. The Wash Buffer Concentrate (25x) was allowed to reach room temperature and mixed to re-dissolve any precipitated salts. This solution was diluted with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 litres, 100 mL may be diluted up to 2.5 litres) and labelled as Working Wash Buffer. This Working Wash Buffer was stored in the refrigerator and used within 14 days. 1x Streptavidin-HRP was prepared within 15 minutes of usage. For each 8-well strip used in the assay, 10µL Streptavidin-HRP (100x) solution was take up (the pipette tip was wiped with a clean absorbent paper to remove any excess solution) and

dispensed into a tube containing 990µL of HRP Diluent and mixed thoroughly. The unused Streptavidin-HRP (100x) solution was returned to the refrigerator and stored. Hu CRP Standard was reconstituted to 8ng/mL with Standard Diluent Buffer. This was swirled to allow the contents to sit for 10 minutes to ensure complete reconstitution. The standard was used within 15 minutes of reconstitution. 300µL of the reconstituted standard was added to a tube containing 1700µL Standard Diluent Buffer and mixed, labelled as 1200pg/ mL Hu CRP. 300µL of Standard Diluent Buffer was added to each of 7 tubes labelled as follows: 600, 300, 150, 75, 37.5, 18.75, and 0pg/mL of Hu CRP. Serial dilutions of the standards were made as described below. Human plasma required a 3000–fold dilution in the Standard Diluent Buffer. This was undertaken using a serial dilution of samples in a dilution series of 1:3000.

This assay has been calibrated against the WHO reference preparation 85/506 (NIBSC, Hertfordshire, UK, EN6 3QG). One nanogram equals 0.98 International Units. Glass or plastic tubes were used for diluting standards. 100µL of Standard Diluent Buffer was added to the zero standard wells with wells reserved for the chromogen blank left empty. 100µL of each of the standards, diluted samples or controls were then added to the appropriate microtiter wells. The plate was then covered with the plate cover and incubated for 2 hours at 37°C. The solution was thoroughly aspirated, and wells were washed 4 times with diluted Wash Buffer. 100µL of Hu CRP Biotin Conjugate solution was then added into each well except chromogen blanks before covering the plate with plate cover and incubated for 1 hour at room temperature. The solution was thoroughly aspirated, and wells were washed 4 times with diluted Wash Buffer. 100µL of 1x Streptavidin-HRP was added into each well except the chromogen blanks before the plate was covered with plate cover and incubated for 30 minutes at room temperature. The solution was thoroughly aspirated, and wells were washed 4 times with diluted Wash Buffer. 100µL of Stabilized Chromogen was added to each well for the substrate solution to begin to turn blue and incubated for 30 minutes at room temperature in the dark. 100µL of Stop Solution was added to each well and mixed gently until the solution in the wells changes from blue to yellow.

The absorbance was read at 450nm within 30 minutes after adding the Stop Solution. The curve-fitting software was used to generate the standard curve. This four-parameter algorithm provides the best standard curve fit. Optimally, the background absorbance was subtracted from all data points, including standards, unknowns and controls, prior to plotting. Concentrations for unknown samples and controls were read from the standard curve. The values obtained for the samples were multiplied by the appropriate factor to correct for the sample dilution and plotted on a graph using GraphPad Prism.

4.2.5 Gating strategy

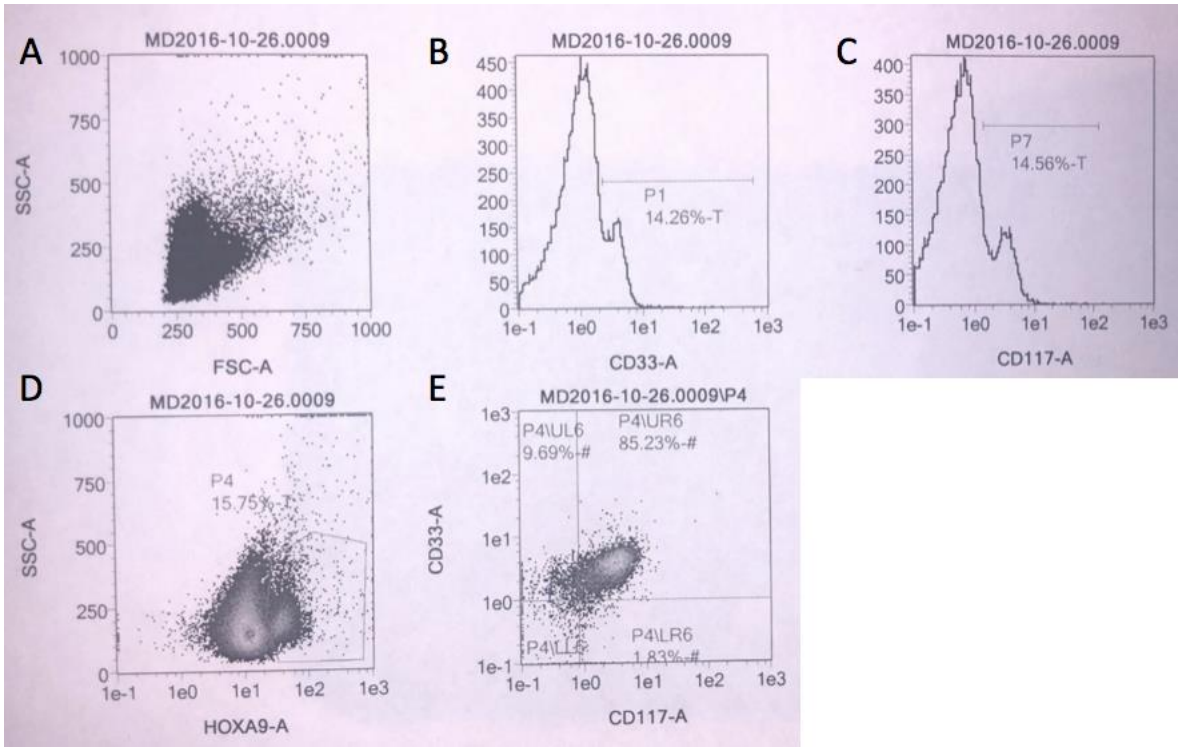


Figure 4.2 *Gating strategy for FACS. A) Isolated myeloid cells from PBMCs. B) Isolating CD33+ cells. C) Isolating CD117+ cells. D) Isolating HOXA9+ cells. E) Gating CD33+ and CD117+ cells that are also HOXA9+.*

4.2.6 FACS staining titrations

Cells were titrated with the relevant antibody in ratios such as: 1:10, 1:20, 1:40, 1:80 and negative (no antibody) to get the final dilutions (Table 4.1). Antibody was made up in PBS with 1% BSA. These were then quenched with media, cells were centrifuged to remove the supernatant and suspended in 500ul PBS and continued as below.

Antibody	***Type***	***Flourophore***	***Isotype***	***Working concentration***	***Supplier***
Primary anti-*HOXA9*	Monoclonal		Rabbit IgG	1/50	Abcam
Primary anti-CD33	Monoclonal		Rabbit IgG	1/80	BioLegend
Primary anti-CD117	Monoclonal		Mouse IgG	1/100	Millipore
Primary anti-CD14	Monoclonal		Mouse IgG	1/50	BioLegend
Primary anti-CD11b	Monoclonal		Rat IgG	1/100	BioLegend
Goat anti-Mouse	Polyclonal		Goat IgG	1/200	Life Technologies
Goat anti-Rabbit	Polyclonal		Goat IgG	1/200	Life Technologies
Goat anti-Rat	Polyclonal		Goat IgG	1/200	Life Technologies

Table 4.1 *A table listing the antibodies used in during FACS.*

4.2.7 Flow cytometry: CD33, CD117, CD14, CD11b

Cells were harvested using a 5mM solution of EDTA before being washed with PBS (Thermo Scientific, UK). A total of $5x10_5$ cells were re-suspended in 100µl of PBS plus 1% BSA (Sigma-Aldrich, UK) and appropriate primary antibody in a flow cytometry tube. Primary antibody for each was used at the required dilution. Further details on antibodies used can be seen in Table 3.2. Cells were incubated for 30 minutes at room temperature. Cells were then centrifuged at 690g for 2 minutes and the supernatant was removed before the cell pellet was then re-suspended in 200µl of PBS. The cell solution was then centrifuged again at 690g for 2 minutes before the supernatant was removed and the cell pellet was re-suspended one last time with the secondary antibody diluted in 100µl of PBS 1% BSA. Secondary antibody was applied at a 1:200 dilution. This was incubated in the dark for 30 minutes at room temperature. Cells were then centrifuged at 2000rpm for 2 minutes. The supernatant was removed, and the cell pellet was then re-suspended in 200µl of PBS before being centrifuged again at 690g for 2 minutes. The supernatant was again removed, and the cells were re-suspended in 200µl of PBS prior to being analysed on a MACS Quant Analyzer 10 (Miltenyi Biotec, UK). All tests were accompanied by a negative (no primary antibody) control. Primary antibody dilution was determined with a positive titre using isolated PBMCs.

4.2.8 Flow cytometry (intracellular): *HOXA9*

The cells used during the above extracellular staining step were re-suspended in 100µl of Fixation medium (Invitrogen, UK) in a flow cytometry tube and incubated for 15 minutes at room temperature. Cells were centrifuged at 690g for 2 minutes. The supernatant was removed, and the cell pellet was then re-suspended in 100µl of Permeabilization medium (Invitrogen, UK) with appropriate primary antibody. Primary antibody was then applied at the required dilution. Further details on antibodies used can be seen in Table 3.2. Cells were incubated for 20 minutes at room temperature before being centrifuged at 690g for 2 minutes. The supernatant was removed, and the cell pellet was then re-suspended in 200µl of PBS before being centrifuged again at 690g for 2 minutes. The supernatant was

again removed and then the cell pellet was re-suspended with a secondary antibody diluted in 100µl of Permeabilization medium. Secondary antibody was applied at a 1:200 dilution. This was incubated in the dark for 30 minutes at room temperature. Cells were then centrifuged at 690g for 2 minutes. The supernatant was removed, and the cell pellet was then re-suspended in 200µl of PBS before being centrifuged again at 690g for 2 minutes. All tests were accompanied by a negative (no primary antibody) control. Primary antibody dilution was determined with a positive titre on the cell lines.

All products used for intracellular staining from Invitrogen were provided in the FIX and PERM Cell Fixation and Permeabilization kit (Invitrogen, UK). Analyses were performed on a FACS-LSRII (BD Biosciences, UK) using the flow-Jo Software (Flow Jo, USA).

4.2.9 FACS Fluorophores

Antibody	***Flourophore***	***Working concentration***
CD11b	PeCy7	1/100
CD14	V450	1/100
CD33	PE	1/80
CD117	APC	1/100
HOXA9	FITC	1/100

Table 4.2 *A table listing the antibodies and fluorophores used during FACS.*

Results

4.3.1 *HOXA9* expression increases with age in healthy volunteers

Blood samples were taken from a randomized group of healthy donors aged between 20 and 86 (n = 132), none of whom were known to be suffering from any haematological abnormality. The expression of *HOXA9* and housekeeper gene, β-actin were quantified using qPCR. *HOXA9* expression remains consistent throughout adult life until the age of c.55-56 (Figure R4.1). From this age onwards, there is a larger variation in *HOXA9* expression. Some patients exhibit a low level of *HOXA9* in these later decades, however the likelihood of outliers has drastically increased. By 55 to 60 years of age, the median expression of *HOXA9* is significantly higher than previous decades, averaging 2.5-3-fold. This age-related change in *HOXA9* expression is supported by Spearman regression analysis that shows there to be a positive correlation. *HOXA9* expression levels of these healthy volunteers were quantified based on decades of age and plotted in the whisker plot as shown in Figure R4.1. Groups divided into decades of age, 20's through to 80's, with numbers of samples in each decade vary between 18 and 19, (Table 4.1). Volunteer samples were analysed in duplicate and tested in experimental triplicate so that each individual sample was analysed six times. These results highlight *HOXA9* expression increasing with age after 50, with an increased median value compared to those within the 20-40-decade groups. The most significant result being that the probability of outliers or the probability of an individual having an increased *HOXA9* expression level increases with age. The lower bars reflect the lowest *HOXA9* expression which can generally be seen in most if not all decade groups.

Changes in *HOXA9* Expression with Age

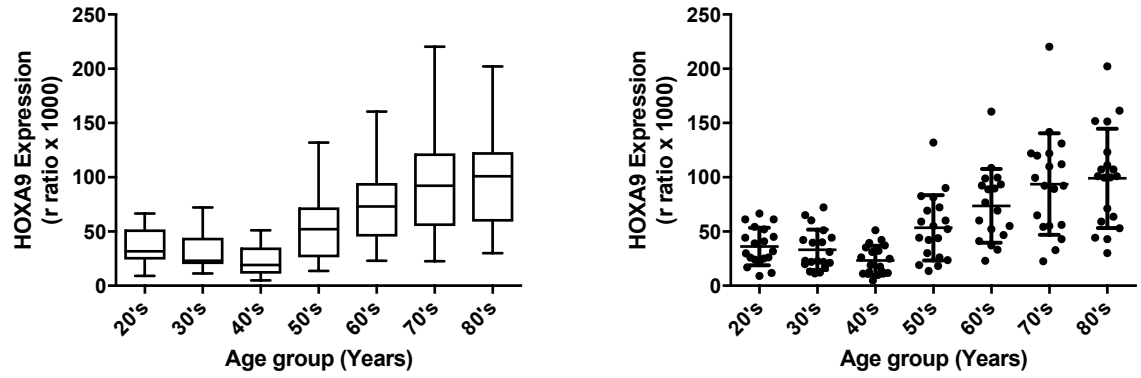


Figure R4.1 *Variation in HOXA9 expression increases with age. Relative HOXA9 expression is represented by the ratio of the expression of HOX genes to the housekeeping gene β-actin (×1000) in mononuclear cells isolated from the peripheral blood of adults of different ages (horizontal axis).* ***A:*** *shows the data as a box and whisker plot. A central bar depicts the median, upper and lower bars represent the 75th and 25th percentiles, and whiskers represent minimum and maximum values.* ***B:*** *shows the data as a scatter dot plot. A central bar depicts the median, upper and lower bars represent the 75th and 25th percentiles. The numbers of samples in each decade are n= 18-19, Table 4.1 where the total n=132.*

Decades of Age	***20s***	***30s***	***40s***	***50s***	***60s***	***70s***	***80s***
Number of Samples	19	19	21	19	18	19	17

Table 4.3 *Representative table of the number of samples in each decade of age.*

4.3.2 Validating outliers for chronic inflammation responsible for the elevated gene expression of *HOXA9*

To validate outliers and assess whether chronic inflammation is responsible for the elevated gene expression of *HOXA9* or if this elevated expression is really increasing with age, whole blood was taken from healthy donors with high *HOXA9* expression levels (outliers). From these outliers, 3 randomised samples were challenged with 1ug/ml of LPS for 3 hours and the expression of *HOXA9* of the treated samples were compared against untreated samples and samples that were treated and left to recover for 24 hours (Figure R4.2.1). As shown, the expression of *HOXA9* in each of the 3 samples decreases significantly when challenged for 3 hours with LPS. Significantly the expression of *HOXA9* in each sample appeared to stay low when left to recover for 24 hours. Western blots confirmed the QPCR findings (Figure R4.2.1). An ELISA was also used to validate and assess whether outliers were suffering from chronic inflammation where 5 outliers were analysed against a positive control. All 5 outliers were in the normal range and supported our LPS analysis where LPS is used in *vitro* to mimic chronic inflammation in cells (Figure R4.2.2).

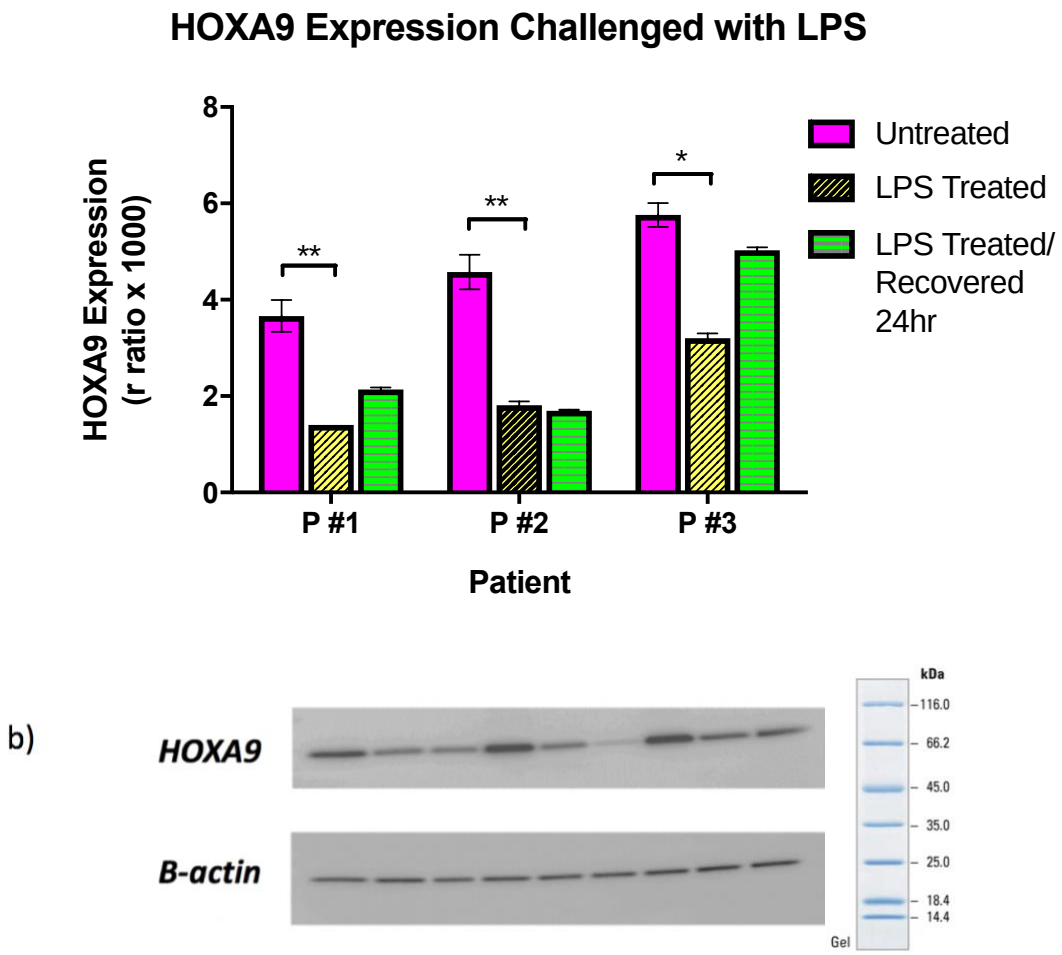


Figure R4.2.1 *A) variation in HOXA9 expression when challenged with LPS. Mononuclear cells were isolated from the whole blood of three healthy adult outliers with a higher than basal HOXA9 expression where cells have been challenged for 3 hours with 1ug/ml LPS. A) The expression of HOXA9 is represented by the ratio of the expression of HOXA9 to the housekeeping gene β-actin (×1000). The mean of three independent experiments are shown and error bars show the SEM in these graphs. *p< .05, **p < .01. B) shows western blot analysis of HOXA9 protein levels in mononuclear cells isolated from the peripheral blood of adult outliers of a HOXA9 expression, after the same treatment.*

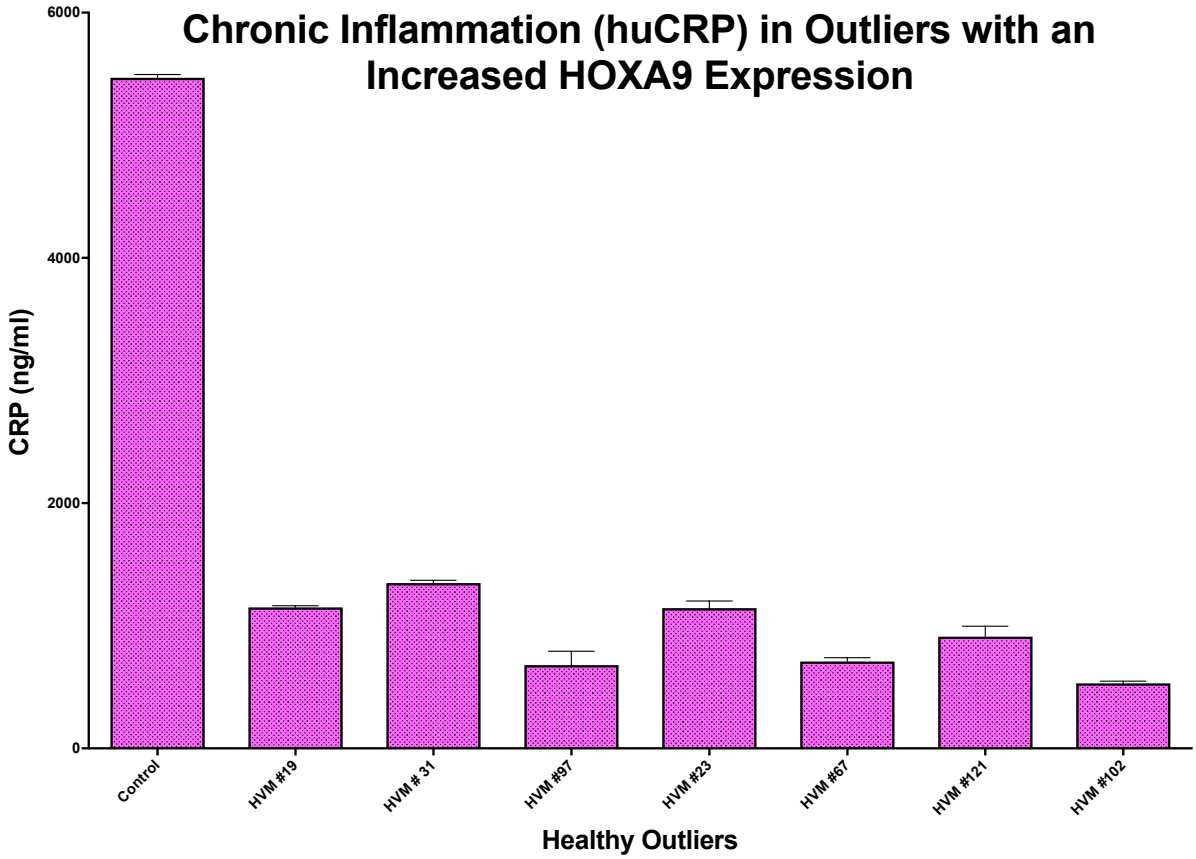


Figure R4.2.2 *Diagram showing huCRP levels in mononuclear cells isolated from the whole blood of healthy adult outliers with a higher than basal HOXA9 expression. These outliers were analyzed and compared to that of the positive control.*

4.3.3 *HOXA9* is expressed extremely highly in myeloid cells, double positive for AML markers CD33 and CD117 in peripheral mononuclear blood

FACS analysis was used to answer if the increase in *HOXA9* expression in peripheral mononuclear blood taken from three outliers is due to an increase in the number of cells already expressing *HOXA9* or indeed an increase in the level of *HOXA9* in these individual cells (n=3). In attempting to answer this question, on whether the elevated level of *HOXA9* in these samples is due to either 1) elevated *HOXA9* expression within a restricted lineage or lineages of cells, and 2) an elevated level of *HOXA9* within those cells already expressing *HOXA9* or 3) is there an increase in number of specific cells expressing high levels of *HOXA9*. FACS analysis was undertaken on healthy volunteers with a higher than normal *HOXA9* expression (outliers) using CD33 and CD117 to observe which cells appear to express *HOXA9* in primary samples (Figure R4.3). As the data shows, three separate peripheral mononuclear blood samples were gated positively for *HOXA9* and many of these *HOXA9* positive cells were double positive for both AML markers CD33 and CD117, 72.69%, 79.05% and 89.61% respectively (Table 4.2). As we have mentioned CD33 and CD117 are markers responsible for the clinical diagnoses of AML, therefore drawing a strong link between the levels of HOXA9 positive cells that are also double positive for both CD33 and CD117.

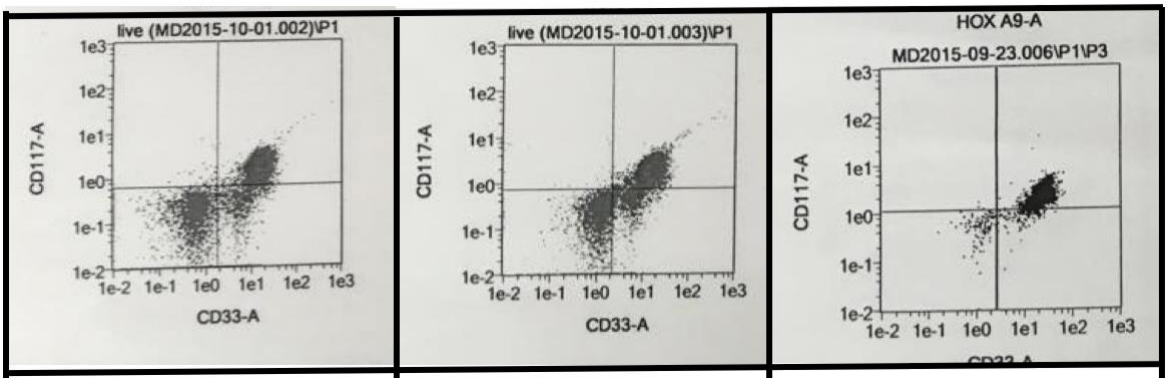


Figure R4.3 *Representative flow cytometry surface staining data from three outliers where HOXA9 positive peripheral mononuclear blood samples (having been intracellularly stained) were also stained with extracellular CD33 and CD117 performed in experimental triplicate (Gating strategy see appendix).*

	1	***2***	***3***
HOXA9 positive cells double negative for CD33 and CD117	20.42% (+/- 0.44%)	15.21% (+/- 0.31%)	4.38% (+/- 0.10%)
HOXA9 positive cells double positive for CD33 and CD117	72.69% (+/- 1.1%)	79.05% (+/- 1.23%)	89.6% (+/- 1.48%)

Table 4.4 *Representative table of data from three outliers where HOXA9 positive peripheral mononuclear blood samples (having been intracellularly stained) were also stained with extracellular CD33 and CD117 where n=3 performed in experimental triplicate.*

4.3.4 *HOXA9* is expressed highly in cells positive for the activation markers CD14 and CD11b.

High *HOXA9* expression was seen in cells that were double positive for both activation markers CD14 and CD11b via FACS analysis on peripheral mononuclear blood taken from two donors with a higher than normal *HOXA9* expression (n=2). Mononuclear cells were isolated and intracellularly stained for *HOXA9* before being stained for the extracellular markers CD14 and CD11b (Figure R4.4). Noticeably cells that were activated (CD11b and CD14 positive) were also positive for *HOXA9*. Both patients exhibited 88.87% and 76.78% positive CD11b expression in their HOXA9 positive cells and 82.9% and 68.21% positive CD14 expression in their *HOXA9* positive cells (Table 4.3). These results indicate a correlation between *HOXA9* expression and cellular activation.

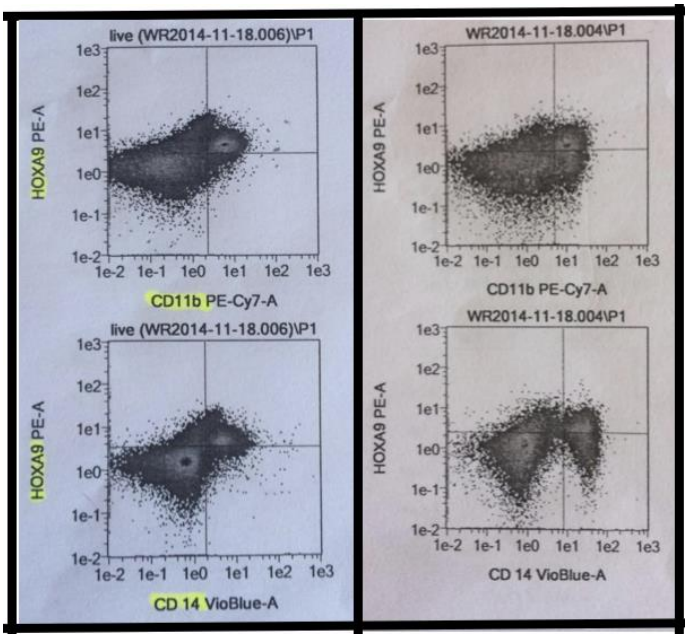


Figure R4.4 *Representative flow cytometry surface staining data of HOXA9 positive peripheral mononuclear blood cells stained with the extracellular activation markers CD14 and CD11b and intracellularly with HOXA9 to analyse if there was a link between cellular activation and high HOXA9 expression (Gating strategy see appendix).*

	1	***2***
HOXA9 Positive cells also Positive for CD11b	*88.87% (+/- 1.27%)*	*76.78% (+/- 1.11%)*
HOXA9 Positive cells also Positive for CD14	*82.90% (+/- 0.98%)*	*68.21% (+/- 0.78%)*

Table 4.5 *Representative table of data where normal peripheral mononuclear blood cells were stained with extracellular CD14 and CD11b and intracellularly with HOXA9 where n=2 performed in experimental triplicate.*

Discussion

Until recently there has been very little evidence for a direct molecular link between a person's age and their risk of developing AML, several age-associated potential haematological factors have been identified. These include MDS and MPN, which like AML, are also classified by WHO and are an extremely heterogeneous group of haematological disorders. MDS and MPD are clonal haematological disorders characterized by ineffective haematopoiesis, bone marrow dysplasia in MDS or hyperplasia in MPN, frequently progressing to AML (15-52% and 7% respectively) and having a poor prognosis. In MPN, HSC dysregulation occurs due to failure of normal cytokine feedback or hypersensitivity. This results in the production of excessive blood cells and involves the 3 main myeloid lineages; erythroid, megakaryocytic/platelet and granulocytic, although predominantly affecting only one lineage. In MDS several factors are believed to contribute to the molecular pathology of this disorder, including increased HSC apoptosis, defective BM stroma, altered response to cytokines and chromosomal abnormalities including deletions of chromosome 5q31–q32. *HOXA9* and *HOXA7* are both often linked to the status of HSC underlying MDS and AML, either through over-expression or reciprocal translocations (the *NUP98-HOXA9* fusion protein for example) although very little, if any profile of HOX expression has been explored for MPD.

The importance of *HOXA9* in homeostasis and disease has led us and others to investigate the way in which its expression changes in a mixed population with age in peripheral, mononuclear blood cells. Blood samples were taken from a randomized group of healthy donors aged between 20 and 86 (n = 132), none of whom were known to be suffering from any haematological abnormality. As the results show, the variation and frequency of high *HOXA9* expression increases in an aging population in support of previous findings (Morgan 2005). *HOXA9* expression remains relatively consistent throughout adult life until the age of c.55-56 (Figure R4.1). From this age a larger variation in *HOXA9* expression is seen perhaps in part due to poorer transcriptional regulation. It is not unusual to see some patients with a low level of *HOXA9* in these later decades, however the likelihood of outliers is drastically increased. By 55 to 60 years of age, the median expression of *HOXA9* higher than previous decades, averaging 2.5-3-fold. *HOXA9* expression increases with age after 50, where there is an

increased median value compared to those within the 20-40-decade groups.

Developmental genes are active in the embryo and in most cases these genes switch off or decrease over time. At this point, highlighting these genes or mechanisms by which the cell reduces the expression of *HOX* genes after development, may pave the way for novel therapeutics or biomarkers of cancers, like AML, heavily implicated by the elevated expression of *HOX* genes. More research into this area could uncover proteins or co-factors as prognostic markers in later years for patients of AML and other haematological malignancies.

Having observed how gene expression changes with age, the next step was to investigate outliers with an increased *HOXA9* expression. This did not support the possibility of chronic inflammation skewing *HOXA9* expression which has been previously shown to affect expression of developmental *HOX* genes. The expression of HOXA9 changed when challenged with LPS, however the results were not an increase of *HOXA9* gene expression as first hypothesized, but rather a universal decrease in *HOXA9* expression (Figure R4.2.2). Having been challenged with LPS, cells were left to recover for 24 hours and the expression of *HOXA9* remained low, relative to initial expression levels. This gene expression data was supported by western blot analysis using the same treatments and parameters. Although this supported the findings by qPCR that outliers express increased level of *HOXA9* it poses many new questions. If inflammation and activation may affect *HOXA9* gene expression *in vitro*, does this hold true *in vitro*? What if cells around an area of chronic inflammation or activation have altered gene expression based on the neighbouring micro-environment and then further effect important fundamental genes responsible for important cellular functions? And if these genes are heavily implicated in cancers as we now know, then what role may chronic inflammation play in the development of human cancer? In summary, having examined these outliers at the protein level using western blotting, we can safely say that chronic inflammation is not responsible for the elevated gene expression of *HOXA9*.

Lineage committed FACS analysis was used to specifically determine which populations express elevated *HOXA9* levels. Here we attempted to address the question, is the elevated level of *HOXA9* in these samples due to elevated *HOXA9* expression within a restricted lineage or lineages of cells. As we would expect, the cells that were positive for *HOXA9* were also very clearly positive for myeloid

markers CD33 and CD117 in three healthy volunteer samples as: 72.69%, 79.05% and 89.6% respectively (Figure R4.3)(Table 4.2). Further work is required to address *HOXA9* across different ages of AML patients and patients of haematological disorders.

Mononuclear cells were isolated and intracellularly stained for *HOXA9* before being stained for the extracellular activation markers CD14 and CD11b (Figure R4.4). Noticeably cells that were activated (CD11b and CD14 positive) were also positive for *HOXA9* but more importantly very few of the negative cells were not activated at all and did not show any signs of expression for CD11b and CD14. Both patients exhibited 88.87% and 76.78% positive CD11b expression in their *HOXA9* positive cells and 82.9% and 68.21% positive CD14 expression in their *HOXA9* positive cells (Table 4.3). This suggests a possible link between the positivity of *HOXA9* expression with CD14 and CD11b expression. The question remains, are activated cells expressing high levels of *HOXA9* or maybe perhaps, high levels of *HOXA9* are causing cells to be activated? It is a difficult responsibility to try and make optimal use of scarce resources and achieve the best outcomes avoiding discrimination purely on grounds of age. To date, treatment options remain limited for these older adults with AML. Even as a bridge to transplant or by themselves, low-intensity approaches may help improve the outlook of elderly patients with AML. However, a limitation for these methods is that additional research on the impact of pre-treatment geriatric assessment and other different therapeutic approaches, like HCT, on the quality-of-life of the elderly, as well as higher social awareness of the huge resources concerned to improve the outcome of this elderly population soon, is urgently required. For all the above reasons, it is essential to tailor the choice of therapy to the patients' condition and the disease characteristics as only a few patients benefit from aggressive approaches. Some novel agents hold promise; however, it is unlikely that they will achieve much benefit as single agents, hopefully, combinations may cover more ground in the future.

In summary, having identified normal individuals with an increased *HOXA9* expression future studies should look to progress onto isolating sub-populations where there is an increased *HOXA9* expression, and being able to highlight or select individuals who may show a higher risk of developing haematological malignancies such as a myeloproliferative neoplasm or a myelodysplastic syndrome

and/or at a high risk of transforming to AML from these often less aggressive disorders and see if these principles hold true, as discussed and investigated in Chapter 5.

Chapter 5: The role of *HOXA9* in the progression of AML from pre-leukemic diseases

5.1.1 *HOX* as a risk factor

The main objective of this section is to determine *HOXA9* gene expression in primary cells and its effect on AML and pre-leukemic malignancies like MPN and MDS. To get a better idea of the progression of AML, it is important not only to analyse AML patients, but also to look closely at other haematological disorders and pre-leukemic disease that may or are known to transform into AML. Importantly, it has been shown over the last decade that *HOXA9* plays a key role and may be used as a risk factor in AML. This is a common finding in many recent studies, for example of 54 AML patients and 20 healthy individuals, *HOXA9* expression was negative in 20 healthy individuals but positive in 22 of the 54 (40.75%) of the AML patients. There was a complete remission (45.45%) for the patients who expressed *HOXA9* which was significantly lower than the 71.86% of patients who did not express this gene after chemotherapy (Li *et al.*, 2013). This supports the now well suggested hypothesis that *HOXA9* may be used as a biomarker for chemotherapy in these patients in the future, however very little is known about patients with pre-leukemic disorders who go on to develop AML. Does *HOXA9* play a role in this progression? If so to what extent could *HOXA9* be targeted in the future as a biomarker for disease but also an intervention therapeutic to prevent the progression to AML from pre-leukemic malignancies like MDS and MPN?

5.1.2 *HOX* genes and cancer

HOX gene expression can affect various pathways that promote tumorigenesis and metastasis.

a) Differentiation: the expression of *HOX* genes involved in the terminal differentiation of normal tissue changes in malignant tissues, resulting in a failure of cellular differentiation, which suggests that the cells maintain an embryonic state. *HOXC8* is a good example it is not usually expressed in prostate tissues but may be upregulated in prostate cancer (Miller *et al*., 2003). This overexpression in prostate cancer cell lines and primary cancer specimens may correlate with a loss of differentiation (Waltregny *et al*., 2002).

b) Apoptosis: *HOX* gene expression may also contribute to tumorigenesis through activating anti-apoptotic pathways. This was first discovered in breast cancer where it was shown that deficient p53 expression in breast cancer cell lines and primary tumours correlated with the methylation of the *HOXA5* promoter and loss of *HOXA5* expression. *HOXA5* binding sites have been found in the promoter region of the TP53 tumour suppressor gene, with transient transfection of *HOXA5* activating the p53 expression in the breast cancer cell lines, MCF-7 and ZR75.1, leading to an increased rate of apoptosis (Raman *et al*., 2000). *HOXA5* has also been shown to induce apoptosis independently of p53 pathways, activating caspase-2 and caspase-8 mediated apoptosis (Chen *et al*., 2004). *HOXA10* has been found in recent years to play a very similar role to *HOXA5* by activating p53 expression in breast cancer cells. The most logical explanation for this is through oestrogen-HOX signalling, whereby oestrogen can upregulate the expression of *HOXA10* in ER+ breast cancer cells (Chu *et al*., 2004).

c) Proliferation: *HOX* genes are known to induce cellular proliferation of cancer cells. In human mammary carcinoma cells, the increased expression of human growth hormone (hGH) also increases the expression of *HOXA1* resulting in a decreased apoptotic response, increased proliferation and metastatic potential (Zhang *et al*., 2003). *HOX* activity appears to show tissue specificity, for example, downregulation of *HOXD10* in breast cancer (Carrio *et al*., 2005) and *HOXB13* in colorectal cancer (Jung *et al*., 2005) have both been shown to increase proliferation. Upregulation of *HOXC9* and *HOXD10* in lung cancers is also linked with increased proliferation (Plowright *et al*., 2009).

d) Invasion: *HOX* genes have been implicated as factors of the invasive properties of cancer cells. *HOXB7* is found to be overexpressed in bone metastasis and some primary breast tumours, and cells transfected with *HOXB7* gained features of EMT. Included in this was the loss of adhesion molecules and changes in cell morphology and cytoskeleton arrangement (Hu *et al*., 2009). EMT is a biological process of the morphogenesis of various tissues that occur during embryogenesis, where polarized epithelial cells undergo multiple biochemical changes that enable them to assume a mesenchymal cell phenotype and facilitate migration through the ECM. It has been hypothesized that cancer cells undergo an EMT-like process to promote ECM invasion and distant metastasis (Thiery, 2009). In breast cancer cells however, HOXB9 was shown to trans-activate the production of transforming growth factor- β (TGF-β), ErbB ligands and several other angiogenic factors that may lead to EMT, invasion, and angiogenesis (Hayashida *et al*., 2010).

It has been almost three quarters of a century since the discovered the primary function of *HOX* genes, however to date, their role in malignance is yet to be fully unveiled. Recent gene expression studies are slowly decoding the genetic antagonists in cancers and have highlighted and identified key molecular targets of which *HOX* genes are one. *HOX* genes have been shown to possess the ability to act as both oncogenes and tumour suppressors, with some possessing both abilities (Morgan *et al*., 2012). *HOX* deregulation has been observed in numerous cancers including bladder, kidney, non-small cell lung, thyroid, melanoma, myeloma, lung, prostate, breast, colon, ovarian cancer and more importantly in various types of leukaemia. In these cancers *HOX* genes have been shown to influence an increase in proliferation, invasion, metastasis, angiogenesis and help in DNA repair. Abate-Shen has proposed three mechanisms of action for the deregulation of *HOX* in cancer (Abate-Shen, 2012). Firstly, temporospatial deregulation, whereby *HOX* gene expression in malignant cells is different to its normal counterpart. Secondly, the mechanism of gene dominance, whereby HOX genes are overexpressed in malignant tissue compared to their adjacent normal parent tissue. Thirdly, the mechanism of epigenetic deregulation, such as methylation, whereby *HOX* genes often present in healthy tissue differ on a molecular level in comparison to their cancer counterpart.

5.1.3 Focusing on *HOXA9*

HOX gene expression over the years has become an important prognostic factor in AML with over-expression of *HOX* genes associated with an intermediate/unfavourable cytogenetic subset of AML. Among 6817 genes investigated in AML patients, the single gene correlated with the worst outcome and relapse of disease with short survival was *HOXA9* (Golub, Slonim *et al*., 1999). Equally, high expression of *HOXA9* is associated with a low CR rate (Li, Li *et al*. 2013). On the other hand, low *HOXA9* expression was found to correlate with some of the best outcomes and responses to therapy (Andreeff, Ruvolo *et al*., 2008). It is also important to note that low expression of both *HOXA4* and *MEIS1* are favourable predictors for AML patient outcome (Zangenberg, Grubach *et al*., 2009). Additionally, a signature of up-regulation of *HOXA6, HOXA9* and *PBX3* combined with a low expression of *MEIS1* is an independent poor prognostic marker in some forms of AML like NK-AML. Simultaneous knockdown of *HOXA6* and *HOXA9* causes cell death and AML cell lines to become significantly more sensitive to cytarabine (Dickson, Liberante *et al*., 2013). Higher expression of the *HOXA7, HOXA9* and *HOXA11* genes along with the cofactor PBX3 is a strong independent prognosis marker of adverse OS in abnormal karyotype AML (Li, Huang *et al*., 2012). The global *HOX* expression levels, also seems to mirror the outcome of disease, possibly mirroring the functional redundancy exhibited by many of the *HOX* genes. For this reason, the highest levels of *HOX* genes are seen in *FLT3* mutation cases, which often have unfavourable outcomes, and generally the *HOX* genes are expressed only at a very low level in favourable subsets of AML. It seems clear that the best biomarker that would assist in finding novel therapeutics for AML and pre-leukaemic malignancies would be *HOXA9* and its potential co-factors along with the mechanisms of action associated with these.

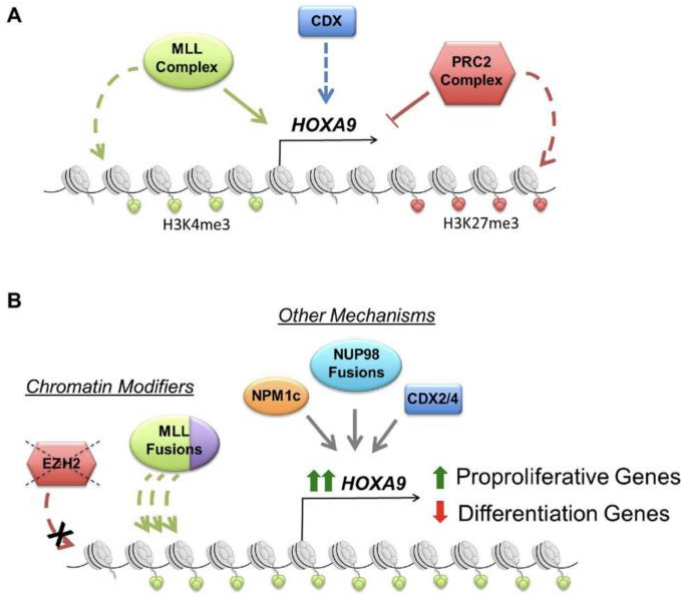


Figure 5.1 *A schematic diagram depicting HOXA9 regulation in normal haematopoiesis and HOXA9 regulation in leukaemia due to upstream genetic alterations or the effects of proteins like NUP98 (Collins and Hess, 2016).*

Recent studies have established the importance of *HOX* genes in the development of AML, although it is still not clear exactly what the functions of some of these genes are beyond general inhibition of differentiation and a significant increase in cell proliferation. There is a large void around the precise mechanistic knowledge for individual *HOX* genes which may also be owing to the functional redundancy showed by many members of this family, making knockdown of single *HOX* genes extremely difficult to interpret, and may also explain the contrast in gene knock-in and knock-out results. In myeloma and some solid malignancies, it has been investigated by targeting *HOX* proteins by antagonizing their interactions with the *PBX* cofactor using the peptide inhibitor *HXR9* (Morgan, Pirard *et al*., 2007; Shears, Plowright *et al*., 2008; Plowright, Harrington *et al*., 2009; Daniels, Neacato *et al*., 2010; Morgan, Plowright *et al*. 2010). As already mentioned, *PBX* binds to the *PBX* motif in the *HOX* paralogous group 1-10. *HOX-PBX* dimers have a higher binding affinity and specificity for target DNA sequences than the *HOX* monomer by itself. *HOX-PBX* dimers however, are strictly involved in the interaction of *PBX* to the conserved hexapeptide sequence WYPWMK found in the N terminal to

the homeodomain of *HOX* proteins from paralogous groups 1-10 for cooperative DNA binding by *PBX* and *HOX* partners (Chang, Shen *et al*., 1995; Shen, Rozenfeld *et al*., 1997; Medina-Martinez and Ramirez-Solis 2003). The HXR9 peptide inhibitor contains a hexapeptide sequence which mimics the *HOX* protein sequences responsible for binding to *PBX* and interferes with the DNA binding of *HOX-PBX*. *HXR9* is extremely cytotoxic to cells, predominantly through the induction of apoptosis in some cancers, which has been shown to depend upon a rapid increase in expression of c-Fos (Morgan, Pirard *et al*., 2007; Shears, Plowright *et al*., 2008; Espinosa, Shinohara *et al*., 2009; Plowright, Harrington *et al*., 2009; Daniels, Neacato *et al*., 2010). A similar approach may be useful in AML for addressing redundant functions of *HOX* genes and analysing the modality of cell death (more detail in Chapter 6).

Aims

To observe the expression of HOXA9 in patients of AML and other related pre-leukemic and haematological malignancies and the role HOXA9 may play in the development of these diseases as well as the molecular mechanisms underpinning the progression from pre-leukemic disorders to AML. Examining the relationship between HOXA9 expression, the cells expressing HOXA9 and the way these may change in peripheral mononuclear cells of patients of various haematological malignancies.

Materials & Methods

To observe the expression of various *HOX* genes in patients with AML and other haematological malignancies, mononuclear cells were isolated from the peripheral blood (Figure R5.1), shown as a ratio to β-actin where n=3 shown by error bars where each patient was analysed in three independent experiments. To assess the variation in expression of *HOX* genes in leukemic patients, several *HOX* genes of most interest were selected based on previous literature: *HOXA5, HOXA6, HOXA9, HOXA10, HOXB4, HOXC4, HOXC9* and *HOXD9*. Blood was taken from patients on the ward or those who appeared in clinic for regular check-ups. FACS analysis was undertaken on the peripheral mononuclear cells isolated from patients selected, who were either suffering from AML or a host of other haematological malignancies using CD33 and CD117 to observe which cells appear to express *HOXA9* in primary cells and to validate the apparent increased expression of *HOXA9* with the progression of AML. Having isolated peripheral mononuclear blood cells from patients of either active AML (n=3), MDS transforming into AML (n=3), MPN (n=2) and AML patients in remission (n=2) can observe from the data that many HOXA9 positive cells were also double positive for both AML markers, CD33 and CD117 (Figure R5.3). In active AML and MDS transforming into AML, we see an average of 90.16% and 82.01% of *HOXA9* positive cells also being positive for the AML markers CD33 and CD117 respectively. On the other hand, we can see the percentage of *HOXA9* positive cells being positive for the AML markers CD33 and CD117 in both MPN patients suffering from both myelofibrosis and polycythemia vera was lower at 61.08% and 54.29% respectively. Most notably, few cells from patients in remission were positive for *HOXA9* let alone positive for *HOXA9* and double positive for CD33 and CD117 (4.08% and 10.88%). As would be expected, the number of *HOXA9* positive cells decreases as we can see from the percentage of cells that are *HOXA9* relative to the total cell number, but we also see a huge drop off in these cells that are double positive for the myeloid markers CD33 and CD117.

Results

5.3.1 Identifying cancer related *HOX* genes in patients suffering from AML and other haematological malignancies

HOX genes are generally low expressed in mononuclear cells isolated from the peripheral blood of healthy adults, however, gene expression increases significantly in patients suffering from AML and other haematological malignancies. The expression of *HOX* genes changes with the progression of AML (Chapter 6), with most AML patients expressing *HOXA9* at a much higher level than other HOX genes and a much higher level than those of other haematological disorders, also supported by previous studies whereby AML patients had a 4.57 or a 1.71 times increase in *HOXA9* expression compared to patients with myelofibrosis (MF) and myelodysplastic syndrome (MDS) respectively. Other noticeable with disease progression include: *HOXA5, HOXA10* and *HOXB4* which are present in the clear majority of patient samples, especially those samples who have progressed towards AML. Samples from patients who are in remission from AML show a very low *HOX* gene expression profile, which is to be expected with some of these patients having had a bone marrow transplant and corrective therapy.

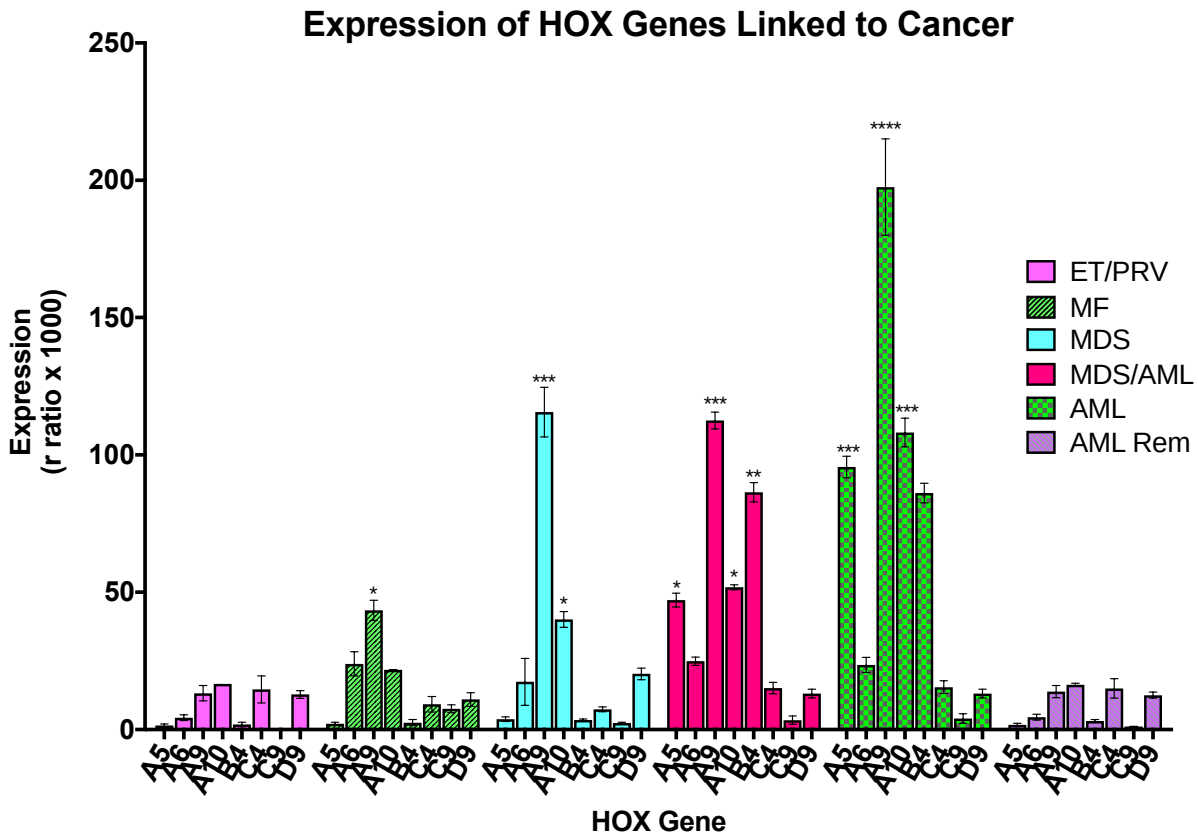


Figure R5.1 *Expression profiling of cancer related HOX genes in mononuclear cells isolated from the peripheral blood of patients with acute myeloid leukaemia and other haematological disorders. The expression of HOX genes is represented by the ratio of the expression of the HOX genes to the housekeeping gene β-actin (×1000). The mean of three independent experiments are shown and error bars show the SEM in these graphs, normal PBMCs were n=3.* *p< .05, **p < .01, ***p <.001 and ****p <.0001.

5.3.2 HOXA9 expression increases in AML from other haematological malignancies

The expression of *HOXA9* was analysed in AML patients and patients with other related pre-leukemic and haematological malignancies. As expected, *HOXA9* expression varies between patient with a significant increase in *HOXA9* expression between patients with mild haematological malignancies such as MPN's like ET and PRV compared to those diagnosed with MDS and AML. Some AML patients express low levels of *HOXA9* though the probability of higher *HOXA9* expression is increased, likely due to discrepancies between differences in AML subtypes which may not express *HOXA9* at all, along with patients who may have had a bone marrow transplant or are currently in remission. When analysing the data, 9 out of the 10 lowest *HOXA9* expression levels analysed were seen in the patients with the mildest haematological disorders suffering from malignancies like ET and PRV or mild anaemia (Figure R5.2.1), on the contrary however, 7 of the 10 highest *HOXA9* expression levels were seen in active AML patients. *HOXA9* has been shown to be necessary for maintaining leukemic transformation; however, the molecular mechanisms through which it promotes leukaemogenesis remain poorly understood. *HOXA9* expression between the individual cohorts changes drastically with AML patients exhibiting an increase of 3.78 and 5.32 times greater *HOXA9* expression than MDS and CML respectively. High expression of *HOXA9* is well associated with low CR rate (Li, Li *et al*. 2013), while low *HOXA9* expression found to correlate with some of the best outcomes and responses to therapy (Andreeff, Ruvolo *et al*., 2008).

When analysing AML patients' *HOXA9* expression, the 22 AML patients were separated either by those in remission/post-transplant patients or active AML sufferers (Figure R5.2.2).

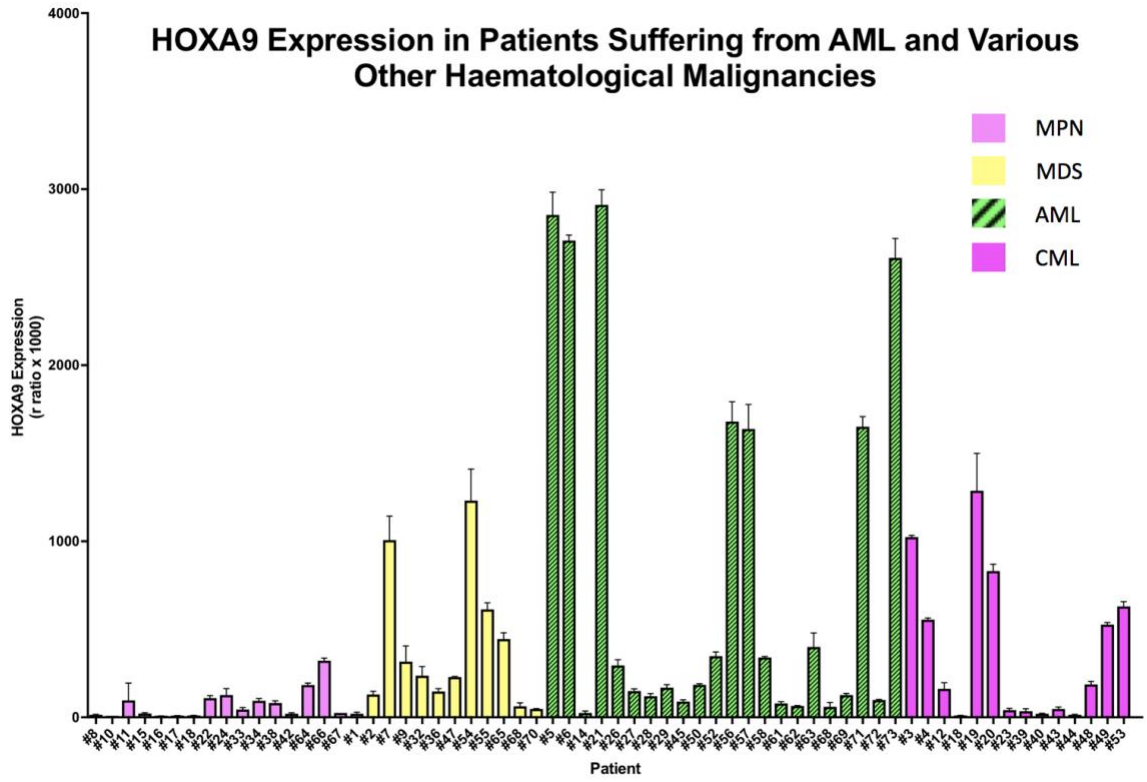


Figure R5.2.1 *Variation in HOXA9 expression in mononuclear cells isolated from whole blood of patients with acute myeloid leukaemia and other haematological disorders. The expression of HOXA9 is represented by the ratio of the expression of HOXA9 to the housekeeping gene β-actin (×1000). The values of seventy-three separate patients were plotted where each patient was analysed in three independent experiments with the SEM for each patient used in this graph. The mean of three independent experiments are shown and error bars show the SEM in these graphs, normal PBMCs were n=3.* *p< .05, **p < .01, ***p <.001 and ****p <.0001.

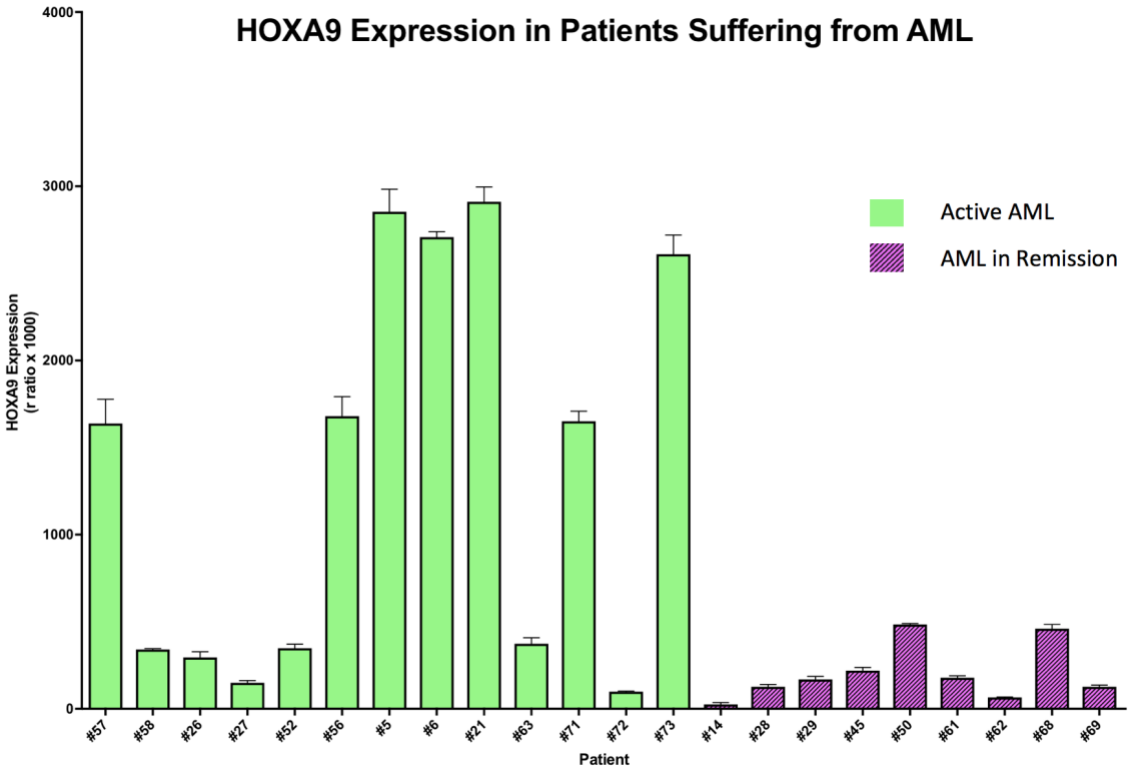


Figure R5.2.2 *Variation in HOXA9 expression of mononuclear cells isolated from the whole blood of 22 patients either with various degrees of active acute myeloid leukaemia (grey) or in active acute myeloid leukaemia in remission (black). The expression of HOXA9 is represented by the ratio of the expression of HOXA9 to the housekeeping gene β-actin (×1000). The mean of three independent experiments are shown and error bars show the SEM in these graphs. The mean of three independent experiments are shown and error bars show the SEM in these graphs, normal PBMCs were n=3.* *p< .05, **p < .01, ***p <.001 and ****p <.0001.

5.3.3 The relationship between *HOXA9* expression, the cells expressing *HOXA9* and the way these may change in peripheral mononuclear cells of patients with haematological malignancies

FACS analysis was undertaken on peripheral mononuclear cells isolated from AML patients and other haematological malignancies using CD33 and CD117 to observe which cells appear to express *HOXA9* in primary cells and to examine the increased expression of *HOXA9* with the progression of AML. RNA was isolated from peripheral mononuclear blood cells of patients with either active AML (n=3), MDS transforming into AML (n=3), MPN (n=2) or AML patients in remission (n=2). We could see from the data (Figure R5.3). Many *HOXA9* positive cells were also double positive for both AML markers CD33 and CD117. Active AML and MDS that transform into AML had an average of 90.16% and 82.01% of HOXA9 positive cells also being positive for the AML markers CD33 and CD117 respectively (Table 5.4). Conversely, the percentage of *HOXA9* positive cells also being positive for the AML markers CD33 and CD117 in MPN patients suffering from both myelofibrosis and polycythemia vera was 61.08% and 54.29% respectively. Of particular interest, very few cells from patients in remission were positive for *HOXA9* let alone positive for *HOXA9* and double positive for CD33 and CD117: 4.08% and 10.88% (Table 5.4).

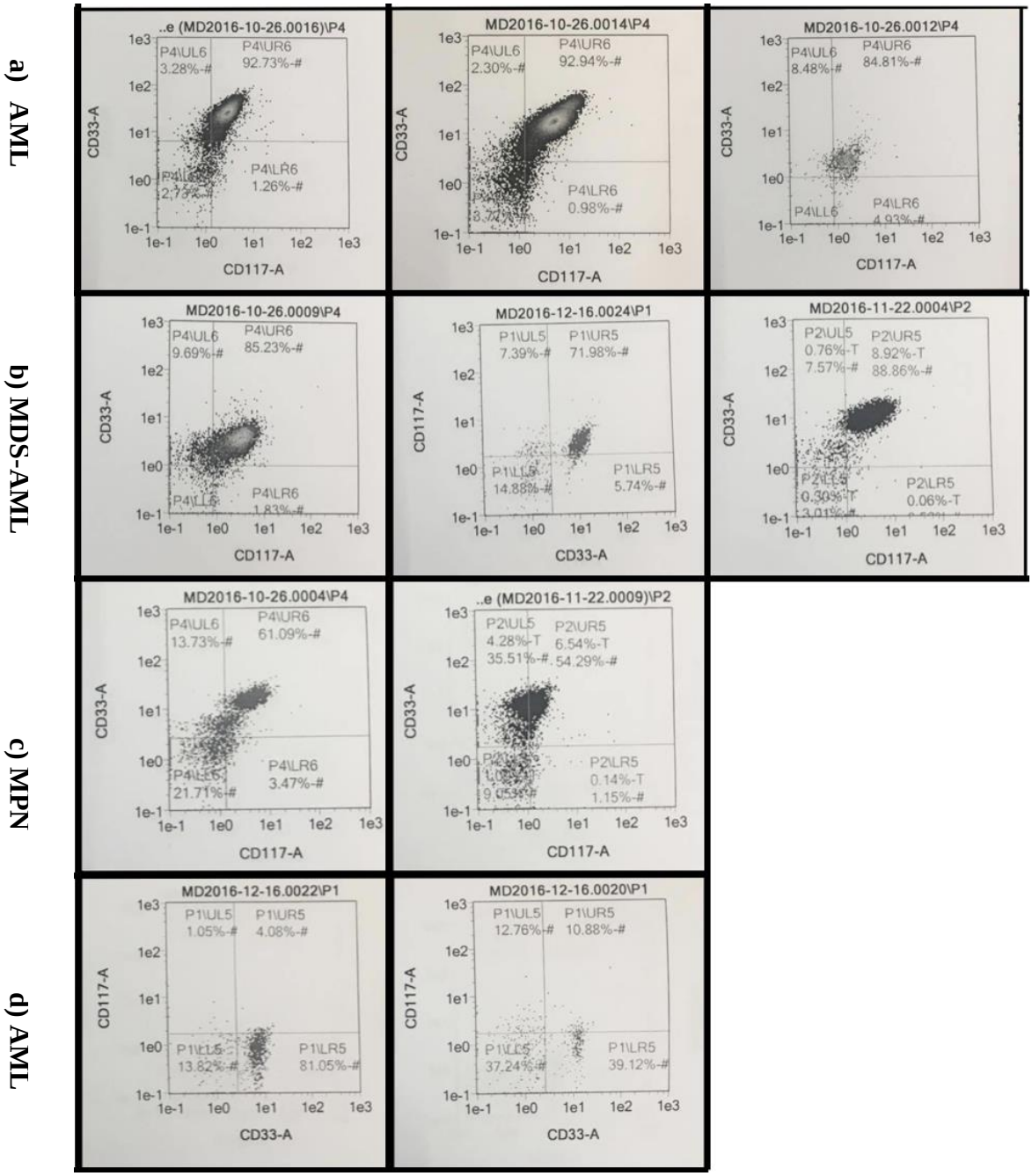


Figure R5.3 *Representative flow cytometry surface staining data of peripheral mononuclear blood cells isolated from patients with a) active AML, b) MDS transforming into AML, c) MPN and d) AML currently in remission stained with extracellular CD33 and CD117 and intracellular HOXA9 antibodies (Gating strategy see appendix).*

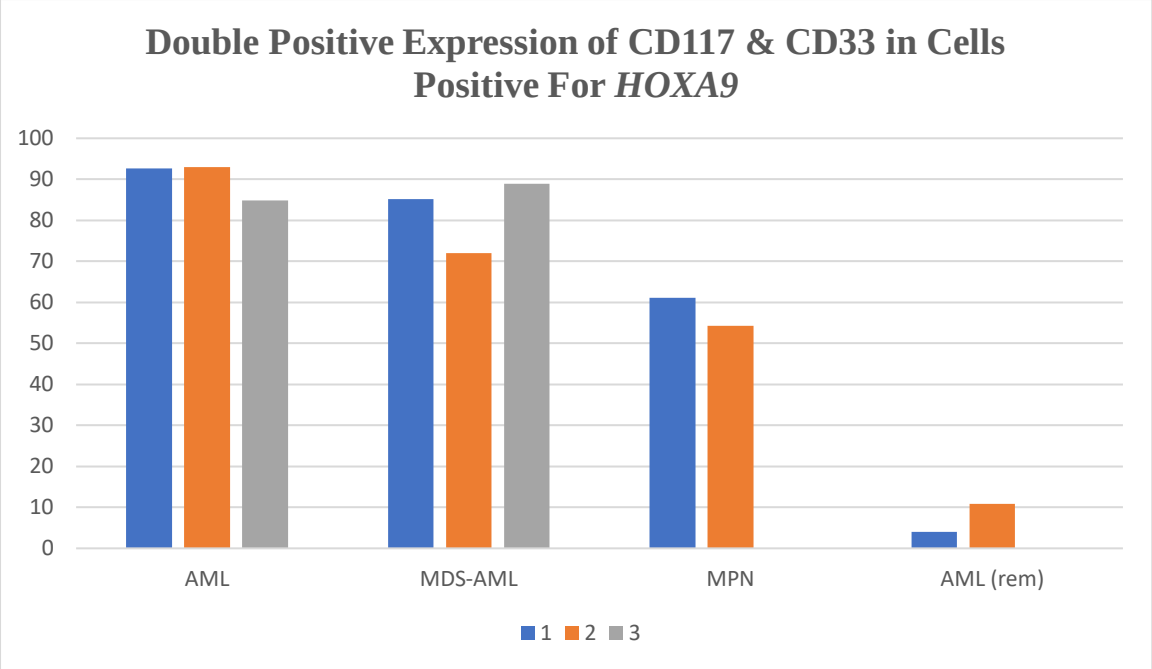


Figure 5.4 *A table listing surface staining data of peripheral mononuclear blood cells isolated from patients with a) active AML, b) MDS transforming into AML, c) MPN and d) AML currently in remission stained with extracellular CD33 and CD117 and intracellular HOXA9 antibodies.*

Disease Progression	***Stained Cells***	***1***	***2***	***3***
AML	HOXA9 positive cells double positive for CD33 and CD117	92.73%	92.94%	84.81%
MDS-AML	HOXA9 positive cells double positive for CD33 and CD117	85.23%	71.98%	88.86%
MPN	HOXA9 positive cells double positive for CD33 and CD117	61.08%	54.29%	
AML in Rem	HOXA9 positive cells double positive for CD33 and CD117	4.08%	10.88%	

Table 5.1 *A table listing surface staining data of peripheral mononuclear blood cells isolated from patients with a) active AML, b) MDS transforming into AML, c) MPN and d) AML currently in remission stained with extracellular CD33 and CD117 and intracellular HOXA9 antibodies.*

5.3.4 *HOXA9* expression changes over time in patients suffering from AML and other haematological malignancies and appears to correlate with progression of disease

To observe the potential change in *HOXA9* expression over time in mononuclear cells isolated from the peripheral blood of patients with various haematological malignancies, blood was taken before and after a 9-month period and gene expression of *HOXA9* was analysed in thirteen separate patients. Evidently, expression of *HOXA9* changes measurably between individuals but we managed to observe a significant change when grouping patients based on their disease progression. High *HOXA9* expression levels were seen in "active" AML patients 27 and 21 which remained significantly high throughout the 9-month period, both patients have a very poor clinical prognosis of AML, one of which has transformed from MDS over the last few years. A low *HOXA9* expression was exhibited in patient 45, 50, 58 and 14, all of which were AML patients in remission or who had received a bone marrow transplant and this expression didn't change hugely over this 9-month period. Most interestingly, *HOXA9* expression increased significantly over time in both patients 29 and 27, who have subsequently transformed from MF and MDS respectively into AML during this 9-month period (Figure R5.4).

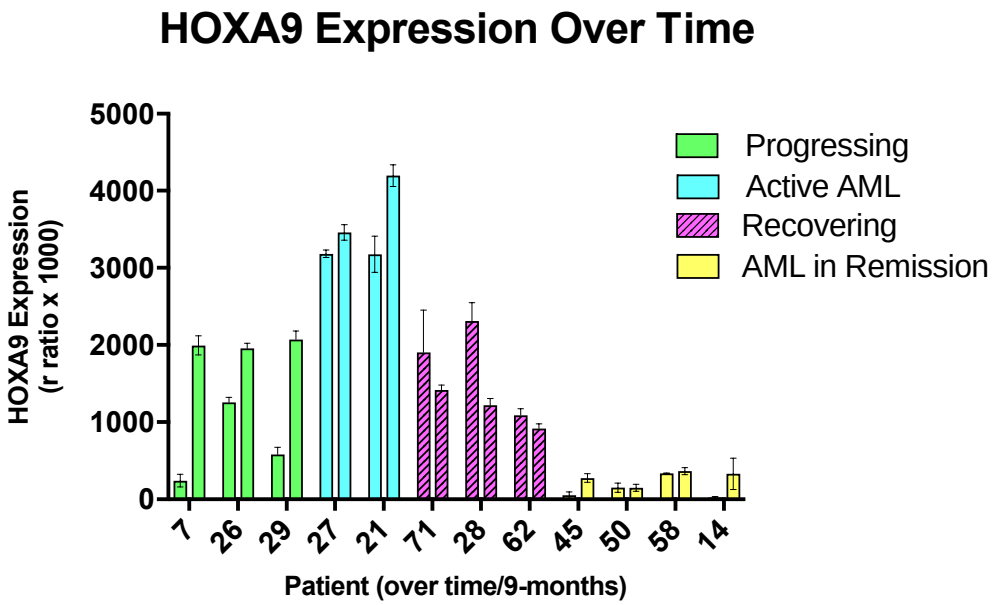


Figure R5.5 *The change (before and after) in HOXA9 expression of mononuclear cells isolated from the whole blood of patients with acute myeloid leukaemia and other haematological disorders over time. Patients were broken up into either: progressing AML, active AML, recovering AML or AML patients in remission based on their clinical diagnosis. The expression of HOXA9 is represented by the ratio of the expression of HOXA9 to the housekeeping gene β-actin (×1000). The values of thirteen separate patients were plotted where each patient was analysed in three independent experiments with the SEM for each patient used in this graph. The mean of three independent experiments are shown and error bars show the SEM in these graphs, normal PBMCs were n=3.* *p< .05, **p < .01, ***p <.001 and ****p <.0001.

Patient	*Gender*	*Age*	*Previous Diagnosis*	*Current Diagnosis*	*Current Cytogenetics*	*Treatments*
7	Female	73	MDS	AML w/ MDS	Transforming High Risk JAK2- (awaiting NM/FLT)	DA2 HDAraC2
26	Female	59	Recent AML	AML	JAK2+	DA1
29	Female	59	AML	AML	High risk AML t(11;19) [NPM1/FLT3-]	DA2 HDAraC1
27	Male	20	AML	Severe AML	Relapse HIGH Risk AML [FLT3+]	Refractory to DA FLAG IDA
21	Male	75	AML (Previously MDS)	Severe AML w/ MDS	Relapse High Risk AML	DA2 & High Cytab2
71	Female	57	AML	NO AML	Post Transplant 150 Days	1DA FLAG IDA
28	Male	63	AML	AML Recovering	[NPM1+ FLT-]	DA2 & High Cytab2
62	Female	50	AML	AML	Relapse AML [FLT3+]	DA3+10 DA3+8 HDAraC2
45	Female	31	AML in Remission	AML in Remission	Post Transplant 19 months	Completed DA
50	Female	61	AML	AML Recovering	Post Transplant 4 years [NPM1+ FLT-]	Completed DA HDAraC2
58	Male	64	AML	NEW AML Recovering	AML-M4 [KMT2A-]	Completed DA
14	Female	59	AML in Remission	AML in Remission	[NPM1- FLT-]	Completed DA

Table 5.2 *A table listing all important data from AML and MDS patients whose HOXA9 expression was analysed both before and after a 9-month period.*

Discussion

Investigating *HOXA9* gene expression in primary cells and its role in acute myeloid leukaemia (AML), myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS) and other pre-leukaemic malignancies will expand on the progression of disease from these disorders towards AML and other haematological cancers. For this reason, it is important not only to analyse AML patients, but also to look closely at other haematological malignancies and pre-leukemic disorders that are thought to, or are known to, transform into AML. It has been shown over the last decade the importance of *HOXA9* and may be used as a prognostic factor in AML. This is a common finding in many recent studies, for example of 54 AML patients and 20 healthy individuals, *HOXA9* expression was negative in 20 healthy individuals but positive in 22 of the 54 (40.75%) of the AML patients. There was a complete remission rate (45.45%) of the patients who expressed *HOXA9* which was significantly lower than the 71.86% of patients who did not express this gene after chemotherapy (Li *et al*., 2013). Does *HOXA9* play a role in this progression? If so to what extent could *HOXA9* be used in the future as a biomarker for disease but also be targeted with intervention therapy to prevent the progression to AML from pre-leukemic disorders like MDS and MPN.

The expression of *HOX* genes linked to cancer were assessed in various patient groups. *HOX* genes selected were those based on previous literature: *HOXA5, HOXA6, HOXA9, HOXA10, HOXB4, HOXC4, HOXC9* and *HOXD9* and analysed in these various cohorts. As we have seen in previous chapters and in the literature thus far, we would normally expect *HOX* genes to be of low expression in mononuclear cells isolated from the peripheral blood of healthy adults. Gene expression vary between individuals, however in terms of *HOXA9*, almost all AML patients analysed express this gene at a much higher level than that of healthy aged matched donors with other mild haematological disorders, which as we know is supported by previous studies. Other noticeable stand-out genes with disease progression include: *HOXA5, HOXA10* and *HOXB4* which are present in the clear majority of patient samples especially those samples which have progressed towards AML. Interestingly however, patients who are in remission from AML showed a very low *HOX* gene expression profile, which is to be expected. Some of these patients have had a bone marrow transplant which would suggest a low or almost normal

HOX gene expression profile. *HOXA9* positively regulates the transcription of other *HOX* genes including *HOXA7* and *HOXA10* and its co-factors *PBX3* and *MEIS1* (Thorsteinsdottir *et al*., 2001) although the effect this may have on the global expression of *HOX* genes currently remains elusive. *HOXA10* resembles *HOXA9* expression to some degree in almost all cohorts of patients and is expressed at high levels simultaneously whereas this was not the case in the mild haematological disorders (Figure R5.1). The *HOX* genes most important for the maintenance of progenitor or stem cell status and promote their proliferation are *HOXA9* and *HOXB4.* The former is the most preferentially expressed *HOX* gene in early hematopoietic progenitors and CD34b HSCs and is downregulated during differentiation (Thorsteinsdottir *et al*., 2001) and both are expressed highly in most samples analysed (Figure R5.1). *HOX* gene expression may also contribute to tumorigenesis through activating anti-apoptotic pathways. It was first discovered in breast cancer with deficient p53 expression in breast cancer cell lines and primary tumours and has also shown to correlate with the methylation of the *HOXA5* promoter and loss of *HOXA5* expression. *HOXA5* binding sites have over recent years been found in the promoter region of the TP53 tumour suppressor gene, and the transient transfection of *HOXA5* activating the p53 expression has been seen in the breast cancer cell lines, MCF-7 and ZR75.1, leading to an increased rate of apoptosis (Raman *et al*., 2000). Recently *HOXA5* has been shown to induce apoptosis independently of p53 pathways, with activation of both caspase-2 and caspase-8 mediated apoptosis (Chen *et al*., 2004). These findings have huge significance and was supported by our observations of high *HOXA5* expression levels in cohorts suffering from AML who also express *HOXA9* at high levels (Figure R5.1). Notably, as we have discussed *HOXA10* is also increased in these cohorts however, the link between *HOXA10* and that of high *HOXA5* expression is relevant. This supports studies that have already shown *HOXA10* to play a similar role to *HOXA5* by activating p53 expression in breast cancer cells. The most logical explanation for this is through oestrogen-*HOX* signalling where oestrogen can upregulate the expression of *HOXA10* in these ER+ breast cancer cells (Chu *et al*., 2004).

To date the relationship between the potentially increased *HOXA9* expression of pre-leukemic disorders and the increased *HOXA9* expression in many AML patients remains elusive. Looking into the expression of *HOXA9* in AML patients at various stages of disease progression and how *HOXA9* changes between AML and pre-leukemic disease such as MDS and MPD will allow us to understand

the molecular roles some genes may have in relation to disease. Not surprisingly we found that there was a significant increase in *HOXA9* expression in patients with AML or some MDS patients especially those close to transforming into AML compared to those who have been diagnosed with very mild haematological disorders such as MPN's like ET and PRV or normal patients. It is important to note, however that there are still various AML patients who express low levels of *HOXA9*. There are several reasons for this, with differences in AML subtypes and the cytogenetic differences between subtypes, it makes it a little more difficult to uncover the exact genes involved. However, we have also found that many of these low level *HOXA9* patents have had a bone marrow transplant or are in remission, which makes things interesting. Having analysed the data, 9 out of the 10 lowest *HOXA9* expression levels were seen in mild haematological disorder patients suffering from malignancies like ET and PRV or mild anaemia (Figure R5.2.1). Most significant for us in this study was that in contrast, 7 of the 10 highest *HOXA9* expression levels were seen in active AML patients.

Attempting to underpin the role *HOXA9* may play in MPN and MPD that results in AML remains elusive, however, recent studies have used haemopoietic chimeras acted as a mouse model for *NUP98-HOXA9* induced leukaemia to reproduce phenotypes observed in human disease. Mice that were transplanted with marrow cells known to be expressing *NUP98-HOXA9* through retroviral transduction went on to develop MPN and eventually succumbed to acute myeloid leukaemia AML. It was the *NUP98* section of this fusion protein that was shown to be responsible for the transformation of a clinically silent pre-leukemic phase into a chronic stem cell derived MPN. Interestingly it was found that the co-expression of this fusion protein as well as *MEIS1* (one of the co-factors highlighted in previous chapters) accelerated this transformation of MPN into AML which highlights an already established genetic interaction between both *HOXA9* and *MEIS1* (Kroon *et al*., 2001).This was the first study that not only highlighted this but was actually able to demonstrate an overlapping yet distinct molecular mechanism for both MPN and AML, highlighting the extreme complexity of leukemic transformations. These Philadelphia chromosomal negative (Ph-) chronic myeloproliferative neoplasms have an inherent tendency to transform into acute myelogenous leukaemia with the long-term rate of transformation in randomised unselected patients being studied extensively in well-defined populations

in Sweden and France. In these studies, a median observation time of 15 years, with 56 subjects of the total 795 (7%) with Ph- transformed from MPN to AML with a yearly incidence rate of 0.38% in PRV, 0.37% in essential ET and almost 1.09% in idiopathic myelofibrosis IMF. The average survival time for these 56 MDS patients that went on to develop AML was 4.6 +-5.5 months and didn't differ substantially to any of the three subtypes of pre-AML MPN. Interestingly 17 of the 18 patients with PRV that went on to develop AML were female as the gender ratio for this group was 146/171 (0.85) compared to ET and IMF showed a slight male dominance of 1.33 and 1.13 respectively.

Looking at the possible progression of AML from pre-leukemic disorders over time and being able to look into each patient's prognosis and clinical pathology with their corresponding *HOXA9* expression change over time gives us a much better understanding of whether these changes are of any significance. Blood was taken before and after a 9-month period and gene expression of *HOXA9* was analysed in thirteen of these patients with access to their medical records and diagnosis, cytogenetics, morphology and disease progression. Expression of *HOXA9* changes between individuals but we managed to observe a significant change when grouping patients based on their disease progression. High *HOXA9* expression levels were seen in some AML patients which remained significantly high throughout the 9-month period, these patients had a very poor clinical prognosis of AML, one of which has transformed from MDS over the last few years. As we know, MDS and MPN are clonal haematological disorders characterized by ineffective haematopoiesis, bone marrow dysplasia in MDS or hyperplasia in MPN, frequently progressing to AML 15-52% and 7% respectively, and incurring a poor prognosis so assessing those patients who may transform was of interest to us. Interestingly then, *HOXA9* expression was increased significantly over time in both patients 29 and 27, who had subsequently transformed from MF and MDS respectively into AML during this 9-month period (Figure R5.4). On the other hand, however, a low *HOXA9* expression was exhibited in patients 45, 50, 58 and 14, all of who were AML patients in remission or who had received a bone marrow transplant and this expression didn't change hugely over this 9-month period. Drawing a link between the expression of *HOXA9* and the progression of disease over time is something that has never been fully investigated. This data suggests to us that a much larger, thorough study would be of huge interest and bear fruit for the potential use of novel therapeutics could target patients suffering from AML and other pre-leukaemic malignancies.

Understanding the affects *HOXA9* may have on AML and other haematological malignancies is of huge importance in the development of novel therapeutics in the future. The high levels of *HOX* gene dysregulation in cancers as we know makes *HOXA9* and the other HOX genes perfect potential targets for therapeutic intervention in theory although it is not always that simple. Targeting of specific *HOX* genes is extremely problematic due to functional redundancy between proteins. However, it has been identified that targeting the *HOX/PBX* dimer is an effective way of impacting the function of multiple *HOX* genes. The logical method was to use a peptide that is responsible for disrupting the interaction between *HOX* proteins and its co-factor *PBX*, to block the functionality of *PBX*-dependent *HOX* functions. This idea led to the production of *HXR9.*

Chapter 6: HXR9 as a potential therapeutic agent for AML and other haematological malignancies

6.1.1 HXR9, the future?

HXR9 is a small peptide that has been designed to mimic the hexapeptide sequence in HOX proteins of paralogue groups 1- 8 (Phelan *et al*., 1995, Chang *et al*., 1995, Shanmugam *et al*., 1997, Piper *et al*., 1999, Morgan *et al*., 2000, Medina-Martínez and Ramírez-Solis, 2003), acting as an extremely specific competitive inhibitor of the HOX/PBX binding site. This peptide has a strong ability to prevent the formation of the HOX/PBX dimer and the subsequent binding to target DNA sequences, essentially inhibiting the transcription of target genes. For HXR9 to enter cells, a polyarginine (R9) sequence is linked to the hexapeptide that has been previously been shown as an extremely effective delivery system (Jiang *et al*., 2006). Previous studies have shown that HXR9 can block this important interaction not only *in vitro* but also *in vivo* (Morgan *et al*., 2007, Plowright *et al*., 2009, Morgan *et al*., 2010) and that antagonising the HOX/PBX interaction induces apoptosis (Morgan *et al*., 2007, Shears *et al*., 2008, Plowright *et al*., 2009, Morgan *et al*., 2010, Morgan *et al*., 2012). Therefore, investigating whether AML cell lines and patient samples were also sensitive to HXR9 was of keen interest. It is also useful to note that there may also be a similar effect when combining HXR9 with commonly used chemotherapies in AML treatment, MTX and DNR (Piccaluga *et al*., 2002, Fernandez, 2010, Larson *et al*., 2012). Molecular agents combined with conventional chemotherapies have given rise to more curative therapies and may provide a rational basis for the selection of a synergistic partner for HXR9, the mechanism by which HXR9 and induces cell death needs to be identified (Figure 6.1).

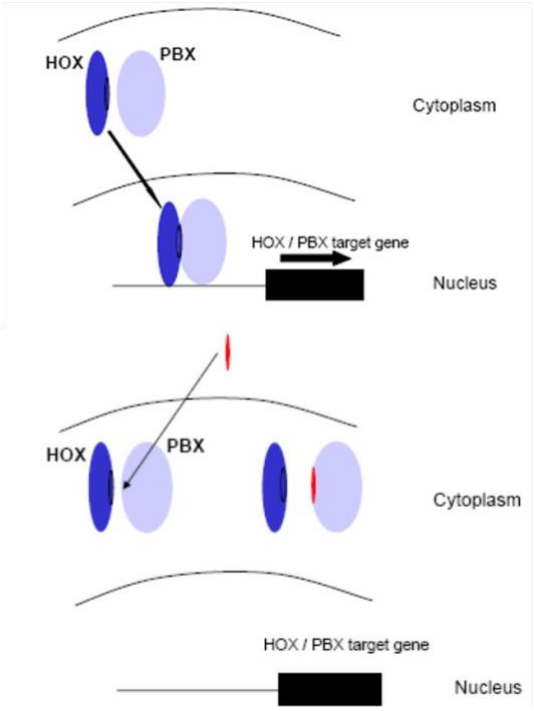


Figure 6.1 *A schematic diagram depicting the blocking of the HOX/PBX dimerization with the small peptide HXR9. HXR9 is a peptide which mimics the hexapeptide sequence of HOX proteins and acts as a competitive inhibitor of HOX/PBX binding. This hexapeptide sequence is linked to nine arginine residues to allow cell membrane penetration (Morgan et al., 2014).*

6.1.2 The HXR9 death pathway

Microarray analysis has revealed over twenty genes that seemed to have a significant increase in transcription levels, this has been confirmed by qPCR. Some of these upregulated genes were the oncogenes c-Fos and c-Jun (Morgan *et al*., 2007) in various cell lines. These oncogenes are transcription factors and members of the bZIP superfamily, that are characterised by a DNA-binding domain with a leucine zipper region (Hess *et al*., 2004). Jun proteins form homodimers or heterodimers with Fos proteins making the activating protein (AP-1) transcription factor. AP-1 mediates regulation of several genes controlling physiological functions including basic functions like cell proliferation and differentiation as well as apoptosis and neoplastic transformation (Ameyar *et al*., 2003, Eferl and Wagner, 2003, Jochum *et al*., 2001, Shaulian and Karin, 2001, Shaulian and Karin, 2002).

The upregulation of c-Fos expression has been well documented in HXR9-mediated apoptosis. It is

currently unknown what the exact mechanism of AP-1 mediated apoptosis is, however, the AP-1 complexes that contain c-Jun promotes the transcription of c-Fos ligand (FasL) which in turn promotes cell death via the FasL/Fas receptor pathway in lymphoid, fibroblast and neuronal cells (Eichhorst *et al*., 2000, Kasibhatla *et al*., 1998, Kolbus *et al*., 2000, Matsui *et al*., 2000). In some prostate cancer cells, c-Jun and c-Fos heterodimerisation has been seen to repress the transcription of cellular-FLICE-inhibitory protein long isoform c-FLIP(L), which is well known as an anti-apoptotic molecule, that has resulted in the sensitisation of extremely resistant prostate cancer cells to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis (Li *et al*., 2007, Zhang *et al*., 2007). Overexpression of c-Fos in hepatocytes has also been widely shown to induce apoptosis (Mikula *et al*., 2003) and is required for Myc-induced cell death of hepatoma cells (Kalra and Kumar, 2004). However, c-Fos re-expression in several established tumour cell lines resulting in the activation of pro-apoptotic genes uses a different mechanism that does not involve Jun (Fleischmann *et al*., 2003). In these prostate cancer cell lines however, as well as c-Fos and c-Jun, other relevant genes were upregulated in response to HXR9 treatment: e.g. Dusp1, Aft3, Transcription factor Krüppel-like factor 4 (KLF4), Mad and Drak2. Dusp1 encodes dual specificity phosphate 1 which in turn dephosphorylates serine, threonine and tyrosine residues from a wide range of substrates, such as mitogen-activated protein kinases (MAPKs), for example which plays important roles in the signalling pathway of MAPK that tightly regulates cell division and cell growth. However, dephosphorylation of these kinases often leads to inactivation of this pathway (Ducruet *et al*., 2005). If AML derived cell lines/patients have an increased c-Fos and c-Jun expression, what about pre-leukemic diseases? Could this mechanism of cellular death help identify what exactly underpins the progression from pre-leukemic disease to AML? Could downstream proteins like c-Fos and c-Jun unlock the answers to HOXA9's dysregulation? Not only this, what if the mode of cell death for AML derived cell lines is not apoptosis, then what role if any may c-Fos and c-Jun play in an apoptosis independent death pathway (Figure 6.4)?

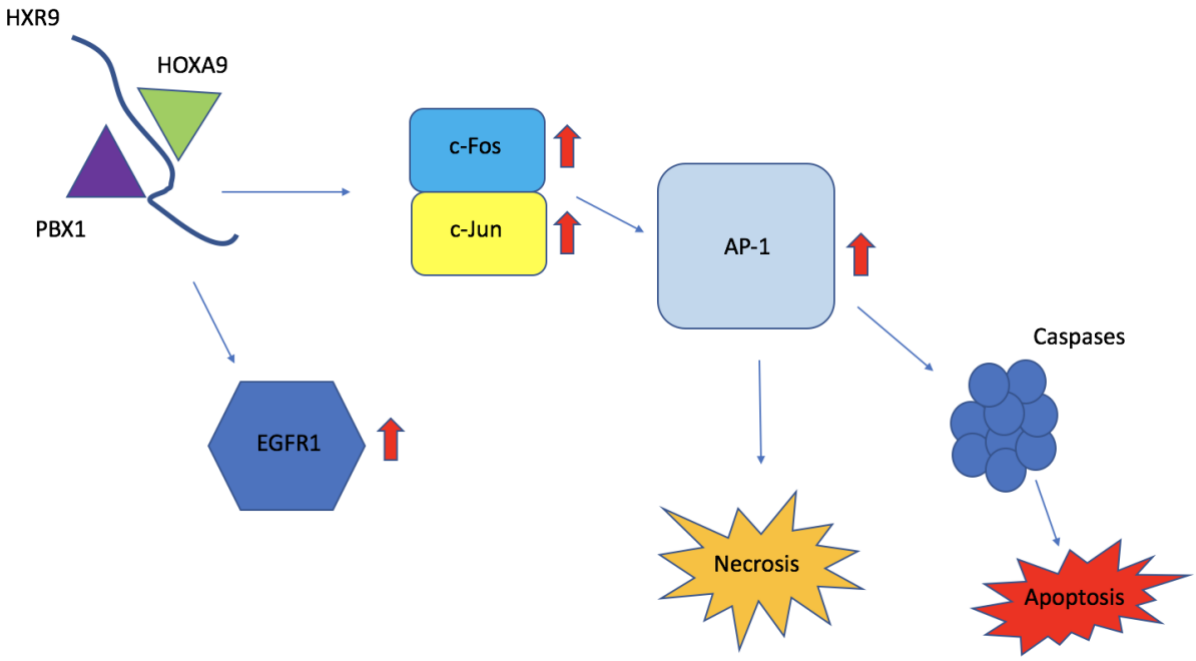


Figure 6.2 *A schematic diagram depicting our currently hypothesised HXR9 Death Pathway including C-Fos, C-Jun (AP-1 complex), EGFR1 and downstream caspases once the HOX-PBX binding site is interrupted by HXR9.*

6.1.3 The importance of c-Fos

Antagonism of the HOX/PBX interaction results in up-regulation in the expression of c-Fos in other cancers. This induction of c-Fos expression by HXR9 has been well documented and is thought to be responsible to some degree, for initiating cell death in human renal, lung, melanoma, ovarian, prostate and breast cancer cell lines (Morgan, Pirard *et al*., 2007; Shears, Plowright *et al*., 2008; Plowright, Harrington *et al*., 2009; Morgan, Plowright *et al*., 2010; Morgan, Boxall *et al*., 2012; Morgan, Boxall *et al*., 2014). It has also been shown that knocking down c-Fos expression has been able to rescue prostate and melanoma cancer cell lines from HXR9-induced apoptosis (Morgan, Pirard *et al*., 2007; Morgan, Boxall *et al*., 2014). The current literature has clearly shown that c-Fos is a key component of the AP-1 transcription activating complex, which dimerizes with Jun (its binding partner), to regulate cell proliferation and even cell cycle progression (Durchdewald *et al*., 2009). It has already been

suggested as a proto-oncogene in several human tumours, which include: bone, brain and skin (Gamberi *et al*., 1998, Silvestre *et al*., 2010, Guinea-Viniegra *et al*., 2012). It is worth noting that the reduction in c-Fos expression in epithelial ovarian carcinoma has shown some potential as an independent marker of overall survival (Mahner *et al*., 2008). It has also been discovered, that c-Fos over-expression may also have a pro-apoptotic function in *vitro* and may cause a delay in tumour growth and the metastasis of ovarian cancer cells (OvCa cells) in *vivo*. The mechanisms for c-Fos have been highlighted by a dysregulation of adhesion proteins (Oliveira-Ferrer *et al*., 2014), which has also be shown to have an involvement in the induction of the human ovarian carcinoma cell line A2780 in response to some fenrtinide treatment (Appierto *et al*., 2004). Interestingly, c-Fos has been subsequently shown to drive pro-apoptotic functions via repressing the anti-apoptotic gene cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (L) (c-FLIP (L)) resulting in an increased sensitivity for prostate cells to the tumour necrosis-related apoptosis-inducing ligand protein (TRAIL) (Zhang, Zhang *et al*., 2007). It has been hypothesized that c-Fos may induce human hepatoma Huh7 apoptosis by mediating c-Myc expression (Kalra and Kumar, 2004). The over-expression of c-Fos results in cell cycle arrest as well as up-regulating the expression of nuclear proteins like CHOP and the inhibitors of the cell cycle $p16^{INK4A}$ and $p57^{KIP2}$ and suppresses rat sarcoma (Ras)-mediated malignancy eventually leading to apoptosis of murine epithelial hepatocytes (Mikula *et al*., 2003). A prolonged expression also suppresses cell cycle entry of murine BM cells (Okada *et al*., 1999). Studies have shown that c-Fos may also enhance hepatocyte apoptosis by directly up-regulating the major isoform of the BIM gene BIMEL (Kitamura, Ogawa *et al*., 2003). For these reasons, c-Fos is one of the more important genes in this pathway and its investigation is warranted for the HXR9 death pathway in AML and other haematological disorder.

6.1.4 The importance of tumour suppressors

Cells undergoing p53-dependant apoptosis demonstrated increased c-Fos transcription (Elkeles *et al*., 1999). HXR9 does not alter the expression of p53 at the mRNA or at the protein level in breast cancer

cells that are p53-independent (Morgan *et al*., 2012). Not only this, but p53 protein interacts directly with anti-apoptotic members of the Bcl-2 family, Bcl-2 and Bcl-XL, therefore resulting in the release of pro-apoptotic members such as Bak and Bax. p53 has also been shown to up-regulate the transcription of, and physically interacts with, Bak, Bax and Bad therefore leading to mitochondrial apoptosis (Chipuk *et al*., 2003, Chipuk *et al*., 2004, Leu *et al*., 2004, Jiang *et al*., 2006, Pietsch *et al*., 2007, Wenzel *et al*., 2012, Lee *et al*., 2008, Saha *et al*., 2014). Interestingly, neither cypD nor PARP1 were seen to be involved in HXR9-induced AML necrosis. Together, these findings clearly suggest that HXR9-induces AML necrosis in a p53-independent manner (Vaseva *et al*., 2012, Montero *et al*., 2013, Pei *et al*., 2014).

p21 is a member of the family of cyclin kinase inhibitors including p27 and p57 (Kwon *et al*., 2003, Masgras *et al*., 2012). It was originally reported to be a key regulator of the progression of the cell cycle (Harper *et al*., 1993). Interestingly, p21 also regulates several other cellular functions such as: apoptosis, cell proliferation and autophagy (Asada *et al*., 1999). Initially it was suggested that the regulation of p21 was totally dependent on p53, however, p21 can in fact be regulated by several transcription factors in a p53-independent manner (Liu and Huang *et al*., 2006; Lafarga, Cuadrado *et al*., 2009; Shin, Kim *et al*., 2011; Han, Kim *et al*., 2012; Masgras, Carrera *et al*., 2012). For example, early growth response-1 protein has been shown to induce p21 transcription independently of p53 (Choi, Kim *et al*. 2008). The early growth response-1 gene is induced in lung cancer cells following HXR9 treatment (Plowright *et al*., 2009). p21 has also been shown to be involved in p53-independent necrotic cell death (Ussat *et al*., 2002, Kwon *et al*., 2003). It is therefore suggested that the induction of p21 in response to HXR9 was p53-independent.

As the expression of some of these tumour suppressor and pro-apoptotic genes increases after HXR9 treatment in malignant cells, this suggests that some of the overexpressed HOX genes found may potentially inhibit intrinsic tumour suppressing pathways and therefore create a pro-tumour environment. Looking to target the HOX/PBX interaction is thus an important target in cancer therapy, with further investigation into HOX deregulation in cancers needed to determine whether HXR9 is a good potential treatment option.

Aims

To observe the cytotoxicity of HXR9 on several AML cell lines along with observing the change in expression of HOX genes (HOXA9), co-factors of HOXA9 and important downstream genes in several AML cell lines, AML patients and patients with other haematological disorders when treated with HXR9 in attempt to answer: Does HXR9 treatment affect downstream genes responsible for cell death in leukemic cell lines? Is HXR9 induced cell death is independent of apoptosis? Does HXR9 treatment affects downstream genes responsible for cell death in patients of AML and other haematological disorders?

Materials & Methods

6.2.1 HXR9 treatment

HXR9/CXR9 powder was taken from -20°C freezer and left in a sterilised hood to slowly warm to room temperature. Once warmed to room temperature, 500mg of HXR9 was reconstituted in 1850µl of sterile water to make up a 100mM solution. CXR9 has almost the same molecular weight, so the same volume was added to make a 100mM solution. Once all the liquid was added it was left in the hood with the lid on for 10 minutes (ensuring not to mix or insert a pipette tip into the liquid once added as this could mean peptide is lost if stuck to the pipette tip). The powder was dissolved and lightly vortexed so the solution was a uniform colour (light yellow) and aliquoted out into 100µl aliquots before being stored at -80°C.

6.2.2 Lactate dehydrogenase (LDH) assay

Lactate Dehydrogenase (LDH) is present in the cytoplasm of cells and is released into culture medium through the membrane of the cell. The amount of this enzyme in culture gives a good indication of the integrity of the cell membrane and provides an estimation of cytotoxicity (Haslam *et al*., 2000). LDH activity is often assessed in two steps. Firstly, LDH oxidises lactate into pyruvate, which reduces NAD^+ to $NADH/H^+$. Having done this, diaphorase, (catalyst), transfers 2H from $NADH/H^+$ to a tetrazolium salt (INT) which results in the formation of red salt (formazan). Therefore, by using this we can gauge the amount of formazan formed and correlate it directly to the amount of LDH in the culture medium and to the number of damaged cells. The LDH cytotoxicity assay (Figure 6.5). LDH catalyses a reduction of NAD+ to NADH+/H+ by converting lactate to pyruvate. Diaphorase reduces yellow tetrazolium salt INT into the red formazan salt with the presence of NADH+/H+. During the process of HXR9 cytotoxicity determination, cells are treated with HXR9 or a negative control (CXR9) for 2 hours as seen in this study. This step is repeated six times. Plates were incubated in a humidified incubator at 37°C, 5% CO_2 for either 2 or 24 hours. After the treatment, plates were centrifuged at 1000rpm for 5 minutes and 100µl of supernatant decanted from each well into a fresh, 96-well flat-bottom plate. The

same amount of LDH reagent is prepared to manufacturer's instructions, added to the supernatants for two minutes, and LDH enzymatic activity was then estimated by reading the absorbance at 492nm, with a plate reader.

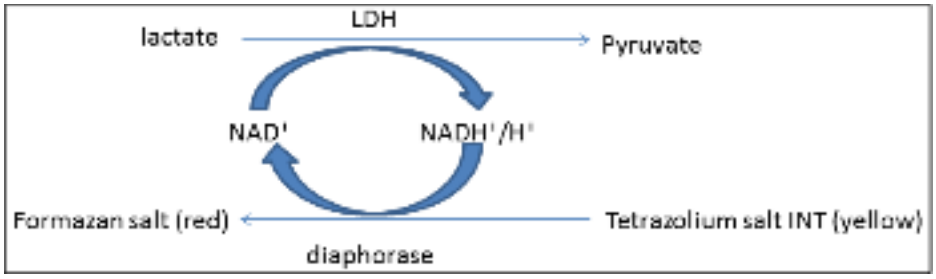


Figure 6.3 *A schematic diagram depicting the mechanism of action of LDH cytotoxicity assay. LDH catalyses a reduction of NAD+ to NADH+/H+ through a conversion of lactate to pyruvate. Then, diaphorase reduces the yellow tetrazolium salt INT to the red formazan salt in the presence of NADH+/H+.*

6.2.3 LDH assay data analysis

To assess the percentage of surviving cells, cytotoxicity percentage was calculated. An average absorbance of all six repeats were used and calculated. The average absorbance value of the background control is then subtracted from the HXR9 treated cells' absorbance average value. Having done this, the cytotoxicity percentage was calculated according to the manufacturer's instructions as per:

$$\text{Cytotoxicity\%} = \frac{\text{treated value} - \text{negative control}}{\text{positive control} - \text{negative control}} \times 100$$

%surviving cells = 100 - cytotoxicity

The % of surviving cells was plotted on a X-Y scatter diagram using GraphPad Prism software (California, USA) after this, to create cytotoxicity curves for each cell line. The surviving fractions of the treated cells was compared to those of untreated cells. Not only this, but the cytotoxicity results of the different cell lines can also be compared. Every individual experiment undertaken was repeated three times (biological replicates) and the valid experimental curve should have a linear correlation coefficient (R^2) $\geq$ 0.95. The cytotoxicity curves were generated using a mean %surviving cells at various drug concentrations. The SEM of each single dose was determined and then included with error bars on the cytotoxicity curves. IC_{50} was then determined by CalcuSyn software (Biosoft, Cambridge, UK) as described in the statistical analysis section. It is also important to note that PBX is not the solitary co-factor for many of these HOX proteins and therefore the inhibition of HOX/PBX interaction may cause alternative cofactors to be utilised.

6.2.4 Inhibition of caspases activity by z-VAD-FMK

Z-VAD-FMK is a cell permeable caspase inhibitor that can prevent the induction of apoptosis by irreversibly binding to caspase protease catalytic sites (Marcelli *et al*., 1999). Z-VAD-FMK was diluted in DMSO to 43mM before being stored at -20°C. Some samples were pre-treated with 50μM z-VAD-FMK. Cells were then suspended in 5% FBS supplemented culture media at a concentration of 1×10^6 cell/ml, seeded in 96-well plates at a concentration of 1×10^5 cells/well. Plates were incubated in a humidified incubator at 37°C, 5% CO2 for one hour. HXR9 and CXR9 were prepared as above, in 5% FBS media with or without 50μM z-VAD-FMK. After an hour pre-incubation, the cells were treated in triplicate with 100μl of HXR9 IC_{50}, or with a CXR9 concentrations that were equivalent to double the IC_{50}. Each plate had an untreated negative control cells and cells treated with Daunorubicin (DNR) as positive control cells. Plates were then incubated in a humidified incubator at 37°C, 5% CO_2 for two hours. Having been treated for two hours, plates were centrifuged at 1000rpm for five minutes and supernatants discarded. Cell pellets were washed twice before RNA was extracted for analysis.

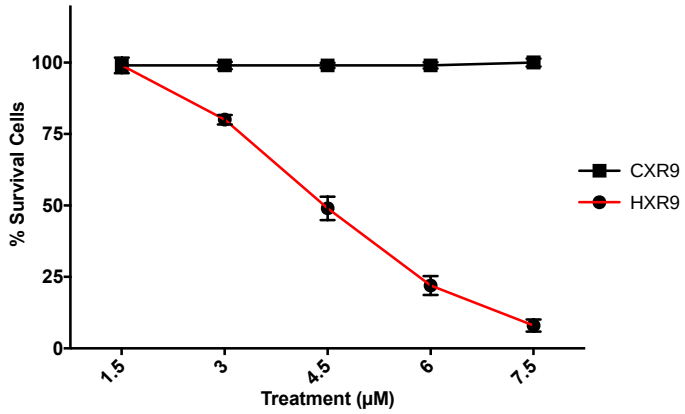
Results

6.3.1 HXR9 is cytotoxic to all tested leukemic cell lines

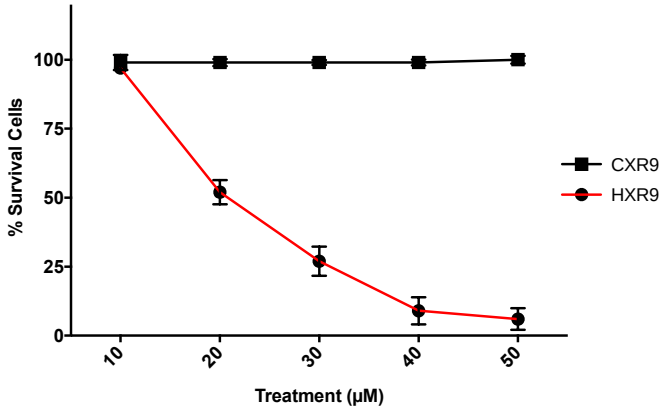
Five AML-derived cell lines including KG-1, HEL 92.1.7 and HL-60, derived from primary AML patients, with KU812F and K562 derived from secondary AML patients were challenged with titrations of HXR9 and its negative control CXR9 for two hours. The cytotoxic effect of HXR9 and CXR9 were subsequently measured by an LDH assay. HXR9 as expected had a large cytotoxic effect on all tested AML cell lines, while CXR9 which does not have a functional domain had no cytotoxicity (Figure R6.1). IC_{50} values were analysed by Calcusyn software and were 4.6μM, 7.2μM, 21.9μM, 9.6μM and 11.4μM for KG-1, HEL 92.1.7, HL-60, KU812F and K562, respectively (Table 6.1).

HXR9 Cytotoxicity in AML Derived Cell Lines

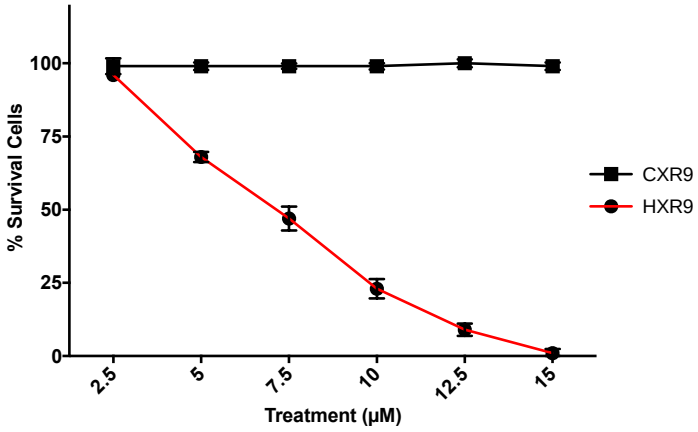
KG-1



HL-60



HEL 92.1.7



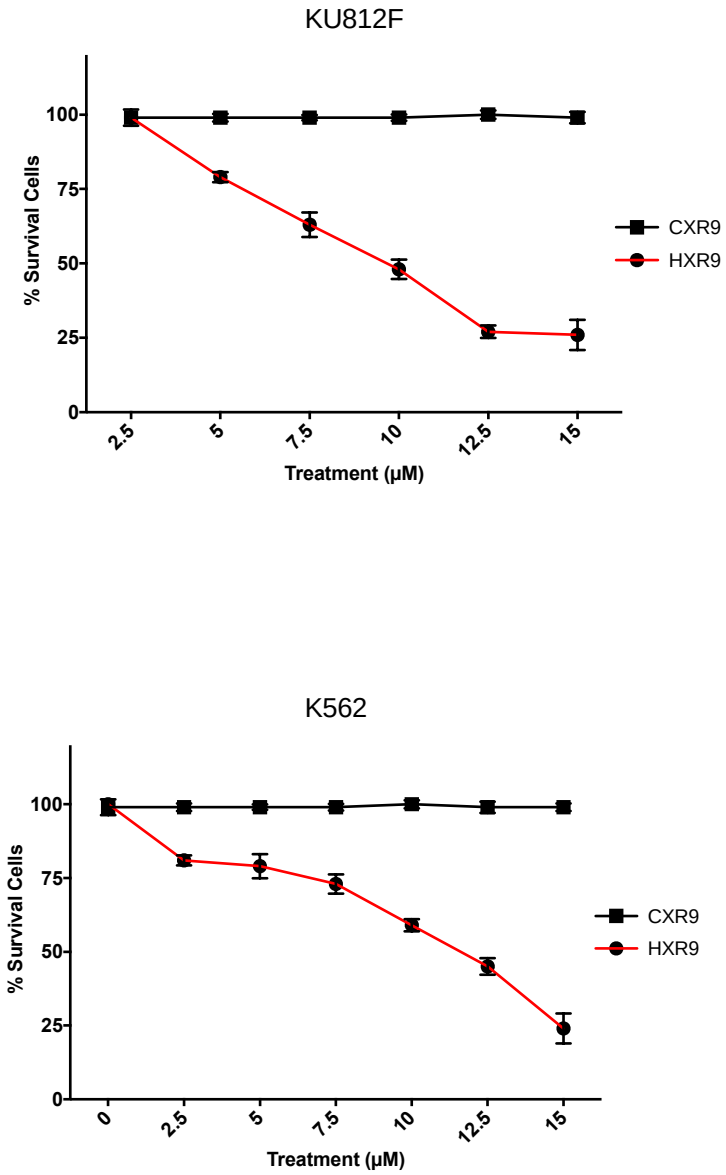


Figure R6.1 *LDH assay for HXR9 and CXR9 cytotoxicity on five AML-derived cell lines. Cell lines were challenged with titrations of HXR9 and CXR9 and LDH enzyme activity was measured in supernatants, indirectly reflecting cell survival.* IC_{50s} *of HXR9 were then calculated using Calcusyn software. Graphs show the mean of three independent repeats that had* $R^2 \geq 0.95$ *where* R^2 *= (0.956), (0.961), (0.959), (0.962), (0.949), (0.962) respectively and error bars show SEM.*

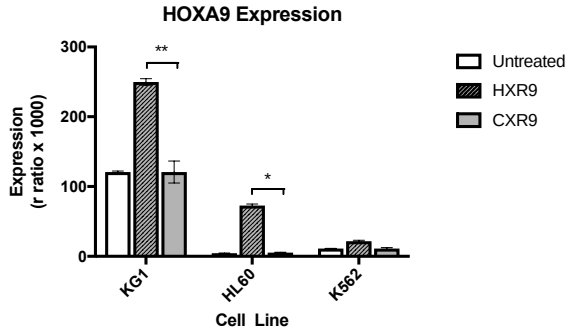
Cell Line	*Source*	*IC50 of HXR9 (uM)*	*SEM*
KG-1	Primary AML patients	4.6	+/- 0.322
HEL 92.1.7		7.2	+/- 0.711
HL-60		21.9	+/- 0.371
KU812F	Secondary AML patients	9.6	+/ 1.676
K562		11.4	+/- 0.267

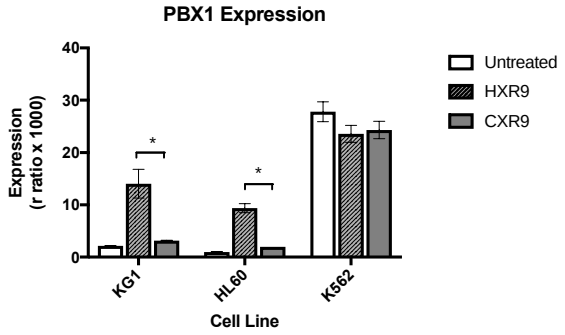
Table 6.1 *A table listing the IC50 values for each of the five AML-derived cell lines that represent the mean IC50 of three experiments, conducted independently and their corresponding SEM.*

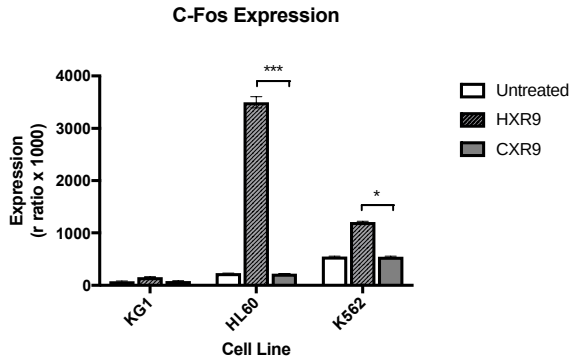
6.3.2 HXR9 treatment affects downstream genes responsible for cell death in leukemic cell lines

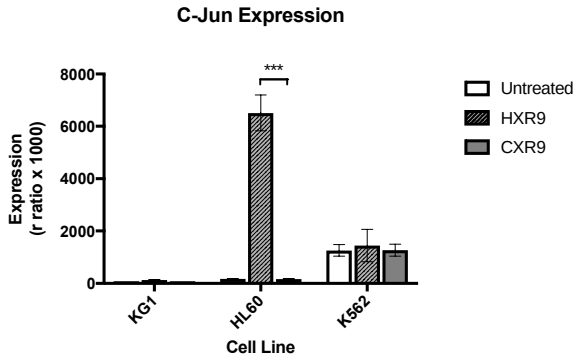
To analyse the effect HXR9 may have on downstream genes hypothesised in our proposed HXR9 pathway, expression candidate genes was analysed in primary AML-derived cell lines HL60 and KG-1 but also in the secondary AML-derived cell line K562, either challenged with titrations of HXR9 and its negative control CXR9 (Figure R6.2.1). HXR9 has an immediate effect on gene expression in genes analysed, varying between cell lines. HXR9 increases HOXA9 and PBX1 expression almost uniformly. Although HL60 expression was not what we had expected. This two-hour treatment with HXR9 resulted in a 1.93, 15.37, and 2.22-fold increase in c-Fos expression in KG-1, HL-60, and K562, respectively, compared to the negative control. A significant increase in both c-Fos, c-Jun and EGFR1 was noted especially in HL60 and K562 cells however virtually no, if any increased expression in either genes were noted in KG-1 cells. Interestingly, it was observed that none of the pro-apoptotic members of the Bcl-2 family including Bcl-XL, nor the anti-apoptotic member Bcl-2, which mediates mitochondrial cell death (Green and Kroemer, 2004), showed any transcriptional changes having been challenged with HXR9. The results obtained via qPCR on both HOXA9 and PBX1 were validated using western blotting which support the data with an increased expression of HOXA9 in all cell lines tested (Figure R6.2.2).

Gene Expression in AML Derived Cell Lines Challenged with HXR9









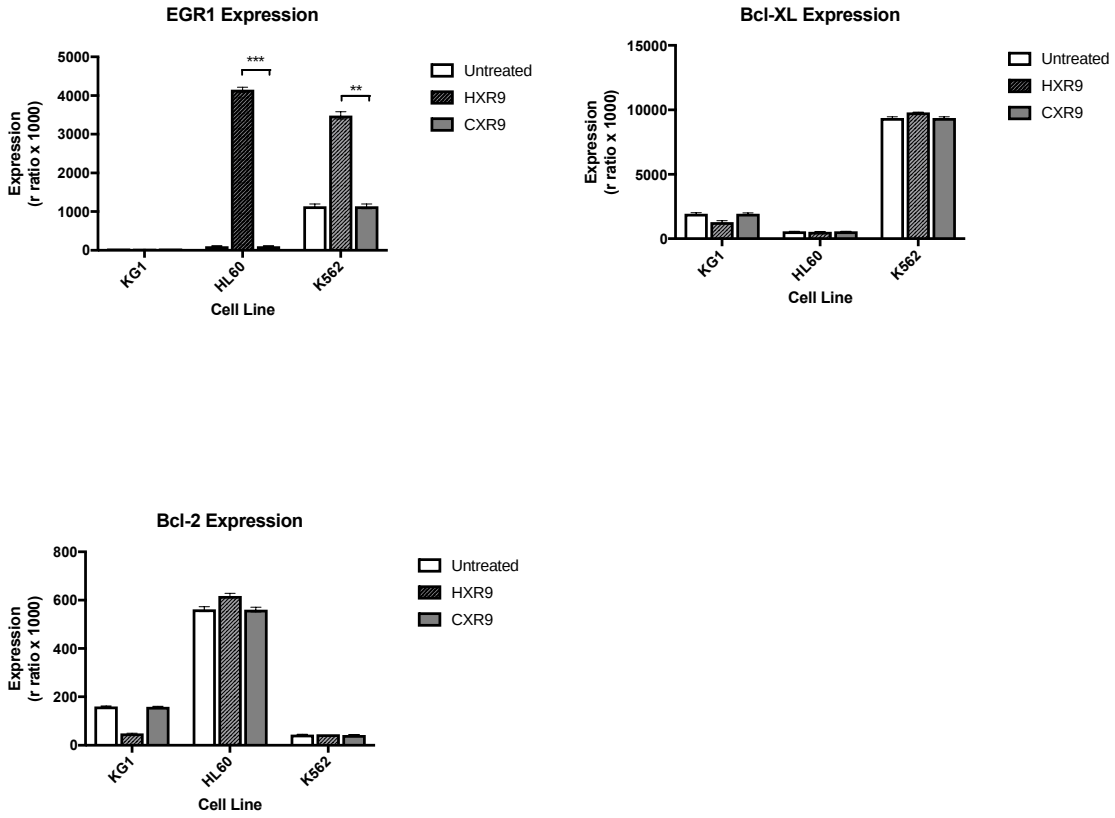


Figure R6.2.1 *Gene expression of HOXA9, PBX1 and their downstream effector proteins in the primary leukaemic cell lines KG-1, HL60 and secondary leukaemic cell line K562 challenged with HXR9 and its control CXR9. Gene expression is represented by the ratio of the expression of genes to the housekeeping gene β-actin (×1000). The values of three separate experiments were plotted shown by error bars where the SEM for each was used in this graph.* *p< .05, **p < .01, ***p <.001.

Protein Expression in AML Derived Cell Lines

Challenged with HXR9

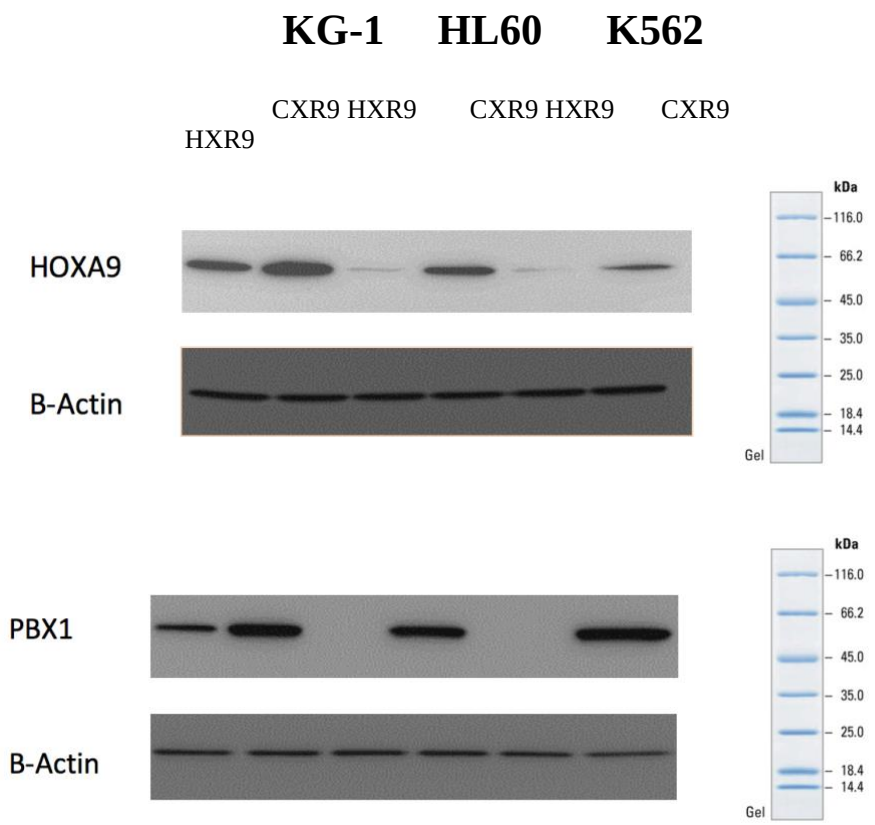


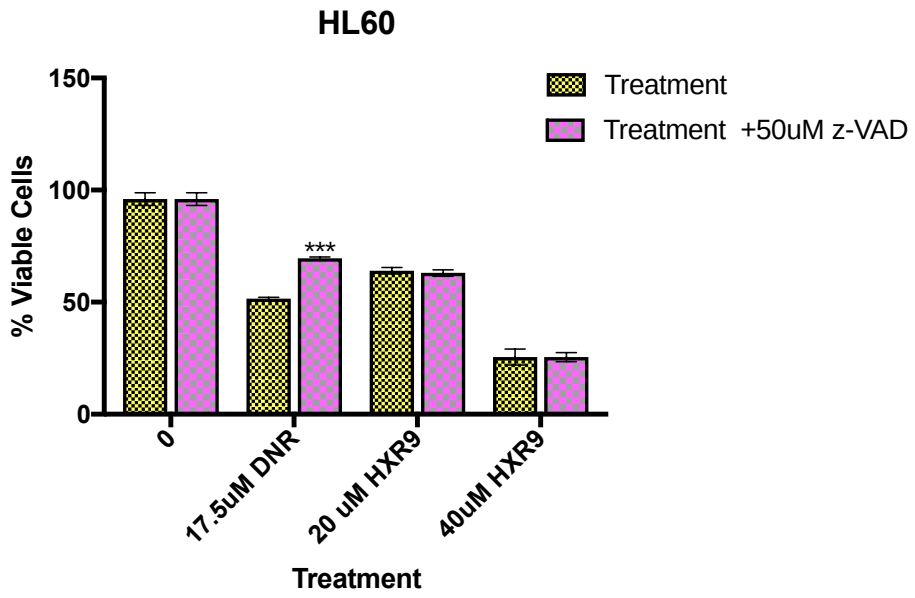
Figure R6.2.2 *Western blot analysis of HOXA9 and PBX1 protein levels isolated from HL60, K562 and KG-1 cell line, where cell lines were challenged with titrations of HXR9 and CXR9, for two hours.*

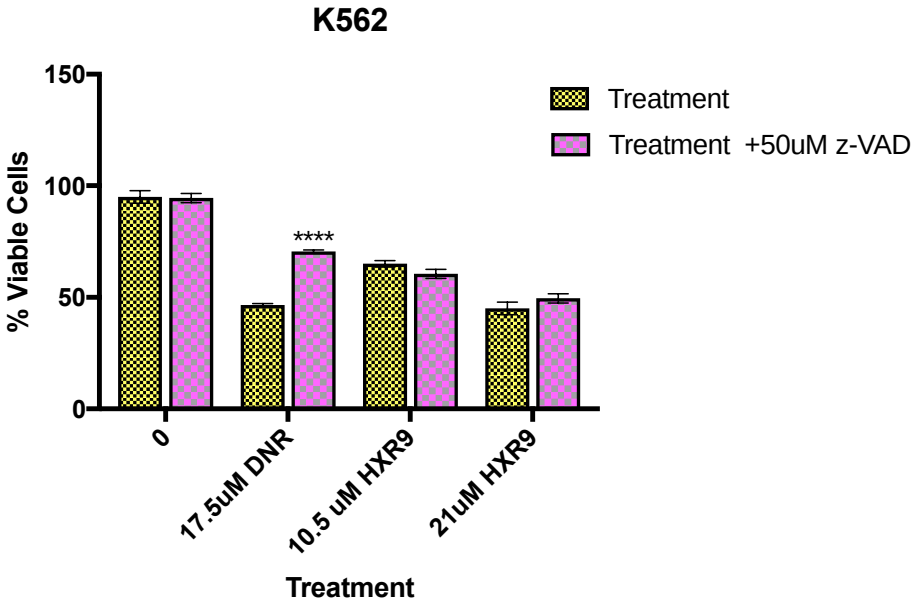
6.3.3 HXR9 induced cell death is independent of apoptosis with no effect on caspase activity or the integrity of their nuclear membrane

To gain a better understanding into the mechanism of cell death and to test the hypothesis, changes were assessed in caspase activity along with assessing nuclear morphology upon HXR9 treatment. KG-1, K562 and HL-60 cells were pre-treated with or without the caspase inhibitor z-VAD-FMK using DNR (shown to activate caspases) as a positive control (Liu *et al*., 2002). Results showed that 50μM z-VAD-FMK pre-treatment dramatically increased the percentage of viable cells in DNR-treated cells, $p < 0.0001$, but saw no statistical difference in terms of the percentage of viable cells between z-VAD-FMK non-treated or pre-treated cells. The percentage of viable cells was 64% and 63% for z-VAD-FMK non- or pre-treated HL60 cells respectively and 65% and 61% for z-VAD-FMK non- or pre-treated K562 cells respectively (Figure R6.3.1).

When AML-derived cell lines were treated with the corresponding IC_{50s} of HXR9, there were no significant changes to the nuclear morphology compared to the untreated cells in all cell lines (Figure R6.3.2). This suggests that HXR9-induces cell death in a nuclear fragmentation-independent pathway and therefore supports the hypothesis that HXR9 kills cells in a caspase-independent pathway.

Inhibition of Caspase Activity in AML Derived Cell Lines Challenged with HXR9





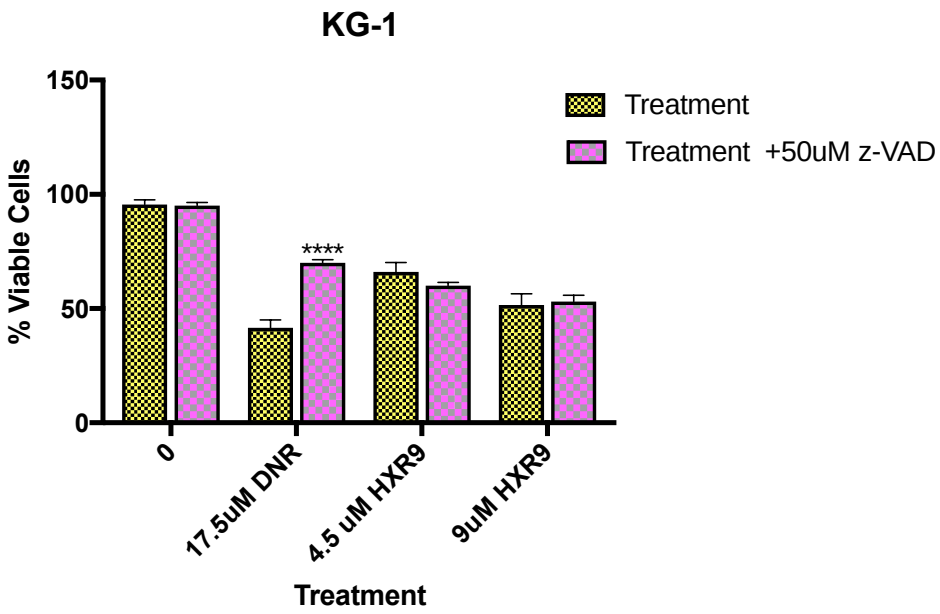


Figure R6.3.1 *The inhibition of caspase activity in HXR9 challenged AML cell lines KG-1, HL60 and K562 by the caspase inhibitor z-VAD-FMK. Cells were pre-treated either with/without 50μM z-VAD-FMK for an hour, before the* IC_{50}*, or 2x*IC_{50} *of HXR9 for two hours or with 17.5μM DNR for 24 hours, as a positive control. Data showed that there was no statistical difference in terms of sensitivity to HXR9 between pre-treated cells with or without 50μM z-VAD-FMK. The mean of three independent experiments are shown and error bars show the SEM in these graphs. *p< .05, **p < .01, ***p <.001 and ****p <.0001.*

Changes in Cellular Morphology when Challenged with HXR9

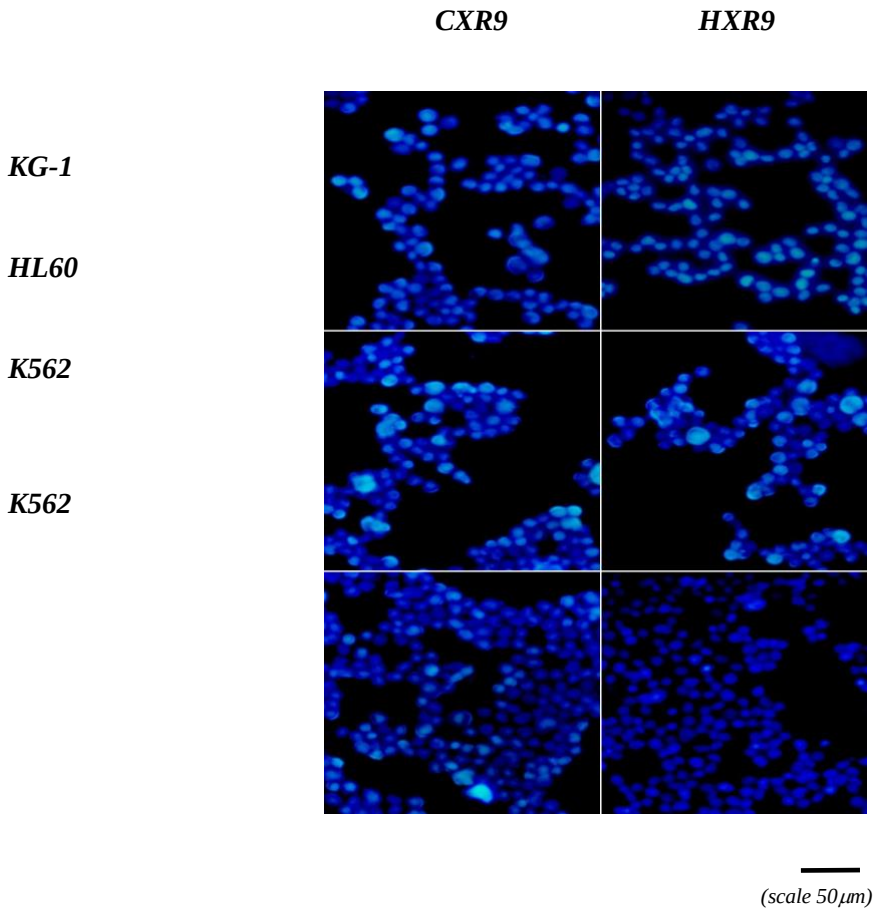
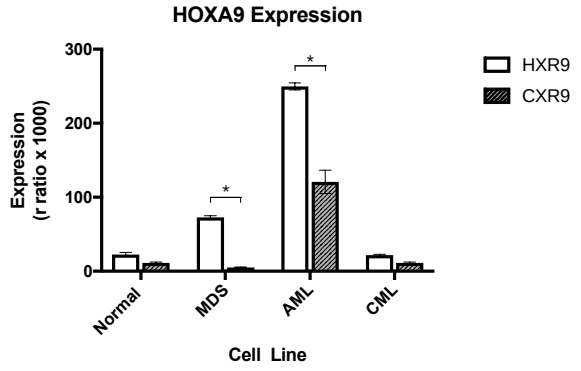


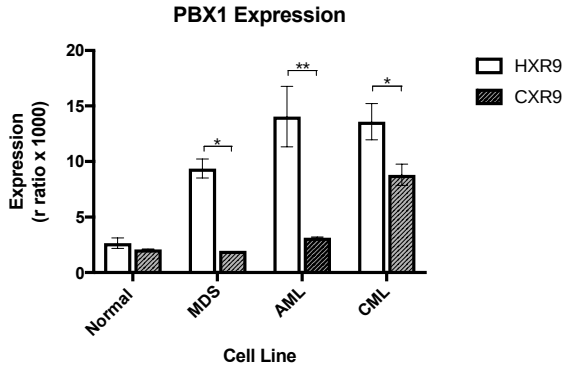
Figure R6.3.2 *DAPI staining of AML cell lines KG-1, HL60 and K562, upon HXR9 treatment. Cells were treated with the* $IC_{50\ values}$ *of HXR9 for 2 hours. Cells were then harvested and fixed with 4% formaldehyde, and cytospan. Fixed cells were stained with DAPI for 5 minutes in the dark. Finally, stained cells were analysed by a fluorescent microscope at 20x magnification.*

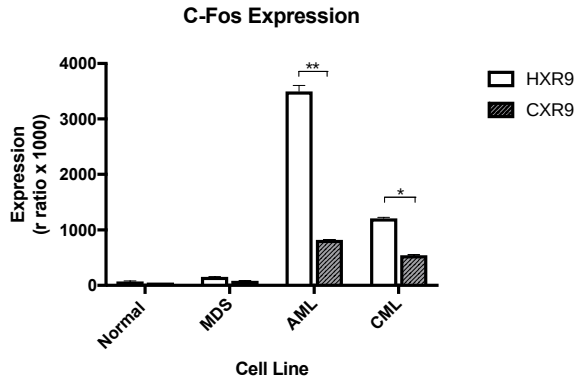
6.3.4 HXR9 treatment affects downstream genes responsible for cell death in patients of AML and other haematological disorders

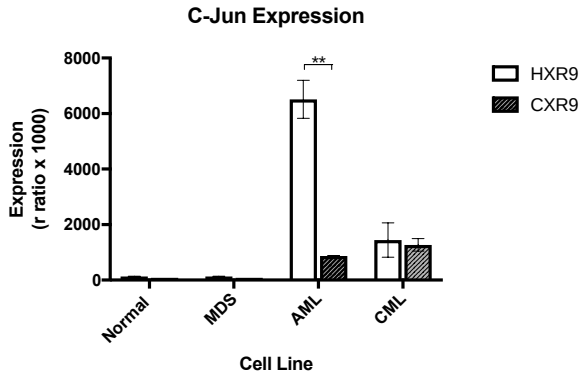
Gene expression was analysed in primary cells of healthy normal donors, MDS, AML or CML patients challenged with either titrations of HXR9 or its negative control CXR9 which did not have a functional domain for 2 hours, where n=3 plotted with error bars and each patient was analysed in three independent experiments (Figure R6.4). HXR9 has an immediate effect on gene expression in several genes analysed, which varies between cohorts. HXR9 increases HOXA9 and PBX1 expression almost uniformly throughout all groups, although this is seen more significantly in AML and MDS patients. A significant increase in both c-Fos, c-Jun and EGFR1 was noted especially in AML patients however there appeared virtually no expression of either genes in MDS patients. Antagonism of this HOX/PBX interaction has been seen to play a role in up-regulation in the expression of c-Fos in other cancers previously mentioned.

Gene Expression in Patients of AML and Other Haematological Malignancies Challenged with HXR9









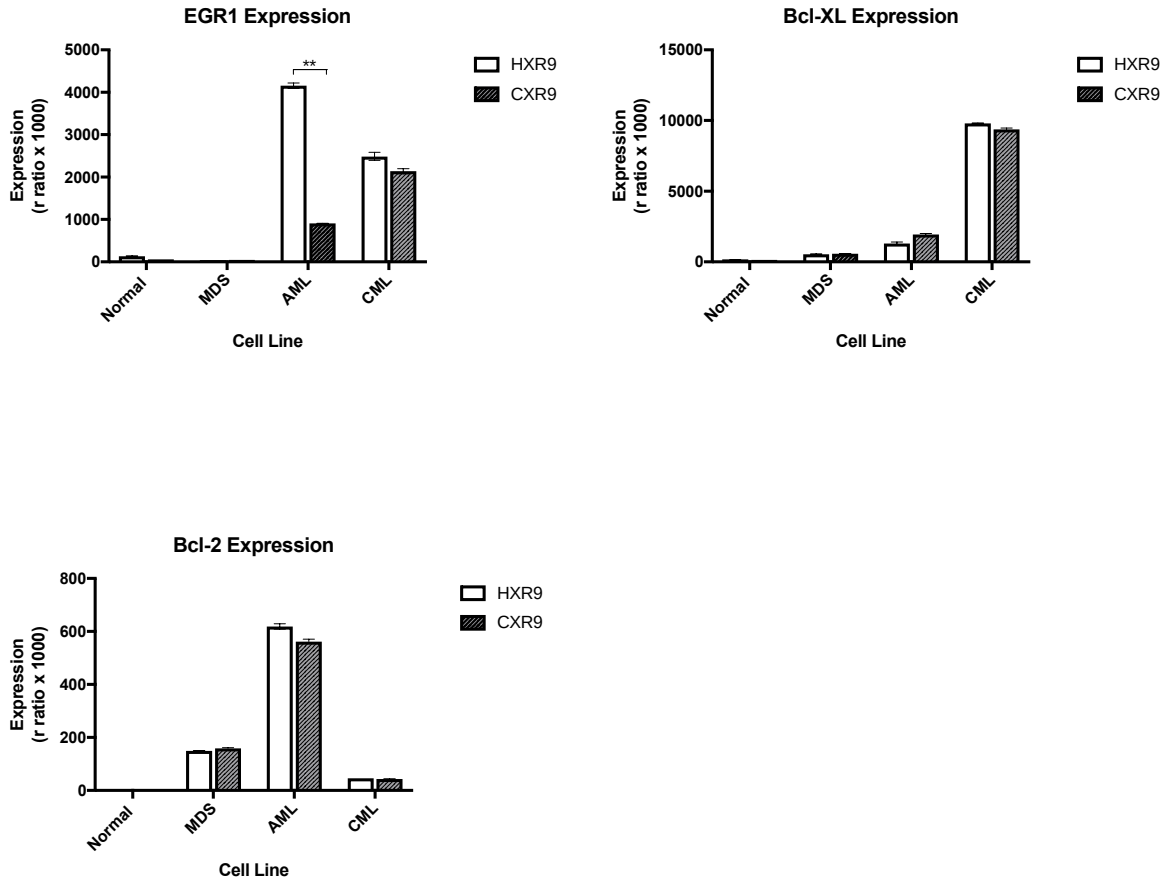


Figure R6.4 *Gene expression of HOXA9, PBX1 and their downstream effector proteins in patients with acute myeloid leukaemia and other haematological disorders challenged with HXR9 and its control CXR9. Gene expression is represented by the ratio of the expression of genes to β-actin the housekeeping gene (×1000). The values of three separate patients were plotted shown by error bars where each patient was analysed in three independent experiments with the SEM for each patient used in this graph.* *p< .05, **p < .01.

Discussion

Here it has been confirmed that high expression of specific *HOX* genes could be used as a therapeutic target in AML and potentially in other haematological malignancies since targeting the *HOX-PBX* interaction with HXR9 causes significant cell death in our tested AML-derived cell lines (Figure R6.1). The IC_{50} of HXR9 for these treated AML cell lines ranged from 4.6μM to 21.9μM after treatment for two hours. The sensitivity of the tested cell lines to HXR9 can be directly correlated to the level of some *HOX* gene expression, for example, KG-1 cells that highly express *HOXA5*, *HOXA6*, *HOXA9* and *HOXA10* were most sensitive to HXR9 with an IC_{50} of 4.6μM after a two-hour treatment whereas HL-60 cells, which show a global down-regulation of HOX genes were the least sensitive to HXR9, with an IC_{50} 21.9μM after a two-hour treatment. However, there was very little difference in terms of HXR9 sensitivity between the HEL92.1.7 cells derived from a primary AML patient and the KU812F and K562 cell lines, derived from CML patients in blast crisis (secondary AML) which may well be due to these cells expressing *HOX* genes at a much lower level. Noticeably, the tested AML cell lines were far more sensitive to HXR9 than any of the other cell lines that were derived from solid malignancies e.g. melanoma, renal, ovarian and lung cancers (Shears, Plowright *et al*., 2008; Plowright, Harrington *et al*., 2009; Morgan, Plowright *et al*., 2010). This difference in sensitivity between AML cells and solid cancers may have something to do with the survival of AML cells depending on the *HOX-PBX* interaction, or because there is an abundance in expression of HOX genes in AML cells compared to solid cancers.

Interestingly and most important to note, over recent years the expression of CD34 is thought to be a good marker of a poor prognosis, an adverse clinical course and a low CR rate of AML (Repp, Schaekel *et al*., 2003). Additionally, $CD34^+$ AML cells have been reported to be resistant to current chemotherapies, however $CD34^+$ predicts high sensitivity to HXR9 since HOX genes are highly expressed in $CD34^+$ cells (Bailly, Muller *et al*. 1995; Bailly, Skladanowski *et al*., 1997). Gene expression profiling of these $CD34^+$ cells in some CML patients that are in a chronic phase has revealed a higher level of expression of *HOXA9*, suggesting that HXR9 could be used in combination with other

drugs like imatinib, in first line treatment for CML, that target the BCR-ABL fusion protein (Diaz-Blanco *et al*., 2007, Palandri *et al*., 2008, Hehlmann, 2012). As seen here, cell lines that express the BCR-ABL fusion like K562, are sensitive to HXR9. It has also been reported that BCR-ABL co-operates with Nup98-HOX proteins that progresses to the ultimate phase of CML (blast crisis) (Mayotte *et al*., 2002, Ito *et al*., 2010, Di Giacomo *et al*., 2014). Sensitivity of Ph+ AML cells to HXR9 in recent studies also suggests that HXR9 could be used in a combination with imatinib to kill ALL cells that express both BCR-ABL fusion and *HOX* genes like HOXA9 (Redaelli *et al*., 2009).

To determine what affect HXR9 has on the up-regulation of c-Fos and other important downstream genes in these AML-derived cell lines, the mRNA gene expression levels of important downstream genes were measured in untreated, CXR9 or HXR9 treated cells (Figure R6.2). *HOXA9, PBX1, c-Fos, c-Jun, EGFR1, Bcl-Xl* and *Bcl-2* expression levels were measured under all 3 conditions, treated for two hours with the IC_{50} of HXR9 or equivalent concentrations of the negative control peptide CXR9 which were 4.6μM, 7.2μM, 21.9μM, 9.6μM and 11.4μM for KG-1, HEL 92.1.7, HL-60, KU812F and K562, respectively (Table 6.1). This two-hour treatment with HXR9 resulted in a 1.93, 15.37, and 2.22-fold increase in c-Fos expression in KG-1, HL-60, and K562, respectively, compared to untreated cells or cells treated with CXR9 (Figure 6.6).

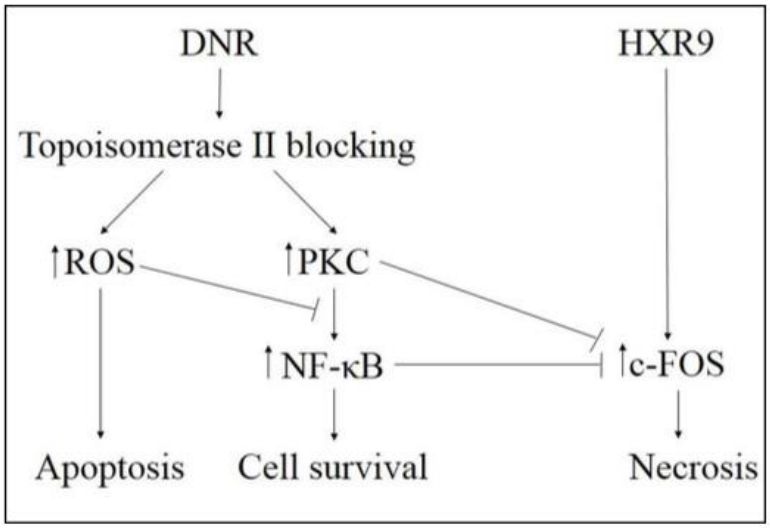


Figure 6.4 *A schematic diagram depicting the current hypothesis of the interaction between the current DNR therapeutic and the newly discovered HXR9. DNR is a topoisomerase II inhibitor leading to the activation of the PKC/NF-kB survival pathway known for reducing necrosis by its ability to dampen c-Fos activity.*

The primary hallmark of apoptosis is caspase activation; which was not found necessary in HXR9-induced cell death (Figure R6.3.1). Recent data appears to be consistent with the likely mechanism of HXR9-induced cell death in renal cancer cell lines such as Caki-2 and 769-P, in malignant B cell lines and some prostate cell lines (Shears, Plowright *et al*., 2008; Daniels, Neacato *et al*., 2010; Morgan, Boxall *et al*., 2014). However, as seen here, the caspase inhibitor z-VAD-FMK had little or no significant effect on the viability of cells challenged with HXR9, nor did the integrity of the nuclear envelope change post treatment (Figure R6.3.2). The general inhibition of caspases in recent studies in other cancers has decreased the cytotoxicity of HXR9 on the tested renal, ovarian, breast and prostate cancer cell lines, although this decrease did not completely abrogate the effect of HXR9 (Shears, Plowright *et al*., 2008; Morgan, Plowright *et al*., 2010; Morgan, Boxall *et al*., 2014). The current hypothesis is that this may be due to the presence of other mediators of HXR9-induced cell death. Findings of recent studies also support this and suggest that mitochondrial apoptosis is not required for

HXR9-mediated cell death. The unchanged ratio found between the anti-apoptotic Bcl-2 and the pro-apoptotic Bax and Bak1 (in previous studies) was supported here and could potentially reflect how intact the mitochondrial membranes is, as the presence of excess pro-apoptotic members of the Bcl-2 family appears to enhance mitochondrial permeabilization (Martin *et al*., 2010). As we have mentioned, we observed that none of the pro-apoptotic members of the Bcl-2 family including Bcl-XL, nor the anti-apoptotic member Bcl-2, which mediates mitochondrial cell death (Green and Kroemer, 2004), showed any major transcriptional changes having been treated with HXR9.

This data ties in nicely with previous studies mentioned above that have also revealed that HXR9 did not cause transcriptional changes in the initiator caspase-9 and Apaf1 that together are known to form an apoptosome complex with cytochrome C (cyt C) which together start the intrinsic apoptosis pathway (Adrain and Martin, 2001). In addition to this, previous unpublished studies have shown that executioner caspases-3, -6 and -7, (key mediators of apoptosis) (Huerta *et al*., 2007) were not up-regulated after HXR9 treatment. To summarise: HXR9 treatment of AML cells did not initiate nuclear fragmentation of apoptotic cells which results from the cleaving of the cytoskeletal protein lamin by caspase-3 or -6 (Kagawa, Go *et al*., 2001; Ruchaud, Korfali *et al*., 2002) suggesting an alternative to apoptosis. An alternative mode of cell death might explain the absence of nuclear fragmentation in HXR9-treated AML cell lines. These changes in nuclear morphology like chromatin condensation and nuclear fragmentation are important markers of late stage apoptosis (Collins *et al*., 1997, Ziegler and Groscurth, 2004, Huerta *et al*., 2007). The treatment of cells with HXR9 did not result in changes to the nuclear morphology after comparing these to untreated cells in all investigated cell lines. This suggests that HXR9-induces cell death in a nuclear fragmentation-independent pathway. The absence of apoptotic-hallmarks of activation, including caspase activation, Bcl-2 family involvement, chromatin condensation and nuclear fragmentation strongly suggests that HXR9 induces AML cell death via necrotic pathways.

Analysing the effect HXR9 may have on primary cells and downstream genes involved in the proposed HXR9 pathway and the variation in these genes depending on the disease/progression of disease of the patient is of great importance. Gene expression was analysed in peripheral mononuclear cells of healthy

normal donors, MDS, AML or CML patients which were challenged with either HXR9 or the control CXR9 for 2 hours. *HOXA9, PBX1, c-Fos, c-Jun, EGFR1, Bcl-Xl* and *Bcl-2* expression levels were measured under all 3 conditions, and it was very clear that the HXR9 peptide has an immediate effect on gene expression in many of these genes varies between cohorts. HXR9 increases *HOXA9* and *PBX1* expression almost uniformly throughout all groups, although this is seen more significantly in AML and MDS patients (Figure R6.4). The significant increase in both *c-Fos, c-Jun* and *EGFR1* was noted when challenged with HXR9 especially in AML patients however there appeared virtually no expression of either gene in MDS patients and suggests how effective this peptide may be in manipulating gene expression of crucial genes responsible for the progression of pathology. Most important seems to be c-Fos upregulation with the antagonism of this HOX/PBX interaction already highlighted as a key a role in up-regulation in the expression of c-Fos in other cancers and the induction of c-Fos expression by HXR9 thought to be responsible for initiating cell death in some human renal, lung, melanoma, ovarian, prostate and breast cancer cell lines (Morgan, Pirard *et al*., 2007; Shears, Plowright *et al*., 2008; Plowright, Harrington *et al*., 2009; Morgan, Plowright *et al*., 2010; Morgan, Boxall *et al*., 2012; Morgan, Boxall *et al*., 2014). Interestingly, it was observed that none of the pro-apoptotic members of the Bcl-2 family including Bcl-XL, nor the anti-apoptotic member Bcl-2, responsible for the mediation of mitochondrial cell death (Green and Kroemer 2004), showed any transcriptional changes having been treated with HXR9. If this is the case, and taking into consideration that in AML-derived cell lines, it has already been shown (as have others) that apoptosis may not be the primary mode of cell death, then how might c-Fos play a role, in the progression to various forms of cell death other than by apoptosis?

Future work aiming to look into the mitochondrial release of cyt C and analyse if this would ultimately lead to caspase activation would be of great benefit to this area of research. In addition to this, the cleavage and activation of Bid that is known to mediate mitochondrial apoptosis depends on the activation of caspase-8, which was not required for HXR9 cytotoxicity. HXR9 seems to induce cell death in a Bax- independent process, since Bax-dependent apoptosis involves either caspase-9 or p53 activity, neither of which was needed for HXR9 mediated-cell killing (Janssen *et al*., 2007, Gogada *et*

al., 2011, Meng *et al*., 2012). CypD knockout studies over the last few years have demonstrated its crucial role in mitochondrial necrosis by Ca^{2+} overload and oxidative stress, however it is not required for apoptosis (Li, Johnson *et al*., 2004; Baines, Kaiser *et al*., 2005; Nakagawa, Shimizu *et al*., 2005; Schinzel, Takeuchi *et al*., 2005). In addition, CypD is involved in Ca^{2+}-independent, RIP3- and p53-dependent mPTP necrosis (Lerch, Halangk *et al*., 2013; Tian, Xu *et al*., 2013; Pei, Shang *et al*., 2014). Accordingly, HXR9 might be a key regulator in mediating necrosis of AML in an mPTP-independent pathway, or at least via a CypD-independent mechanism. Recently published was data suggesting similarly that HXR9 leads to cell death through a necrotic pathway, at least in part, through RIP1 in HL-60 cells, however K562 cell death was RIP1-independent (Alharbi *et al*., 2017). It is possible however, that the effect of blocking RIP1 HXR9-induced HL-60 cell death may reflect a sub-effective dose of the RIP1 inhibitor, because Nec-1 has shown some toxic effects at higher doses. The latter is a good explanation that seems consistent with the fact that Nec-1 partially protected HL-60 cells from HXR9. On the other hand, some mediators in addition to RIP1 may be required in HXR9-induced cell death. It is important to note that a RIP1-independent cell death pathway does not rule out a potential role for necroptosis since it was reported that viral infection resulted in RIP3-dependent but RIP1-independent necroptosis through TNF mediated necroptosis (Zhang, Shao *et al*., 2009; Upton, Kaiser *et al*., 2010), suggesting that the role of RIP3 in HXR9-induced K562 cell death requires further investigation and draws a very strong link between HXR9 and necroptosis.

Looking closely into some of the downstream pathways strongly related to cell death ought to help understand the mechanism of actions involved when cells are challenged with HXR9. It has been recently suggested that the presence of ATP may also be crucial for caspase-dependent apoptosis as recent studies have suggested, but also for PARP1 hyper activation. In cases of caspase-dependent apoptosis, ATP involvement can be seen in the formation of the apoptosome complex. This apoptosome complex mediates apoptotic signalling by cleaving and activating various executioner caspases (Jochen, Richter *et al*., 2002). It has been reported that ATP depletion by fructose in some primary rat and mouse hepatocytes inhibits cyt C release and the activation of executioner caspases along with DNA fragmentation by TNF-mediated apoptosis as well this, these events were restored after ATP repletion

(Latta *et al*., 2000). Not only this but it is well established that ATP is essential for chromatin condensation and nuclear fragmentation (Tsujimoto, 1997). ATP depletion by fructose has been shown to prevent caspase activation and subsequently shifts cell death in the direction of necrosis in leukaemic Jurkat and K562 cells (Leist, Single *et al*., 1999; Verrax, Dejeans *et al*., 2011). Similarly, PARP1 requires ATP during the transfer of poly (ADP-ribose) from NAD^+ (Devalaraja-Narashimha and Padanilam *et al*., 2009; Ethier, Tardif *et al*., 2012). Together, the depletion of this ATP initiates the role of caspases and PARP1 hyper-activation in HXR9-induced cell death of AML cells. It is well understood that at extremely high concentrations, fructose may trap intracellular ATP and maintain sufficient cytoplasmic concentrations that prevents cell necrosis (Latta *et al*., 2000, Latta *et al*., 2007, Speicher *et al*., 2012). The results of recent work like this has revealed that ATP depletion by a high concentration of fructose is unable to prevent HXR9-induced necrotic death on tested AML cells. This also supports a caspase- and PARP1- independent mechanism for HXR9-induced cell death.

Despite a critical role in TNF-induced necrosis, PARP1 has been shown in TNF-mediating necroptosis (Xu *et al*., 2006). For this reason, TNF may activate necroptosis and necrosis in specific pathways, relevant to the results, although PARP1 has also been shown to be a downstream effector of the necrosome in TRAIL-induced necroptosis (Jouan-Lanhouet *et al*., 2012). Although the JNK pathway is heavily involved in PARP1-induced necrosis, HXR9-induces necrosis of AML cells is JNK-independent (Degterev *et al*., 2005, Xu *et al*., 2006, Jog *et al*., 2009, Éthier *et al*., 2012). PARP1 is a key player in mPTP, and results of this PARP1 activation have supported a mitochondria-independent pathway for HXR9-induced necrosis (Abramov and Duchen, 2008) supporting the findings and highlights again that necrosis (necroptosis) is most likely the mode of cell death in this pathway. Further downstream of this however, the MAPK family of protein kinases is vital in a lot of key cellular processes. This family consists of four members; ERK, p38, JNK and the mitogen activated protein kinase. MAPK proteins are known to regulate various cellular pathways as mentioned above, including cell survival, proliferation and cell death (Chen and Sommer, 2009). Importantly, the JNK pathway has been reported to be a firm inducer of clonal evolution of Fanconi anaemia to AML (Li *et al*., 2007) and a sustained activation of the JNK pathway with AKT/FOXO signalling has been shown to maintain

AML cells in an undifferentiated state (Sykes *et al.*, 2011). Of interest here, in FL15.12 cells (a haemopoietic pro-B cell line), IL-3 promotes cell survival by phosphorylating BAD through the JNK signalling pathway (Yu *et al.*, 2004). Since this discovery, co-inhibition of the TNF-JNK pathway has been shown to increase sensitivity of primary human AML cells in *vitro* to NF-κB inhibitors (Volk *et al.*, 2014), not only this but the JNK pathway promotes proliferation and metastasis in several cancers including liver and skin (Chen *et al.*, 2001, Bettermann *et al.*, 2010, Ebelt *et al.*, 2013).

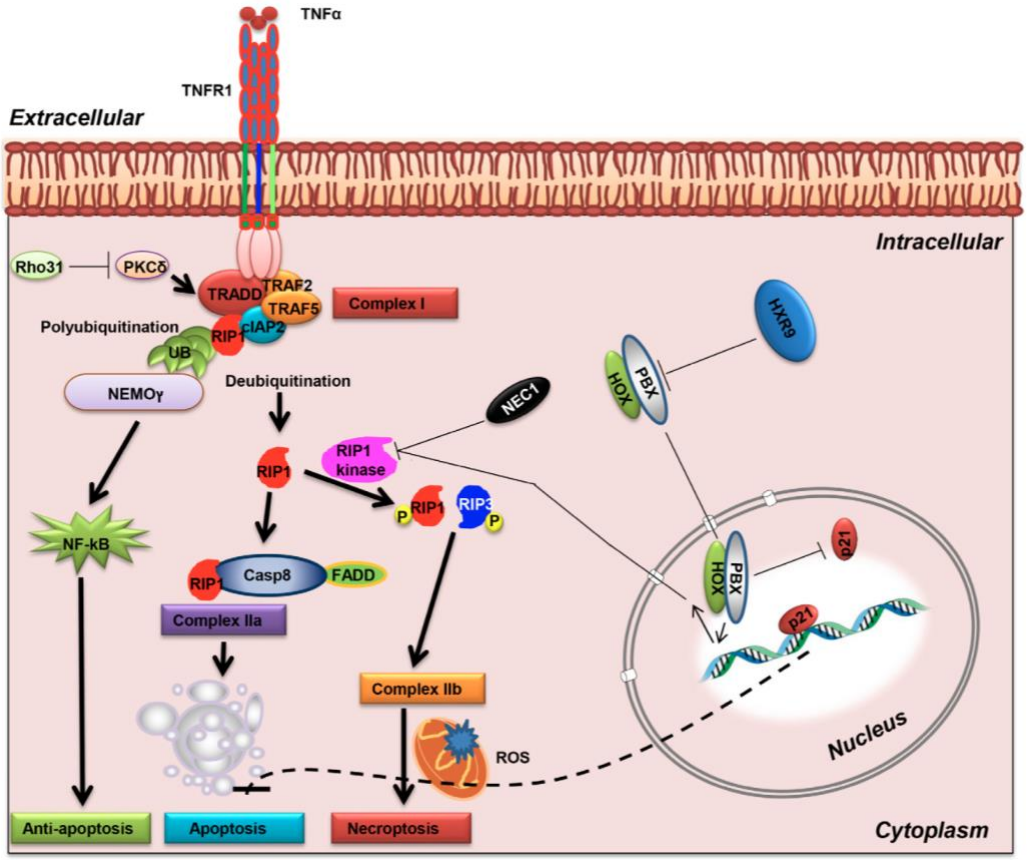


Figure 6.5 *Activation of RIP1-mediated necroptosis which clearly shows HXR9 (peptide of interest) inhibiting the interaction of PBX and HOX before the complex translocates into the nucleus and inhibits p21 (Alharbi* et al.*, 2017).*

To summarise high expression of *HOX* genes could form the basis of individualised medicine in AML and progressive pre-leukaemic malignancies by antagonising the interaction between *HOXA9* and its co-factor *PBX* with the integration of a small cell-permeable peptide, HXR9 resulting in cell death. A strong link between the susceptibility of the tested AML cell lines to HXR9 and their potential correlation with their expression levels of *HOXA9* has also been seen. The most susceptible cell line KG-1 has been shown to highly express a set of *HOX* genes of great importance, while HL-60 has showed very low *HOXA9* gene expression and has the lowest susceptibility to HXR9 as expected other than in a few experiments which go against previous literature. Although we have thoroughly analysed this discrepancy, further analysis should be undergone in the future to explore why this is the case.

Finally, cell death was analysed and hypothesised by assessing changes in various cellular compartments after HXR9 treatment. The most obvious method of cell death in AML-derived cell lines would, on the above data, appear to through an apoptosis-independent pathway that involves programmed necrosis. Nuclear morphology was assessed using DAPI staining, with the only although significant limitation of this assay being the lack of a positive control for nuclear fragmentation. As mentioned already, the cytosolic changes have been previously studied by analysing changes in different proteins including the pro-apoptotic member of the Bcl-2 family, XIAP, Apaf1 and caspase activation. Over recent months, more studies have emerged that suggest the most obvious method of cell death in AML-derived cell lines is through an apoptosis-independent pathway that involves programmed necrosis and the results of this study support this theory. This is newly termed necroptosis.

These *in vitro* results show that the HOX-PBX dimer in theory could be a potential therapeutic target in a subset of AML that over-express *HOX* genes. The comprehensive analysis of the mechanism of HXR9 cytotoxicity would guide us in the selection of the potential combination drugs. For example, avoiding agents that cause NOX, CaM, PKC activation ought to be avoided if possible. However, a proper *in vivo* systemic model should be used in future studies to assess the *in vivo* efficacy of HXR9 cytotoxicity, as well as conducting more *in vitro* assays to investigate the mechanism of HXR9 to choose potential synergistic agents.

Chapter 7: Discussion & Future Directions

7.1 Discussion

Microarray analysis has Acute myeloid leukaemia is well known as the most frequent acute leukaemia that affects adult patients around the globe. Generally, AML can be classified cytogenetically according to cell karyotypes in three main prognostic groups: which includes favourable prognostic groups t(8;21), inv(16) and t(15;17), adverse prognostic groups like complex karyotypes, trisomy 8 and CML-blast crisis, and also in an intermediate prognostic group that includes other abnormalities but also NK-AML (Roche *et al*., 2004). Noticeably, 50% of all AML cases exhibit normal karyotypes (Marcucci, Mrozek *et al*., 2005). The most standard treatment for any of these prognostic groups has not changed dramatically over the last few decades, other than for APML until the last few years. Current induction therapy is a combination therapy which consists of DNR for 3 days followed by cytarabine for a week. This treatment is termed the "3+7 regimen". This is strategic and usually achieves 72%-76% CR in young (< 60 years), and just over half (51%) CR in older patients. It seems very plausible then that a post-induction or consolidation regimen that consists of a high cytarabine dose or an allogeneic transplantation could significantly improve the outcome of these AML patients. However, unfortunately, the outcome is heterogonous and many of these patients relapse and die even if they have achieved clinical CR (Burnett, 2012; Patel and Levine, 2012). The significant heterogeneity in outcomes of these AML patients could be caused by various other factors. It would be important firstly, for us to address the current CR criteria, which as we know can be very broad, is very much dependent on a microscopic count of blast cells in the bone marrow and using this to determine whether these patients' make-up the 5% of the cell population which has not changed measurably since 1956 (Hourigan and Karp, 2013). Evidently this is a massive limitation and it is a hindrance that could be easily overcome by including the detection of AML-specific molecular stable targets such as *HOXA9* and various other biomarkers using some of the findings addressed in this study like PCR or flow cytometry in CR criteria. The detection of AML cells post chemotherapy, which are at levels under the sensitivity of microscopic detection have been coined minimal residual disease (MRD) (Ossenkoppele and Schuurhuis, 2013). There is a very low specificity of the current treatment regimen which is well known to play a large role in the heterogeneity of response. In overcoming this limitation, AML patients

may now be treated with drugs that are a lot more gene or cell specific as suggested here with HXR9. Finally, the heterogeneity of some AML cells in respect to treatment sensitivity can be of huge importance, and as we have seen, favourable prognostic groups often appear to respond better than poor prognostic groups to current therapeutics and chemotherapy.

To date, current evaluation of relapse risk in AML, apart from APML patients, is based around the average clinical results of some large historical populations that have had the same pre-treatment, chromosomal and molecular abnormalities at the time of presentation (Mrózek *et al*., 2012, O'Donnell *et al*., 2012). However, this analytical assessment can lack evaluation and induction treatment efficiency due to patient-individualised-evaluation. There is no doubt however that this could be massively improved by including MRD detection with the ability to improve decisions when deciding on the most-suitable post-induction treatment available. MRD is currently well known and is applicable in some cases for example in CML, where it can be used to detect BCR-ABL fusion proteins that present in virtually all CML cases (Cross *et al*., 2012, O'hare *et al*., 2012). Recently it has been shown that detection of the $NPM1^{mut}$ MRD preceded haematological relapse by almost 8-9 weeks (Schnittger *et al*., 2009). Interestingly, therapeutic intervention often used after the detection of a molecular relapse by MRD is known and shown to significantly improve clinical outcomes (Rubnitz *et al*., 2010, Inaba *et al*., 2012). It is therefore of huge significance for us to bear in mind that *HOX* gene over-expression is quite common in many AML subtypes, now strongly associated with a poor prognosis as previously mentioned in the introduction, thus clearing potential targets in MRD as we have discussed with HOXA9. Future studies aimed at detecting which AML subtypes are more sensitive to HOX over-expression in a larger widespread study ought to highlight the relationship between the various subtypes of AML and *HOX* genes like *HOXA9.*

The heterogeneity of AML has been underpinned by the presence of various cytogenetic abnormalities in over half of AML cases that include numerical aberrations, structural aberrations and balanced translocations (Buccisano *et al*., 2012). Some studies have revealed a diversity of molecular markers that may be further sub-classified in many NK-AML patients (Marcucci, Mrozek *et al*., 2005) and more

recent discoveries of molecular markers in NK-AML strengthens the hypothesis that leukaemogenesis may result from an interaction of many mutations leading to either an increase in cell proliferation (class I mutations) or the block of cell differentiation (class II mutations), ultimately leading to AML (Döhner and Döhner, 2008b, Renneville et al., 2009, Betz and Hess, 2010). The ever advancing and ever-increasing molecular understanding of diseases like AML will be extremely valuable, including further sub-classification of disease and being able to develop a more personal prognostic prediction that will hopefully form the cornerstone for future therapeutic procedures. The current literature seems to suggest that using all-trans retinoic acid (ATRA) is the only widely used molecularly targeted therapy in AML in the treatment of APML. APML is characterised by the presence of t(15;17)/PML-RARA. It is only by having a better understanding of this molecular mechanism, signalling pathways and co-operating mutations could now pave the way for new potential molecular drugs that will lead to more personalised treatment for patients of AML and pre-leukaemic malignancies in the future.

Recently in-depth analysis of AML subtypes suggests that HOX genes can be one of the common denominators of a lot of the different cytogenetic and molecular aberrations involved in AML, however does this hold true for haematological malignancies that develop into AML? As we know, HOX gene over-expression has been universally reported in AML with chromosomal rearrangement that include MLL fusion proteins and an *MYST3-CREBBP* translocation (Ayton and Cleary, 2003, Camós *et al*., 2006, Slany, 2009). The MLL fusion protein has been found to function in AML by collaborating with signal transduction protein pathways like Ras that are responsible for driving an over-expression of *HOXA9* and activate Raf (Ono *et al*., 2009). HOX proteins also form fusion proteins with the *NUP98* protein and go on to mediate AML formation in combination with *FLT3* (Borrow *et al*., 1996, Nakamura *et al*., 1996, Raza-Egilmez *et al*., 1998, Kroon *et al*., 1998, Fujino *et al*., 2002, Suzuki *et al*., 2002, Taketani *et al*., 2002b, Pineault *et al*., 2004, Palmqvist *et al*., 2006). In addition to this, the *NUP98-HOXA9* protein has been strongly linked with KRAS and Wilms' tumour gene 1 mutations (Chou *et al*., 2009). *HOX* genes are regulated by the Cdx family in AML (Bansal *et al*., 2006, Thoene *et al*., 2008), with some gene profiling studies having shown strong association between *FLT3* mutations and the *HOX* genes investigated in this study (*HOXA7*, *HOXA9* and *HOXA10* expression) (Roche *et al*., 2004,

Eklund, 2007). Over-expression of some of these *HOX* genes has also been seen in the presence of *NPM1* mutations (Mullighan *et al*., 2007, Vassiliou *et al*., 2011). All-in-all, these studies point at *HOX* genes being key novel targets for molecular targeted therapies in various AML subtypes.

In summary, our in *vitro* results have shown that the HOX-PBX dimer may be very useful when looking at potential therapeutic targets in subsets of AML and pre-leukaemic malignancies that over-express *HOX* genes and *HOXA9* which would help the development of personalised medicine for patients suffering from AML diseases in the future. A more comprehensive analysis of the mechanism of action around HXR9 cytotoxicity may guide us in the selection of new potential combination drugs attempted here. Based on results and previous studies on the subject, perhaps some agents that cause NOX, CaM, PKC activation ought to be completely avoided with a more robust in *vivo* systemic model to be used in future, to assess in *vivo* efficacy of HXR9 cytotoxicity, as well as conducting significantly more in-*vitro* assays that may further investigate the mechanism of HXR9. Combination therapy is becoming increasingly important in cancer treatment. These findings along with other studies reveal that HXR9 could trigger necrosis of K562 and KU812F cells that express the BCR-ABL fusion protein; and HL-60 that expresses PML-RARA. It is therefore extremely desirable in the future to assess some of these combination therapies with HXR9 and some other first line treatment drugs like imatinib and ATRA that are known to already target BCR-ABL and PML-RARA fusion proteins, respectively for not only patients of AML but CML as well.

It would be logical that the resistance to cell killing by HXR9 may involve some anti-necrotic proteins, meaning that agents targeting these proteins are potential synergistic agents to HXR9. For example, tamoxifen and trifluoperazine, CaM antagonists, could also be used in combination with HXR9 (Ahn, Pan *et al*. 2003; Wang, Li *et al*. 2010). Equally, enzastaurin which was previously known to inhibit PKC activity and increase p21 levels in multiple myeloma cells may also have some potential synergistic effects with HXR9 (Raab, Breitkreutz *et al*. 2009). Arsenic trioxide has been shown in recent years to exert its cytotoxicity by inhibiting the JNK pathway and therefore increasing the activity of our gene of interest, c-Fos and the p21 protein which will also be valuable in assessing its combinatory effect with HXR9 (Huang *et al*., 2012, Liu *et al*., 2009). The recently suggested hypothesis of silencing

c-FLIP in combination with HXR9 treatment could also be an interesting combination, since c-FLIP is negatively regulated by c-Fos (Zhang, Zhang *et al.*, 2007). In addition to this c-FLIP is a known downstream effector for CaM and PKC (Hwang *et al.*, 2009, Kaunisto *et al.*, 2009), thought to also inhibit apoptosis and/or necrosis by suppressing procaspase-8 and RIP1, respectively (Day, Huang *et al*. 2008; Wang, Du *et al*. 2008; He and He 2013).

The mechanism of cell death by HXR9 of course requires further investigation in the future. A better understanding of how RIP1 activity may cause c-Fos over-expression after HXR9 treatment in HL-60 cells for example may help, and the upstream regulators of c-Fos along with downstream effectors of RIP1 (like apoptosis signal-regulating kinase 1) could be involved in this mechanism (Liu, Nishitoh *et al*. 2000; Vanlangenakker, Vanden Berghe *et al*. 2012). Some of these investigations may also include c-Fos upstream regulators, with c-Fos related kinase or ribosomal S6 kinase 2 for example (Zhang, Zhang *et al*. 2007). Notably, p21 downstream effectors could also be examined. It is essential to highlight how important it is to assess the role of RIP3 in HXR9 cytotoxicity if necroptosis is involved, at least in HL-60 cells. AML cells may be co-cultured in direct or indirect contact with the stromal cells like human MS-5 cells to evaluate the influence of stromal cells and the secretome in the efficacy of HXR9. A systemic in *vivo* model is needed in the future however, to be able to assess the efficacy of HXR9 on AML disease. Recently, nude mice strains have been used, although growth rate was low, likely due to immune activity. Additionally, previous C57BL/6 and SCID flank models did not represent AML disease. For this reason, it is important to try and establish a systemic in *vivo* model in other mouse-mouse strains including NOG or NOD, known as the most common AML models, characterised by a lack of T and B lymphocytes, some impaired NK cells and antigen-presenting cells (Shan and Ma, 2013).

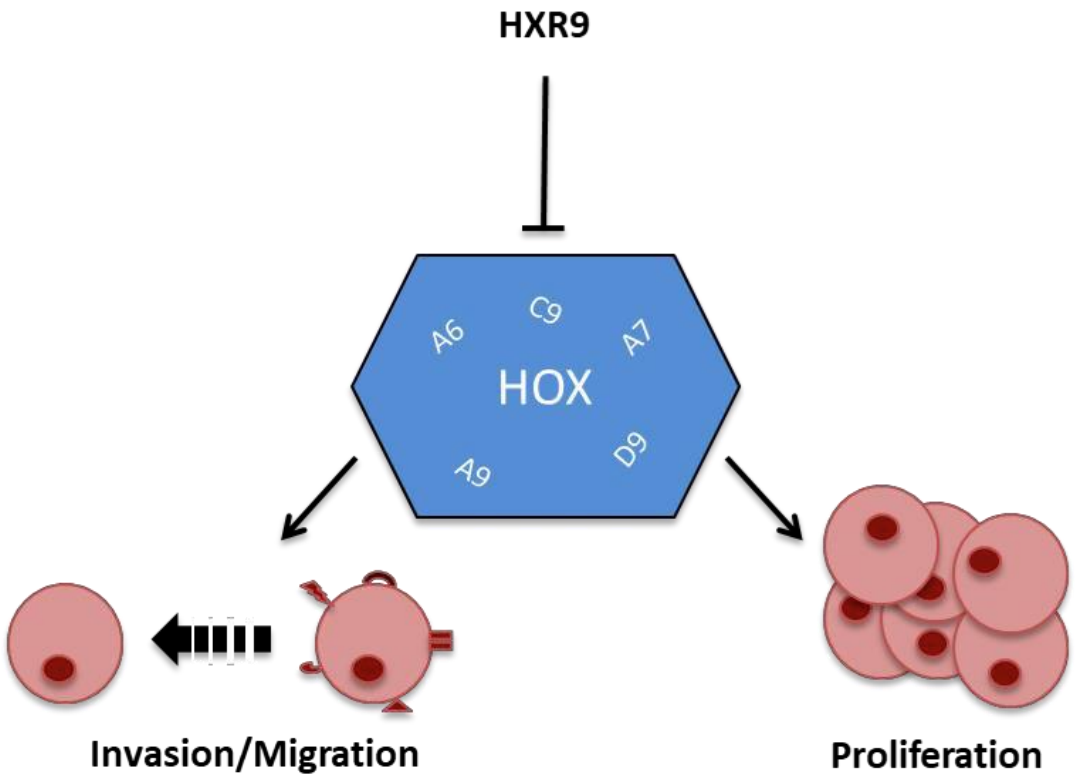


Figure 7.1: HOX protein such as HOXA6, HOXA7, HOXA9, HOXC9 and HOXD9 have been linked to increased proliferation rates as well as increased migration and invasion potentials. HXR9 is able to block HOX-PBX dimers, which in turn suppresses proliferation and invasion/migration.

HOX genes have been shown to influence a variety of cellular functions including cell migration and proliferation in both normal and malignant cells (Morgan, 2014; Breau 2013). Our findings demonstrate that HXR9 has anti-proliferative and anti- migratory effects. As HXR9 targets multiple HOX and PBX proteins, it is not possible to establish which specific set or sets of HOX-PBX dimers are required for these activities, although the high level of functional redundancy amongst HOX proteins would indicate that a broad disruption of dimer formation is necessary. However, previous studies have shown that loss of HOXD9 expression reduces cellular proliferation, while suppression of HOXD10 causes an increase in invasion and migration (Tabuse, 2011). In addition, HOX genes HOXA6 and HOXA9 have been linked to increased invasion and migration, and HOXA7, HOXA9 and HOXC9 are all implicated in

cellular proliferation (Figure 7.1). Notably these are all targetable by HXR9 therapy. Further assessments of the mechanism behind HXR9's anti- proliferative and migratory abilities are to be explored but the broad suppressive effects of HXR9 on HOX proteins, as well as their inherent functional redundancies, seriously hamper the likeliness of success.

Investigations into HXR9's anti-invasion potential resulted in a less than satisfactory outcome. This came as a surprise as HOX proteins have been implicated in the regulation of various MMPs. A potential explanation for this result is the short half-life HXR9. HXR9's half-life in serum has been calculated to be 12 hours (Morgan, 2007). Though it must be noted that HXR9 incubated with cells will be actively ubiquitinated and degraded. Thus, the half-life on cells will be low. With this in mind the 12 hour time point for the migration assay will provide positive results as by the assay end point is reached less HXR9 would be degraded compared to invasion assay, 24 hours, which would have almost no HXR9 left. This would mean that once HXR9 had been degraded, HOX suppression would be lowed allowing for cells to invade. Potential adding a second IC25 dose of HXR9 at the 12-hour time point for invasion assay might have resulted in more positive outcome, and is currently under investigation.

HXR9 treatment was shown to induce expression of cleaved caspase 3, C-Fos and DUSP1 in parental and CSC tumours. This indicates that HXR9 induces apoptosis though caspase 3 activation, like that seen in vitro. In addition to this, results showed that C-Fos, DUSP1 and cleaved caspase 3 staining all correlated. This provided a strong indicator that the 3 aforementioned stains are interconnected, and that C-Fos and DUSP1 could have either a joint or apposing apoptotic effect on HXR9 treated cells (Figure 7.2).

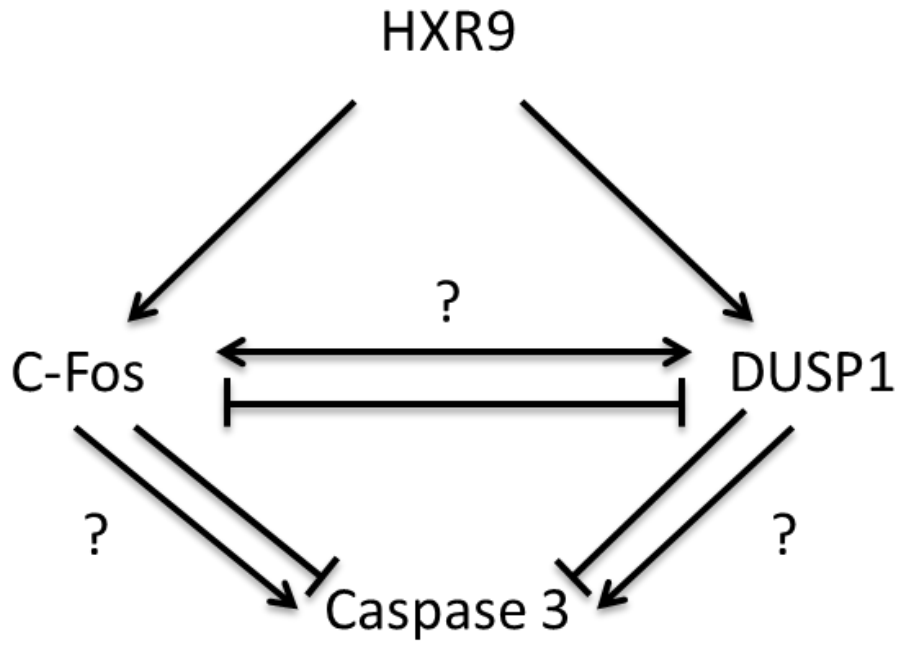


Figure 7.2: HXR9 treatment causes an increase in C-Fos and DUSP1 protein levels. C-Fos and DUSP1 activation and or suppression of caspase 3 ensue. In addition to this, C-Fos and DUSP1 transactivation and suppression is also possible.

7.2 Future work

This study has provided additional knowledge of HOX and TALE expression in healthy and malignant brains in adults and children. It has also proven that these interactions can be inhibited with potent cytostatic and cytotoxic results. However, there are still areas surrounding the body of work presented here which needs further investigating. Here we outline proposed future short- and long-term objectives we have currently set out to complete

A major benefit of ICT9119 treatment over HXR9 and HTL00-1 is its increased half-life and thus sustained reactivity. The addition of amino acids to HXR9 and HTL00-1 can help increase the half-life, but cannot be larger enough to hinder peptide binding. Using the half-life prediction software ProtParam (Wilkins, 1999), which predicts that in mammalian cells, HXR9 and HTL00-1 have half-life's of 2.8

hours. To improve this, we are currently trialling the use of a proline added to the N- terminus of HXR9 and HTL00-1. This theoretically should increase peptide half- life to >20 hours. Initial results have shown no change in cytotoxicity with the added proline, with additional experiments using mass spectrometry planned.

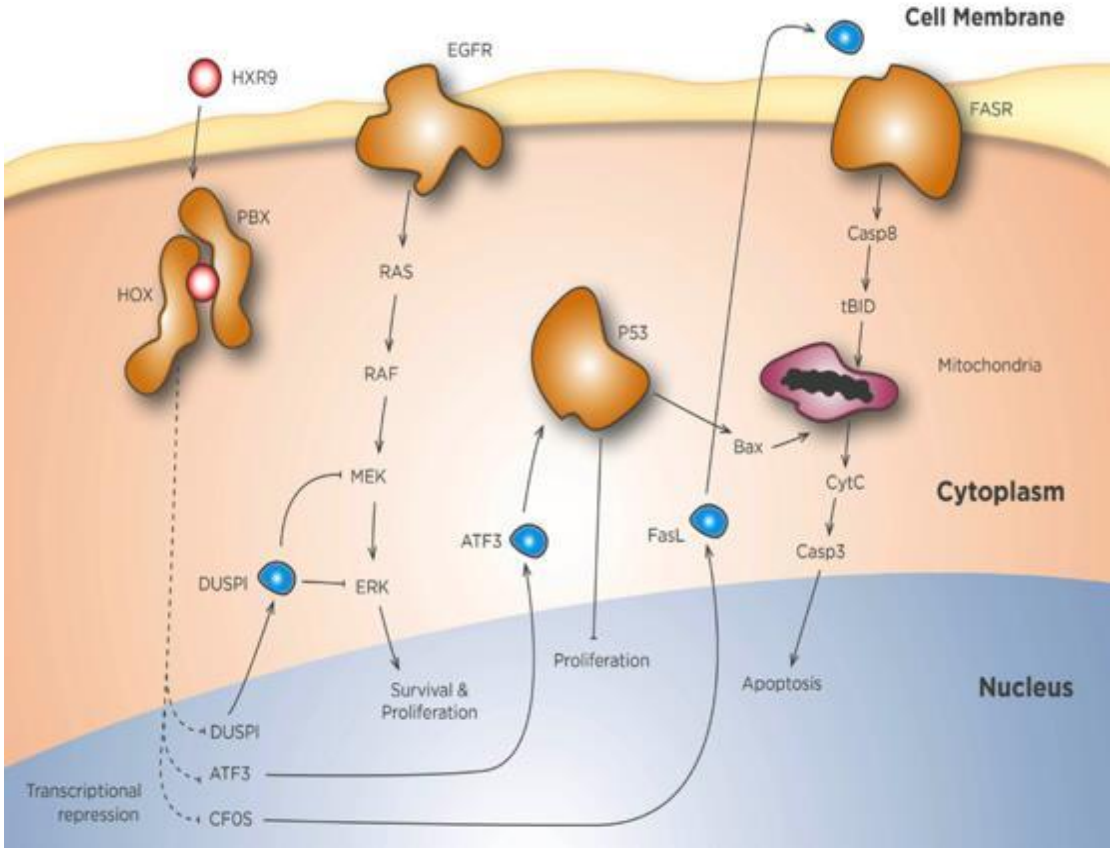


Figure 1.33 *Unhindered HOX-PBX dimers are able to translocate and enter the nucleus, thus transcribing and repressing their target genes. Potential repressor targets are DUSP1, ATF3 and C-Fos. HXR9 mediated inhibition of HOX-PBX causes an increase in DUSP1, ATF3 and C-Fos expression. The current consensus is that increased C-Fos protein levels activate Fas ligand (FasL) transcription. FasL protein in turn binds and activates Fas receptor (FasR) triggering the extrinsic apoptosis pathway. ATF blocks ubiquitination of p53, which promotes mitochondria mediated apoptosis and blocks proliferation through cell cycle arrest. DUSP1 dephosphorylates MEK and ERK, causing a loss of RAS mediated signalling (Morgan, 2017).*

The use of an additional N-terminus proline reduces proteasome activity. This again is seen as an important area of research. We currently plan on using proteasome activity assay that utilise fluorescently tagged peptides to see is peptide sensitivity correlated with proteasome activity. If positive results are obtained, the use of proteasome inhibitors in combination with HXR9 and HTL00- 1 will be explored. The importance of DUSP and MAPK activity in response to ICT9119 and RT treatment has been previously discussed in Chapter 5 Discussion. We plan to experimental test our hypothesis that DUSP1 and MAPK activity influences potential synergy of HOX-PBX inhibits with RT. This will be achieved with various knockdown and overexpression assays in combination with RT and ICT9119 treatments. We also plan to explore the use of DUSP inhibitors with ICT9119-RT to rescue potential synergy between treatments in this sequence. Though HXR9 and HTL00-1 has been shown to have an identical death pathway to ICT919, their potential to replace ICT9119 in synergy treatments is questionable. This is mainly due to their shorter half-life, and the ability of ionising radiation to target and degrade them. Nonetheless additional therapeutic combination assays are planned to include HOX-PBX inhibitors with TMZ. This will be evaluated both in vitro and in vivo.

Work in this study has proved that CSCs express elevated levels of HOX and TALE genes compared to parental cells. This elevated expression equates to an increase in sensitivity to HOX-PBX inhibition. It must be noted that these CSC are enriched using growth factors and represent a unique type of GBM CSC. Hypoxic induced CSC's express different genes compared to their growth factor counterparts, and also behave differently. We envision the use of a hypoxic chamber to enrich parental cell lines for hypoxic induced CSCs. We then plan to full HOX and TALE prolife to be able to compare their expression pattern to both parental and growth factor induced CSCs. These hypoxic CSCs will also be assessed for sensitivity to HOX-PBX inhibitors and compare results to that obtained from parental and growth factor CSCs.

Peptide based therapies contain unique properties as they are based from naturally occurring molecules. These properties include high target specificity, low off-site binding, low toxicities and targeted degradation by proteasomes. As previously described the latter point means that half-life of peptide

therapies is low than that seen from inorganic compounds. This can be overcome by peptide modifications, modulation of the cell's proteasome activity, or by providing patient's with multiple drug doses. We propose that viral based drug delivery could provide a more enticing opportunity. The use of a virus such, as herpes simplex virus (HSV), can be genetically manipulated with exogenous genes inserted. This allows for a gene encoding HXR9 or HTL00-1 to be inserted into a virus. Once the virus enters the cancer cell, it shuts down cellular process, and 'hijacks' them for the production of new viral components. Once these are produced, the newly inserted HXR9 and HTL00-1 gene will be transcribed and translated. This thus allows newly formed peptides to target HOX-PBX dimers, causing cellular death (Figure 7.1).

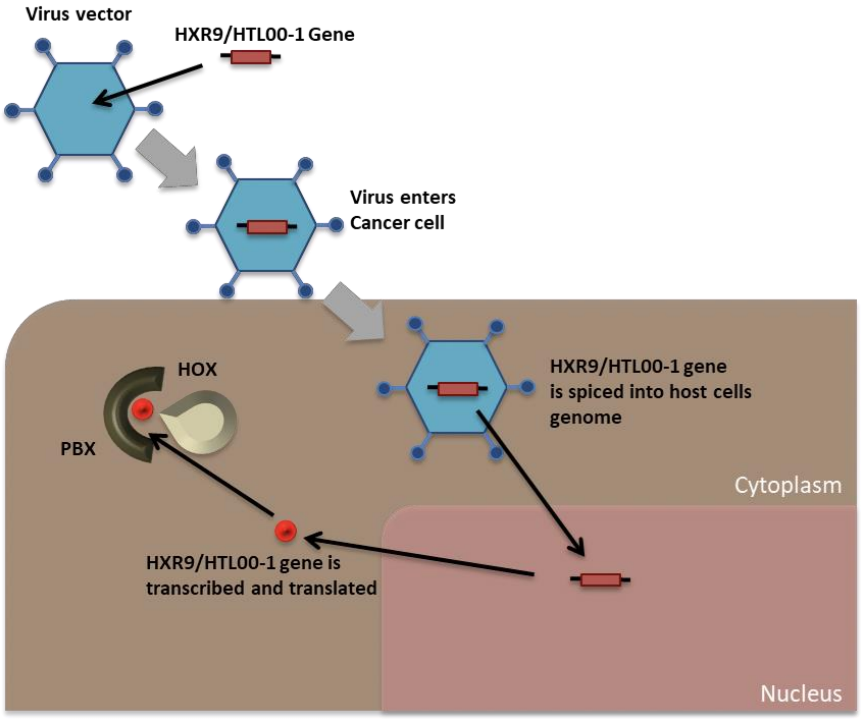


Figure 7.1: Genes encoding HXR9 or HTL00-1 can be inserted in virus vectors such as HSV. These modified viruses can then be used to transfect neoplastic cells. Once inside host cells, viruses utilised host's cellular machinery to transcribe viral genes. HXR9/HTL00-1 molecule will be transcribed and translated, and then go on to target HOX-PBX dimers. If viral particles are able to full replicate before host cell death, they will be released and allowed to infect neighbouring cells.

HXR9 and HTL00-1 are encoded by relatively small gene allowing for individual viruses to be able to contain thousands and possible millions of copy numbers. Furthermore, it is possible to insert HXR9 or HTL00-1 genes into late cycle gene loci, allowing for virus particles to be able to fully replicate before transcribing peptide drugs. This would have the added benefit of continuing the viral cycle, allowing new viral particles to infect neighbouring neoplastic cells. Thus, starting a new cycle replicating additional viral particles and peptide agents. This concept is still under review, with preliminary tests commencing in the near future.

Finally, we are currently investigating HOX-PBX inhibitors immunogenicity and inflammatory potential. Preliminary results show that HOX-PBX inhibitors do not induce inflammatory markers such as calreticulin, Fas ligand, Heat shock protein- 70KDa (HSP-70), Programmed death-ligand 1 (PD-L1), Cluster of differentiation 80 (CD80) and Human leucocyte antigen (HLA). Though these results are not definitive, if correct it proves that HOX-PBX inhibitors are non-inflammatory. This is a highly revered characteristic in brain tumour research, due to the adverse effects of inflammation and oedema. Inflammation and oedema cause an elevation in intracranial pressure of the brain, potential resulting in loss of cognitive functions, and even death. In addition to this, oedema causes an increase in hydrostatic pressure of the tumour causing an increase in drug efflux from the tumour site. These positive preliminary results are currently being followed up with additional in vitro experiments, with in vivo experiments being scheduled.

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Appendix

Reagent	*Supplier*
Protoporphyrin IX	Sigma (Dorset, UK)
Carestream® Kodak BioMax® light film	
Fixer and replenisher	
Developer and replenisher	
Cloned AMV first-strand synthesis kit	Invitrogen, Thermoscientific (Paisley, UK)
Glutamine	
FBS	
IMDM medium	
NuPAGE® LDS sample buffer	
Benchmark™ prestained protein ladder	
NuPAGE novex 12% Bis Tris	
DMSO	
Pierce® RIPA buffer	
Protease inhibitor cocktail	
NuPAGE® sample reducing agent	
NuPAGE® MES SDS running buffer	
Invitrolon™ PVDF filter paper sandwich	
Novex cryoTube™ vials	
SuperSignal® West Pico Chemiluminescent substrate	
Cytotoxicity detection kit	Roche (Mannheim, Germany)
RNeasy mini kit and RBC lysis solution	Qiagen (West Sussex, UK)
Gene Primers	Eurgentec (Seraing, Belgium)
Mycoplasma detection kit	Lonza (Slough, UK)

Reagent	*Supplier*
SYBR® green jumpstat™ Taq ready mix™	Sigma (Dorset, UK)
Penicillin-streptomycin	
μI-1640	
DMEM	
Trypan blue	
HBSS	
Histopaque®-1077 Hybri-Max	
β-mercaptoethanol	
DAPI stain	
z-VAD-FMK	
EDTA	
DPI	

Table A1*: A table listing reagents that were used in this study and their suppliers.*

Media and Supplements	***Abbreviation***	***Supplier***
Minimal Essential Medium Eagle (With Earle's salts, non-essential amino acids and sodium bicarbonate)	MEME	Sigma-Aldrich
Dulbecco's Modified Eagle's Medium (With 4500 mg L-glucose, 110 mg L-sogium pyruvate, pyridoxine hydrochloride and sodium bicarbonate)	DMEM	Sigma-Aldrich
RPMI-1640 Medium	RPMI	Sigma-Aldrich
Foetal Bovine Serum	FBS	Gibco
Penicillin-Streptomycin (10,000 units of penicillin and 10 mg/ml streptomycin in 0.9% NACl)	Pen/Strep	Sigma-Aldrich
L-Glutamine 200mM	L-Glu	Sigma-Aldrich
Minimal Essential Medium Non-Essential Amino Acids	MEM NEAA	Gibco
Sodium Pyruvate 100mM	NaP	Sigma-Aldrich
N-2 Supplement 100x	N/A	Gibco
Heparin Salt Solution 0.2%	Hep	StemCell Technologies
Epidermal Growth Factor 100µg	EGF	Invitrogen
Basic Fibroblast Growth Factor 50µg	bFGF	Gibco
Hanks Balanced Salt Solution	HBSS	Sigma-Aldrich

Table A2: *A table listing all the media and supplements used in tissue culturing throughout the entire study.*

RT-qPCR Primer Design, Sequences and Validation

Forward and reverse primers were designed using the Primer3web software version 4.0 or Primer-BLAST, both of which are web-based software. Primer sequences can be seen in Table A.3. The following parameters were used, when possible, to design primers:

1. A PCR product size of between 100 to 400 base pairs
2. A primer melting temperature between 55°C to 65°C
3. Primers that span exon-exon junctions
4. A primer length between 20 to 30 bases
5. A primer with a GC% of >55%
6. Self-complementarity between 2 to 6
7. Self 3' complementarity between 0 to 3

All primers were purchased from Sigma-Aldrich, UK and were provided at a concentration of 100µM and desalted. Prior to delivery all primers are quality control checked by electrospray ionization mass spectrometry. Diluted and undiluted primers were stored at -20°C. All primers were assessed to prove single product amplification via melt curve analysis. In addition to this a 'no template (cDNA) control' was performed for all sets of primers to identify the possibility of primer dimer formation. Any primers that fail these validation tests were discarded and primers were redesigned.

Gene Target	*Forward and Reverse Primer Sequences*	*Supplier*
HOX A1	*U10421; 511 to 663; 153bp* *F: 5' CTGGCCCTGGCTACGTATAA 3'* *R: 5' TCCAACTTTCCCTGTTTTGG 3'*	*Eurogentec, Belgium*
HOX A2	*NM_006735; 1027 to 1202; 176bp* *F: 5' TTCAGCAAAATGCCCTCTCT 3'* *R: 5' TAGGCCAGCTCCACAGTTCT 3'*	*Eurogentec, Belgium*
HOX A3	*NM_030661; 1525 to 1751; 227bp* *F: 5' ACCTGTGATAGTGGGCTTGG 3'* *R: 5' ATACAGCCATTCCAGCAACC 3'*	*Eurogentec, Belgium*
HOX A4	*NM_002141; 633 to 903; 271bp* *F: 5' CCCTGGATGAAGAAGATCCA 3'* *R: 5' AATTGGAGGATCGCATCTTG 3'*	*Eurogentec, Belgium*
HOX A5	*NM_019102; 796 to 988; 193bp* *F: 5' CCGGAGAATGAAGTGGAAAA 3'* *R: 5' ACGAGAACAGGGCTTCTTCA 3'*	*Eurogentec, Belgium*
HOX A6	*NM_024014; 361 to 518; 158bp* *F: 5' AAAGCACTCCATGACGAAGG 3'* *R: 5' TCCTTCTCCAGCTCCAGTGT 3'*	*Eurogentec, Belgium*
HOX A7	*NM_006896; 38 to 322; 285bp* *F: 5' TGGTGTAAATCTGGGGGTGT 3'* *R: 5' TCTGATAAAGGGGGCTGTTG 3'*	*Eurogentec, Belgium*
HOX A9	*NM_152739; 653 to 855; 203bp* *F: 5' AATAACCCAGCAGCCAACTG 3'* *R: 5' ATTTTCATCCTGCGGTTCTG 3'*	*Eurogentec, Belgium*

HOX A10	*NM_018951; 1040 to 1198; 159bp* *F: 5' ACACTGGAGCTGGAGAAGGA 3'* *R: 5' GATCCGGTTTTCTCGATTCA 3'*	*Eurogentec, Belgium*
HOX A11	*NM_005523; 800 to 1078; 279bp* *F: 5' CGCTGCCCCTATACCAAGTA 3'* *R: 5' GTCAAGGGCAAAATCTGCAT 3'*	*Eurogentec, Belgium*
HOX A13	*NM_000522; 1061 to 1236; 176bp* *F: 5' GGATATCAGCCACGACGAAT 3'* *R: 5' ATTATCTGGGCAAAGCAACG 3'*	*Eurogentec, Belgium*
HOX B1	*NM_002144; 176 to 332; 157bp* *F: 5' TTCAGCAGAACTCCGGCTAT 3'* *R: 5' CCTCCGTCTCCTTCTGATTG 3'*	*Eurogentec, Belgium*
HOX B2	*NM_002145; 9 to 267; 259bp* *F: 5' CTCCCAAAATCGCTCCATTA 3'* *R: 5' GAAAGGAGGAGGAGGAGGAA 3'*	*Eurogentec, Belgium*
HOX B3	*NM_002146; 1970 to 2268; 299bp* *F: 5' TATGGCCTCAACCACCTTTC 3'* *R: 5' AAGCCTGGGTACCACCTTCT 3'*	*Eurogentec, Belgium*
HOX B4	*NM_024015; 593 to 747; 155bp* *F: 5' TCTTGGAGCTGGAGAAGGAA 3'* *R: 5' GTTGGGCAACTTGTGGTCTT 3'*	*Eurogentec, Belgium*
HOX B5	*NM_002147; 1543 to 1731; 189bp* *F: 5' AAGGCCTGGTCTGGGAGTAT 3'* *R: 5' GCATCCACTCGCTCACTACA 3'*	*Eurogentec, Belgium*

HOX B6	*NM_156037; 151 to 334; 184bp* *F: 5' ATTTCCTTCTGGCCCTCACT 3'* *R: 5' GGAAGGTGGAGTTCACGAAA 3'*	*Eurogentec, Belgium*
HOX B7	*NM_004502; 143 to 391; 249bp* *F: 5' CAGCCTCAAGTTCGGTTTTC 3'* *R: 5' CGGAGAGGTTCTGCTCAAAG 3'*	*Eurogentec, Belgium*
HOX B8	*NM_024016; 964 to 1228; 265bp* *F: 5' GTAGGCTTCAGCTGGGACTG 3'* *R: 5' GGGAGCCTTTGCTTAAATCC 3'*	*Eurogentec, Belgium*
HOX B9	*NM_024017; 533 to 730; 198bp* *F: 5' TAATCAAAGACCCGGCTACG 3'* *R: 5' CTACGGTCCCTGGTGAGGTA 3'*	*Eurogentec, Belgium*
HOX B13	*NM_006361; 154 to 387; 234bp* *F: 5' CTTGGATGGAGCCAAGGATA 3'* *R: 5' CCGCCTCCAAAGTAACCATA 3'*	*Eurogentec, Belgium*
HOX C4	*NM_014620; 1121 to 1396; 276bp* *F: 5' CGCTCGAGGACAGCCTATAC 3'* *R: 5' GCTCTGGGAGTGGTCTTCAG 3'*	*Eurogentec, Belgium*
HOX C5	*NM_019953; 555 to 822; 268bp* *F: 5' CAGTTACACGCGCTACCAGA 3'* *R: 5' AGAGAGGAAAGGCGAAAAGG 3'*	*Eurogentec, Belgium*
HOX C6	*NM_004503; 774 to 963; 190bp* *F: 5' AAGAGGAAAAGCGGGAAGAG 3'* *R: 5' GGTCCACGTTTGACTCCCTA 3'*	*Eurogentec, Belgium*
HOX C8	*NM_022658; 390 to 539; 150bp* *F: 5' CTCAGGCTACCAGCAGAACC 3'* *R: 5' TTGGCGGAGGATTTACAGTC 3'*	*Eurogentec, Belgium*

HOX C9	*NM_006897; 704 to 893; 190bp* *F: 5' AGACGCTGGAACTGGAGAAG 3'* *R: 5' AGGCTGGGTAGGGTTTAGGA 3'*	*Eurogentec, Belgium*
HOX C10	*NM_017409; 982 to 1270; 289bp* *F: 5' CGCCTGGAGATTAGCAAGAC 3'* *R: 5' GGTCCCTTGGAAGGAGAGTC 3'*	*Eurogentec, Belgium*
HOX C11	*NM_014212; 353 to 538; 186bp* *F: 5' CGGAACAGCTACTCCTCCTG 3'* *R: 5' CAGGACGCTGTTCTTGTTGA 3'*	*Eurogentec, Belgium*
HOX C12	*NM_173860; 654 to 833; 180bp* *F: 5' CAAGCCCTATTCGAAGTTGC 3'* *R: 5' GCTTGCTCCCTCAACAGAAG 3'*	*Eurogentec, Belgium*
HOX C13	*NM_017416; 1840 to 2009; 170bp* *F: 5' GTGGAAATCCAAGGAGGACA 3'* *R: 5' TTGTTGAGGGACCCACTCTC 3'*	*Eurogentec, Belgium*
HOX D1	*NM_024501; 929 to 1160; 232bp* *F: 5' TTCAGCACCAAGCAACTGAC 3'* *R: 5' TAGTGGGGGTTGTTCCAGAG 3'*	*Eurogentec, Belgium*
HOX D3	*NM_006898; 492 to 667; 176bp* *F: 5' CAGCCTCCTGGTCTGAACTC 3'* *R: 5' ATCCAGGGGAAGATCTGCTT 3'*	*Eurogentec, Belgium*
HOX D4	*NM_014621; 23 to 195; 173bp* *F: 5' TCAAATGTGCCATAGCAAGC 3'* *R: 5' TCCATAGGGCCCTCCTACTT 3'*	*Eurogentec, Belgium*
HOX D8	*NM_019558; 1167 to 1456; 290bp* *F: 5' TCAAATGTTTCCGTGGATGA 3'* *R: 5' GCTCTTGGGCTTCCTTTTTC 3'*	*Eurogentec, Belgium*

HOX D9	*NM_014213; 1803 to 2038; 236bp* *F: 5' TCCCCCATGTTTCTGAAAAG 3'* *R: 5' GGGCTCCTCTAAGCCTCACT 3'*	*Eurogentec, Belgium*
HOX D10	*NM_002148; 364 to 517; 154bp* *F: 5' GCTCCTTCACCACCAACATT 3'* *R: 5' AAATATCCAGGGACGGGAAC 3'*	*Eurogentec, Belgium*
HOX D11	*NM_021192; 302 to 554; 253bp* *F: 5' GGGGCTACGCTCCCTACTAC 3'* *R: 5' GCTGCCTCGTAGAACTGGTC 3'*	*Eurogentec, Belgium*
HOX D12	*NM_021193; 113 to 313; 201bp* *F: 5' CGCTTCCCCCTATCTCCTAC 3'* *R: 5' CTTCGGGCGCATAGAACTTA 3'*	*Eurogentec, Belgium*
HOX D13	*NM_000523; 868 to 1132; 265bp* *F: 5' GGGGATGTGGCTCTAAATCA 3'* *R: 5' AACCTGGACCACATCAGGAG 3'*	*Eurogentec, Belgium*
β-Actin	*F: 5' ATGTACCCTGGCATTGCCG 3'* *R: 5' GACTCGTCATACTCCTGCTTG 3'*	*Sigma-Aldrich, UK*

Table A3: *Forward and reverse primer sequences for all GoI quantified by RT-qPCR and their National Center for Biotechnology Information (NCBI) accession number.*

Grouped HOXA9 Expression:

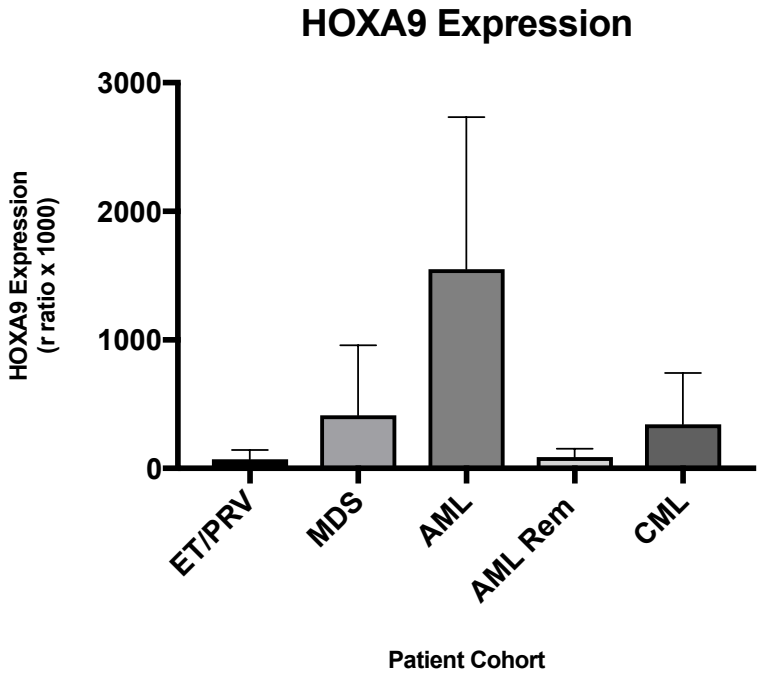

Figure A1*: Variation of HOXA9 expression in mononuclear cells isolated from the peripheral blood of patients of various hematological disorders grouped and shown as a ratio of beta-actin to the gene of interest signal (vertical axis) the expression of which is represented of the ratio of the expression of the gene of interest to the housekeeping gene to β-actin (×10 000). The variation of expression between patients is shown by error bars in this graph.* *p< .05, **p < .01, ***p <.001 and ****p <.0001.