Regulation of the LIFR and gp130 genes by the RUNX1 transcription factor

by

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Oakford PC, James SR, **Qadi A**, West AC, Ray SN, Bert AG, Cockerill PN, Holloway AF. Transcriptional and epigenetic regulation of the GM-CSF promoter by RUNX1. Leuk Res. 2010 34(9):1203-13.

AA Qadi, PC Taberlay[1], A Young, RN Brown, AC West[2], JL Dickinson and AF Holloway. Regulation of LIFR and gp130 genes by RUNX1. Manuscript under review.

Abstract;

The RUNX1 transcription factor is an important regulator of haematopoiesis and has been found to influence gene activity at both the transcriptional and chromatin levels. In leukaemia, particularly Acute Myeloid Leukaemia, RUNX1 activity is frequently altered by point mutations and chromosomal rearrangements, the most common being the t(8;21) translocation that produces a RUNX1-ETO fusion protein. While RUNX1 is generally associated with gene activation, RUNX1-ETO mainly acts as a transcriptional repressor and its presence is therefore associated with altered expression of RUNX1 target genes.

Previous microarray analysis performed in our laboratory identified both the LIFR and gp130 genes as novel putative RUNX1 targets. LIFR together with gp130 forms a heterodimeric receptor complex that mediates LIF signalling, and in doing so controls various cellular processes including cellular development, differentiation and inflammatory responses. However, despite their important biological roles little is known about regulation of the LIFR and gp130 genes. Bioinformatic analysis identified potential RUNX1 binding sites in the promoters of both the LIFR and gp130 genes, and this study therefore examined the hypothesis that LIFR and gp130 are RUNX1 target genes and their altered expression in leukemic cells in which RUNX1 is disrupted may therefore contribute to leukaemia.

The LIFR gene is regulated by alternate promoters, with a so called ‘general’ and ‘placental’ promoter previously described. Analysis of LIFR mRNA across a number of cell types demonstrated that activity of the placental promoter is limited to cell lines of placental origin, while the general promoter is active in a range of cell types, including myeloid cells. This was confirmed by analysis of the chromatin status of the two promoters, which found that the general, but not placental promoter is assembled into highly acetylated histones in myeloid cells. The expression of the gp130 mRNA was detected across all cells lines examined.

Reporter analysis demonstrated that both the placental and general promoters can be activated by RUNX1 in placental and myeloid cell lines respectively. In addition, the gp130 promoter was activated by RUNX1 in myeloid cell lines. In contrast, RUNX1-ETO repressed both the LIFR and gp130 promoters in myeloid cells. Further, binding of RUNX1 to the endogenous LIFR and gp130 promoters was confirmed by chromatin immunoprecipitation, suggesting that the endogenous promoters are targeted by RUNX1. In support of this RUNX1 knockdown reduced LIFR and gp130 expression in myeloid cell lines, and decreased expression of the placental LIFR transcript in placental cells.

Put together, the data presented in this thesis demonstrates that RUNX1 regulates expression of the LIFR and gp130 promoters, suggesting that activity of the LIFR/gp130 receptor complex is likely to be altered in leukaemic cells in which RUNX1 is altered.

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ABBREVIATIONS;

ALL	Acute lymphoblastic leukemia
AML	acute myeloid leukemia
ANXA1	calcium/phospholipid-binding protein
bp	base pairs
BRG1	brahma-related gene 1
BSA	bovine serum albumin
C/EBPα	CCAAT/enhancer-binding protein alpha
CBF	core binding factor
CBFβ	core Binding Factor Beta
ChIP	chromatin immunoprecipitation
CpG	cytosine guanine dinucleotide
MYB	myb proto oncogene protein
c-myb	myb transcription factor
CML	chronic myeloid leukemia
CSF1R	colony stimulating factor 1 receptor
dpc	days post coitum
DBD	DNA binding domain
E	embryonic days
ERK	Extracellular Signal-Regulated Kinases
ETO	eight twenty one protein
FPD-AML	familial platelet disorder-acute myelocytic leukaemia
FCS	foetal calf serum
GAPDH	glyceraldehydes phosphate dehydrogenase.
GH	growth hormone
H	histone
HAT	histone acetyltransferase
hr	hour
I	calcium ionphore
IL	interleukin
IL-3	interleukin-3

K	lysine residue
LIFR	Leukaemia inhibitory factor receptor
MDS	myelodysplastic syndrome
MEF	myeloid elf-1-like factor
MIP-α	macrophage inflammatory protein-1α
MPO	myeloperoxidase gene
MYB	transcriptional activator Myb
MYH11	myosin, heavy chain 11, smooth muscle gene
PEBP	polyomavirus enhancer-binding protein
NCoR	nuclear receptor corepressor 1
NFAT	nuclear Factor of Activated T cells
NHR	nervy homology region
NMR	Nuclear Magnetic Resonance
NMTS	nuclear-matrix-targeting signal
NLS	Nuclear localization signals
RUNX	Runt Related Transcription factor
RHD	Runt Homology Domain
SMMHC	smooth muscle myosin heavy chain
STAT-1	Signal Transducers and Activators of Transcription Factor 1
TEL	Transducin-like Enhancer Split
EVi1	ecotropic viral integration site 1
HDACs	Histone deacetylases
JAK/STAT	Janus kinase/Signal transducer and activator of transcription

Chapter 1

INTRODUCTION

1.1.1 Overview;

RUNX transcription factors are important regulators of various developmental pathways. RUNX proteins share a common domain, which is highly conserved within species and homologous to the DNA binding domain of the *Drosophila melanogaster* Runt transcription factor (Rennert et al., 2003). The drosophila Runt protein regulates various functions including; sex determination, embryonic segmentation and neuronal differentiation, as well as having essential roles in eye development and haematopoiesis (Levanon et al., 2003). In vertebrates there are three RUNX related transcription factors; RUNX1, RUNX2 and RUNX3.

1.1.2 RUNX Nomenclature;

Runt related transcription factors were first identified in 1990 as DNA binding molecules that bind to a specific sequence in the enhancer of retroviruses and were therefore termed core binding factor (CBF) [Zaiman et al.,1995]. The term PEBP was also used to describe these factors after the isolation of murine cDNA for the polyoma enhancer binding protein (Ogawa et al., 1993). The designation of AML arose following analysis of chromosomal translocations in acute myeloid leukemia (AML) which identified one of these genes, termed AML1 at the t(8,21) breakpoint (Ogawa et al., 1993). Numbers were assigned to the different family members based on the identification and cloning order of the gene (See Table 1.1). In 1999 the Nomenclature Committee of the Human Genome Organization (HUGO) designated the 'RUNX' term to describe the runt related transcription factors (van Wijnen et al., 2004), and this terminology will be adopted in this thesis (Table1.1). The dimerisation partner of the RUNX proteins is termed CBFβ throughout.

	RUNX Nomenclature and gene Locus				
Gene	**Protein Name**	**Core binding factor (CBFα)**	**Acute Myelogenous Leukemia**	**Polyoma Enhancer Binding Protein 2α subunit (CBFβ)**	**Locus**
RUNX1	RUNX1	CBFA2	AML1	PEBP2alphaB	21q22
RUNX2	RUNX2	CBFA1	AML3	PEBP2alphaA	6p21
RUNX3	RUNX3	CBFA3	AML2	PEBP2alphaC	1p36

Table 1.1 Nomenclature for mammalian RUNX transcription factors. The current nomenclature of RUNX was approved by the Committee of the Human Genome Organization (HUGO).

1.1.3 Biological functions of RUNX proteins;

In mammals RUNX proteins have overlapping functions (Smith et al., 2005), with RUNX proteins acting as key transcriptional regulators in several tissues. RUNX1 has a well characterized role in haematopoiesis and neurogenesis, RUNX2 regulates osteogenesis while RUNX3 has an essential role in gastric epithelium development and neurogenesis (Levanon et al., 2004). The importance of RUNX proteins in mammalian development has been demonstrated by gene targeting studies in mice. RUNX1 disruption in mice results in embryonic lethality with embryos dying at E12.5 dpc due to central nervous system defects and soft tissue bleeding. The mice display normal primitive haematopoietic development, but lack liver derived haematopoiesis with the absence of cells from a definitive haematopoiesis origin **(**Okuda et al., 1996). The role of RUNX1 in definitive haematopoiesis was further confirmed by the rescue of $RUNX1^{-/-}$ embryonic stem cells with a RUNX1 knock-in approach (Okuda et al., 2000). A biological role for RUNX2 has been described in osteoblast differentiation (Smith et al., 2005). Mice deficient in RUNX2 completely lack bone tissue and die from respiratory failure caused by thoracic instability (Otto et al., 2002). The biological role of RUNX3 was first described in gastrointestinal tract cell development. $RUNX3^{-/-}$ mice display gastric hyperplasia and also suffer from limb ataxia due to a neural defect (Li et al., 2002; Inoue et al., 2002; Kramer et al., 2006).

RUNX proteins have also been linked to tumourigenesis. Studies have described tumour suppressor activity of RUNX3 in several tissues (Bae et al., 2004). In addition changes in RUNX2 have been associated with prostate cancer (Lim et al., 2010), while alteration to RUNX1 due to point mutation or chromosomal translocation is commonly associated with leukaemia (Preudhomme et al., 2000).

1.2 Structural domains and isoforms of RUNX1;

1.2.1 DNA Binding Domain (DBD);

The role of RUNX1 as a transcription factor was suggested by the characterisation of the Runt homology domain (RHD) as a functional DNA binding domain shared between RUNX proteins. The RHD is a highly conserved 128 amino acid domain that is located in the N-terminal region of the protein and regulates the DNA binding affinity of the protein (Rennert et al., 2003). RUNX1 recognizes a specific DNA sequence motif referred to as the core element which has the consensus sequence TGPyGGTPy, where Py is pyrimidine. RUNX1 binds to DNA as a heterodimeric complex with CBFβ. X-ray crystallography studies have described the structure of the RUNX1 RHD as having a β barrel architecture that is characterized by an s-type immunoglobulin fold. The β barrel architecture is mostly comprised of 12 β strands coiled in antiparallel fashion to form a β structure that has several folds utilized in DNA binding (Bravo et al., 2001). NMR spectroscopic studies indicate that the s-type Ig fold of RUNX1 has homology to DNA binding domains of NFAT, p53, NF-Kappa B and STAT-1 (Bravo et al., 2001). The interaction of the RHD with DNA is mainly initiated by the β barrel strands which interact with the major and minor groove of the DNA.

1.2.2 RUNX1/ CBFβ Heterodimer;

RUNX proteins bind to DNA as a heterodimer of an α subunit (RUNX1, 2 or 3) and the β subunit, CBFβ. RUNX1 hetrodimerization with CBFβ stabilizes and increases its DNA binding affinity by 10 fold (Ogawa et al., 1993). CBFβ is a transcriptional co-activator that is localized in the cytoplasm and translocates to the nucleus as a RUNX1-CBFβ heterodimer (Lu et al., 1995). Further analysis has demonstrated the ability of CBFβ to unmask RUNX1 negative regulatory regions (NRDB) in the N-terminal and the C-terminal domains of the protein to increase the RUNX1 DNA

binding ability (Kanno et al., 1998). The functional role of CBFβ was further investigated in mouse models. CBFβ deficient mice die between E11.5-13.5 dpc due to hemorrhage in the central nervous system. The CBFβ mutant embryos display primitive erythropoiesis in the yolk sac but display an absence of fetal liver haematopoiesis with a similar phenotype RUNX1$^{-/-}$ mice (Sasaki et al., 1996). CBFβ null mice can be rescued from midgestational lethality by re-expressing CBFβ in the haemopoietic system. Expression of the CBFβ-Tek, transgene in CBFβ null mice was able to rescue haematopoiesis, with CBFβ expression detected in all endothelial cells, small subsets of haematopoietic progenitors including; c-kit^{+} and sca1^{+} progenitors, B220+ fetal liver cells at E17.5 dpc and in most of the fetal liver cells after E13.5 dpc (Miller et al., 2002). CBFβ null mice were also rescued by expressing CBFβ under the control of the GATA1 promoter. CBFβ-GATA1 transgenic mice survived until birth, with cells from erythroid and myeloid but not lymphoid lineages detected (Yoshida et al., 2002).

1.2.3 Transactivation Domain;

The C-terminal region of the RUNX1 protein contains a number of functional elements (Figure1.1). The C-terminal domain contains a putative proline serine threonine rich region (PST) which is essential for the transcriptional activity of RUNX1 (Tanaka et al., 1996). A repressor domain within the C-terminal region has also been described in several studies. This VWRP repressor motif is conserved between the RUNX proteins and interacts with the co-repressor Groucho (Okuda et al., 2000). An adjacent inhibitory domain masks the transactivating surface in the C-terminal domain and reduces its activity (Kanno et al., 1998) [Figure1.1]. In addition the C-terminal region contains a nuclear localization signal (NLS) which regulates the nuclear import of the RUNX1 protein (Choi et al., 2001). A matrix association element was also described in a region distinct from the NLS. This element, consisting of a 31 amino acid segment in the C-terminal region was later identified as a Nuclear Matrix Targeting Signal (NMTS) which is required for subnuclear localization of RUNX1 and gene targeting (Zeng et al., 1997).

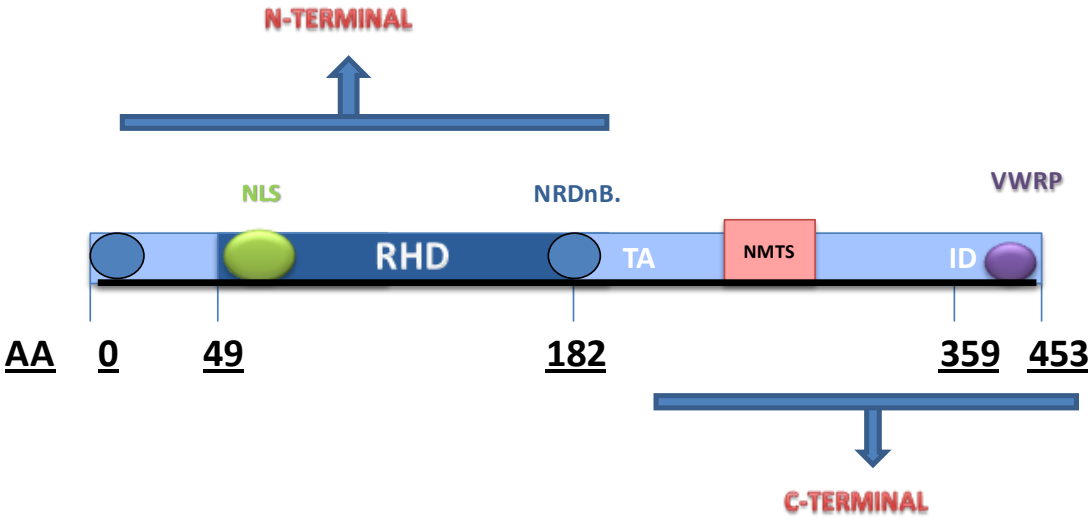


Figure 1.1 Schematic representation of RUNX1 protein (453AA, MW = 48737 Da). The various structural domains and functional motifs are shown [NLS (Nuclear localization signal), NRDnB (Negative regulatory region of DNA binding), TA (Transactivation domain), NMTS (Nuclear matrix targeting signal), ID (Inhibitory domain) and VWRP (repressor motif). The RUNX1 structure is depicted showing the RHD (49-182 AA) conserved region, C-terminal domain and N-terminal domain.

1.2.4 RUNX1 transcripts and variants;

Promoter analysis of the RUNX1 gene has identified alternative promoter activity, with the two promoters described as the proximal (P1) and distal (P2) promoters. RUNX1 transcription is initiated at the 5′ end of the gene by both promoters (Levanon et al.; 2001). The two promoters have different structures and regulatory elements.

The distal promoter (P2) is located at a large evolutionary conserved CpG island that is not found across the proximal promoter (P1). Promoter activity during adult and embryonic stages was analyzed by RUNX1 allele targeting (Pozner et al., 2007), with mice diminished in P2 activity failing to develop definitive haematopiesis, although the role of the two promoters in embryonic development is still unclear. Alternative splicing gives rise to multiple forms of RUNX1 protein, which vary in size and functional properties. The expression of these different isoforms is based on promoter regulation, with the P1 promoter producing long transcripts with extended coding regions containing the transactivation domain, while P2 transcripts are shorter and lack sequences coding the transactivation domain (Levanon et al., 2001).

Three distinct forms of RUNX1 have been characterized. The longer forms of RUNX1 have been designated RUNX1b (453 amino acid) and RUNX1c (480 amino acid), while the RUNX1a isoform (250 amino acid) is a shorter protein that lacks the C-terminal region (Miyoshi et al., 1995). RUNX1b and RUNX1c contain the PST motif in the C-terminal region which has transactivational activity. These isoforms differ in a small region in their N-terminal domain and have both been described as transcriptional regulators (Okuda et al., 2000). The short form of the protein is functionally distinct. For example, while overexpression of RUNX1b can stimulate myeloid cell differentiation, overexpression of RUNX1a that lacks the C-terminal region leads to a complete block in differentiation (Tanaka et al., 1995).

However, the negative effect of RUNX1a on cell differentiation can be cancelled by overexpression of RUNX1b. These findings suggest that RUNX1a is not a transcriptional activator of RUNX1 target genes. However RUNX1a has a higher affinity for DNA and therefore might block and antagonize the transcriptionally active forms of RUNX1 (Tanaka et al. 1995).

1.3 RUNX1 in leukemogenesis;

1.3.1 Overview;

The deregulation of RUNX1 has been shown to induce a block in myeloid cell differentiation (Dunne et al., 2010; Zaidi et al., 2009). Three forms of alteration to the RUNX1 gene have been associated with leukemogenesis; point mutations, amplification and translocation (reviewed in Niebuhr, 2008). The RUNX1 gene is a common breakpoint in several chromosomal translocations described in acute myeloid leukemia (AML) and hence was originally named AML1, after its association with the disease.

1.3.2 Acute Myeloid Leukaemia;

AML is a heterogeneous disease characterized by the accumulation of hematopoietic precursors in the bone marrow. AML patients are clinically classified according to the French-American-British (FAB) classification system into 8 subgroups, M0-M7, based on leukemic cell differentiation and morphology. Due to the variability of the disease within these AML subgroups several diagnostic tools are used as indicators for disease progression, such as cytogenetic findings, white-cell count, and the haematological phenotype. The cytogenetic tools have enabled further subdivision of AML patients into three prognoses: low, intermediate and high risk groups. Each risk group is treated differently ranging from chemotherapy treatment to allergenic stem cell transplantation. Despite this a large proportion of AML are classified into the intermediate risk group with no identifiable cryptogenic abnormalities (reviewed in Gregory et al., 2009). Recently cytogenetic and molecular analysis, in combination with other features classically used such as prior therapy and history has led to an improvement in the clinical diagnosis of AML patients (Bonadies et al., 2011).

1.3.3 RUNX1 translocations;

Chromosomal abnormalities are reported in 58% of all AML cases (Cheng et al., 2009). The identification of the RUNX1 gene at the breakpoint of chromosome 21 has led to the identification of several common translocations involving RUNX1 associated with leukemia (Figure 1.2 B). These include t(8;21)(q22;q22) which is associated with acute myeloid leukaemia (Miyoshi et al. 1991), t(12;21) (p13;q22) which is a frequent translocation in pediatric B-lineage leukemia (Golub et al., 1995) and t(3;21)(q26;q22) identified in therapy-related myelodysplastic syndrome (MDS/AML) and chronic myeloid leukaemia (CML) in blast crisis (Yamamoto K et al., 2000). The molecular consequences of these chromosomal re- arrangements is the production of a fusion protein that generally acts as a dominant negative of RUNX1 function, recruiting repressor molecules that alter the normal function of the gene. In the t(8,21) translocation, RUNX1 on chromosome 21 is fused to the Eight Twenty One gene on chromosome 8 generating the RUNX1/ETO fusion protein associated with 5-10 % of all AML cases. Other examples of fusion proteins generated from the chromosome 21 breakpoint includes the t(12;21) translocation which generates RUNX1/TEL found in AML and also associated with ALL with B cell precursor phenotype. A less common translocation is t(3;21) that generates the RUNX1-MDS/EVi1 fusion protein associated with MDS and CML. Mutations in the RUNX1 gene have also been described in congenital diseases such as familial platelet disorder (Hart et al., 2002).

The CBFβ gene is also a common target of chromosomal aberrations in leukemia. CBFβ is rearranged and fused to the MYH11 gene that encodes for the smooth muscle myosin heavy chain (SMMHC) in inv(16). This rearrangement generates the CBFβ-SMMHC fusion protein which appears in 15-18% of the subtype M4Eo in AML cases (Poirel et al., 1995). CBFβ-SMMHC knock-in mice display a block in embryonic definitive haematopoiesis and these mice have a similar phenotype to the

RUNX1$^{-/-}$ or CBFβ$^{-/-}$ mice (Kuo et al., 2006), suggesting that expression of this fusion protein disrupts normal RUNX1 and CBFβ function.

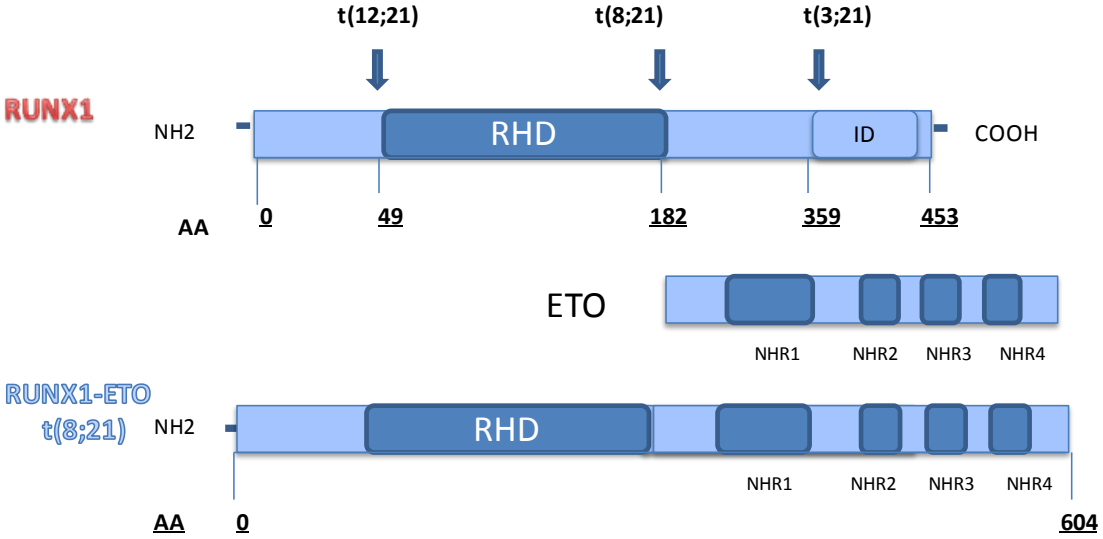
1.3.4 RUNX1-ETO fusion protein;

In 1990 the RUNX1-ETO fusion protein was identified from the t(8;21) translocation as a breakpoint cluster across both the RUNX1 and ETO genes. Mapping of the breakpoint on chromosome 8 identified the ETO gene which was found to consist of 13 exons located across 87 kb with breakpoint regions that create a fusion with intron 5 in the RUNX1 gene (Kozu et al., 1993). The resultant RUNX1-ETO protein contains the RUNX1 DNA binding domain with complete replacement of the C-terminal domain with the ETO protein [Figure 1.2 A]. The Nervy Homology Domain is an evolutionary conserved domain described in ETO proteins which consists of four Nervy Homology Regions (NHR1, 2, 3 and 4). The four NHR have functions and homology related to the *Drosophila* Nervy protein. Two predicted zinc fingers motifs have been described in the NHR4 region of the ETO protein. These motifs are involved in protein–protein interactions, which facilitate the interaction of the ETO protein with repressor molecules such as NCoR and SMART molecules (Ahn et al., 2008). Therefore RUNX1-ETO can bind to RUNX1 target genes but recruits different transcriptional co-regulators including NCoR, SMART and HDACs (Liu et al., 2007). The RUNX1-ETO protein is generally described as a transcriptional repressor. For example, RUNX1-ETO represses the NP3 promoter by recruiting the N-CoR co-repressor. A single amino acid substitution in the N-CoR binding site in the RUNX1-ETO protein impairs its ability to repress the NP3 promoter (Lutterbach et al.,1998). RUNX1-ETO binds to the RUNX1 consensus site and therefore binds to RUNX1 target genes. RUNX1-ETO efficiently blocks RUNX1 transcriptional activation and has been found to repress various RUNX1 target genes including the TCRβ gene (Meyers et al., 1995).

The role of RUNX1-ETO in leukemogenesis was demonstrated by gene targeting experiments. RUNX1-ETO knock-in embryos lack definitive haematopoiesis in the

fetal liver, which is similar to the RUNX1$^{-/-}$ phenotype, suggesting that RUNX1-ETO neutralizes RUNX1 function in vivo through a dominant negative mechanism (Okuda et al., 1998). However, regulation of gene expression by RUNX1-ETO is complex, as analysis of the M-CSFR promoter revealed that RUNX1 can activate the promoter in cooperation with RUNX1-ETO (Rhoade et al., 1996; Shimada et al., 2002). Further, co-expression of RUNX1-ETO in murine derived bone marrow has identified a role for RUNX1-ETO in the partial differentiation of myeloid progenitors (Okuda et al., 1998).

A.



B.

Chromosomal translocation Fusion protein	Associated diseases	Reference
t(8;21)(q22;q22) ETO (MTG8)	AML-M2	Miyoshi et al. (1993)
t(12;21)(p13;q22) TEL (ETV6)	B-lineage ALL	Golub et al. (1995); Romana et al. (1995)
t(12;21)(q12;q22) t(12;21)(q24;q22)	AML-M2 AML	Ramsey et al. (2003) Roulston et al. (1998)
t(3;21)(q26;q22) MDS1	T-MDS/AML CML	Yamamoto K et al.,2000

Figure 1.2 RUNX1 is a frequent target for chromosomal rearrangement. The t(8,21) translocation generates a RUNX1-ETO fusion protein (604AA, MW=67566 Da) in which the C-terminal of RUNX1 is replaced by the ETO protein with 4 NHR [Nervy Homology Regions] (A), as shown. A brief list of chromosomal translocations described in AML that disrupt the RUNX1 gene is shown (B).

1.4 Transcriptional regulation by RUNX1

1.4.1 Overview;

RUNX1 acts as sequence specific transcription factor regulating gene expression programs within cells. Transcription factors generally regulate gene expression by recruiting transcriptional machinery and by altering chromatin structure and thus influencing gene transcription (Suganuma et al., 2011; Kadonaga et al., 2004).

1.4.2 Regulation of gene expression by chromatin structure;

In eukaryotic cells, genes are packed in long strands of DNA that is tightly coiled around four core histone proteins (H2A, H2B, H3 and H4). The core histones form an octamer arrangement around which the DNA is wrapped to form a complex referred to as the nucleosome (Lorch et al.,2006). The nucleosomes are the basic building blocks for chromatin which are then assembled into higher order chromatin structures. Each of the core histones has a globular domain essential for histone-histone interactions and the formation of the nucleosome. Histone tails are extended from the core histones, rich in amino acid residues often involved in post-translational modifications. These changes often alter nucleosome packaging that restricts DNA accessibility for regulatory proteins. Such alteration of DNA accessibility is a dynamic process that contributes to transcriptional regulation, DNA replication and repair. The molecular mechanisms involved in altering DNA accessibility are well characterized in the literature and include histone post-translational modifications such as acetylation and methylation and ATP dependent chromatin remodeling (Suganuma et al., 2011; Narlikar et al 2002).

1.4.3 Genes Regulated by RUNX1;

Gene expression profiling as well as studies of individual genes has now identified several pathways and a large number of genes downstream of RUNX1 (Otto et al., 2003). During transcription RUNX1 recognizes a specific regulatory element in the promoters and enhancers of genes, with a large number of RUNX1 targets now described with important roles in the hematopoietic system, and the best described of these are noted in Table 1.2. These genes include those encoding granulocyte macrophage colony-stimulating factor (GM-CSF, Oakford et al., 2010; Takahashi et al., 1995), myeloperoxidase, neutrophil elastase (Nuchprayoon et al., 1994), macrophage colony-stimulating factor receptor (M-CSFR, Zhang et al., 1997), interleukin 3 (IL-3, Cameron et al, 1994), and T-cell antigen receptor (Giese et al., 1995).

Microarray analysis has further described several biological processes controlled by RUNX1, with the immune response and cell cycle significantly correlated to RUNX1 activity (Michaud et al., 2008). In this study genomic and bioinformatic analysis was performed to identify genes regulated by RUNX1 using three models in which RUNX1 activity was altered; RUNX1 haploinsufficiency in cells obtained from FPD-AML patients B cells, overexpression of RUNX1 in Hela cells and RUNX1 deficiency using cells obtained from mouse $RUNX1^{-/-}$ embryos at E8.5-12 dpc. The study also considered the presence of RUNX1 binding sites in regulatory regions and previous gene expression studies. As mentioned before the analysis characterized several RUNX1 targets involved in cellular development. Genes well described in haematopoiesis such as CSF1R, MYB and MPO were differentially expressed in the $RUNX1^{-/-}$ embryo model. In addition, enrichment for genes involved in cell proliferation was observed in FPD-AML and RUNX1 overexpression datasets. All data sets showed differential expression of the Annexin I (ANXA1) gene. ANXA1 is a protein encoded by an immune gene involved in cell differentiation and proliferation. Differential expression of cyclin D3 gene was observed in both the FPD-AML and RUNX1 overexpression datasets, and several studies have previously described the role of RUNX1 in

regulating cell cycle related genes such as p21 (Lutterbach et al., 2000; Peterson et al., 2007). In addition mutations in the cyclin D3 gene were previously described in acute myeloid leukemia patients samples (Smith et al., 2005). A number of other studies have similarly conducted genome wide screens for RUNX1 target genes (Wotton et al., 2008; Valk et al., 2004; Ichikawa et al., 2006) but few of the identified targets have been extensively characterized (Table 1.2).

1.4.4 Transcriptional regulation by RUNX1;

In myeloid cells RUNX1 synergizes with several transcription factors to regulate gene expression. RUNX1 binds directly to MEF to transactivate the IL-3 promoter (Mao et at., 1999). RUNX1 can also synergize with and recruit other transcription factors binding to adjacent sites on the DNA. The role of RUNX1 in regulating the M-CSF receptor promoter involves synergy with PU.1 and C/EBPα, both of which have binding sites adjacent to RUNX1 in the M-CSFR promoter (Zhang et al., 1996). In addition RUNX1 has been shown to synergize with c-myb to activate the myeloperoxidase promoter in myeloid cells (Britos-Bray et al., 1997 and Friedman et al., 1997). Mutation of both RUNX1 and c-myb binding sites disrupts this synergism. RUNX1 is also involved in T-cell specific gene regulation (Bruhn et al., 1997), where it co-operates with PU.1 and c-myb to regulate activity of the TCR enhancer (Giese et al., 1995). In order to assemble the transcriptional machinery on the TCR enhancer RUNX1 recruits ALY enhancer molecules to influence its functional collaboration with other transcription factors. ALY proteins were first described as enhancers of transcriptional proteins that facilitate the interaction between RUNX1 and c-myb on the TCR enhancer (Bruhn et al., 1997).

In addition, during transcription RUNX1 recruits several classes of chromatin modulators that have the ability to induce histone modifications. For example RUNX1 acts as an activator by recruiting co-activator molecules such as p300, CBP and MOZ to gene promoters. This has been described at the M-CSF promoter at which p300 co-activators facilitate the functional synergy of RUNX1 with C/EBPα (

Kitabayashi et al, 1998). Later studies have also described synergy between RUNX1 and MOZ at the MIP-1α promoter (Bristow et al., 2003). These co-activator molecules act as adaptor proteins with HAT activity and are able to induce histone acetylation which is linked to chromatin remodeling and gene activation (Kitabayashi et al, 1998; Aikawa et al., 2006). In addition an association between RUNX1 and the SWI/ SNF chromatin remodeling complex core subunits BRG1 and INII was reported at the GMCSF and IL-3 promoters (Bakshi et al., 2010). In a different context RUNX1 can act as a repressor of transcription by recruiting co-repressor molecules such as Groucho/transducin-like Enhancer of split and mSinA (Aronson et al., 1997). Further, mSin3A has been found to interact with the inactive RUNX1 protein and protect RUNX1 from proteasomal degradation (Imai et al., 2003) [Figure 1.3].

Gene	Expressed	Up/Down regulation	References
TCR	T cells	Up regulated by Runx1	Hsiang et al., 1993; Giese et al. 1995; Hernandez-Munain and Krangel 1995; Meyers et al., 1995; Bruhn et al. 1997.
CD3	T cells	Up	Hallberg et al., 1992
IL-3	T cells		Cameron et a., 1994; Mao et al. [1999]
Granzyme B	T cells	Up	Wargnier et al. 1995
GM-CSF	T cells	Up	Takahashi et al., 1995; Oakford et al., 2010
M-CSFR	Monocytes	Up	Zhang et al.,1994; Zhang et al., 1996; Petrovick et al.,1998
MPO	Immature myeloid cells	Up	Britos-Bray and Friedman 1997; Nuchprayoon et al., 1994
CD36	Macrophage	Up	Armesilla et al., 1996
CD53	Primitive myeloid cell line L-G	Down	Shimada et al., 2000
Pim-2	Primitive myeloid cell line L-G	Down	Shimada et al., 2000

Table 1.2 RUNX1 target genes. Table of well described targets regulated by RUNX1 in the hematopoietic system.

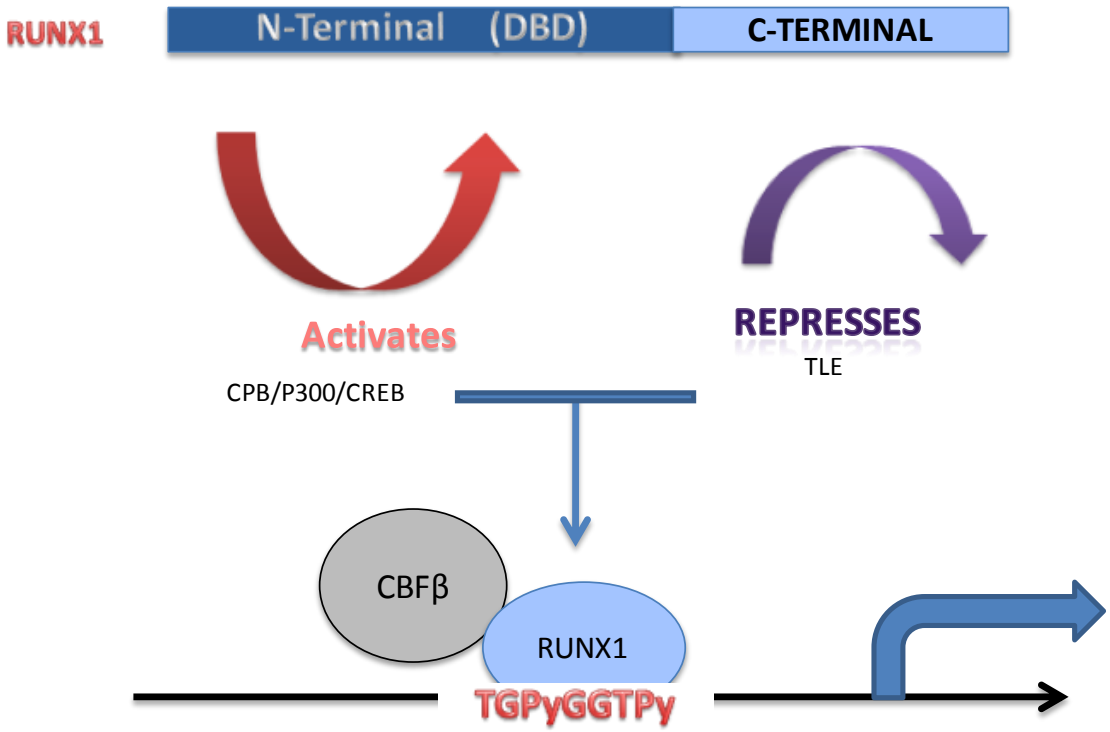


Figure 1.3 Transcriptional regulation by RUNX1 is context dependent. RUNX1 can act as either an activator or repressor depending on the co-regulatory proteins it recruit to target genes.

1.4.5 RUNX1 post translational modifications;

Several post–translational modifications to RUNX1 have been reported, including acetylation, methylation and phosphorylation. RUNX1 can be activated through phosphorylation induced by several pathways (Tanaka et al., 1996). Cytokine and growth factor signaling has been found to induce several modifications to RUNX1 through the Mitogen Activated Protein Kinase (MAPK) signaling pathway which mediates phosphorylation of RUNX1. Two phosphorylation sites (S-294 and S-266) were described within the Proline Serine and Threonine (PST) region of RUNX1. Mutations in the PST region reduce phosphoylation of RUNX1 (Tanaka et al., 1996). In addition, hematopoietic stem cell stimulation with IL-3, which activates the ERK pathway induces phosphorylation of RUNX1 and stimulates its transcriptional activity (Tanaka et al., 1996). ERK-dependent phosphorylation of RUNX1 was also reported as a mechanism by which RUNX1 is released from interactions with repressors such as mSin3A that inhibit proteasomal degradation. Further, notch signaling in murine embryonic stem cells has been demonstrated to have effects on several lineage specific transcription factors including RUNX1 (Meier-Stiegen et al., 2010). Activation of RUNX1 through acetylation by co-activators such as p300 has been described (Yamaguchi et al., 2004). p300 acetylates RUNX1 at two conserved lysine residue (K24 and K43), mutation of these sites impairs RUNX1 DNA binding and activity (Yamaguchi et al., 2004). In addition methylation of RUNX1 through interaction with SUV39H1 has been described as a repressive mechanism that modulates the affinity of RUNX1 for the DNA (Chakraborty et al., 2003). The SUV39H1 histone lysine methyltransferase is associated with gene silencing, and co-expression of SUV39H1 lowers RUNX1 DNA binding affinity and abrogate the transactivation of M-CSFR by RUNX1(Chakraborty et al., 2003).

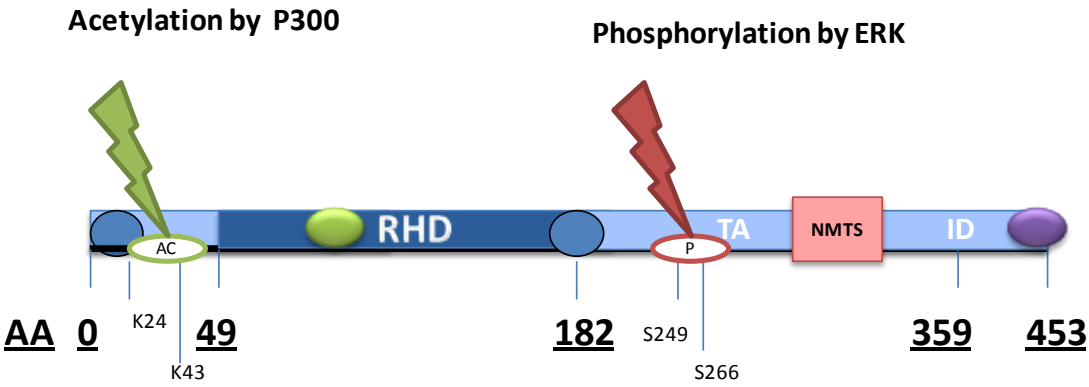


Figure 1.4 RUNX1 post translational modifications. RUNX1 transcriptional activity is enhanced by phosphorylation of two serine (S249, S266) residues and the acetylation of lysine (K24, K43) residues.

1.4.6 Rationale for this study;

RUNX1 is expressed in hematopoietic cells and it is clear from a number of studies that RUNX1 plays an important role in the commitment and transition of haematopoietic cells into different lineages. There is a strong evidence that disruption of RUNX1 by point mutation or chromosome alterations contributes to the development of leukaemia.

It is proposed that dysregulation of RUNX1 disrupts normal haematopoiesis due to abnormal expression of RUNX1 target genes. While a number of RUNX1 targets have been identified as outlined above, the set of genes that are regulated by RUNX1 and critical for haematopoietic development has not been fully elucidated. Therefore in order to further understand how RUNX1 regulates haematopoiesis our laboratory aimed to identify targets of RUNX1 which likely contribute to hematopoietic development.

Previously our laboratory undertook microarray experiments to identify RUNX1 targets (Oakford and Holloway, unpublished). Potential targets were identified by altering RUNX1 expression in T cells by introduction of RUNX1 siRNA. Among the differentially expressed genes were the Leukemia Inhibitory Factor Receptor (LIFR) and gp130 genes suggesting these are potential novel targets of RUNX1.

This study aimed to examine the role of RUNX1 in the regulation of LIFR and gp130 and this thesis propose that RUNX1 regulation of both genes (LIFR and gp130) is altered in leukemia in which RUNX1 is disrupted.

1.5 The LIFR and gp130 Receptor Complex

1.5.1 Overview;

The IL-6 cytokine family consists of functionally related members which have roles in the acute phase response, inflammation and the immune response. The main members of the IL-6 cytokine family are; Interleukin-6 (IL-6), IL-11, Leukaemia Inhibitory Factor (LIF), Oncostatin (OSM), Cardiotrophin (CT-1) and Ciliary Neurotrophic Factor (CNTF). Beside their main role as immune mediators the IL-6 cytokines also have well described roles in haematopoiesis, as well as in liver and immune regulation (reviewed in Auernhammer and Melmed, 2000). The biological activity of the cytokines is regulated by their interaction with a specific receptor complex initiating intracellular signaling. The receptor complex consists of a non-signalling alpha subunit and a common signal transducer shared between the IL-6 cytokines, known as gp130 (or IL-6ST, IL6 signal transducer). However, some of the α subunit receptors such as LIFR and the OSM receptor can also act as signaling transducers.

Deregulation of signaling through these receptor complexes contributes to a range of diseases (reviewed in Taga, 1997).

1.5.2 Leukaemia inhibitory factor (LIF);

LIF is a glycoprotein originally identified for its ability to induce differentiation of the murine myeloid leukemic cell line M1 down the macrophage lineage and terminate self renewal of this highly clonogenic cell line (Tomida et al., 1984). The LIF gene has since been described in human as well as a range of other species (Willson et al., 1991). Cloning of the murine and human LIF nucleotide sequences revealed homology in their coding regions (Stahl et al., 1990). LIF appears to be a

pleiotropic cytokine having well described roles in hematopoietic cell proliferation, platelet formation, bone formation, hormone production, neuronal development and blastocyte implantation (reviewed in Metcalf, 2003). Immunoassay analysis of the LIF protein detected expression in a range of organs including thymus, skeletal muscle, pancreas, kidney and neural tissue (Fukada et al., 1997). Although LIF $^{-/-}$ is not lethal in mice, knock-out mouse models confirmed a role for LIF in the hematopoietic system. LIF deficient mice have a pronounced reduction in pluripotent hematopoietic stem cells in spleen and bone marrow and have impaired thymic maturation (Escary et al., 1993). In addition LIF has a critical role in mammalian pregnancy. Female mice lacking the LIF gene do not develop pregnancy due to failure of their blastocysts to implant. However LIF$^{-/-}$ blastocytes can be implanted in wild type pseudopregnant females and form normal embryos, demonstrating that this is an implantation defect (Stewart et al., 1992).

1.5.3 Cytokine Receptors;

IL-6 cytokine receptors consist of several receptor subunits that initiate the interaction needed to form homo or hetrodimeric complexes during signalling. IL-6 receptors vary in their overall structural arrangement but share a conserved 200 amino acids in their extracellular region (Bravo et al., 2000). The conserved amino acids are essential for cytokine-receptor interaction and this region is known as the cytokine homology domain (CHD). Two tandem Fibronectin type III (FNIII) folds are arranged in the CHD, the first FNIII fold contains a conserved cysteine motif, while a highly conserved WSXWS motif is located in the C-terminal of the second FNIII fold (Hibi et al, 1990). Signalling receptors such as gp130, LIFR and OSMR have the ability to engage with the JAK/STAT signalling pathway through their cytoplasmic domain (O Sullivan et al., 2007).

1.5.4 Biological function of LIFR;

LIFR acts as a low affinity signaling receptor for LIF (Gearing et al, 1991). LIFR heterodimerizes with gp130 to form a high affinity complex that also has the ability to signal for other cytokines including oncostatin M (OSM), and cardiotrophin (CT-1) (Gearing et al., 1992) [Figure 1.5]. The receptor complex then initiates downstream signaling cascades ultimately resulting in downstream gene activation [Figure 1.6]. The ability of a cell to respond to the cytokine is mainly dependent on the expression of the receptor. LIF: LIFR signaling is known to control significant cellular processes such as cellular development, growth promotion of hematopoietic cells, neuron maintenance, bone growth, hepatic development and pregnancy. $LIFR^{-/-}$ mice die after birth (Ware et al., 1995), with $LIFR^{-/-}$ animals displaying a reduction in bone mineralization, liver glycogen levels, as well as spinal cord and brain stem astrocytes (Ware et al., 1995).

1.5.5 gp130 receptor subunit;

gpl30 was originally cloned and identified as an essential signaling receptor shared between the IL-6 cytokine family. The administration of IL-6 cytokine upregulates murine gp130 and induces growth in IL-3 dependent cell lines (Saito et al., 1995). The gp130 glycoprotein belongs to the class I cytokine receptors and shares homology with the LIF and OSM receptors (Bravo et al., 1998). Although gp130 shares structural homology in 200 amino acid of the FNIII domain with class I cytokine receptors, several structural analysis suggest that different mechanisms are involve in the activation of the receptor. gp130 utilizes its Ig domain to stabilize and neutralize the activity of IL-6 but not LIF and OSM (Hammacher et al.,1998).

1.5.6 gp130 Biological function;

gp130 has a broad expression pattern among different tissues, and the shared usage of gp130 by a number of the IL-6 family cytokines, may explain the overlapping function of the IL-6 cytokine family [Figure 1.5]. gp130 is expressed in a range of organs including brain, spleen, kidney, lung, liver and placenta as determined by analysing gp130 mRNA levels upon IL-6 administration in mice (Saito et al., 1995). Mice deficient in one of the IL-6 cytokines generally display a mild phenotype. For example IL-6 $^{-/-}$ mice display defects in haematopoiesis, acute phase protein synthesis, antigen – specific antibody production and increased susceptibility to infection. CT$^{-/-}$ mice have an increased loss of motor neurons, while LIF$^{-/-}$ female mice are sterile due to the defect in the blastocyste implantation (Fasnacht et al., 2008). On the other hand, the inactivation of gp130 is lethal. Mice homozygous for the gp130 mutation die at E12.5 dpc, and have severe anemia and reduced numbers of committed hematopoietic progenitors in the liver. gp130$^{-/-}$ mice also display hypoplastic ventricular myocardium which suggest a role for gp130 in the normal development of the heart tissue (Akira et al., 1995).

1.5.7 LIFR/gp130 receptor signaling;

Receptor signaling results in a signaling cascade that initiates a cellular response, triggered by ligand molecules such as cytokines and hormones. Chimeric LIFR and gp130 receptor signaling models were studied by different groups, using chimeric receptors based on the cytoplasmic region of either gp130 or LIFR with the extracellular region of the human granulocyte macrophage colony stimulating factor (h GM-CSFR) (Hammacher et al., 2000). The role of LIFR and gp130 cytoplasmic regions in the signaling was evident. In a complementary experiment mutational analysis of the chimeric gp130 molecule described the role of the membrane proximal region in FNIII in gp130 in LIF dependent signaling (Hammacher et al.,2000). Mutation of the two membrane distal tyrosines affect LIFR- STAT3 activation (Tomida et al.,1999) . IL-6 stimulation of the cytokine

signaling transducer receptor including; LIFR, gp130 and OSMR can trigger the JAKs molecules (Giese et al., 2005). JAK-STAT3 is a common signaling pathway shared by several receptors of the cytokine I and II families [Figure 1.6]. Tyrosine phosphorylation of the gp130 cytoplasmic tail is an essential mechanism that leads to activation of both STAT transcription factors and the protein tyrosine phosphatase enzyme SHP2 [Figure 1.6]. Activation of gp130 is dependent on the tyrosine motif Y759 which interacts with activator proteins such as SHP2 and suppressor of cytokine signaling molecules (SOCS3). Transgenic mice deficient in gp130-STAT3 or gp130-SHP2 pathways were challenged with gp130 human mutant cDNA. The SHP2 deficient mice were born normal with splenomegaly and lymphadenopathy and enhanced acute phase reactions, while the STAT3 deficient mice died perinatally. Mice deficient in the gp130-SHP2 pathway showed prolonged gp130-STAT3 activation indicating a negative regulatory role for SHP2 (Ohtani et al., 2000). This confirms the role of gp130 Y795 in the phosphorylation by SHP2 and ERK.

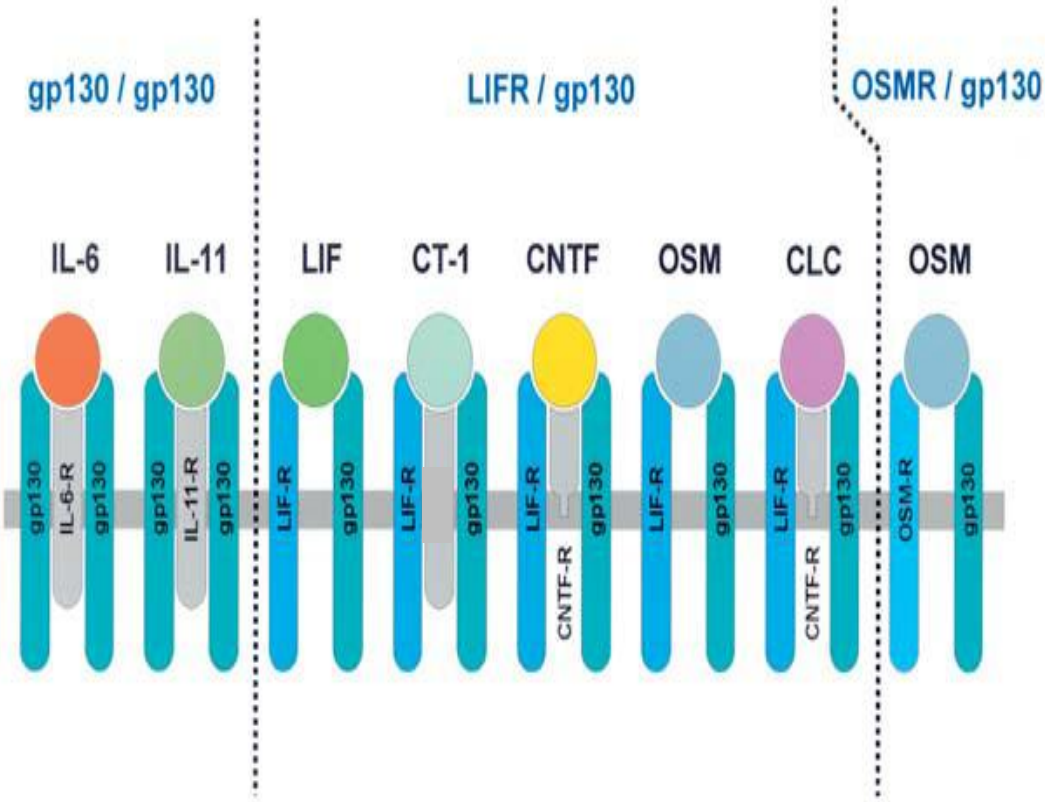


Figure 1.5 Schematic representation of the IL-6 family cytokines and the receptor complexes through which they signal (Heinrich et al., 2003).

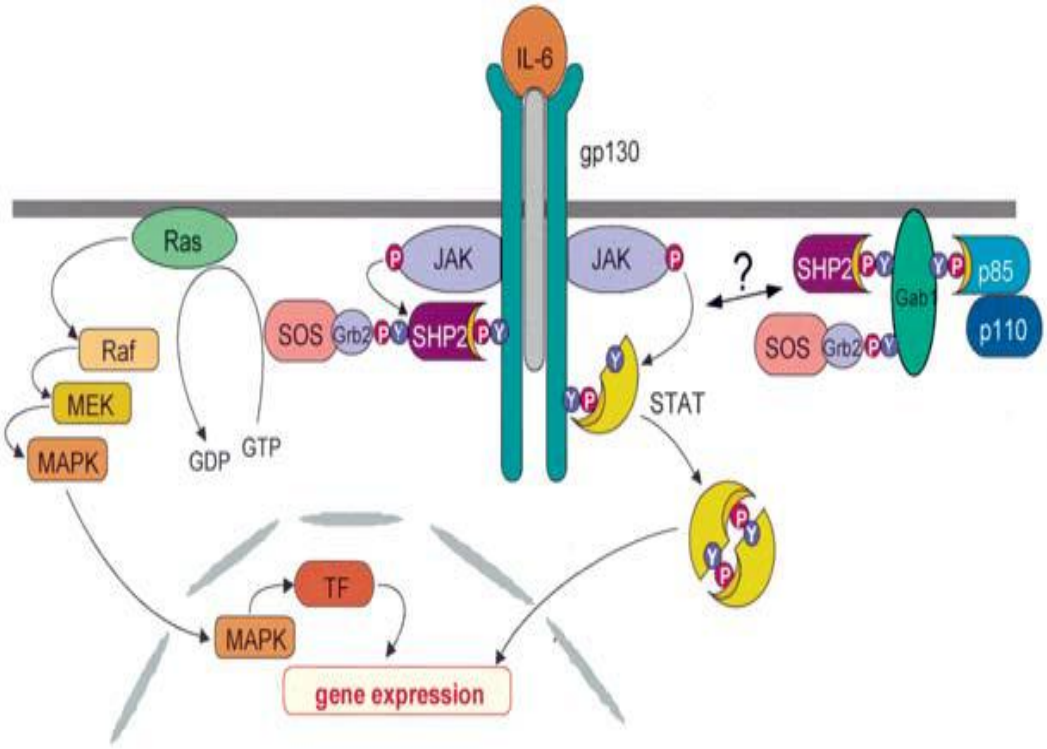


Figure 1.6 Schematic of the signalling pathway downstream of the IL-6 family receptors (Heinrich et al., 2003).

1.6 Regulation of LIFR gene expression;

I.6.1 Leukemia Inhibitory Factor Receptor Gene;

The Leukemia Inhibitory Factor Receptor (LIFR) gene was cloned and characterized in 1991 (Gearing et al., 1991). The human LIFR gene is located on Chromosome 5 and spans more than 70 Kb with 20 exons (Gearing et al., 1993). Sequencing of the gene has further described the gene structure, revealing that the 5' untranslated region is encoded by exon 1 and 2, the cytoplasmic domains CH1 and CH2 are encoded by exons 3-6 and 8-11 respectively, while the extracellular, Ig domain is encoded by exon 7 and the FNIII domains are encoded by exons 12, 17 and 18 (Tomida et al., 1996). LIFR has now been cloned in a range of species. The murine gene is located on Chromosome 15 in a region that shares homology with human chromosome 5. The LIFR gene locus is linked to a cluster of cytokine receptor genes including IL-7, Prolactin and GH receptors (Gearing et al., 1993).

1.6.2 Regulation of LIFR;

While the role of LIFR signaling in a range of biological processes is well described in the literature little is known about regulation of the LIFR gene. The enhancer was first cloned by Wang and colleagues (Wang and Melmed, 1998). Using 5′ RACE were also detected two transcription start sites suggestive of alternative promoter usage, identifying one promoter that was active in placental tissue and a second in non placental tissue. In addition an enhancer with potential transcription factor binding sites including for CRE, GATA, NF-kB and OCT-1 was described (Wang and Melmed, 1998). However these promoters have remained uncharacterised as far as transcription factor binding. Subsequent analysis confirmed the placental promoter but was unable to confirm the general promoter sequence by genomic or database analysis (Blanchard et al., 2000), but confirmed the existence of an alternative promoter associated with a CpG island (Blanchard et al., 2000) [Figure 1.7].

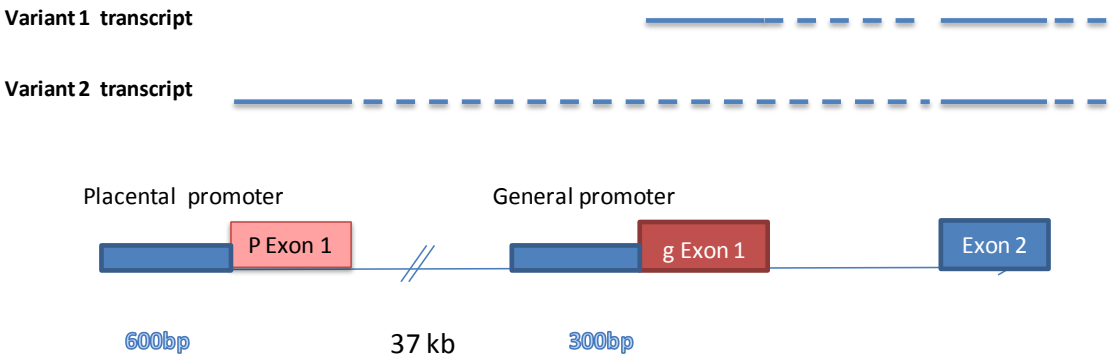


Figure 1.7 Schematic of the LIFR promoters. The placental and general promoters and promoter specific exon 1 are shown, as well as exon 2. Transcripts generated from each promoter are shown.

This LIFR promoter was shown to be subject to chromatin modification with high levels of acetylated histones observed at the LIFR promoter after LIF cytokine stimulation. Further chromatin modification was induced by treatment with HDAC inhibitors (FR901228) which increase the cellular response to LIF cytokine (Blanchard et al., 2002). Therefore alteration in LIFR expression levels is correlated with chromatin remodeling and maybe a target for alteration during tumourgenesis. (Blanchard et al., 2002).

1.6.3 Regulation of gene expression by alternative promoter;

Alternative promoter usage has now been well described in controlling gene expression. Large scale analysis of the human genome indicates 14-58% of genes are subject to regulation by putative alternative promoters (Kimura et al., 2006). The role of alternative promoter usage appears to generate diversity of protein structure, tissue distribution, subnuclear localization and function. Alternative promoters have a well described role in tissue specific expression. For example the mouse Prkar1 gene encodes for the type1 alpha c-AMP dependent protein kinase which regulates cell cycle during growth and differentiation. Transcripts generated from this gene are produced from three different promoters known as P1, P2 and P3. The Prkar1 gene generates four transcripts during late postnatal stage which are distributed across different tissues (heart, liver, brain and skeletal muscle). In addition each transcript differs in their 5′ UTR with variation of CpG island content. While P1 is non-CpG related both P1 and P3 have CpG islands, suggesting different mechanisms associated with their regulation (Bandy et al., 2011). Alternative promoter usage may in some instances control the level of expression, which is most commonly described for genes encoding signaling molecules and enzymes. For example, the amylase gene has a weak promoter which is active in liver tissue and a second promoter which is a strongly expressed promoter in the parotid gland and pancreas (Schibler et al., 1983). In some genes one promoter directs generation of transcripts with ubiquitous expression and an alternative promoter directs cell type specific expression. The gene encoding for the porphobilinogin deaminase (PBGD) produces two transcripts. An upstream promoter produces a transcript expressed in all tissues, while the second transcript

is produced from a downstream promoter and is only expressed in erythroid cells (Schibler et al., 1983). This appear to be the case for the LIFR gene also with the placental promoter so far found to be active only in placental tissue and other promoter active in all tissues so far examined (Wang and Melmed, 1998; Blanchard et al., 2002).

1.7 Aims;

Previous microarray studies in our laboratory have identified the LIFR and gp130 genes as putative RUNX1 target genes and therefore we hypothesize that the LIFR and gp130 genes are targets of RUNX1 and their regulation is altered during leukaemia in which RUNX1 normal function is altered. As mentioned above LIFR is controlled by alternative promoters, the so called general and placental promoter. So far there is no clear description of either promoter or the transcripts they generate in the hematopoietic system and therefore the first aim of this thesis is to determine which of the LIFR promoters is active in myeloid cells. The second aim of this thesis is to investigate regulation of both the LIFR and gp130 promoters by RUNX1.

Chapter 2

MATERIALS AND METHODS

2.1 Cell culture;

All cell lines were sourced from the ATCC and maintained for less than six months of continuous culture.

2.1.1 Myeloid Cell line Culture;

The myeloid cell line Kasumi-1 (originally derived from a 7-year-old Japanese boy with AML; Asou H et al., 1991) was cultured in RPMI 1640 medium (Gibco BRL), supplemented with 20% FCS, 100U/ml penicillin and 100µg/ml streptomycin (JRH Biosciences). The KG-1 and KG-1a cell lines (derived from a Caucasian 59 year old male with AML; Furley AJ et al., 1986) were both maintained in RPMI 1640 medium, supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin. All of the cell lines were incubated at 37^{o}C and 5% CO_2 to maintain their growth. The density of the cells was determined every 72 hours using a haemocytometer and adjusted to $5x10^5$ cells/ml by the addition of fresh medium.

2.1.2 Myeloid cell stimulation;

Myeloid cell lines were cultured in RPMI medium with required supplements for each cell line. Cells were maintained at $5X10^5$ cells per ml and stimulated by adding 20 ng/ml phorbol 12-myristate13-acetate (PMA; Boehringer-Mannheim) and 1µM calcium ionophore A23187 (I; Sigma-Aldrich).

2.1.3 Adherent cell line culture;

The human placental choriocarcinoma JAR cell line established by R.A. Pattillo (derived directly from a 24 year old female with a placental tumour; Pattillo et al.,1972) was maintained in RPMI 1640 medium (Gibco BRL), supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin (JRH Biosciences). The human placental choriocarcinoma cell line JEG-3, originally isolated by Kohler (Kohler et al., 1971) was maintained in alpha minimum essential medium (α MEM; Gibco BRL). The medium was supplemented with 10% FCS, 100U/ml penicillin and 100µg/ml streptomycin (JRH Biosciences). Both of these cell lines were subcultured every three days, when at 70-100% confluence. The adherent cells were detached from the flask by incubation with 2% trypsin solution (Gibco BRL) at 37^{o}C and 5% CO_2 for 10 minutes. The cells were diluted in medium, recovered by centrifugation, washed with PBS and replated in fresh medium at a 1:3 dilution.

2.1.4 Thawing Cells;

Cell line stocks were thawed at 37^{o}C then transferred to a 15ml centrifuge tube and 10 ml of medium added. The cells were collected by centrifugation at 500 g for 5 minutes. The medium was aspirated, replaced with fresh medium and the cells transferred to tissue culture flasks then maintained at 37^{o}C and 5% CO_2.

2.1.5 Freezing Cells;

Cells were collected by centrifugation at 500g for 5 minutes and re-suspended at a density of 1X10^{7} cells/ml in culture medium contain 20% DMSO (Dimethylsulfoxide; Sigma). The cells were transferred to cryovials and stored at -

80^{o}C in an insulated box with cotton wool to slow freezing, and then transferred to the vapour-phase of liquid nitrogen.

2.2 Extraction of RNA and synthesis of cDNA from cultured cells;

2.2.1 RNA extraction from cell lines;

Cultured cells at a density of 5x10^{5} cells/ml were centrifuged at 500g for 5 minutes, and the cell pellet lysed in TRI-Reagent (500µL, Sigma-Aldrich, U.S.A) for 5 minutes at room temperature. The cell lysate was treated with chloroform (100 µL; AnalaR, BDH Chemicals, Australia), mixed vigorously for 15 seconds and left at room temperature for 10 minutes. The mixture was centrifuged at 500 g for 15 minutes to allow phase separation. The top aqueous layer was collected and the RNA precipitated overnight with isopropanol (500µL, Merck Australia). The RNA was recovered by centrifugation at 500g for 20 minutes at 4^{o}C. The pellet was washed with 70% ethanol, air-dried and resuspended in 20 µL of sterile MilliQ water, then stored at -80^{o}C.

2.2.2 RNA quantification;

RNA concentration was quantified using the Nanodrop ND-1000 UV-vis spectrophotometer (Nanodrop Technologies, U.S.A). Sample concentration (ng/µL) was calculated based on absorbance at 260 nm. RNA quality was determined by ratio of the sample absorbance at 260/280 nm, with a ratio of 2 accepted as indicative of high quality RNA.

2.2.3 cDNA synthesis;

A total of 1µg RNA was used for each cDNA reaction. The RNA was treated with DNase I (1 unit, Invitrogen U.S.A) to remove any DNA contamination in 1X First

strand buffer (Invitrogen) in a total reaction volume of 10µL. The reaction was incubated at 37^{o}C for 30 minutes followed by 75^{o}C for 5 minutes to inactivate the DNase I. Oligo dT (0.1nmol) was added to the RNA sample, and was left to anneal for 10 minutes at 70^{o}C. The sample was treated with superscript reverse transcriptase (100 units; Invitrogen, USA) in 1X First strand buffer, supplemented with DTT (0.1M dithiotheritol; Invitrogen, USA) and dNTPs (0.5 mM, Invitrogen). cDNA was generated by reverse transcription at 42^{o}C for 50 minutes, followed with incubation at 70^{o}C for 15 minute to inactivate the reverse transcriptase enzyme.

2.3 PCR analysis;

2.3.1 DNA and cDNA amplification by PCR;

cDNA or DNA samples were amplified by PCR using Go-Taq Green Master Mix (Promega Corporation,USA). Primers were designed by analysing the relevant sequence using the Primer 3 website (http://frodo.wi.mit.edu/primer3/). Primers were sourced from Geneworks and those used in this thesis are outlined in Table 2.1.

Primer Set	Primer Name	Sequence	Description
V2pLIFRm	V2pLIFRmF	AGCCTCTGCGACTCATTCAT	m RNA primers
	E2LIFRmR	ATCCAGGATGGTCGTTTCAA	
V1gLIFRm	V1gLIFRmF	GCAGGGGATGGCAAGATAG	m RNA primers
	E2LIFRmR	ATCCAGGATGGTCGTTTCAA	
h LIFR	h LIFR F	CAGGCTATAAGCCACAGATGC	Primers amplify the 3UTR of the LIFR gene
	h LIFR R	AGCCCAGGATGCCCTTGAGGG	
gapdh	h GAPDH F	AAATATGATGACATCAAGAAGGTGGT	m RNA primers
	h GAPDH R	AGCCCAGGATGCCCTTGAGGG	
RUNX1	AML1 F	CACCTACCACACAGAGCCATAC	m RNA primers
	AML1 R	CTCGGAAAAGGACAAGCTCC	
RUNX1-ETO	ETO F	AATCACAGTGGATGGGCCC	m RNA primers
	ETO R	TGCGTCTTCACATCCACAGG	
LIFR2	LIFR2 F	CCTAACCTGGGTTGGACTCA	Placental promoter primers for ChIP
	LIFR2 R	TGACTGAATGCATCAGCAGTGC	
LIFR4	LIFR4 F	TAGAAAACCGAGGCCAAGTG	General LIFR promoter primers for ChIP
	LIFR4 R	GGCTTATTTGTGCGGAGAAG	

Table 2.1 Quantitative PCR primer sequences.

cDNA or DNA (50ng) was amplified in 25µl reactions containing Go-Taq Green Master Mix (1X), forward and reverse primers (300nM) and MilliQ water to the required volume. No template control reactions (NTC) were conducted in parallel. All reactions were amplified using the following conditions:

95°C for 2 minutes

95°C for 30 seconds
60°C for 30 seconds
72°C for 1 minute
} 30 Cycles

72°C for 5 minutes

Amplified product was analysed by 2% agarose gel electrophoresis to confirm the PCR product size.

2.3.2 DNA and cDNA analysis by quantitative PCR (q PCR);

cDNA or DNA analysis was performed by qPCR using a SYBR Green PCR kit (Qiagen). PCR was conducted using the Corbett Rotor Gene (Corbett PC-960C, Australia). Samples were prepared by adding 5µL cDNA or DNA (50ng) to 1 X SYBR Green master mix, forward and reverse primers (300nM) and PCR water to a final volume of 25 ul. No template control reactions were prepared and amplified in parallel. All reactions were amplified using the following conditions:

95°C 15 Minutes

94°C 15 Seconds
60°C 60 Seconds
} 35 Cycles

These cycles were followed by a Melt analysis from 65ºC to 95ºC, increasing by 1ºC every 5 seconds and acquiring at every temperature increment.

Threshold values were set through the linear region of the amplification curve to correlate the amount of product generated with Ct value. Data was quantitated using standard curves generated from each primer set. The GAPDH housekeeping gene was amplified in parallel to normalize for variation in cDNA synthesis or the amount of starting material. Melt curve analysis was used to determine that a single PCR product was generated in each reaction. Amplified products for each primer set were also subjected to agarose gel electrophoresis analysis to confirm the presence of single products of the expected size.

2.3.3 Standard Curve generation;

Standard curves were generated for each PCR product to allow precise quantification of PCR amplification. Amplified PCR product was subjected to gel electrophoresis, products were then extracted from the gel. Purification of the extracted DNA was performed using the Qiagen gel extraction Kit according to the manufacturer's instructions. DNA was phenol/chloroform extracted and precipitated by ethanol.
Purified product concentration was determined by Nanodrop, and known copies of DNA analysed by qPCR to generate a standard curve of copy number relative to Ct.

2.4 Generation of plasmid constructs for reporter assay analysis;

2.4.1 Plasmid DNA isolation;

L-broth medium (1ml) containing ampicillin (50mg/ml) was inoculated with the required plasmid and incubated overnight at 37^{o}C with shaking. The overnight

culture was then inoculated into 100 ml of L-broth containing ampicillin and incubated overnight in the same conditions. The plasmid was harvested using the Pure Link ^{TM} Hi Pure Plasmid Filter Purification Kit (Invitrogen) according to the manufacturer's instructions.

2.4.2 Plasmids;

The LIFR general promoter reporter construct was generated by amplifying a region of the general LIFR promoter from -305 to +24 relative to the transcription start site, as determined by Blanchard (Blanchard et al., 2002). General LIFR promoter sequence was amplified using the following primers;
For 5'-CTACGGATTCCAAGGTGTGTCTGTAGAGTCCTGA-3' and

Rev 5'-CATCCTCGAGAGTATCCTGGAGCCATCTAGTCTTG-3',

which contain *Bam*H1 and *Xho*I restriction enzyme sites respectively. The PCR product generated was cloned into the pXP1 vector at the *Bam*H1 and *Xho*I restriction sites using T4 DNA ligase (New Englad Biolabs, U.S.A).

The placental LIFR promoter sequence was previously described by Melmed (Wang and Melmed, 1998). A region containing the placental promoter from -608 to +107 relative to the transcription start site was amplified with the following primers which contain *Bam*HI and *Sac*I restriction enzymes sites respectively:

For 5'-CTACGGATCCCTGTCATCCCAGCACTTTGG- 3' and

Rev 5'-CATCGAGCT CAGCTGGGATTAGGAGTTGAT- 3'

The PCR product generated was cloned into the pXP1 vector at the *Bam*H1 and *Sac*I restriction sites using T4 DNA ligase (New Englad Biolabs, U.S.A).

The gp130 promoter reporter construct was generated by amplifying the promoter region (526bp) described previously by Blanchard and O Brien using Go Taq

Green and the following primers which contain *Bam*H1 and *Sac*I restriction sites, respectively:

For 5'-CGC GGATCCCTTCACTTTCCCCAAGAGGC- 3' and

Rev 5'-CGGGAGCTCCACTCTAACTCCAGCTACGC- 3'

PCR product was cloned into the pXP1 vector to generate the placental LIFR reporter construct. Both plasmid and insert were digested with *Bam*H1 and *Sac*I restriction enzymes and ligated using T4 DNA ligase (New Englad Biolabs, U.S.A).

RUNX1 and RUNX1/ETO expression constructs were obtained from the Addgene plasmid repository, and have been described previously (Meyers et al., 1995). The pGL3-control plasmid containing the SV40 promoter and enhancer linked to the luciferase gene was purchased from Promega. The pcDNA 3.1 vector was purchased from Invitrogen.

The RUNX1 DNA binding domain construct was generated by amplifying the region equivalent to 1-242 amino acids of RUNX1b, and cloning it into the pSG5 expression vector and was provided by Phillippa Oakford (Oakford et al., 2010). The RUNX1 antisense construct was generated by amplifying the sequence equivalent to 242-453 amino acid of RUNX1b and cloning this region into pSG5 in the reverse orientation, and was provided by Phillippa Oakford.

2.4.3 Generation of LIFR mutant and deletion constructs;

Site directed mutagenesis of RUNX1 binding sites in the LIFR promoters was performed using the QuickChange II XL Kit (Stratagene, Agilent Technologies, U.S.A).

The DNA construct with the insert of interest (general LIFR promoter in pXP1 or placental LIFR promoter in pXP1) was mutated using two oligonucleotides containing the desired mutation. Mutagenesis primers were designed using primer

X software (http://www.bioinformatics.org/primerx/) based on the QuickChange II XL Kit specifications (primers between 25-45bp length and melting temperature >78^{o}C).

Poly A tract regions around the first RUNX1 binding site made it difficult to design primers cover the placental first site using primer X, and therefore a deletion of the placental promoter RUNX1 binding site 1 was performed by sub-cloning to generate the deletion construct using primers which contain *Bam*H1 and *sac*I restriction sites;

For 5'- CTACGGATCCGATTACTCTAATACTAG- 3' and

Rev 5'- CAT CGAGCTCAGCTGGGATTAGGAGTTG- 3'

RUNX1 binding sites in the general and placental promoter (site2, site3) were mutated using the following primers;

RUNX1 binding site Mutation	**Primers**	**Sequences with mutations**
General LIFR RUNX1 TGCGGA to TGCCCA	**LIFRgm1For**	GAGGCCAAGTGAAACTGCCCAAATGGGAACAATGTAC
	LIFRgm1Rev	GTACATTGTTCCCATTTGGGCAGTTTCACTTGGCCTC
Placental LIFR site2 ACCACA to AGGTCA	**LIFRpm2For**	CCCATTACTAGTTCCTTGAAGGTCAATCCAAGACACAATTTGG
	LIFRpm2Rev	CCAAATTGTGTCTTGGATTGACCTTCAAGGAACTAGTAATGGG
Placental LIFR site3 ATGCCA to ATGGGT	**LIFRpm3For**	CTCCAGTCCCTGGGCAATGGGTCACATGTATATATAGCCTC
	LIFRpm3Rev	GAGGCTATATATACATGTGACCCATTGCCCAGGGACTGGAG

Table 2.2 Primers used for site directed mutagenesis.

2.4.4 Site directed mutagenesis;

The mutagenesis primers anneal to the parental DNA template and replicate the plasmid DNA with incorporation of the mutation. PCR was performed using 10ng of the plasmid template in 1 X Quickchange master mix, dNTP (1µl), 125 ng of forward and reverse primer and 3µl volume QuikSolution™ reagent to enhance amplification and 2.5 Units pfu Turbo DNA polymerase in a total volume of 50 ul.

PCR amplification was performed using the following cycle parameters;

95°C 1 minute

95°C 50 seconds
60°C 50 seconds
68°C 7 minutes
} 18 cycles

68°C 7 minutes

The reaction containing parental and mutant DNA was then treated with 2.5 U/µl Dpn I enzyme. Mutated construct was analysed by gel electrophoresis to confirm construct size then transformed into XL10 Gold Ultracompetent cells (Stratagene, Agilent Technologies, U.S.A). Colonies were then screened for the mutated construct using two sets of primers that detect the normal and mutated RUNX1 binding sites. Mutated constructs were also sequenced to confirm the mutation.

2.4.5 Sequence analysis;

Cloned fragments were analyzed by sequencing using Big Dye terminator (Applied Biosystems, U.S.A). Reactions were prepared by adding 200-500ng of plasmid DNA obtained using the Miniprep DNA purification system (Promega), T7 primer (3µM; 5'-TAATACGACTCACTA–3'; Novagen, U.S.A.) and Big dye terminator

sequencing reagents under the following conditions: 95^{o}C for 10seconds, 50^{o}C for 5 seconds, 60^{o}C for 4 minutes for a total of 25 cycles. DNA was purified using 10 µl of CleanSEQ system magnetic beads (Agencourt Product, USA). DNA samples were resuspended in 42µl 85% ethanol and recovered using a magnetic plate. Samples were washed twice with 100µl 85% ethanol and eluted with MilliQ H_2O. Sequences were analysed in-house using the ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

2.5 Reporter assays;

2.5.1 Transfection protocol;

Cell lines were transfected by electroporation as follows. Myeloid cell lines were grown to a density of 1X10^5 cells/ml before transfection. Cells were harvested by centrifugation at 500g for 10 minutes, and then resuspended at a density of 1.5x10^7 cells/ mL in RPMI 1640 culture medium.
Adherent JEG-3 cells were grown to near confluence in 75cm^2 culture flasks. Cells were washed with 10 ml warm 1X PBS followed by 2% trypsin solution (Gibco BRL). Once cells were detached medium with 10% FCS was added to the trypsin solution, transferred to a 50 ml centrifuge tube and washed. The cell pellet was resuspended in 1ml medium and cell concentration was adjusted to 1.5x10^7 cells/ ml in α MEM medium.

Plasmid DNA and 300µl of cells was added to 0.4 cm electroporation cuvettes (BioRad) and electroporated at 270V and 960µFarad. Cells were rested for 5 minutes, fresh culture medium (1ml) added and transferred to flasks containing 5ml medium. Cells were maintained at 37^{o}C and 5% CO2 for 24 hours before luciferase assay analysis for myeloid cells cultures. Adherent cell were maintained for 48 hours before analysis.

2.5.2 Luciferase assay;

Transfected cells were harvested by centrifugation at 500g for 10 minutes then washed twice with 10 ml Phosphate Buffer Saline (PBS) (MP Biomedical Inc.). The cell pellet was resuspended in 100µL of 1x lysis buffer (Promega). Protein concentrations were determined by Bradford assay (Bio-Rad) and 20µg of cell lysate was added to 100 µL of luciferase assay reagent (Promega). Luciferase activity was analysed using the Varitas microplate luminometer (Turner Biosystem).

2.5.3 Bradford Assay;

Protein concentrations of cell lysates were determined by Bradford assay. Bradford stock solution (Bio-Rad) was diluted 1:5 and 1ml added to 10µl of cell lysate. Sample absorbance was determined at 595nm wavelength. Protein concentration of the samples was determined following analysis using a standard curve obtained from BSA standards.

2.6 siRNA transfections;

2.6.1 Transfection of myeloid cell lines with siRNA;

KG-1 myeloid cells maintained at a density of $5x10^5$ cells per ml were harvested at 500g for 5 minutes and the pellet resuspended at $1.5X10^7$ cells/ml in RPMI containing 20% FCS. RUNX1 siRNA (Ambion, U.S.A) targeting RUNX1b (RUNX1 #2904, Sense 5'-GGGAAACUGUGAAUGCUUCTT-3'; Antisense 5'-GAAGCAUUCACAGUUUCCCTC-3') was added to sterile electroporation cuvettes with 300µl of KG-1 cells. siRNA was transfected into the cells at 270V and a capacitance of 975µF using the Gene Pulser Xcell Electroporation Unit (Bio-Rad, U.S.A.). Electroporated cells were incubated in 1ml of RPMI1 1640 medium supplemented with 20% FCS for 5 minutes. Cells were transferred into culture

flasks with fresh RPMI 1640 (10% FCS) and maintained at 37^{o}C, 5% CO2 for 24 hours. Samples were harvested for RNA extraction and cDNA synthesis.

2.6.2 Transfection of adherent cell lines with siRNA;

JEG-3 placental cell lines maintained in 75cm^2 culture flasks (70-100% confluence) were washed and detached with 2% trypsin. Cells were collected in 10 ml centrifuge tubes and harvested at 500g for 5 minutes. The pellet was resuspended in 1XPBS to remove any residual trypsin then resuspended in α MEM media. JEG-3 cells were seeded in 24 well plate at a total density of 2X10^5 cells per well in 500µl medium and left to rest for 5 minutes before transfection. siRNA pools (ON-Target plus) targeting the RUNX1 sequence were purchased from Dharmacon.

ON-TARGET plus SMART pool	Sequence
si RNA J-003926-08	CAAAUGAUCUGGUGGUUAU
si RNA J-003926-07	CGAUAGGUCUCACGCAACA
si RNA J-003926-06	GAAACUAGAUGAUCAGACC
si RNA J-003926-05	UGACAACCCUCUCUGCAGA

Table 2.3 ON-TARGET RUNX1 siRNA sequence

Transfection efficiency was determined using the si*GLO* Transfection Indicator (Dharmacon) and transfection efficiency of 70% routinely achieved.

All sample and controls was treated under similar transfection conditions. To achieve high efficiency in adherent cells siRNA transfection was performed using Attractene Transfection reagent (Qiagen). siRNA (10µM) was diluted in serum free

alpha MEM medium to obtain a final concentration of (100 nM). Attractene (1.5µl) reagent was added to the diluted siRNA in a total volume of 60µl and incubated for 15 minute at room temperature. The siRNA:attractene complex was added to the cells and incubated at 37^{o}C for 48 hours. Each well was washed and harvested by trypsinization, and RNA extracted for cDNA synthesis.

2.7 Chromatin Immunoprecipitation (ChIP) Assay;

2.7.1 ChIP

Myeloid cell lines were grown to a density of 5x10^{5} cells / ml and a total of 2x10^{6} cells collected by centrifugation. Adherent JEG-3 cells were grown to near confluence in 75cm^{2} culture flasks. Cells were washed with 10 ml warm 1X PBS followed by 2% trypsin solution (Gibco BRL). Once cells had detached medium with 10% FCS was added to the trypsin solution transferred to a 50 ml centrifuge tube and the pellet collected by centrifugation at 500g for 5 minutes. The supernatant was aspirated from the pellet and the cell pellets were washed with 1X PBS (10ml). The pellet was then collected by re-centrifugation and resuspended in 15ml warm culture medium.

Cells were treated with 1% formaldehyde to cross link proteins to the DNA on an orbital mixer at room temperature for 10 minutes. Crosslinking was halted by addition of glycine (Sigma-Aldrich, USA) to a final concentration of 0.125M. Samples were incubated for an additional 10 minutes on the orbital mixer at room temperature. Cells were collected by centrifugation at 500g for 5 minutes, the supernatant was aspirated and the pellet washed in cold 1 X PBS (10ml). The pellet was then collected and resuspended again in 1XPBS. A total of 2X10^{6} crosslinked cells were centrifuged at 3000g for 2 minutes at 4^{o}C. Cells were resuspended in 0.250 ml of SDS lysis buffer (final concentration of 1% sodium dodecylsulphate, 10 mM EDTA and 50mM Tris) supplemented with 5µl protease inhibitor cocktail (Roche), and incubated for 10 minutes at 4^{o}C. Cells were sonicated 4 times at 3,300 output for 30 seconds (Microson XL2000 Small Volume

Ultrasonic Processor, Misonix Inc., U.S.A) to generate fragments between 200-400bp. Sonicated DNA was diluted with 1 ml ChIP dilution buffer (0.01 % SDS, 1.2mM EDTA, 16.7 Tris-HCL, 1% TritronX and 167 mM NaCl) supplemented with protease inhibitor cocktail (1µl/1ml, Roche) and pre-cleared with 60µl salmon sperm DNA/protein A agarose slurry (Upstate Biotech) for 2 hours at 4^{o}C. The mixture was cleared by centrifugation at 2000 g for 1 minute, then aliquoted (300 ul) and incubated with Acetyl H3 antibody (2µg; Santa Cruz Biotechnology) or with H3 antibody (2µg; Santa Cruz Biotechnology) or RUNX1 (5µg; Santa Cruz Biotechnology) or RUNX1-ETO (2µg; Santa Cruz Biotechnology) antibodies for . An additional sample of 100 µl of crosslinked DNA was aliquoted as total DNA input and stored at -80^{o}C. A no antibody control sample was prepared in parallel. Aliquoted no antibody and Ab treated samples were then incubated overnight on the orbital mixer at 4C. Antibody bound DNA was then precipitated with 60µL salmon sperm DNA/protein A agarose slurry for 2 hours at 4^{o}C. The agarose was collected by centrifugation at 2000g for 1 minute followed by single washes in each of low salt buffer (0.01% SDS, 2.0mM EDTA, 20mM Tris-HCl,1% Titron X and 150mM NaCl), high salt buffer (0.01% SDS, 2.0mM EDTA, 20mM Tris-HCl, 1% Titron X and 500 mM NaCl), and lithium chloride buffer (1.0 mM EDTA, 10 mM Tris-HCl, 250mM Li Cl, 1% Igepal and 1% deoxycholate). The pellet was then washed twice with Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA). All buffers were supplemented with protease inhibitors (Roche). The DNA was eluted in 200ul elution buffer (1% SDS and 1mM NaHCO3) for 15 minute at room temperature, and the agarose beads removed by centrifugation at 2000g for 1 minute. The elute was collected then treated with 0.2M NaCl and proteinase K overnight at 65^{o}C to reverse the crosslinks. The total input samples were treated in parallel. Samples were purified by phenol/chloroform extraction and recovered by ethanol precipitation, then washed with 70%, air dried and resuspended in 50 µL MilliQ H_2O. The DNA was then analysed by qPCR.

Chapter 3

CHARACTERIZATION OF THE LIFR AND GP130 PROMOTERS

3.1 Introduction;

LIF is a member of the IL-6 cytokine family, which are important modulators of cellular differentiation and the immune response. LIF signals via a heterodimeric complex consisting of a LIF-specific subunit, LIFR, and gp130, which is a subunit shared by other IL-6 family receptors (reviewed in Taga, 1997). Interaction of LIF with its receptor induces activation of the JAK-STAT and ERK signaling pathways leading to gene activation (Boulton et al., 1994). LIF is a polyfunctional cytokine which was initially described as a factor able to induce differentiation of a highly clonigenic murine leukaemic cell line and has since had roles described in the development and activation of certain haemopoietic lineages, osteogenesis, endocrine function and neuronal formation and survival (reviewed in Metcalf, 2003).

The cellular response to LIF is mainly regulated by the expression pattern of its receptor LIFR/gp130 which is influenced by cellular differentiation programs (Blanchard et al., 2002). However, surprisingly, given its broad biological roles, little is known about the regulation of the LIFR gene.

The LIFR gene is regulated by alternate promoters that produce different transcripts (Figure 3.1). The transcriptional regulation of the LIFR gene was first investigated over ten years ago, leading to the identification of a placental specific promoter and enhancer (Wang and Melmed, 1998). The study failed to detect the

placental transcript from non-placental tissue and therefore the existence of an alternative general promoter with broad tissue specificity was predicted.

A subsequent study was able to confirm the placental transcript and promoter but failed to identify the start of the alternate transcript described by Wang and Melmed, 1998. However a CG rich region and alternative transcription start site was identified relative to the predicted sites described by Wang and Melmed (Blanchard et al., 2002). Further analysis of this region led to the description of a general LIFR promoter that is active in a range of cell types and is subject to regulation by epigenetic modifications (Blanchard et al., 2002). Promoter activity was elevated by HDAC inhibition by a mechanism involving CBP/p300-dependent targeting of histone acetylation at the general promoter.

Besides the description of these two promoters, there little known about the regulation of the LIFR gene. The placental LIFR enhancer element contains a number of potential transcription factor binding sites including for GATA, Sp1, NF-κB and OCT-1 (Wang and Melmed, 1998), although functional analysis of these sites has not been conducted. However, a microarray screen performed in our laboratory identified both the LIFR and gp130 genes as novel targets of the RUNX1 transcription factor in immune cells. RUNX1 is an important regulator of haematopoiesis, particularly myeloid differentiation and development and LIF signalling has well described roles in the haemopoietic system, but the expression and regulation of the LIFR and gp130 genes in the haemopoietic system has not been examined.

The aim of this chapter was therefore to examine expression of the LIFR and gp130 genes in the myeloid lineage and investigate LIFR promoter usage in this cell type.

3.2 RESULTS;

3.2.1 LIFR gene and promoter homology;

Previous microarray analysis in our laboratory identified the LIFR and gp130 genes as putative RUNX1 target genes, as expression of both of these genes was decreased in Jurkat T cells in which RUNX1 was knocked down using siRNA (Oakford and Holloway unpublished). To determine whether these genes are likely to be direct targets of RUNX1 a search was undertaken for RUNX1 binding sites in the gene promoters. The LIFR gene is found on human chromosome 5 and as mentioned previously is reported to be regulated by two promoters. Bioinformatic analysis of the two promoters using the Gene2Promoter (Version 2.4) program in the Genomatix Software suite, interrogating -510 to +50 relative to the respective transcription start sites for V$HAML1.01 consensus motifs identified potential RUNX1 binding sites in both promoters. Three potential RUNX1 binding sites were identified in the placental LIFR promoter and a single site in the general promoter (Figure 3.1 and Table 3.1). In addition four potential RUNX1 binding sites were identified in the gp130 promoter (Figure 3.1 and Table 3.1). Further analysis of each binding site using the MatInspector tool was undertaken to gauge the likely affinity of the binding sites, and provides a measure of the similarity of the 'core' region of the binding site, ie the region with the greatest conservation. In addition this analysis provides a 'matrix' similarity score which also considers the nucleotides either side of the core region. Scores greater than 0.90/0.75 for 'core'/'matrix' values generally represent 'good' matches to the consensus sequence and are potentially high affinity sites. Of note, all sites identified in the LIFR/gp130 promoters meet these minimum requirements for potentially high affinity sites (Table 3.1).

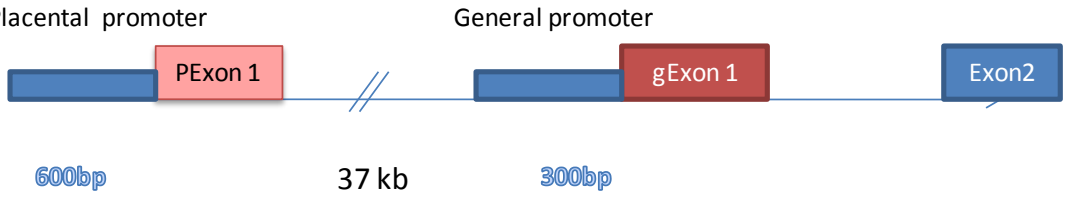
There is increasing evidence that functionally important regulatory elements tend to be conserved across species (Loots and Ovcharenko, 2006). Therefore to provide some insight into the likely functional significance of the RUNX1 binding sites in the LIFR promoters, the evolutionary conservation of the LIFR general and placental promoters was examined using the Evolutionary Conserved Region

browser (ECR, http://ecrbrowser.dcode.org/). The general LIFR promoter has one putative RUNX1 binding site which was aligned across a range of mammalian species (human, monkey, mouse, dog, frog), as well as opossum, chicken and fugu. Sequence analysis indicates complete conservation of the general LIFR RUNX1 binding site across mammals suggesting it is likely to be a functionally significant regulatory element (Figure 3.2). However, analysis of the three predicted RUNX1 binding sites in the placental promoter revealed that these sites are less evolutionarily conserved with only the second site displaying conservation in any of the other species examined (Figure 3.3).

3.2.2 LIFR expression analysis;

LIFR expression has been reported in placental tissue and a range of cell types, but expression of the LIFR gene or promoter usage in haemopoietic lineages has not previously been described. The two LIFR promoters generate different mRNA transcripts; the placental transcript, (variant 2; NM_002310) and the general transcript, (variant 1; NM_001127671). These transcripts contain different non-coding first exons (Figure 3.4) but are otherwise identical. To examine LIFR expression in myeloid cells RNA was isolated from Kasumi-1 myeloid cell line. For comparison RNA was also isolated from HeLa cells, a human cervical carcinoma cell line with epithelial cell-like characteristics which has previously been shown to express high levels of LIFR (Blanchard et al., 2002), and from two placental cell lines (JAR and JEG-3). RNA was reverse transcribed to cDNA and analyzed for LIFR expression by quantitative reverse transcriptase PCR (qRT-PCR), using primers designed to detect the 3'UTR of both LIFR transcripts. In parallel, samples were analyzed with primers designed to the GAPDH housekeeping gene to normalize for variation in cDNA synthesis or the amount of starting material. Amplification was detected by SYBR Green incorporation and data was quantitated using standard curves generated for each PCR product. For each primer set, PCR product was examined by melt curve analysis and agarose gel electrophoresis to ensure the presence of a single PCR product, representative PCR curves and melt curves are shown in Figure 3.7.

A.



B.

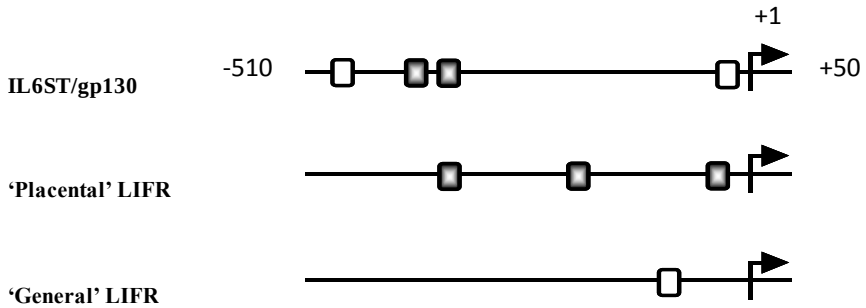


Figure 3.1 (A) Schematic of the LIFR promoters and exon 1 and 2. **(B)** RUNX1 binding sites as predicted using MatInspector (Genomatix). Sites with core similarity score of 1 (black boxes) and 0.9 (white boxes) are shown. The transcription start site is indicated by an arrow.

Gene, promoter	Site	Position	Strand	Core Similarity	Matrix Similarity	Sequence
LIFR; general	1	-98 to -82	(+)	0.909	0.811	aactGCGGaaatggg
LIFR; placental	1	-329 to -315	(+)	1	0.916	tattGTGGtttcag
LIFR; placental	2	-174 to -160	(-)	1	0.809	gattGTGGttcaagg
LIFR; placental	3	-57 to -43	(-)	1	0.767	atgtGTGGcattgcc
gp130	1	-458 to -444	(-)	0.909	0.817	gcctGCGGcctctct
gp130	2	-376 to -362	(-)	1	0.827	ttctGTGGaataacg
gp130	3	-343 to -329	(-)	1	0.820	ctctGTGGgcggaaa
gp130	4	-40 to 026	(+)	0.909	0.811	ctctGCGGagaagga

Table 3.1 Bioinformatically determined RUNX1 binding sites in the LIFR and gp130 promoters identified using the MatInspector tool (Genomatix software suite). Binding sites were identified on both the (+) and (-) strand as indicated, and orientation is not expected to influence the ability of RUNX1 to bind to and activate the promoter

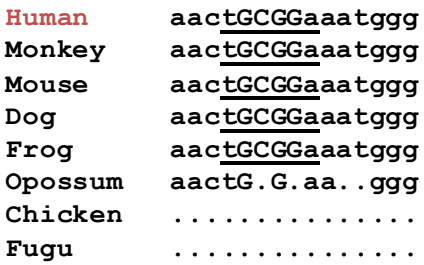


Figure 3.2 Conservation of the RUNX1 binding site in the general LIFR promoter as determined using the Evolutionary Conserved Region Browser (ECR, http://ecrbrowser.dcode.org/) is shown across the indicated species.

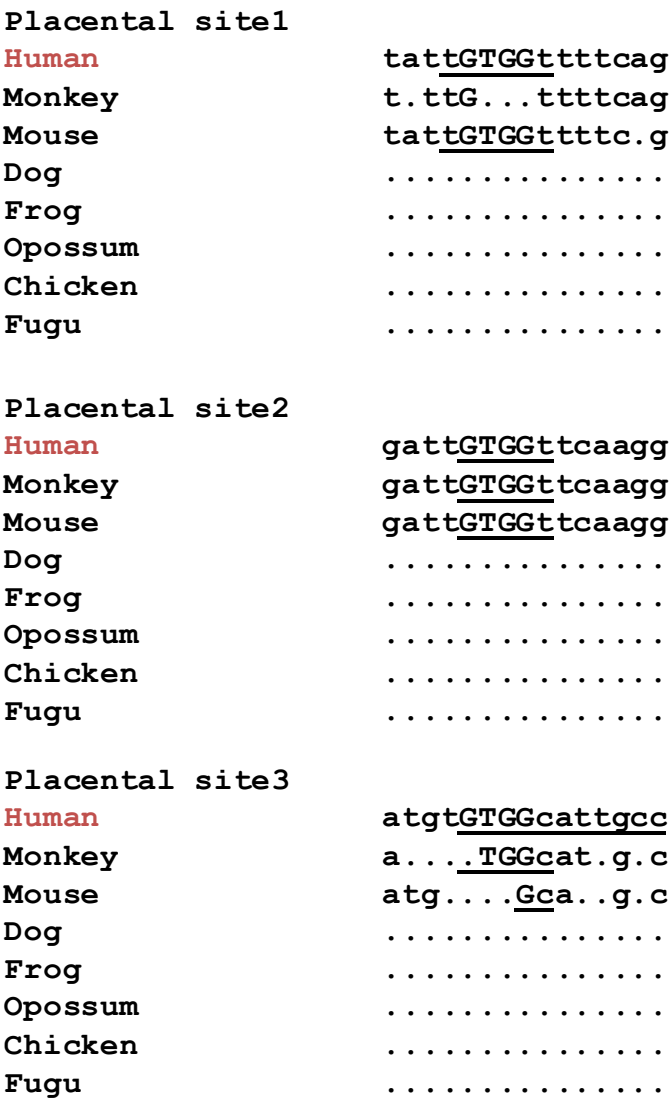


Figure 3.3 RUNX1 binding sites in the placental LIFR promoter. RUNX1 binding site sequences were compared using the Evolutionary Conserved Region Browser (ECR, http://ecrbrowser.dcode.org/) across the indicated species.

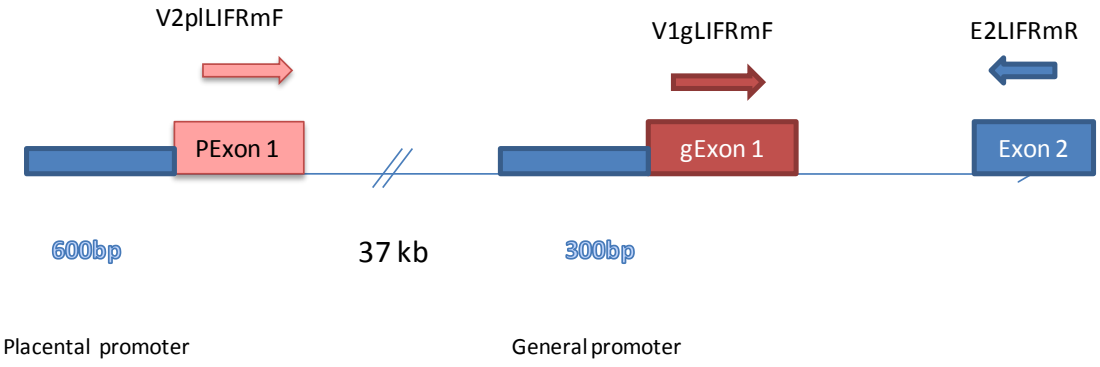


Figure 3.4 The LIFR alternate promoters produce different transcripts. The transcripts can be distinguished by PCR using a forward primer specific to the promoter specific exon 1 and reverse primers designed to the shared exon 2, as shown.

Analysis of the cDNA with primers that detect the 3' UTR of the human LIFR gene (3′UTR hLIFR) and therefore detect LIFR transcript derived from both promoters is shown in Figure 3.5. LIFR mRNA was detected in all cell lines examined, at high levels in the placental cell lines (JAR and JEG-3) compared to HeLa cells. While the Kasumi-1 myeloid cells expressed the LIFR mRNA, levels were low compared to HeLa cells and the placental cell lines (Figure 3.5).

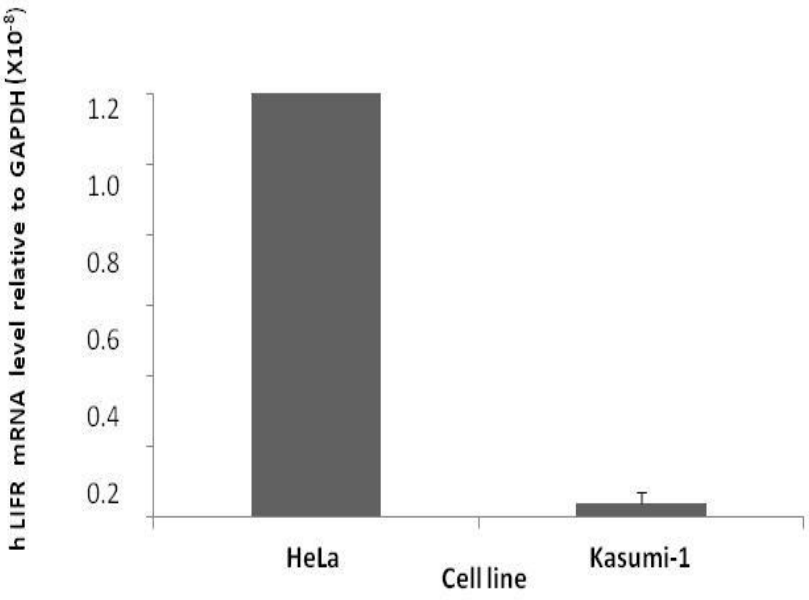
3.2.3 The Variant 1 LIFR transcript is expressed across a range of cell lines;

Expression of LIFR Variant 1 mRNA which is produced from the general promoter was then examined in the different cell lines. RNA isolated from the placental cell lines (JAR and JEG-3), HeLa cells, and the Kasumi-1 cell line, was reverse transcribed and analysed by qPCR using primers specific to the LIFR Variant 1 transcript, with the forward primer designed to the Variant 1 specific exon1 and the reverse primer designed to exon 2 (Figure 3.4). Ct values for samples analysed for LIFR Variant 1 and GAPDH were quantified using standard curves generated from each primer set. GAPDH was analysed as an internal control for each reaction as it is a commonly used housekeeping gene for the analysis of inducible gene expression in the haemopoietic system (eg Oakford et al., J Leuk Res 2010; Lefevre et al., Mol Cell 2008; Hoogenkamp et al., Mol Cell Biol., 2007). GAPDH mRNA levels remained constant in samples treated with PMA and calcium Ionophore (Figure 3.8). Melt curves generated in each run were analyzed for single product generation in each reaction. A representative PCR curve and melt curve are shown in Figure 3.7. High levels of the Variant 1 mRNA were detected in the JAR placental cell line and in HeLa cells, with lower levels detected in the JEG-3 and Kasumi-1 cells. These data therefore suggest that the general LIFR promoter is active in all these cell types, including myeloid and placental cell lines (Figure 3.6).

Expression of the LIFR Variant1 mRNA was then examined across a range of cell lines of myeloid origin including KG-1 and KG-1a (AML), NB4 (APL), K562

(erythroleukaemia), as well as Kasumi-1 cells (AML with t8; 21). LIFR Variant1 mRNA was detected in all cell lines examined (Figure 3.9). In addition a significant increase in LIFR mRNA was detected in Kasumi-1 (p value, 0.0008) and K562 cells, but not the other cell lines, in response to the differentiating agent, PMA and Calcium Ionophore. These observations suggest that LIFR mRNA is expressed in myeloid cells and that this expression is derived from the general LIFR promoter (Figure 3.9).

A.



B.

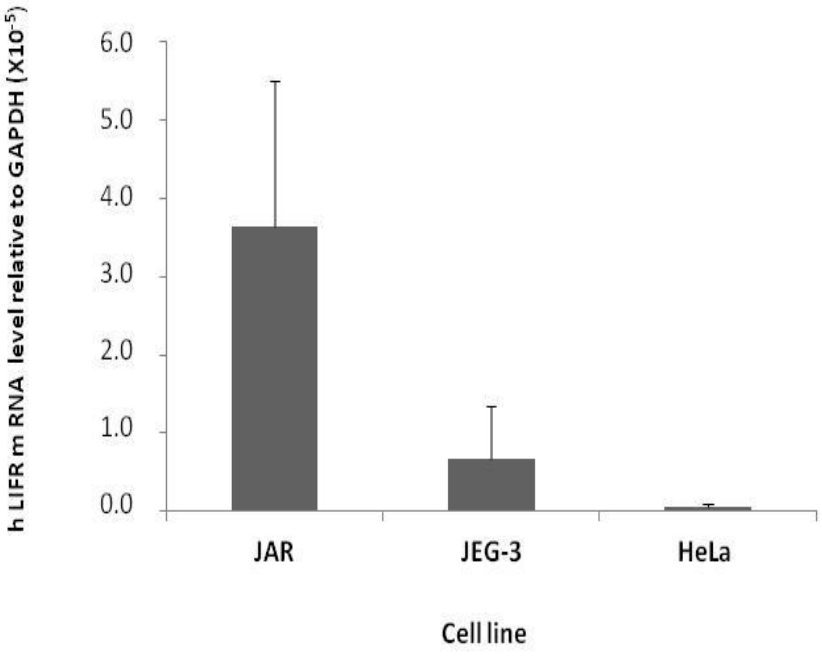


Figure 3.5 Expression of the LIFR gene using primers designed to amplify the 3UTR of both transcripts of the LIFR gene in HeLa and Kasumi-1 cell lines (A) and JAR, JEG-3 and Hela (B), was determined by qRT-PCR. The mean and standred error of the three individual experiments is shown.

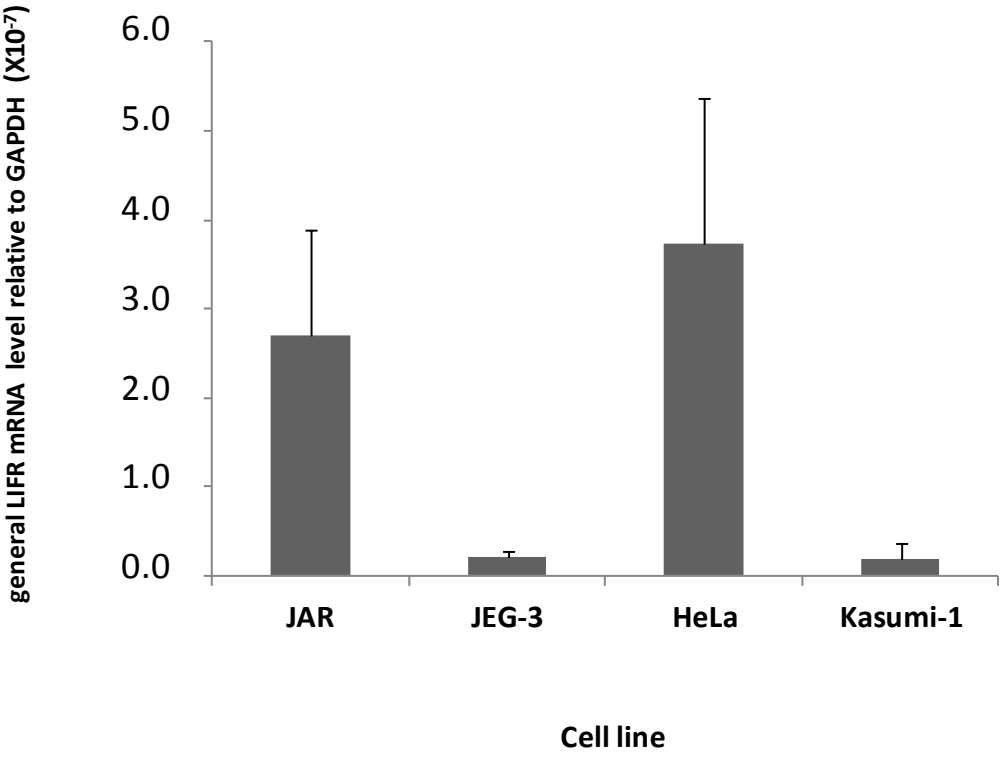
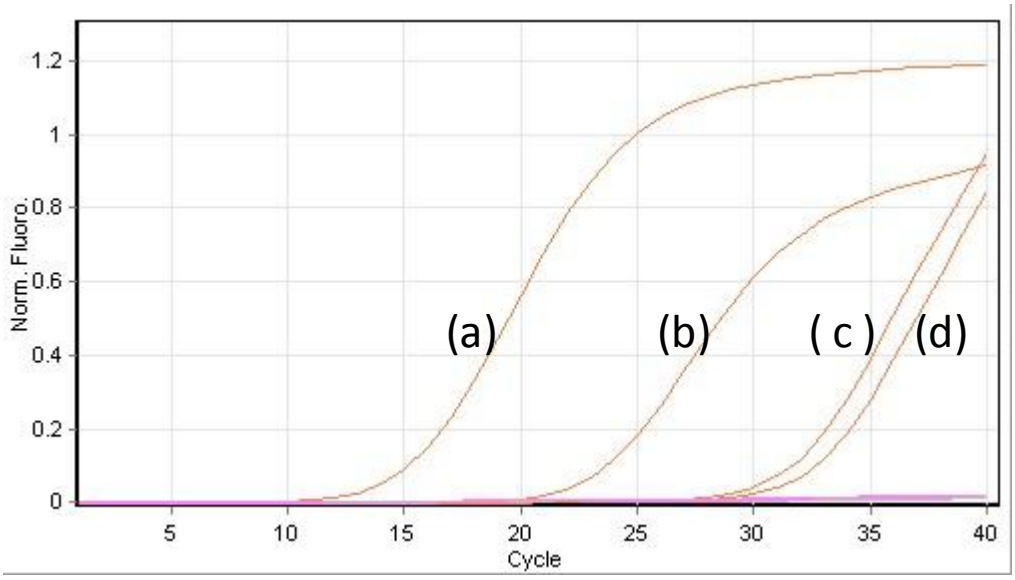


Figure 3.6 Expression of the LIFR Variant1 mRNA in different cell lines. LIFR Variant1 mRNA levels were determined by qRT-PCR in a range of cell lines as specified . Levels of the mRNA of interest were normalized to GAPDH, The mean and standard error of three independent experiments is shown.

A.



B.

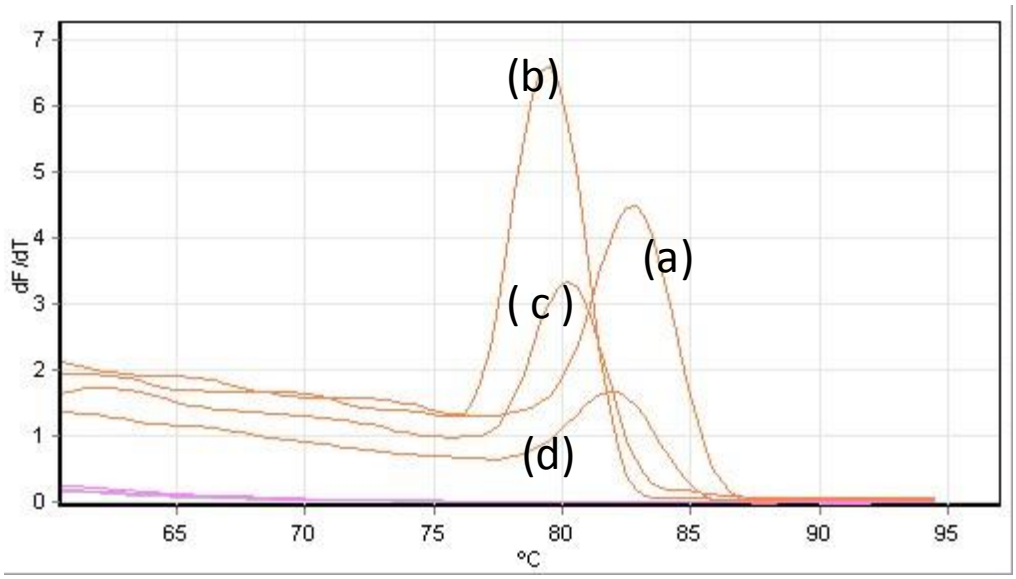


Figure 3.7 qRT-PCR data for the LIFR and GAPDH primer sets. (**A)** Representative qRT-PCR curves are shown for each primer set following analysis of JEG-3 mRNA. (a) GAPDH, (b) 3UTR, (c) placental LIFR, (d) general LIFR. **(B)** Representative melt curves for each primer set are shown following analysis in **A**

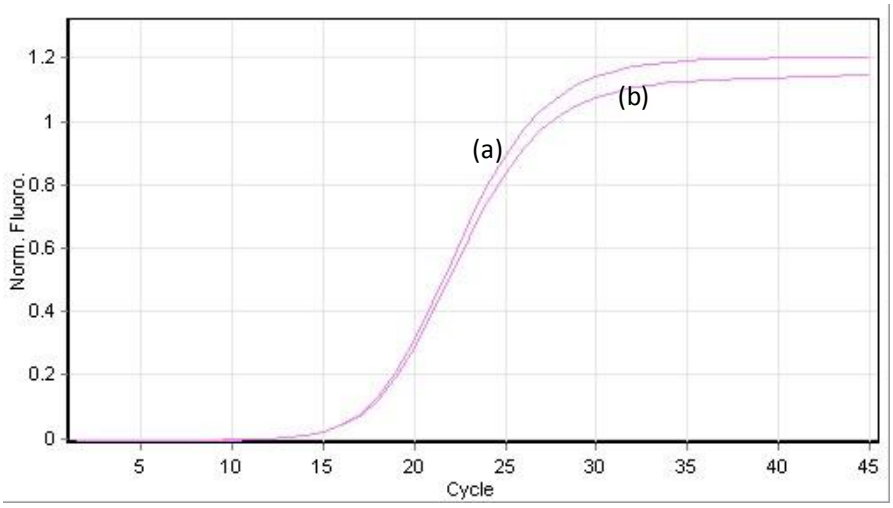


Figure 3.8 Representative qRT-PCR curves for GAPDH primer sets. mRNA from Kasumi-1 cells either unstimulated (a) or stimulated with calcium Ionophore (b) was analyzed by qRT-PCR using GAPDH primers.

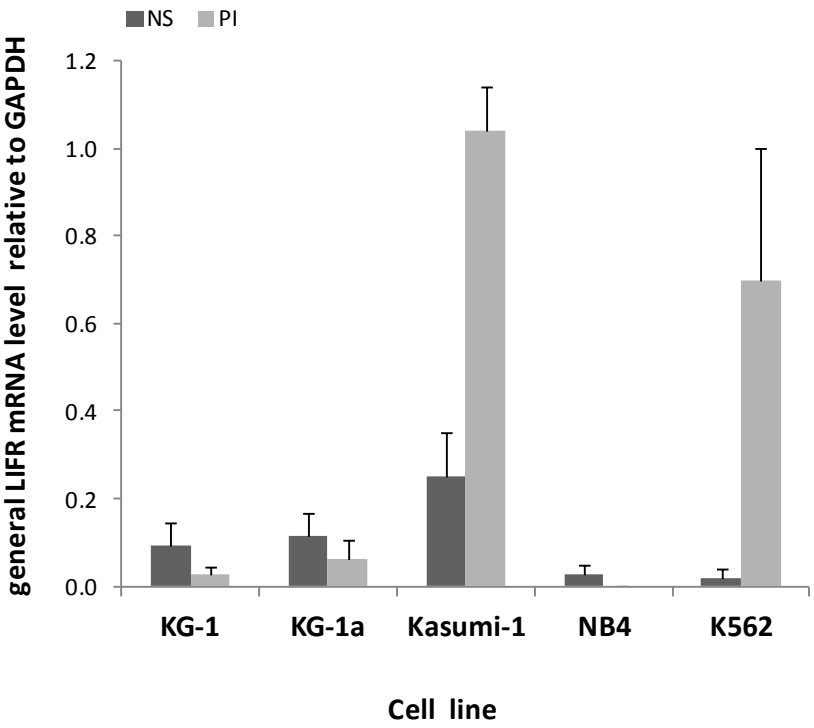


Figure 3.9 Expression of the LIFR Variant1 mRNA in myeloid cell lines. mRNA Levels were determined by qRT-RCR in a range of myeloid cell lines as indicated. Cells were either stimulated (PI) with PMA and calcium Ionphore or unstimulated. The mean and standard error of three independent experiments is shown.

3.2.4 LIFR variant 2 is only expressed in placental cell lines.

LIFR Variant 2 expression was then examined in different cell lines. RNA from the various cell lines was analyzed with primers specific to Variant 2; with the forward primer designed to the Variant 2 specific exon1 and the reverse primer in exon2 (Figure 3.4). A representative PCR curve and melt curve are shown in Figure 3.7.

Variant 2 mRNA was detected at high levels in the placental derived JAR cell lines, at lower levels in the placental JEG-3 cell lines, but was not detected in either HeLa cells or the Kasumi-1 cells (Figure 3.10). These data therefore suggest that, as has been suggested previously (Wang and Melmed, 1998), Variant 2 mRNA is specific to cells of placental origin.

3.2.5 gp130 expression in myeloid cell lines;

gp130 is a component of the LIFR/gp130 receptor complex and its expression is essential for LIF signaling. gp130 expression and promoter activity were first confirmed in HepG2 cells (O Brien et al., 1997). To investigate gp130 expression in myeloid cells, gp130 mRNA was examined in a range of cell lines of myeloid origin including KG-1, KG-1a, NB4, K562, as well as Kasumi-1 cells. Cells were either left unstimulated or stimulated with the differentiating agent PMA and calcium ionophore for 8hrs. cDNA was analysed for gp130 mRNA by qPCR and expression determined relative to the GAPDH housekeeping gene. gp130 mRNA was detected in all of the myeloid cell lines (Figure 3.11). Further, expression levels increased upon stimulation.

Put together these data indicate that both LIFR and gp130 are expressed in myeloid cell lines and that LIFR expression is directed in these cells by the general LIFR promoter.

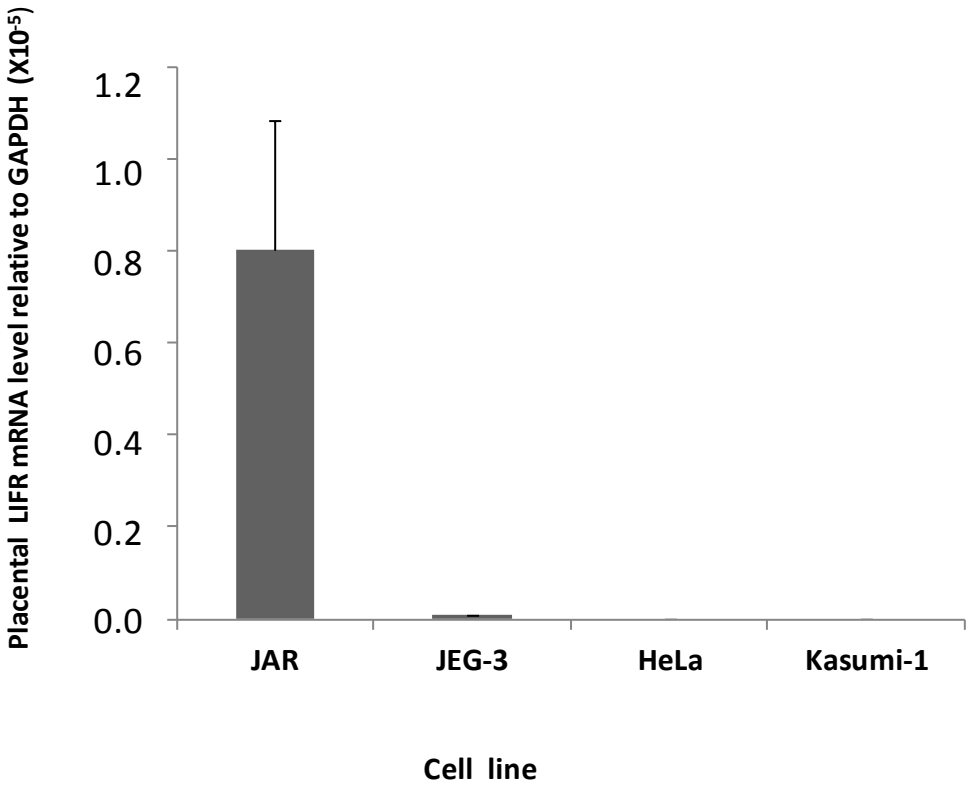


Figure 3.10 Expression of the LIFR Variant 2 mRNA in different cell lines. LIFR mRNA level were determined by qRT-PCR in a range of cell lines as shown. Levels of mRNA of interest were normalized to GAPDH. The mean and standard error of three independent experiments is shown.

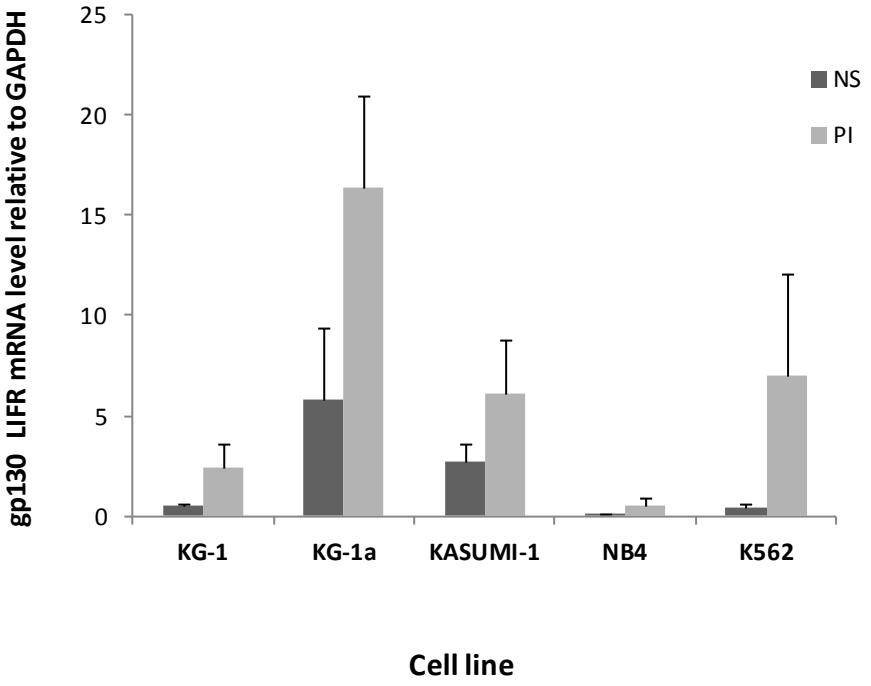


Figure 3.11 gp130 mRNA is expressed in myeloid cell lines. gp130 mRNA was detected in unstimulated cells or stimulated with PMA and calcium Ionophore . The mean and standard error of three independent experiments is shown.

3.2.6 Active chromatin marks are associated with the general LIFR promoter in myeloid cell lines;

The expression analysis outlined above suggests that the general but not placental LIFR promoter is active in myeloid cells. Chromatin structure and core histone modifications can influence the promoter activity with active promoters often associated with particular chromatin features, such as specific histone marks including, lysine acetylation of H3 and H4 (Kurdistani et al.,2004; Hawkin et al., 2006). To determine whether expression of the LIFR transcripts are reflected by the presence of active chromatin features at the respective promoters, the status of the chromatin associated with the LIFR promoters was examined in Kasumi-1 myeloid and JEG-3 placental cells by chromatin immunoprecipitation (ChIP). These cells express the Variant 1 transcript from the general promoter at similar levels (Figure 3.6), but JEG-3 also express Variant 2 mRNA. Cells were treated with formaldehyde to cross link DNA and proteins and the DNA then sonicated into 200-400 bp fragments. The DNA was then precipitated with acetyl histone H3 and core histone H3 specific antibodies, or with no antibody (NA) as a negative control. Precipitated DNA was quantitated then examined by qPCR analysis with primer sets that amplify specific regions within the general and placental LIFR promoters. GAPDH primer sets that amplify an unrelated region of the genome (GAPDH) was analysed as a control (Chapter 2, table 2.1). No antibody samples were amplified with LIFR and GAPDH primers to demonstrate specificity of precipitation of DNA with the histone antibodies, as shown in (Figure 3.12).

To evaluate the level of enrichment at the LIFR promoters in Kasumi-1 cells, DNA samples precipitated with Acetyl H3 (AcH3) were compared to the No Antibody (NA) control sample which represents the level of background binding, precipitated DNA was quantitated relative to the total DNA before precipitation. AcH3 levels at the general LIFR promoter demonstrate approximately 5 fold increase above the NA sample. Precipitated DNA was also amplified by GAPDH primers as a control region, in order to demonstrate AcH3 binding in regions not related to LIFR. These primers amplify an exonic region of the GAPDH gene and

therefore would be expected to have lower acetyl H3 levels than an active promoter. Acetyl H3 (Figure 3.13) and H3 (Figure 3.14) levels were then determined taking into account no antibody controls and total input.

Acetyl H3 levels (Figure 3.13) H3 levels (Figure 3.14) and acetylH3/H3 (Figure 3.15) at the placental promoter were similar in both cell types. However, the general promoter appeared to be hyperacetylated in both cell types, with higher acetylH3 levels detected at the general compared to the placental promoter in both cell lines (Figure 3.13) Although there were lower acetyl H3 levels at the general LIFR promoter in Kasumi cells compared to JEG-3, further analysis showed that this was not statistically significant. Both promoters were analysed for Histone 3 occupancy in the different cell lines. Low H3 histone density across both the general and placental LIFR promoters was observed in Kasumi-1 cells. On the other hand the density of H3 histones was higher in the general LIFR promoter compared to the placental promoter in JEG-3 cells (Figure 3.14). Analysis of acetylated histones relative to the amount of histone H3 across the promoters demonstrate high acetylated histones levels at the general LIFR promoter compared to the placental promoter in Kasumi-1 cells. Similarly higher acetylated histone levels at the general compared to placental promoter in JEG-3 cells were observed although levels were lower than Kasumi-1 cells (Figure 3.15). Therefore increased histone acetylation levels at the general promoter in Kasumi-1 correlates with the expression of only the Variant 1 transcript in these cells. In contrast hyperacetylation of the placental promoter is not observed even in JEG-3 cells in which it is active, suggesting an alternative mechanism of regulation of the promoter.

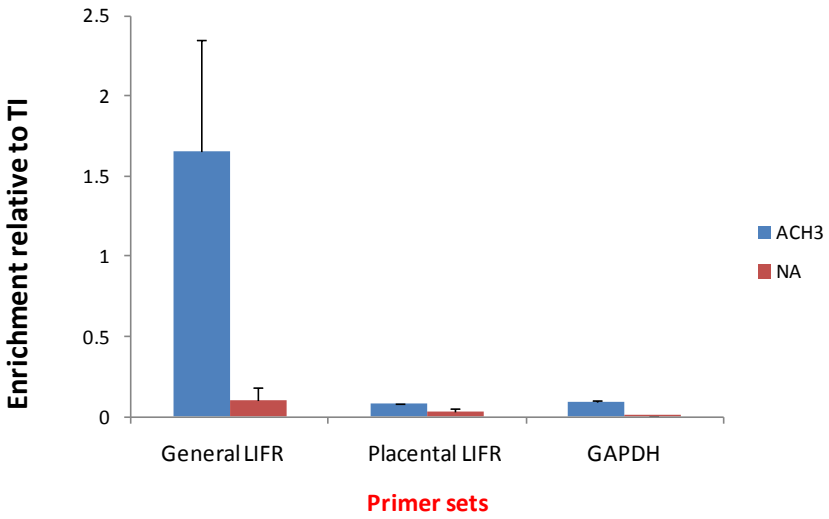


Figure 3.12 ChIP analysis of the LIFR promoters and an exonic region of the GAPDH gene for acetyl H3 (AcH3). Precipitated DNA was measured by qPCR and levels expressed relative to total input DNA, AcH3 enrichment was compared to No antibody (NA) and GAPDH as a region control.

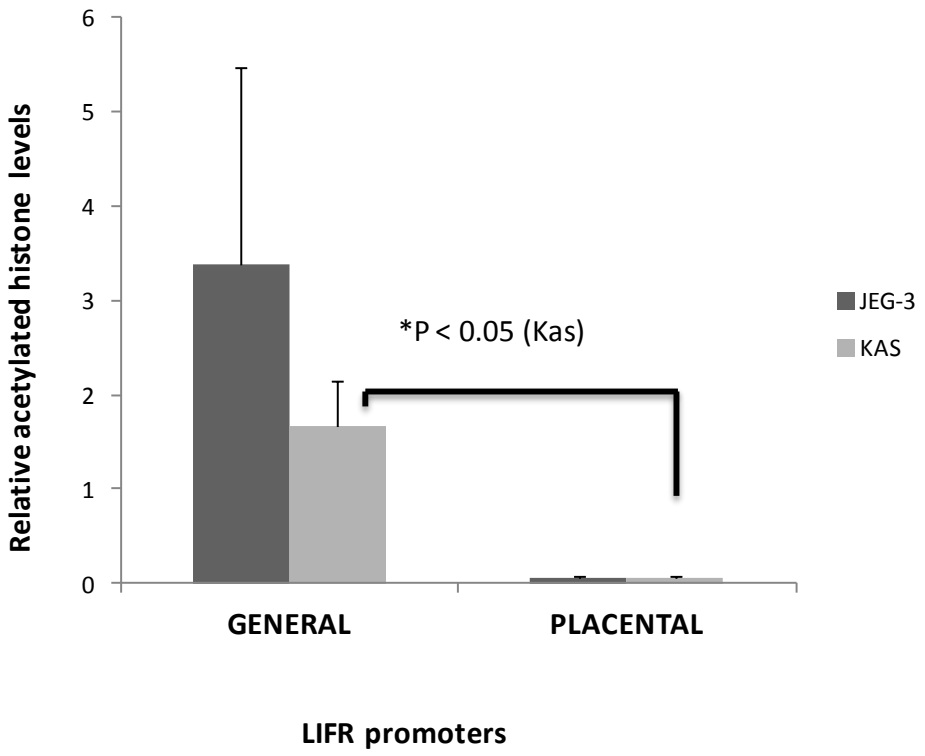


Figure 3.13 The general LIFR promoter is associated with acetylated histones. ChIP analysis was conducted with Acetyl H3 antibodies to monitor histones at the LIFR promoters. Kasumi-1 myeloid cells and JEG-3 placental cells precipitated DNA was measured by qPCR and expressed relative to the no antibody control and total input DNA. The mean and standard error of three independent experiments is shown. *P < 0.05 Student T-test

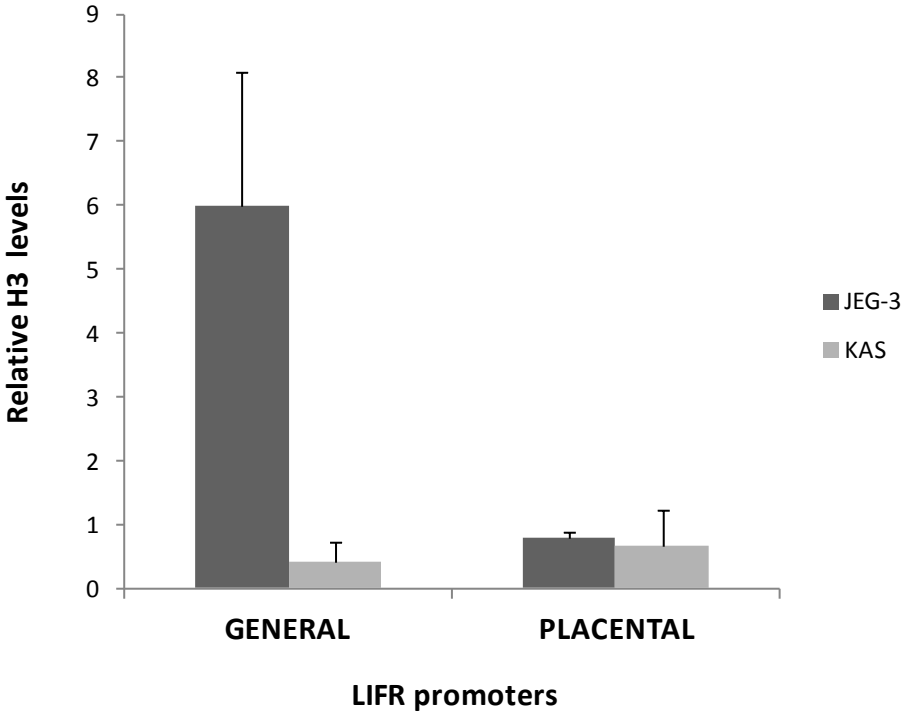


Figure 3.14 LIFR promoter H3 occupancy. ChIP analysis with H3 antibodies was used to monitor histones at the LIFR promoters. Precipitated DNA was measured by qPCR and levels expressed relative to the no antibody control and total input DNA. The mean and standard error of three independent experiments is shown.

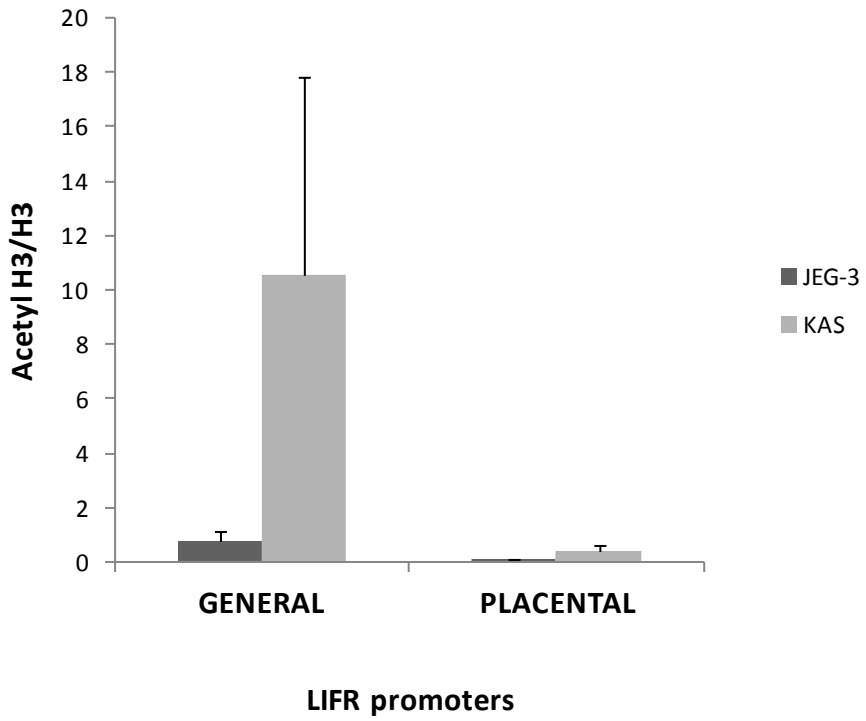


Figure 3.15 Histone acetylation relative to histone density at the LIFR promoters. Histone acetylation (as determined in Figure 3.13) was measured relative to density (as determined in Figure 3.14) in the indicated cell lines. The mean and standard error of three independent experiments is shown.

3.3 DISCUSSION;

Previous studies in our laboratory identified the LIFR and gp130 genes as putative RUNX1 target genes following microarray analysis of cells in which RUNX1 was depleted by siRNA. LIFR has two promoters, and here both of these, as well as the gp130 promoter were shown to contain potential RUNX1 binding sites suggesting they might be direct targets of RUNX1. A single RUNX1 binding site was predicted in the general LIFR promoter, three RUNX1 binding sites in the placental promoter and four predicted sites in the gp130 promoter. Sequence analysis of the RUNX1 binding sites in the LIFR promoters indicate conservation of the general LIFR promoter RUNX1 binding site across mammals suggesting it is likely to be a functional regulatory element, and therefore subject to selective pressure ensuring its evolutionary conservation. Interestingly the three RUNX1 binding sites predicted in the placental LIFR promoter are better matches to the RUNX1 consensus sequence, but are less conserved across species. However, the entire general promoter is relatively conserved between human and mouse (approximately 80% nucleotide conservation) while the placental promoter is not well conserved across species. This may reflect the highly tissue-specific regulation of the LIFR placental promoter and the fact that there is considerable variation in implantation and placentation between species. While LIF has been shown to play an important role in implantation in a number of species, uterus reactions and responses to the LIF cytokine vary between species (reviewed by Vogiagis, 1999; Carter et al., 2010).

Previous studies suggest that the LIFR placental promoter is only active in cells of placental origin, and the general LIFR promoter is relatively ubiquitously active (Wang and Melmed, 1998; Blanchard et al., 2002). Surprisingly though, despite its well characterised role in the haematopoietic system, regulation of the LIFR gene has not been examined in myeloid cells, or any other cell type of the haematopoietic system. The data presented here show that LIFR mRNA is expressed in myeloid cell lines, and further demonstrate for the first time that the general LIFR promoter which drives expression of the LIFR Variant 1 mRNA is active in myeloid cell lines. Expression of the placental LIFR promoter was only

detected in placental cell lines, which confirms its description by Wang and Melmed as a placental-specific promoter (Wang and Melmed, 1998). Interestingly though both cell lines examined expressed the Variant 1 and Variant 2 transcripts, indicating that both the placental and general promoters are active in these cells and suggesting that their regulation is independent.

Here we employed chromatin immunoprecipitation assay to measure histone occupancy and level of histone3 acetylation (AcH3). Two different controls were used; a no antibody control (NA), which many studies use in place of an IgG control (Chen et al., 2005) , and analysis of the GAPDH gene as a control region. High levels of AcH3 were observed at the general LIFR promoter compared to the No antibody control and GAPDH control region. Suggesting an enrichment of Ach3 associated at the LIFR prompter but not the control samples.

Characterization of the chromatin associated with the LIFR promoters in myeloid and placental cell lines by ChIP assay support the interpretations of the transcript expression analysis. Hyperacetylation of the general LIFR promoter was observed in both Kasumi-1 myeloid and JEG-3 placental cell lines, in which the Variant 1 transcript is expressed. While the Variant 1 transcript was expressed at similar basal levels in these two cell types, the chromatin was not identical. While the promoter was hyperacetylated in both cell types, the promoter had reduced histone density in the Kasumi-1 cells, which may reflect the ability of the gene to be upregulated in this cell type in response to stimulation. Despite the differential regulation of the placental promoter in the myeloid and placental cell types, the histones associated with the promoter were less acetylated in both cell types. This suggests that the expression of the two promoters is subject to different regulatory mechanisms. The general LIFR promoter has previously been shown to be subject to epigenetic regulation, with increased expression from this promoter observed in response to HDAC inhibitor treatment (Blanchard et al., 2002) Further, analysis of the LIFR gene identified a large CpG island associated with the general LIFR promoter, which has been show to be regulated by methylation (Blanchard et al., 2002). In contrast, while there is a GC-rich region upstream of the placental promoter, this promoter itself is not located within a CpG island, and in fact is remarkably devoid of CpG dinucleotides, suggesting that it may be less responsive

to epigenetic modification. This is also in keeping with the suggestion that CpG islands are less likely to be associated with highly tissue-specific genes (Illingsworth and Bird, 2009). There are a number of examples in the literature of alternative promoters which are regulated by different mechanisms, with one tissue-specific promoter and a second ubiquitously active promoter. For example, the Prkar 1 gene generates four transcripts that are differentially expressed and regulated by CpG island content in their promoters (Bandy et al., 2011). Analysis in our lab described a large CpG island associated with the general LIFR promoter but not the placental which is suggestive of differential promoter regulation. While the data presented here demonstrate that the general LIFR promoter is active in myeloid cells, there is a dearth of information regarding the transcription factors that regulate this promoter in myeloid or any other cell type. Similarly very little is known about the transcriptional regulation of the placental LIFR promoter or in fact the gp130 promoter.

Chapter 4

REGULATION OF LIFR AND GP130 BY RUNX1

4.1 Introduction;

The RUNX1 transcription factor is an important regulator of haematopoietic gene expression programs, particularly in myeloid cell differentiation. To provide insight into the gene expression programs regulated by RUNX1, our laboratory previously undertook microarray analysis to identify novel RUNX1 targets in the haematopoietic system.

The LIFR and gp130 genes were thus identified as potential RUNX1 targets, and the data presented in Chpater 3 demonstrates that the promoters that control expression of both of these genes contain putative RUNX1 binding sites. Further, both the LIFR and gp130 genes are expressed in myeloid cells (Chapter 3), and analysis of the promoter activity in myeloid cells demonstrates that LIFR expression is derived from the general promoter.

Therefore in this Chapter the aim was to determine whether the LIFR and gp130 genes are regulated by RUNX1 and further whether these genes are direct targets of this transcription factor.

4.2 RESULTS:

4.2.1 The LIFR general promoter is regulated by RUNX1;

The general, but not the placental, LIFR promoter was found to be active in myeloid cell lines (Chapter3), and a potential RUNX1 binding site was detected bioinformatically in the general LIFR promoter (Figure 4.1). Therefore, to determine whether RUNX1 can regulate the general LIFR promoter in myeloid cells, the general LIFR promoter a region of approximately 500bp as characterized previously (Blanchard et al., 2002) was cloned into the pXP1 luciferase reporter vector (section 2.4.2) and introduced into the KG-1 myeloid cell line. Cells were transfected with the general LIFR promoter reporter construct alone or co-transfected with a RUNX1b expression construct (Meyers et al., 1995), which has been shown in these and many subsequent studies to result in increased RUNX1 expression in myeloid cell lines. Cells were harvested 24hr after transfection and luciferase activity determined, with changes in luciferase activity providing a measure of changes in promoter activity. Transfections were performed with reporter alone to detect the basal level of the LIFR promoter and transfection efficiency control (PGL3) in each experiment.

Luciferase activity was compared to the activity of the reporter construct alone. A low basal level of reporter activity was detected from the general LIFR promoter construct alone, with an approximately 3 fold increase in reporter activity detected following co-transfection with the RUNX1b plasmid, suggesting that RUNX1 activates the general LIFR promoter in myeloid cells (Figure 4.2).

Data obtained from each experiment can be graphed as an average of three repeats obtained from one representative experiment (Figure 4.2), or by comparing readings of each experiment to luciferase activity of a control set to 100 value. Since that basal line reading vary between experiments, reporter assay data in this thesis and other similar studies often normalized to control adjusted to100 value (Oakford et al., 2010).

The data in Figure 4.2 is three repeat analyses of one experiment. In reporter assays, particularly in cells that transfect with low efficiency, there is often variability in the basal levels of the reporter between experiments and therefore it is common practice to either present one representative experiment (Holloway et al., 2000), or alternatively graph data obtained from different experiments relative to the control value, (set to at 1 or 100), to normalize the variation between individual experiments (eg Oakford et al., 2010). This second approach was taken here, and data are represented in Figure 4.3. Again a significant increase in reporter activity was observed following transfection of RUNX1 ($P < 0.05$, Student T-test).

The RUNX1 transcription factor is composed of a number of functional domains that influence its transcriptional activity. RUNX1 binds to DNA through the DNA binding domain located at the N-terminal region of the promoter, while a range of transcriptional co-activator molecules are recruited by the C-terminal transactivation domain (Kanno et al., 1998). Previous studies have demonstrated that truncated forms of the protein such as the RUNX1a isoform, lack transactivation activity and can act to block myeloid differentiation by competing with RUNX1 and blocking its normal function (Tanaka et al., 1995).

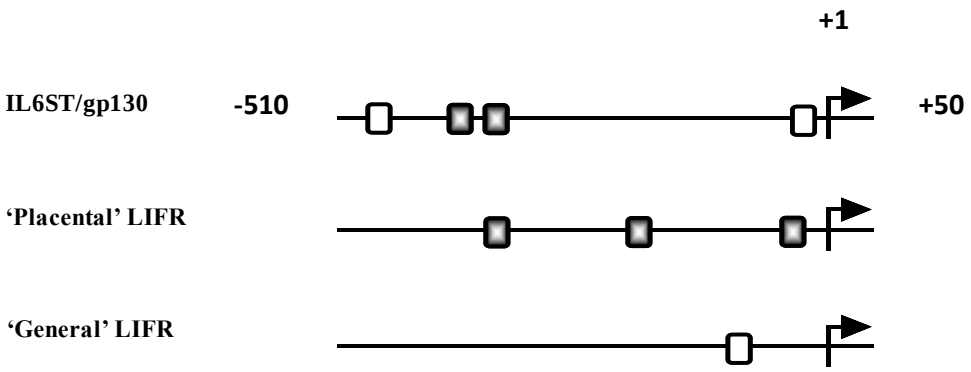


Figure 4.1 Schematic of LIFR/gp130 promoters showing RUNX1 binding sites as predicted using MatInspector (Genomatix). Sites with core similarity score of 1 (black boxes) and 0.9 (white boxes) are shown. The transcription start site is indicated by an arrow.

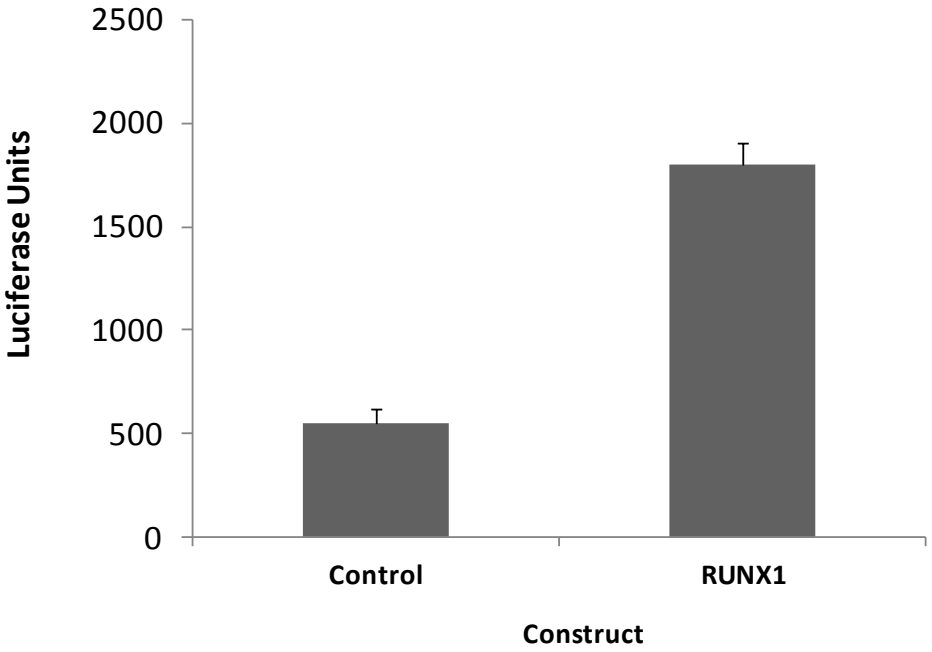


Figure 4.2 RUNX1 activates the general LIFR promoter in myeloid cells. KG-1 cell lines were transfected with the LIFR general promoter luciferase plasmid along with RUNX1 and luciferase activity measured. Un-normalized data was graphed from one representative experiment. The mean and standard error of three readings included in the experiments is shown .

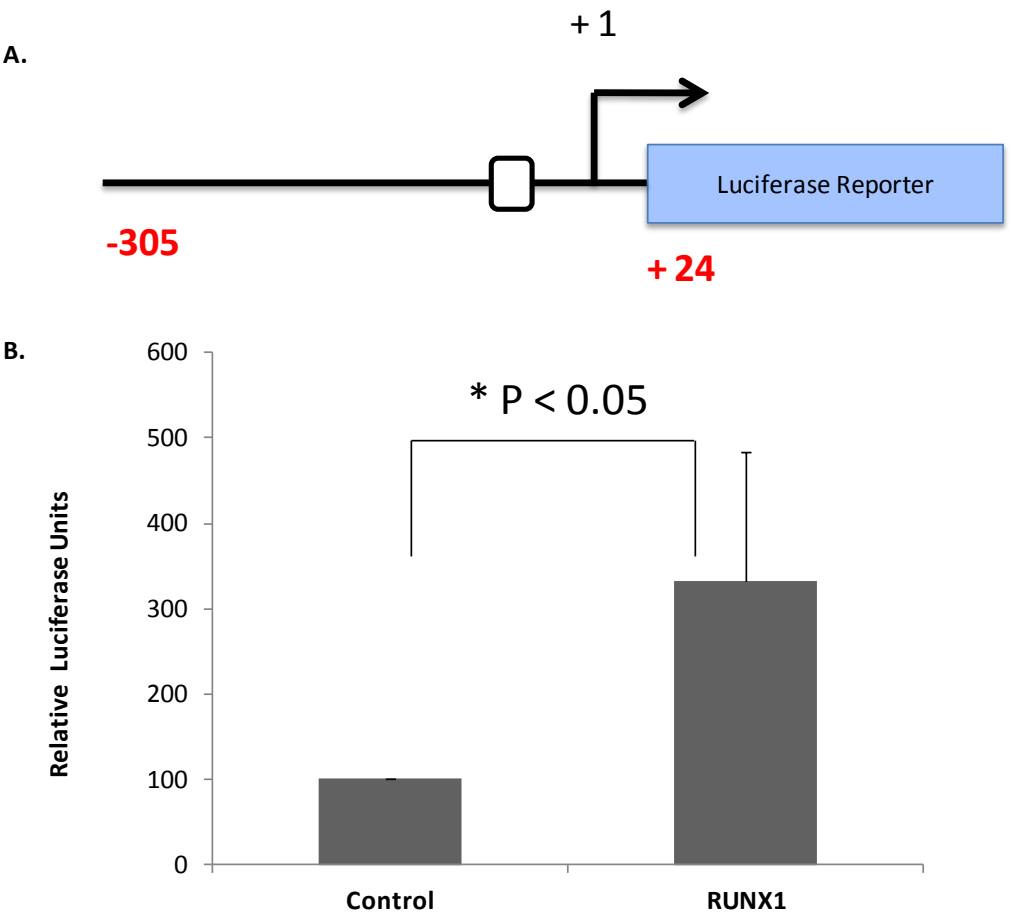


Figure 4.3 **(A)** RUNX1 activates the general LIFR promoter in myeloid cells. **(A)** The general LIFR promoter was cloned into the pXP1 luciferase reporter. **(B)** KG-1 cell lines were transfected with the LIFR general promoter luciferase plasmid along with RUNX1 and luciferase activity measured. *P < 0.05, Student T-test. The mean and standard error of three independent experiments is shown.

To investigate the requirement for the different RUNX1 domains for general LIFR promoter activation by RUNX1, KG-1 cells were transfected with the general LIFR promoter reporter construct along with plasmid expressing only the DNA binding domain of RUNX1 which has been characterized previously (amino acids 1-242 of RUNX1; Oakford et al., 2010). Cells were analysed by reporter assay 24 hr after transfection. As seen before overexpression of the full-length RUNX1b construct activated the general LIFR promoter in the KG-1 myeloid cell line (Figure 4.2, 4.3). In contrast overexpression of the RUNX1 DNA binding domain had no effect on the LIFR promoter with activity of the general LIFR promoter remaining similar to the basal levels observed for the promoter construct alone (Figure 4.4). To confirm the effect of RUNX1 on the general LIFR promoter, an antisense construct containing the DNA sequence equivalent to RUNX1 transactivation domain of RUNX1b (amino acids 242-453) in reverse orientation (Oakford, 2008) was transfected in KG-1 cells. Co-expressing the antisense RUNX1construct decreased general LIFR promoter activity by approximately 2 fold ($P < 0.05$, Student T-test) confirming that the promoter is responsive to RUNX1 levels (Figure 4.4)

The reporter assay data presented above suggests that RUNX1 regulates the LIFR promoter. RUNX1 regulates its target genes by recognizing and binding to a specific DNA sequence, the consensus motif being 5-TGT/CGGT-3, in gene promoters. The general LIFR promoter contains a single putative RUNX1 binding site (Figure 4.5; Chapter 3 Table3.1) and therefore it is likely that RUNX1 regulates the LIFR promoter via this site. To test this, site directed mutagenesis of the putative RUNX1 binding site in the general LIFR promoter luciferase construct was performed (Figure 4.5A). Mutation of the site from TGCGGA to TGCCCA was confirmed by sequencing. The KG-1 cell line was then transfected with the wild-type and mutated general LIFR promoter reporter construct, either alone or with the RUNX1 expression construct. Luciferase activity was compared to the basal activity of the wild-type general LIFR construct alone. Basal activity of the

general LIFR reporter construct was not altered by mutation of the putative RUNX1 binding site.

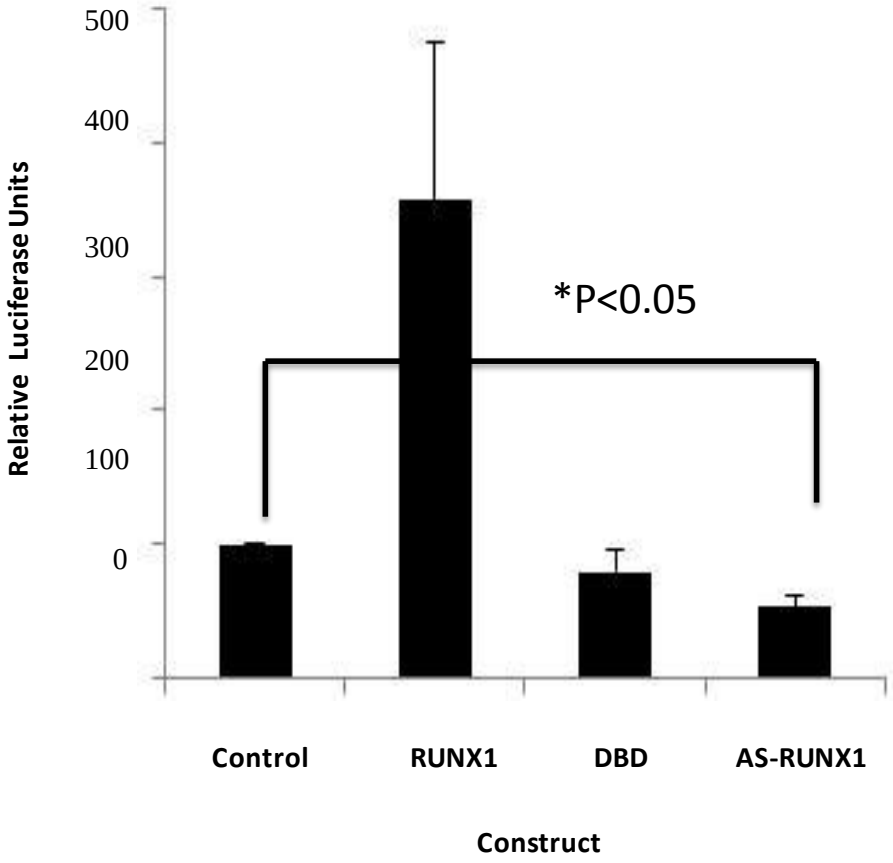


Figure 4.4 Regulation of the LIFR general promoter by RUNX1 is dependent on the transactivation domain. The KG-1 cell line was transfected with a LIFR general promoter luciferase plasmid along with RUNX1 , RUNX1 DNA Binding Domain (DBD) and RUNX1 antisense construct (AS-RUNX1), luciferase activity was measured. The mean and standard error of three independent experiments is shown.

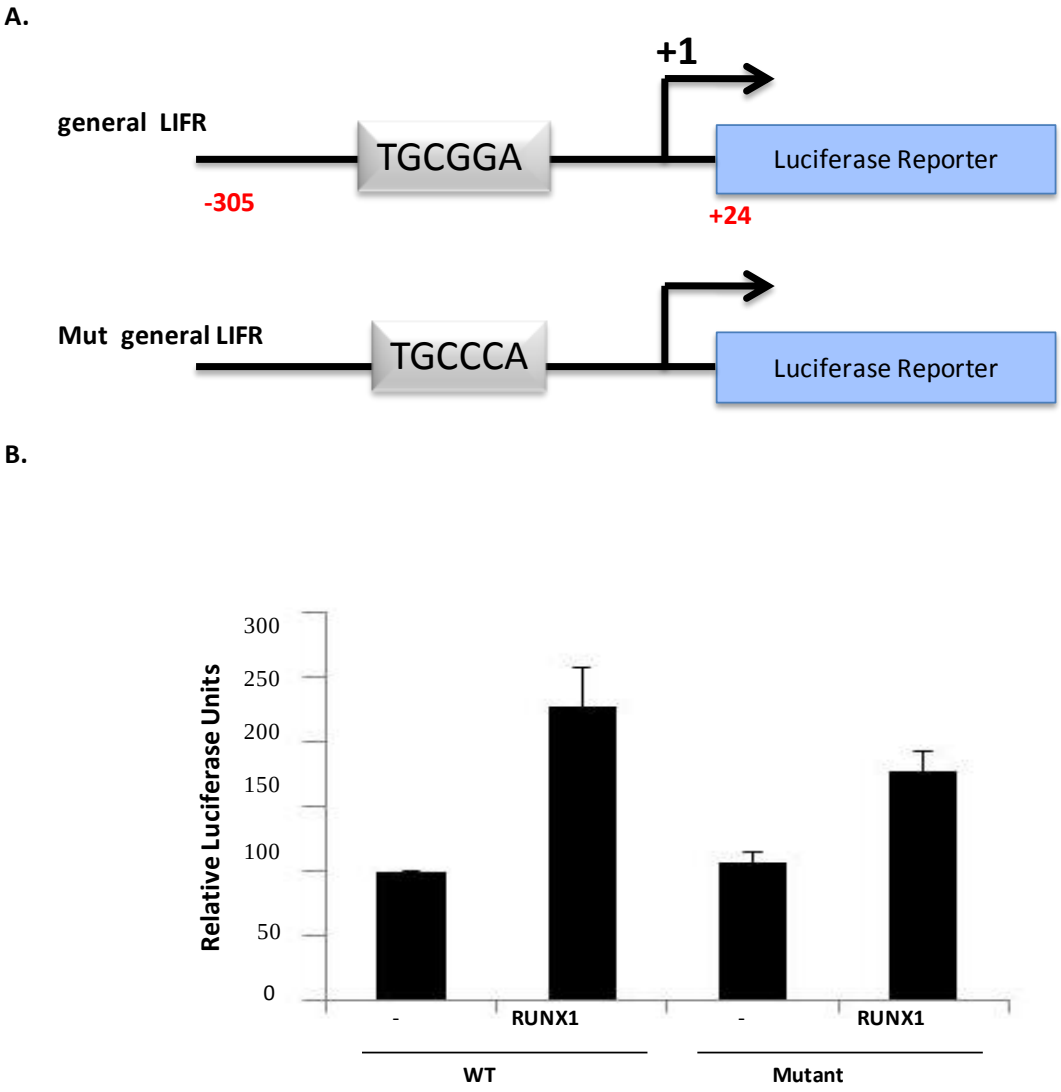


Figure 4.5 The RUNX1 binding site in the general LIFR promoter contributes to activation by RUNX1. **(A)** Schematic of the wildtype (WT) and mutated (Mut) general LIFR promoter reporter construct**. (B)** KG-1 cells were transfected with a LIFR general promoter luciferase plasmid along with RUNX1. In parallel, cells were transfected with reporter construct in which the RUNX1 binding site was mutated as shown in (A). Luciferase activity was measured in the transfected cells. The mean and standard error of three independent experiments is shown.

However activation of the LIFR promoter by RUNX1 decreased when the putative RUNX1 binding site was mutated, although it did not completely abolish activation of the promoter by RUNX1 (Figure 4.5B). The responsiveness of the mutated promoter to RUNX1 is most likely due to the fact that many cryptic TGTGG RUNX1-like elements exist in the pXP1 backbone vector or that the two base pair mutation did not fully abolish RUNX1 binding. This could be further examined using band shift assays. This data therefore suggests that the bioinformatically determined binding site is functional and that RUNX1 can activate the general LIFR promoter by binding to this RUNX1 binding site in the promoter.

4.2.2 RUNX1-ETO protein represses the general LIFR promoter;

In leukaemia RUNX1 is a frequent target of mutation and chromosomal rearrangement, the most common being the t(8,21) translocation in acute myeloid leukaemia. The translocation generates the RUNX1-ETO fusion protein which is expressed concurrently with RUNX1 in leukemic cells. RUNX1 has generally been described as an activator and RUNX1-ETO as a constitutive repressor, however RUNX1-ETO can activate gene expression in certain circumstances and for example has been found to activate the M-CSFR promoter (Shimizu K et al., 2000). The effect of RUNX1-ETO on the general LIFR promoter was therefore explored in reporter assays. KG-1 myeloid cells were again transfected with the general LIFR promoter luciferase construct either alone or along with RUNX1 or RUNX1-ETO expression constructs (Meyers et al., 1995). Cells were harvested after 24hrs to determine the effect of RUNX1 and RUNX1-ETO on LIFR promoter activity. As described before RUNX1 was able to activate the general LIFR promoter. In contrast co-expressing RUNX1-ETO with the general LIFR promoter decreased its activity by approximately two fold ($P < 0.05$, Student T-test), below the basal level of the general LIFR promoter alone (Figure 4.6). These data suggest that while RUNX1 activates the LIFR promoter, RUNX1-ETO has a repressive effect on the general LIFR promoter.

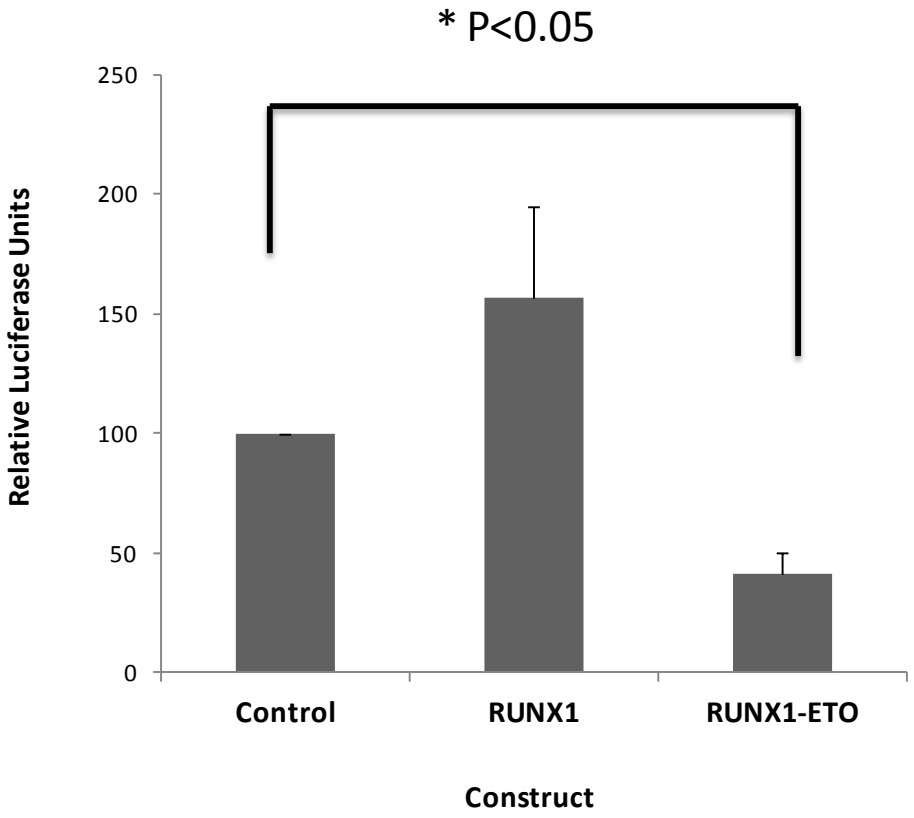


Figure 4.6 The general LIFR promoter is regulated by RUNX1 and RUNX1-ETO. KG-1 cells were transfected with a LIFR general promoter luciferase plasmid either with RUNX1 or RUNX1-ETO expression plasmids and luciferase activity measured. The mean and standard error of three independent experiments is shown.

The Kasumi-1 cell line was originally derived from a 7-year-old Japanese boy with AML (Asou H et al., 1991) and contains the t(8,21) translocation and therefore is assumed to expresses both RUNX1 and RUNX1-ETO. To confirm this RUNX1 mRNA levels were examined in Kasumi-1 as well as KG-1a and KG-1 cell lines using primers designed to detect mRNA transcript of RUNX1 and RUNX1-ETO (Chapter 2; Table 2.1). As expected RUNX1 mRNA was detected in all cell lines (Figure 4.7A). In contrast RUNX1-ETO transcript was only detected in Kasumi-1 cells (Figure 4.7B). RUNX1-ETO protein expression was confirmed by western blot in Kasumi-1 cells (Oakford et al., 2010).

To determine whether RUNX1 can relieve the repressive effect of RUNX1-ETO on the general LIFR promoter, Kasumi-1 cells which contain the t(8,21) translocation and expresses RUNX1-ETO were transfected with the general LIFR promoter and increasing concentrations of RUNX1 expression plasmid. Over expression of RUNX1 in Kasumi-1 cells increased general LIFR promoter activity in cells containing RUNX1-ETO, in a dose dependent manner. This suggests that RUNX1 can compete with RUNX1-ETO in leukemic cells to activate the LIFR promoter (Figure 4.8).

The RUNX1 dose response curve in Figure 4.8 was not linear, but this would not be expected since the cells already contain endogenous RUNX1. Analysis of the effect on the endogenous LIFR promoter was not possible due to the low transfection efficiency of these cells.

4.2.3 RUNX1 regulates the gp130 promoter;

gp130 is an essential component of the receptor complex that mediates LIF signaling. Like the LIFR gene, gp130 was identified as a putative RUNX1 target gene in microarray experiments in which expression of gp130 was found to be reduced in cells transfected with RUNX1 siRNA. In addition a number of putative RUNX1 binding sites were identified in the gp130 promoter. The effect of RUNX1 on the gp130 promoter in myeloid cells was therefore analysed. The human gp130

promoter; a region of 526bp described previously (O Brien et al., 1997) was cloned into the pXP1 luciferase reporter vector (section 2.4.2). The gp130 promoter reporter construct was transfected into KG-1 myeloid cells and luciferase activity was determined alone or following co-transfection with the RUNX1b expression construct. While low luciferase activity was observed by expressing gp130 reporter alone, gp130 reporter activity was increased significantly following co-expression of RUNX1b plasmid ($P < 0.05$, Student T-test) and therefore RUNX1 can activate the gp130 promoter in myeloid cells (Figure 4.9).

Further, the gp130 promoter construct was transfected alone into KG-1 myeloid cells or with constructs expressing either RUNX1-ETO or RUNX1b. Luciferase activity was compared with the basal activity of the gp130 promoter alone. While as before RUNX1 increased the gp130 promoter activity, co-expressing RUNX1-ETO decreased gp130 activity by 2 fold ($P < 0.05$, Student T-test) and therefore while RUNX1 activates the gp130 promoter RUNX1-ETO represses gp130 promoter activity (Figure 4.10).

To investigate whether RUNX1 regulates the gp130 promoter in leukemic cells. Kasumi-1 cells containing RUNX1-ETO fusion protein were transfected with gp130 construct and different concentrations of RUNX1 expression construct. RUNX1 activated the gp130 promoter in cells containing the RUNX1-ETO fusion protein in a dose dependent manner suggesting that RUNX1 can compete with RUNX1-ETO to activate the gp130 promoter (Figure 4.11).

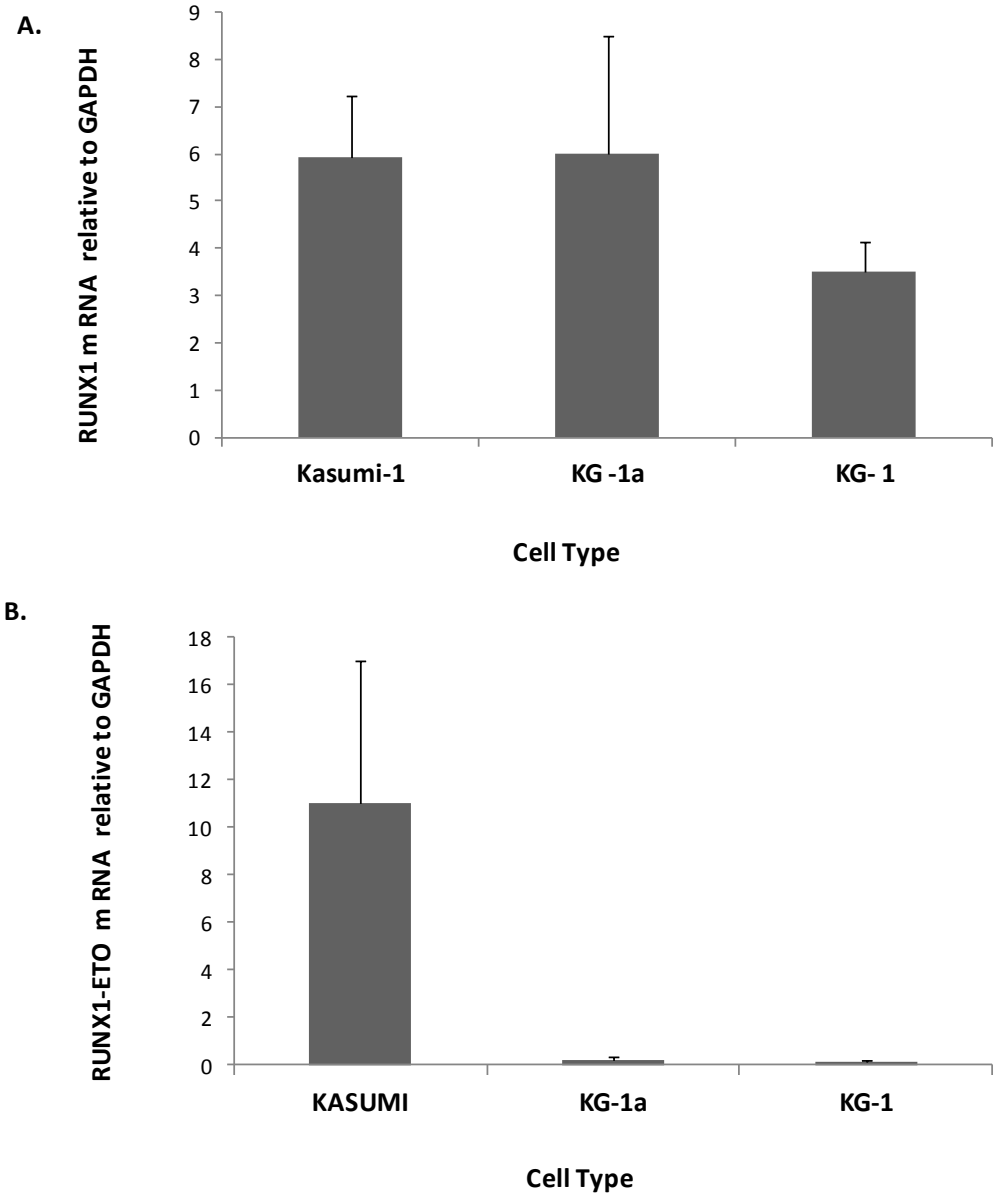


Figure 4.7 mRNA levels of **(A)** RUNX1 and **(B)** RUNX1-ETO were determined by q RT-PCR . Levels of the mRNA of interest were normalised to GAPDH. The mean and standard error of three independent experiments is shown in both cases.

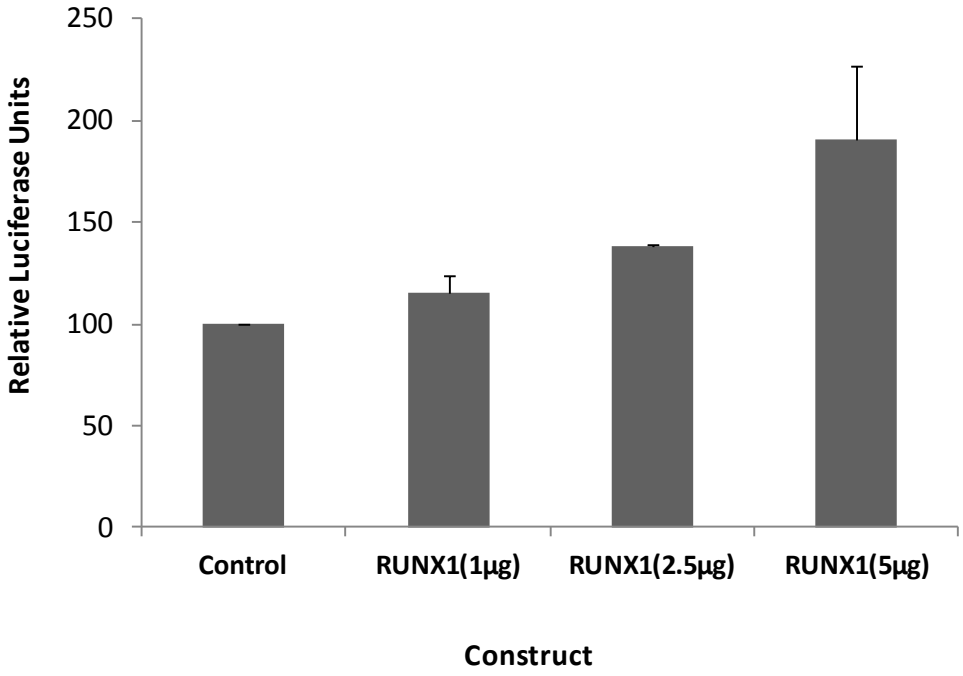


Figure 4.8 Overexpresion of RUNX1 increases LIFR promoter activity. Kasumi-1 cells were transfected with a LIFR general promoter reporter plasmid and different amounts of RUNX1 expression plasmid and luciferase activity measured. The mean and standard error of three independent experiments is shown.

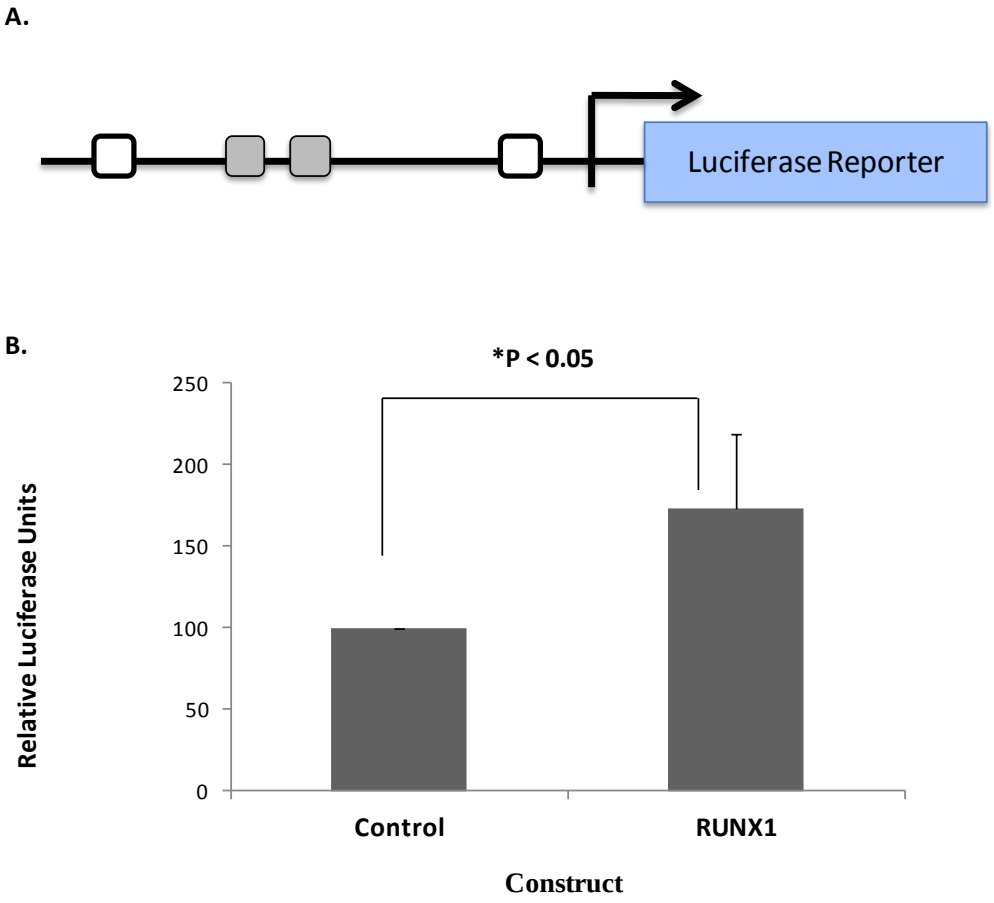


Figure 4.9 RUNX1 activates the gp130 promoter in myeloid cells. **(A)** The gp130 promoter was cloned into pXP1 luciferase reporter**. (B**) KG-1 cell were transfected with the gp130 promoter luciferase plasmid along with RUNX1. The mean and standard error of three independent experiments is shown, *P < 0.05 Student T-test

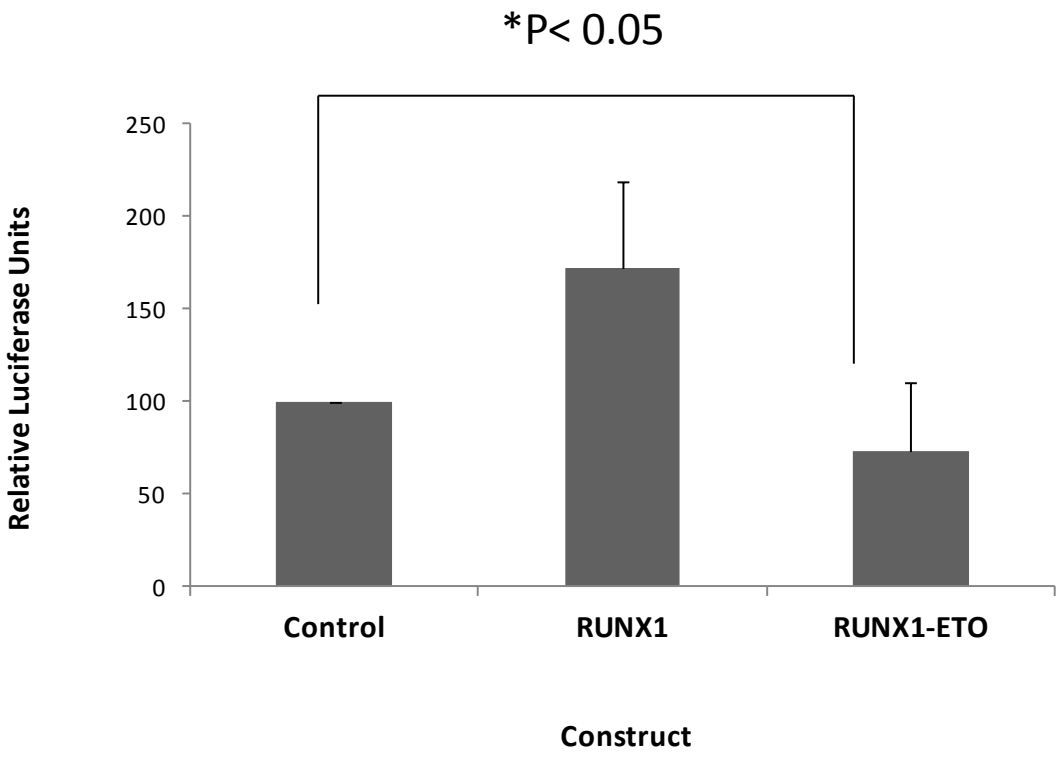


Figure 4.10 RUNX1-ETO represses the gp130 promoter in myeloid cells. KG-1 cells were transfected with gp130 promoter reporter plasmid alone or with RUNX1 or RUNX1-ETO expression plasmid and the luciferase activity measured. The mean and standard error of three independent experiments is shown.

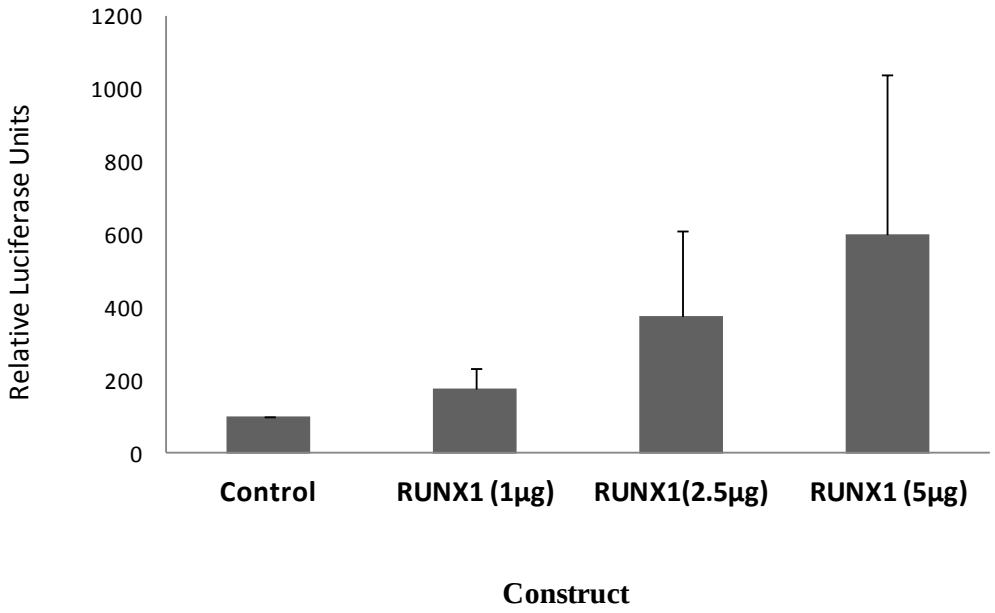


Figure 4.11 Overexpression of RUNX1 activate the gp130 promoter in myeloid cells. Kasumi-1cells were transfected with the gp130 promoter reporter plasmid and different amounts of RUNX1 expressing plasmid and the luciferase activity measured. The mean and standard error of three independent experiments is shown.

4.2.4 The endogenous LIFR and gp130 genes are regulated by RUNX1 in myeloid cells;

The data presented thus far demonstrates that both the general LIFR promoter and the gp130 promoter are regulated by RUNX1, suggesting that the LIFR receptor complex is likely to be subject to regulation by this transcription factor.
To confirm the effect of RUNX1 on the endogenous LIFR promoter, siRNA mediated knock down of RUNX1 was established in myeloid cells (Figure 4.12). KG-1 cells were transfected with RUNX1 siRNA specifically designed to target RUNX1b, or with siRNA control (Ambion, USA). A control siRNA which does not target any known human genes. Cells were transfected with 100nM siRNA by electroporation then RNA harvested after 24hrs. RUNX1 mRNA levels were determined by quantitative RT-PCR and normalized to GAPDH. A reduction of RUNX1 mRNA of 70% was detected in the RUNX1 siRNA treated samples compared to the cells transfected with siRNA control (Figure 4.12). Samples were then analysed with primers that detect the 3′ UTR of the LIFR gene, to determine the effect of RUNX1 knockdown on LIFR expression. Depletion of RUNX1 decreased LIFR promoter expression by 3 fold in stimulated KG-1cells (Figure 4.13), suggesting that RUNX1 regulates endogenous LIFR gene expression in myeloid cells.

RUNX1 effect on endogenous gp130 gene expression was also analysed in myeloid cells that had been treated with RUNX1 siRNA. mRNA from RUNX1 and control siRNA transfected cells was analyzed by qRT-PCR with primers to detect gp130 mRNA. The data demonstrate that depletion of RUNX1 decreases gp130 expression by almost 2 fold suggesting that the endogenous gp130 promoter is also regulated by RUNX1 in myeloid cells also (Figure 4.14).

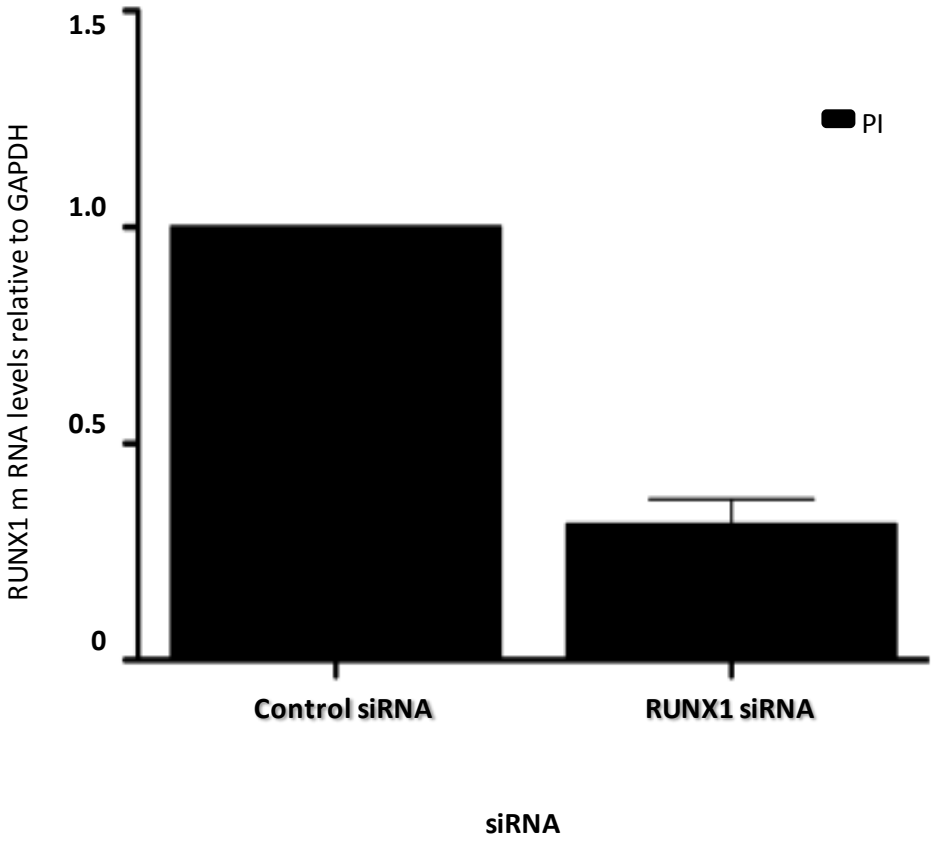


Figure 4.12 Percentage knock down of RUNX1 in myeloid cell lines. KG-1 myeloid cells were transfected with RUNX1 siRNA. RUNX1 mRNA expression was analyzed by qRT-PCR in cells stimulated with PMA and calcium ionphore for 8 hour. RUNX1 levels are shown compared to the control siRNA transfected cells which was set to 1, the mean and standard error of three independent experiments is shown.

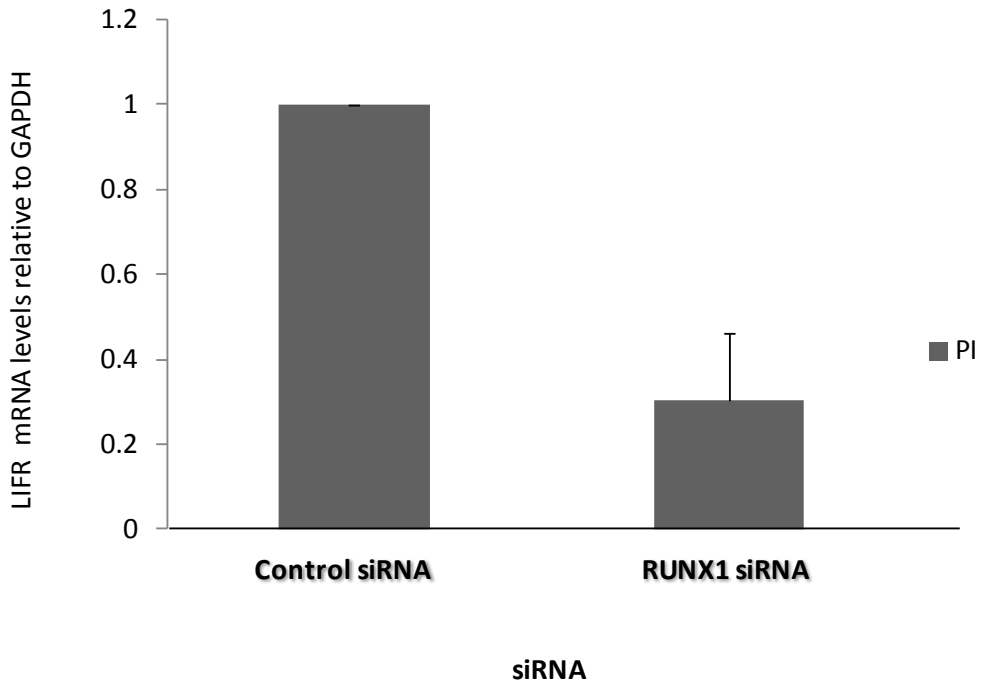


Figure 4.13 Regulation of the LIFR endogenous gene by RUNX1. KG-1 myeloid cells lines were transfected with RUNX1 siRNA . LIFR mRNA expression was analyzed by qRT-PCR in cells stimulated with PMA and calcium ionphore for 8 hours. LIFR levels are shown relative to control siRNA transfected was set to 1, the mean and standard error of three independent experiments is shown.

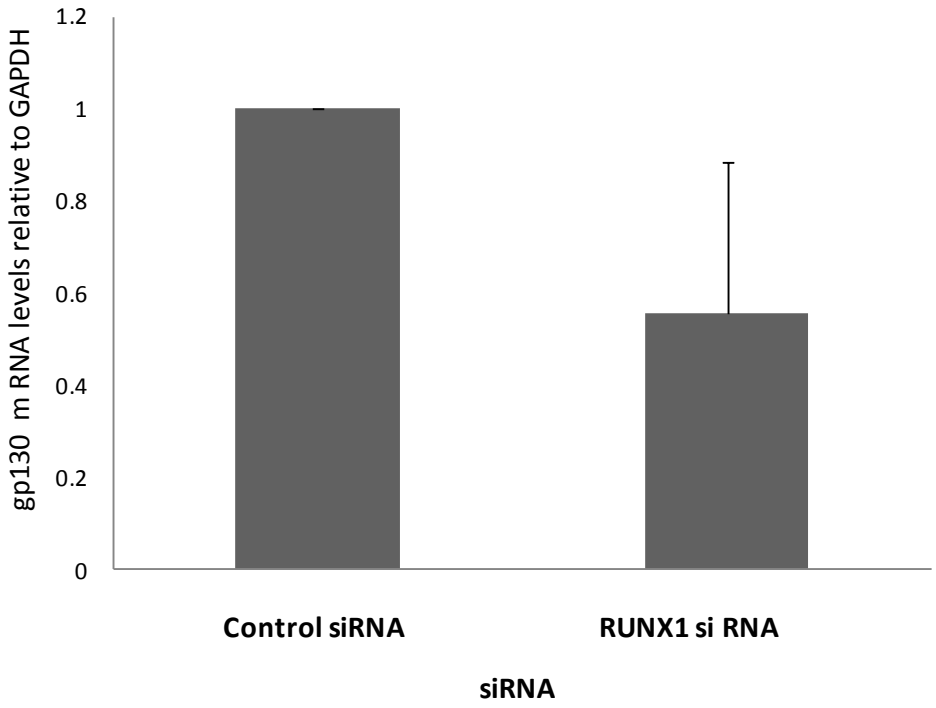


Figure 4.14 Regulation of the gp130 endogenous gene by RUNX1. KG-1 myeloid cells lines were transfected with RUNX1 siRNA . gp130 mRNA expression was analyzed by qRT-PCR in cells stimulated with PMA and calcium ionphore for 8 hours. gp130 levels are shown relative to control siRNA transfected was set to 1, the mean and standard error of three independent experiments is shown

The LIFR general promoter is active in range of cell types including placental cell lines. To determine whether RUNX1 regulates the general LIFR promoter in other cell types, expression of RUNX1 across different cell lines was investigated. RNA was isolated from placental cells (JAR, JEG-3) , HeLa cells and Kasumi-1 myeloid cells. RNA was reverse transcribed to cDNA and analysed for RUNX1 mRNA levels using qRT-PCR. In parallel, samples were analyzed with primers designed to the GAPDH housekeeping gene to normalize for variation in cDNA synthesis or the amount of starting material. All samples expressed RUNX1 mRNA, including the placental cell lines (Figure 4.15), although RUNX1 is expressed in the placental cell lines and HeLa cells at lower levels than Kasumi-1 myeloid cells (Figure 4.15).

To confirm regulation of the general promoter by RUNX1 in placental JEG-3 cells in which the general promoter is also active cells were transfected with siRNA specifically designed to target RUNX1b and control siRNA (Dharmacon, USA). Cells were seeded in 24 well plates and transfected with 100nM siRNA pools of 4 siRNA. Treated cells were incubated for 48hr before RNA preparation. RUNX1 mRNA levels were determined by qRT-PCR and revealed a reduction of RUNX1 mRNA of 70% in the RUNX1 siRNA treated samples compared to the cells transfected with siRNA control (Figure 4.16). RUNX1 depleted samples were then analysed with primers designed to LIFR Variant 1 mRNA to determine RUNX1 effect on the LIFR general promoter of RUNX1 depletion in JEG-3 cells. Decreased expression of the LIFR Variant 1 transcript by 2 fold was observed (Figure 4.17), suggesting that RUNX1 regulates the general LIFR promoter in placental cells, as well as myeloid cells.

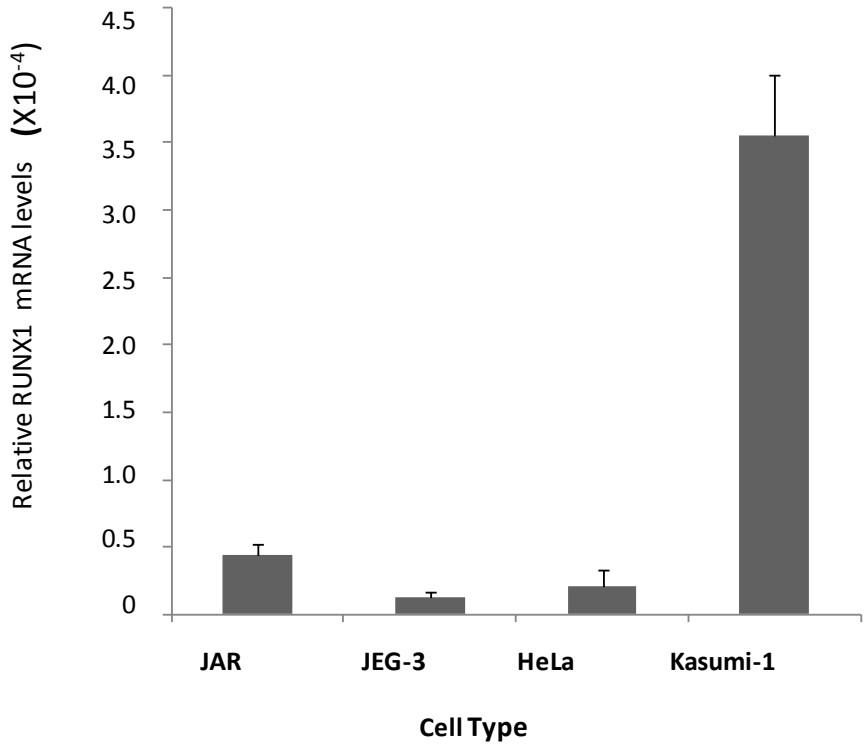


Figure 4.15 RUNX1 expression in different cell lines. RUNX1 mRNA level were determined by qRT-PCR in Kasumi-1 myeloid cell lines, JAR and JEG-3 placental cell lines and HeLa. Levels of the mRNA of interest were normalized to GAPDH. The mean and standard error of three independent experiments is shown.

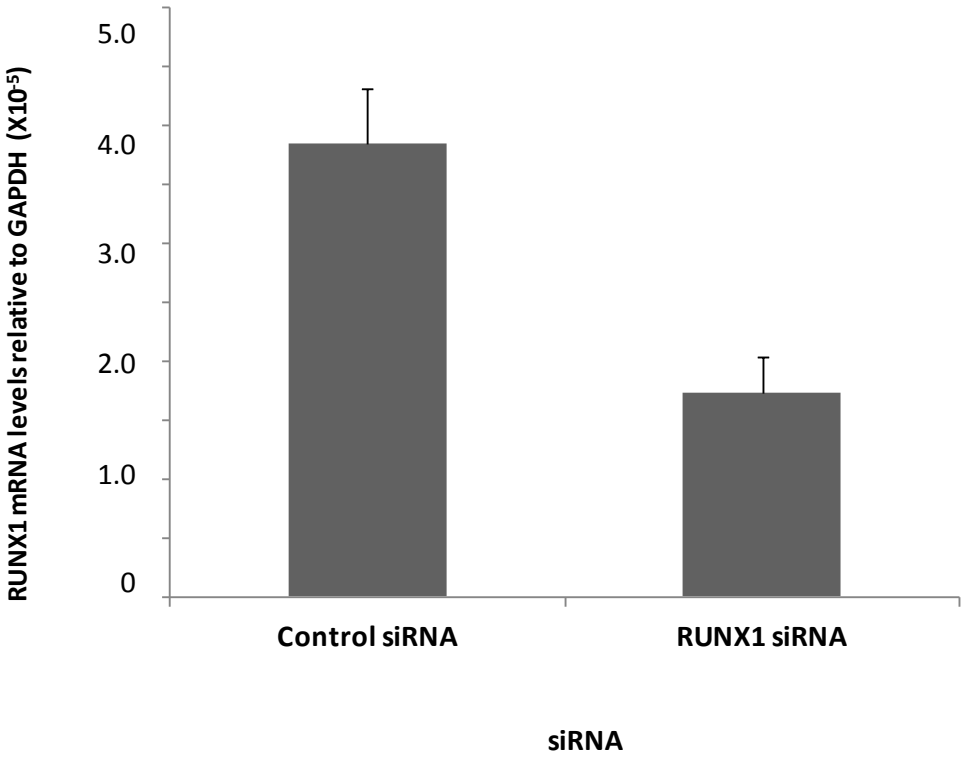


Figure 4.16 Percentage knock down of RUNX1 in placental cell lines. JEG-3 cells were transfected with RUNX1 siRNA . RUNX1 mRNA expression was analyzed by qRT-PCR . The mean and standard error of three independent experiments is shown.

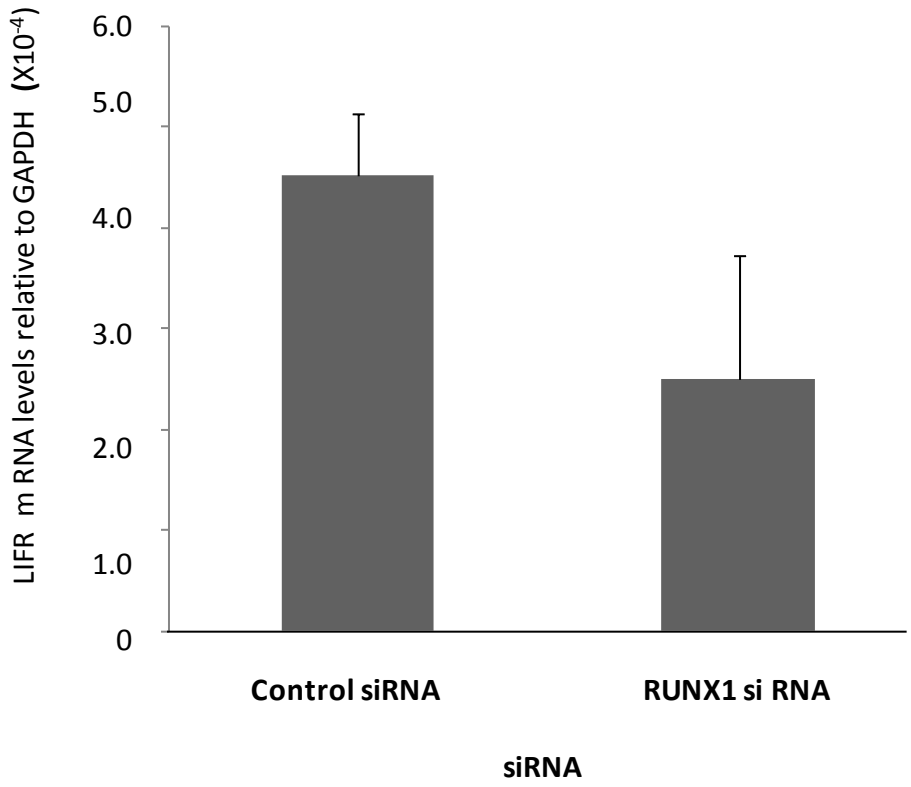


Figure 4.17 Regulation of the general LIFR promoter by RUNX1 in placental cells. JEG-3 cell lines were transfected with RUNX1 siRNA. Variant 1 LIFR mRNA expression was analyzed by qRT-PCR. The mean and standard error of three independent experiments is shown.

4.2.5 LIFR and gp130 are direct targets of RUNX1;

The data presented thus far suggest that the LIFR and gp130 promoters are regulated by RUNX1 and the endogenous promoters are RUNX1 responsive. To confirm the general LIFR and gp130 promoter as direct targets of RUNX1, Kasumi-1 myeloid cells were subjected to ChIP analysis with either RUNX1or RUNX1-ETO antibodies.

Precipitated DNA was analysed by qPCR using primers that amplify regions in the general and placental LIFR promoters (Chapter 2; Table 2.1). In this experiment precipitated DNA was compared to the No Antibody (NA) negative control to demonstrate specificity of precipitation of DNA with RUNX1 antibodies, as for previous ChIP experiments.

Enhanced binding of RUNX1 was associated with the general LIFR promoter, compared to the placental LIFR promoter which suggests that RUNX1 binds to the active general LIFR promoter in myeloid cells (Figure 4.18).

Kasumi-1 cells contain RUNX1-ETO fusion protein and therefore RUNX1-ETO occupancy was determined at the LIFR promoters in Kasumi-1 cells. RUNX1-ETO binding was detected at the general LIFR but not the placental promoter suggesting that RUNX1-ETO also targets the general LIFR promoter in leukemic cells (Figure 4.18).

The same precipitated DNA was also subjected to qPCR analysis using primers which amplify a region within the gp130 promoter to analyse RUNX1 association with the gp130 promoter. Binding of RUNX1 and RUNX1-ETO was detected to the gp130 promoter region which suggests regulation of gp130 by RUNX1 (Figure 4.19).

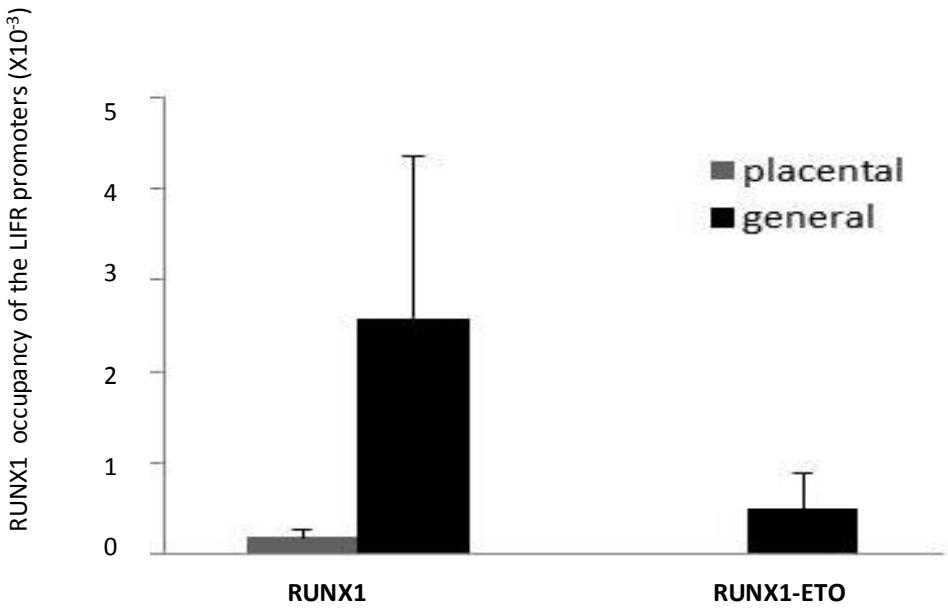


Figure 4.18 RUNX1 occupies LIFR promoter in myeloid cell lines. ChIP assays were used to monitor RUNX1 and RUNX1-ETO binding to the LIFR promoters in Kasumi-1 cells. ChIP DNA was analysed by qPCR and normalized to the total input DNA. The mean and standard error of three independent experiments is shown in.

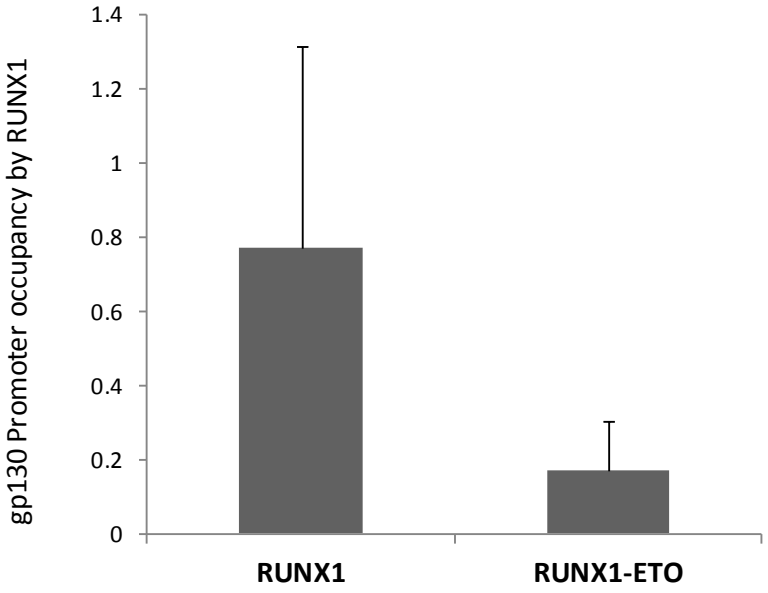


Figure 4.19 RUNX1 occupies the gp130 promoter in myeloid cell lines. ChIP assays were used to monitor RUNX1 and RUNX1-ETO binding to the gp130 promoter in Kasumi-1 cells. ChIP DNA was analysed by qPCR and normalized to the total input DNA. The mean and standard error of three independent experiments is shown

Put together the data presented here suggest that the LIFR and gp130 genes are regulated by RUNX1 in myeloid cell lines. LIF was originally identified as a factor that prevented blast formation of the highly clonogenic murine M1 cell line (Tomida et al., 1984). However these effects were not replicated in other cell lines (reviewed in Metcalf, 2003). Despite this the effect of LIF on myeloid cells or cell lines has not been extensively studied. The data presented here shows that myeloid cells express LIFR/gp130 mRNA therefore the effect of LIF on myeloid cell proliferation was investigated. Kasumi-1 myeloid cells containing RUNX1-ETO were treated with LIF cytokine (10ng/ml) for 3 days or left untreated. Cells numbers were determined each day to examine cell survival and response to LIF treatment. Proliferation of the cells was reduced over the 3 days of treatment with LIF (Figure 4.20), suggesting reduced proliferation of the Kasumi-1 cells in response to LIF cytokine treatment.

4.2.6 RUNX1 activates the placental promoter;

LIFR gene expression is derived from alternate promoters that produce two different transcripts. While the general promoter is active in myeloid cells, both the general and placental promoters are active in placental cell lines (Figure 3.9 and 3.10). Like the general LIFR promoter the placental promoter is potentially regulated by RUNX1, containing 3 putative RUNX1 binding sites (Figure 3.1B).

To determine if RUNX1 can regulate the placental LIFR promoter, a region of 500bp corresponding to the placental promoter as previously described in the literature (Blanchard et al., 2002; Wang and Melmed, 1998) was cloned into the pXP1 luciferase reporter vector (Figure 4.21 A) and introduced into the JEG-3 placental cell line by electroporation. Cells were transfected with the placental LIFR promoter reporter construct alone or co-transfected with increasing concentrations of RUNX1b expression construct. Cells were harvested 48 hr after transfection to determine luciferase activity. Changes in LIFR placental promoter activity would therefore be directly proportional to luciferase activity produced from the construct.

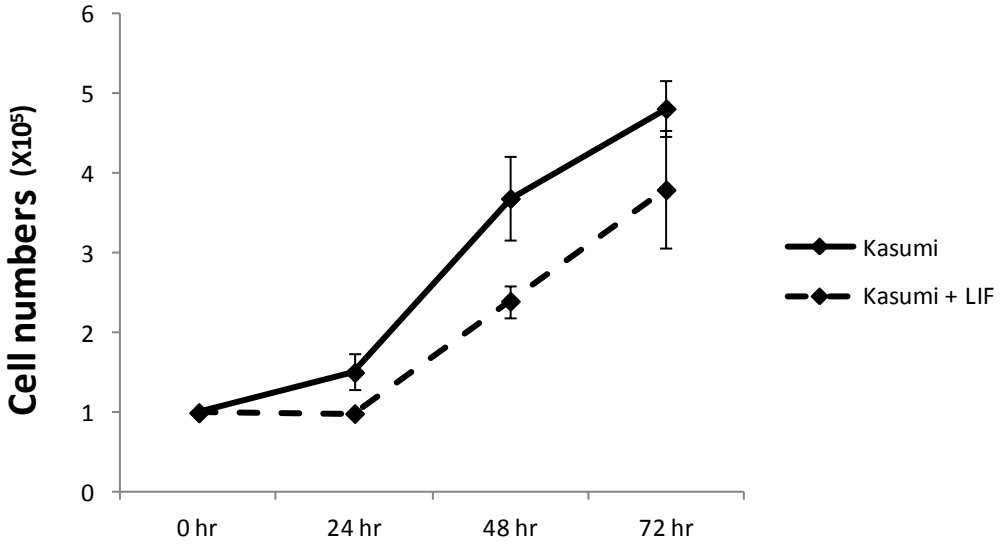


Figure 4.20 Effect of LIF on Kasumi-1 cells. Kasumi-1 cells were treated with 10ng/ml LIF for 3 days as indicated. Viable cells were monitored by trypan blue exclusion and quantitated using haemocytometer. The mean and standard error of three independent experiments is shown

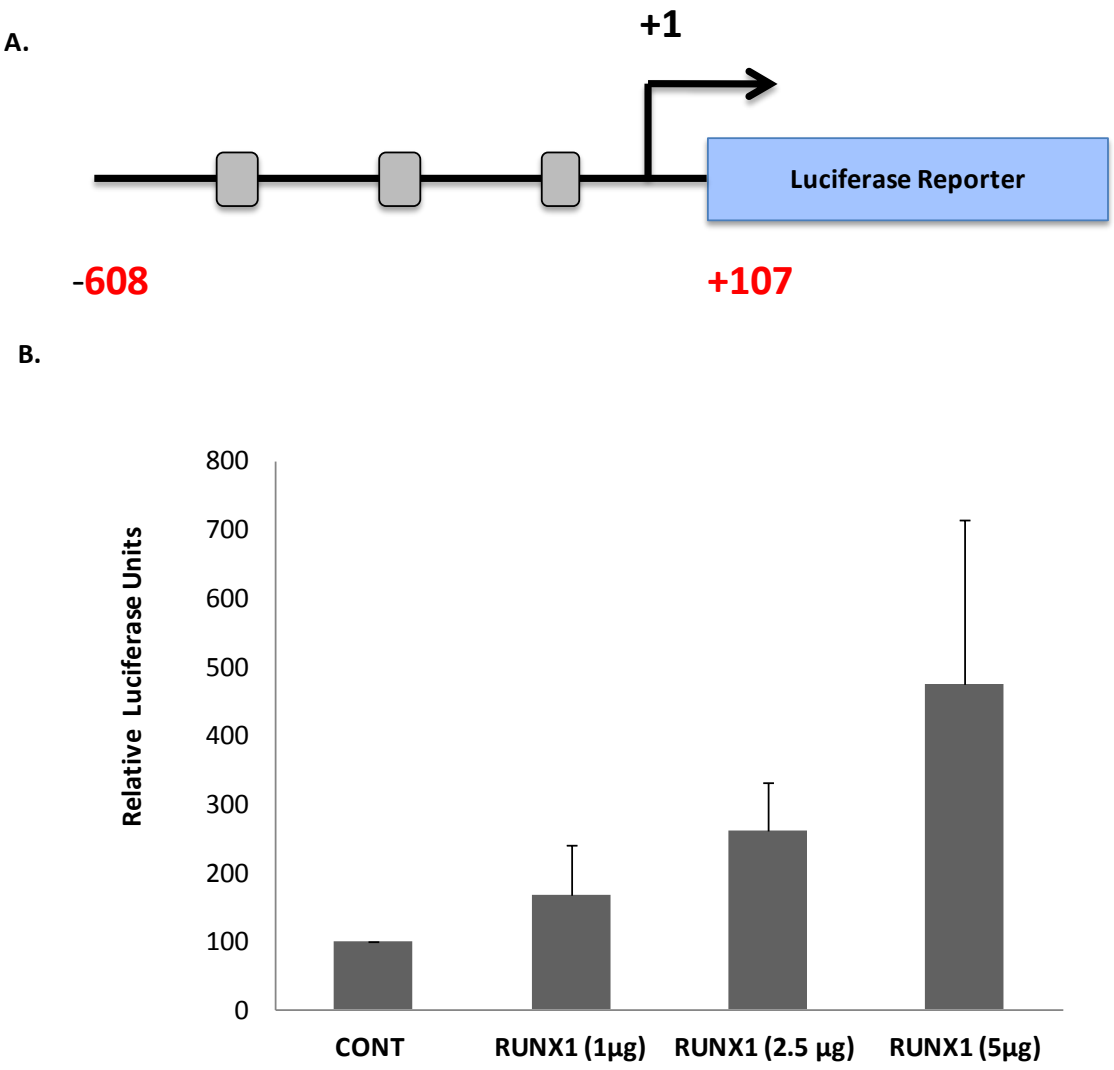


Figure 4.21 RUNX1 activates the placental LIFR promoter. **(A)** The placental LIFR promoter was cloned into pXP1 luciferase reporter.**(B)** JEG-3 cells were transfected with the placental LIFR promoter reporter plasmid and different amounts of RUNX1 expression plasmid and luciferase activity monitored. The mean and standard error of three independent experiments is shown

Co-expression of RUNX1b activated the placental LIFR promoter in a dose dependent manner (Figure 4.21 B), suggesting that the promoter is regulated by RUNX1.

To determine which of the three RUNX1 binding sites contribute to activity of the promoter, reporter constructs were generated in which each site was deleted or mutated independently (Figure 4.22). These constructs were then transfected into JEG-3 cells either alone or with RUNX1b expression construct, cells were harvested 48 hrs after transfection and luciferase activity measured. Basal activity of the placental LIFR reporter construct was not altered by deletion of the distal RUNX1 binding site and activation of the LIFR promoter by RUNX1 was unchanged when the distal RUNX1 binding site was deleted (▲ site1; Figure 4.23). Similarly mutation of the proximal RUNX1 binding site had no effect on either basal promoter activity or activation by RUNX1 (mSite3, Figure 4.23). In contrast, while mutation of the middle RUNX1 binding site did not affect basal activity, it significantly reduced activation by RUNX1 (mSite2; Figure 4.23). These data suggest that the placental LIFR promoter is regulated by RUNX1 and that this effect is mediated by the second bioinformatically determined RUNX1 binding site. Interestingly, this is the only RUNX1 binding site in the placental promoter that showed any conservation in other mammalian species (Figure 3.3).

As described earlier both the general and placental LIFR promoters are active in placental cell lines (Figure 3.9 and Figure 3.10). Therefore to determine whether both of these promoters are regulated by RUNX1 in placental cells, ChIP analysis was conducted in JEG-3 cells with RUNX1 antibodies. Precipitated DNA was analysed by qPCR with primers that amplify either the general or placental promoter. In these experiments, binding of RUNX1 was detected to both the general and placental promoters (Figure 4.24).

Put together the data presented here suggest that both the LIFR general and placental promoters as well as the gp130 promoter is regulated by RUNX1.

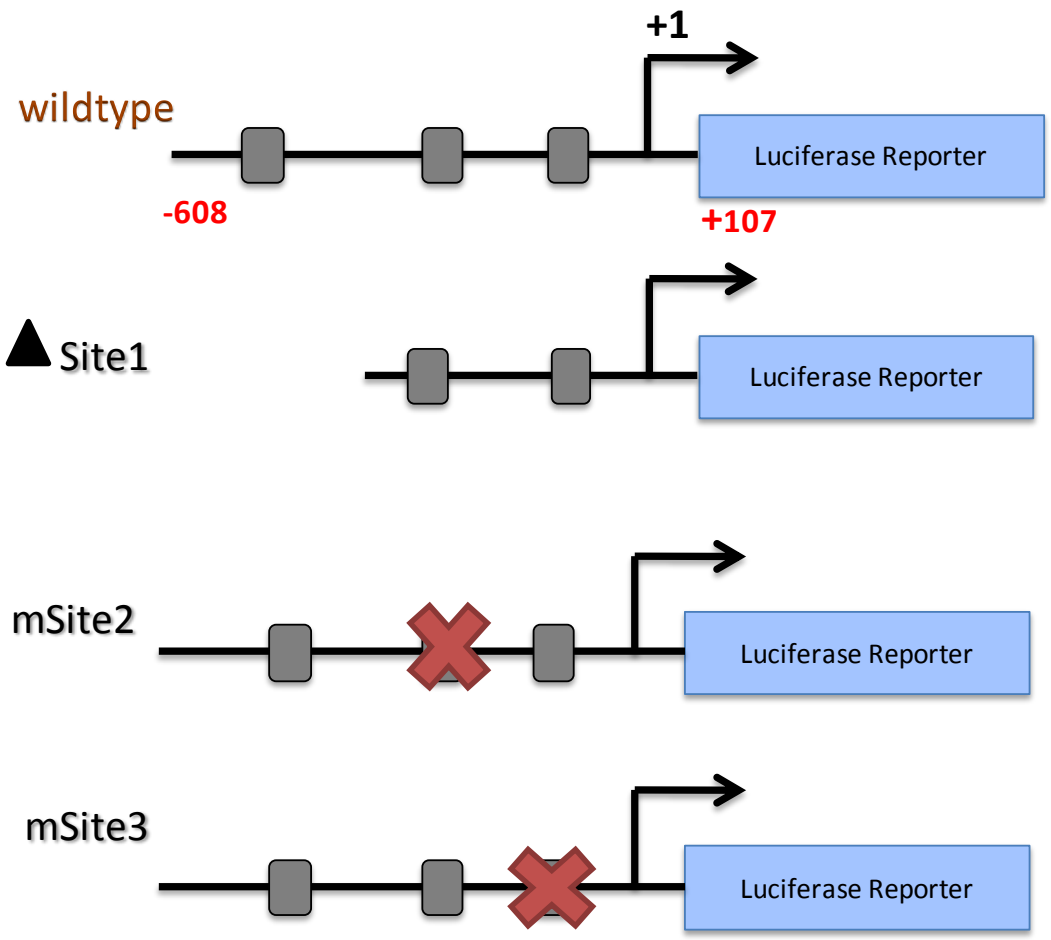


Figure 4.22 Schematic of the mutated and deleted RUNX1 binding site in the LIFR placental promoter reporter construct..

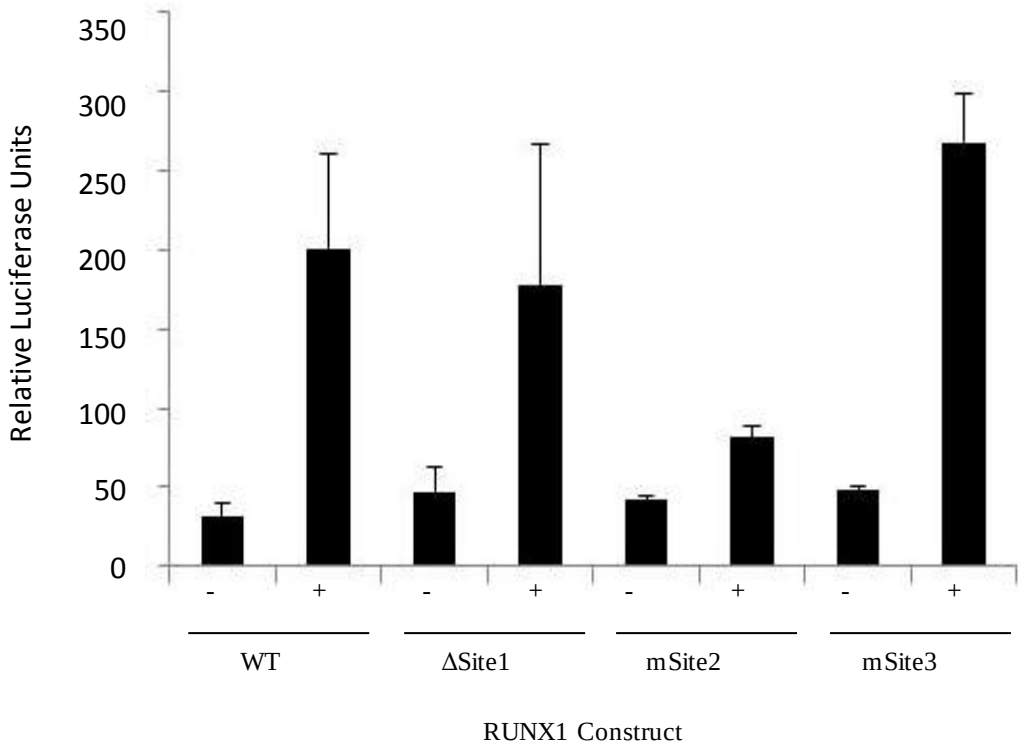


Figure 4.23 Mutational analysis of the RUNX1 binding sites in the placental LIFR promoter. JEG-3 cells were transfected with a wildtype LIFR placental promoter luciferase plasmid along with RUNX1 or plasmids in which the three RUNX1 binding sites were deleted or mutated as described in Figure 4.22. Luciferase activity was measured 24 hours after transfection, The mean and standard error of three independent experiments is shown

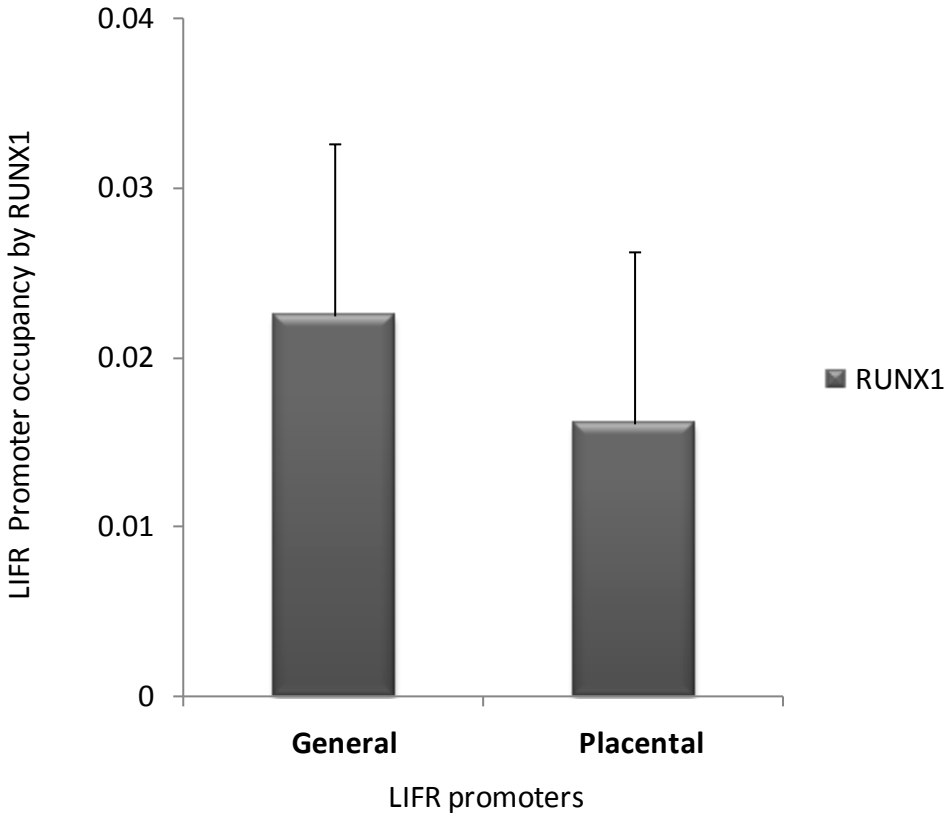


Figure 4.24 RUNX1 occupies the LIFR promoters in placental cell lines. ChIP assays were used to monitor RUNX1 binding to the LIFR promoters in JEG-3 cells. ChIP DNA was analysed by qPCR and normalised to total input DNA. The mean and standard error of three independent experiments is shown.

4.3 DISCUSSION;

Data in this chapter presents a number of lines of evidence that the LIFR and gp130 genes are direct targets of RUNX1 in myeloid cells. Firstly, RUNX1 is able to activate a general LIFR promoter and a gp130 reporter construct in myeloid cell lines. Secondly, depletion of RUNX1 by siRNA in myeloid cell lines decreased expression from the general LIFR and gp130 genes. Thirdly, RUNX1 binding was detected at both the general LIFR and gp130 promoters using ChIP assays in myeloid cells. Put together these finding indicate that RUNX1 directly targets and regulate the general LIFR and gp130 endogenous genes in myeloid cells.

Bioinformatic analysis identified potential RUNX1 binding sites in the promoters of both the LIFR and gp130 genes. RUNX1 binding was confirmed by ChIP analysis in both promoters, in which higher levels of RUNX1 was associated with the gp130 compared to the general LIFR promoter. This finding may be explained by the fact that the gp130 promoter contains four RUNX1 binding sites compared to one site in the general LIFR promoter.

Normal RUNX1 gene function is commonly disturbed by chromosomal rearrangements and mutations in acute myeloid leukemia, most frequently involving the t(8,21) translocation which generates the RUNX1-ETO fusion protein. Expression of RUNX1-ETO in myeloid cells repressed activity of both the general LIFR promoter and the gp130 promoter. This is in agreement with the usually described role of RUNX1-ETO acting as a repressor of RUNX1 target genes (Rio-Machin et al., 2011) and its role in blocking myeloid differentiation (Shimada et al., 2002). This means that RUNX1-ETO compete with RUNX1 and modulate its function on target genes in leukemic cells. However RUNX1 was able to activate both the general LIFR and the gp130 promoters in Kasumi-1 cells which contain the RUNX1-ETO protein. This suggests that RUNX1 can compete with RUNX1-ETO to activate the general LIFR promoter in Kasumi-1 leukemic cells, and that RUNX1 gene dosage is therefore important in determining effects on target genes in the presence of RUNX1-ETO.

RUNX1 regulates expression of a number of cytokine and cytokine receptor genes including GMCSF, TCR, IL3 and M-CSFR in myeloid cells. The LIFR:gp130 complex is therefore an addition to a cytokine receptors that their gene expression program is regulated by RUNX1 in myeloid cells.

Alteration in expression of the LIFR/gp130 receptor complex in cells in which RUNX1 is disrupted may therefore influence LIF signaling. LIF was originally identified as a cytokine with a role in inhibiting leukemic cell survival and self renewal (Tomida et al., 1984; Metcalf, 2003). However this observation has remained largely unconfirmed. Here preliminary examination of the effect of LIF on the growth of human myeloid cell lines was undertaken. Treatment of Kasumi-1 cells with LIF cytokine decreased the number of viable cells in the culture compared to untreated cells suggesting an inhibition of cell proliferation over a three day period. This data supports the idea of LIF signaling influencing myeloid cell proliferation, and suggests that decreased expression of the LIFR;gp130 complex in myeloid cells may confer a growth advantage to these cells, in the presence of LIF. Further analysis of cell viability and proliferation, is essential to determine the nature of the effect of LIF on the Kasumi cells, as the reduced cell numbers may be due to effects on cell cycle or apoptosis. Further, LIF appeared to influence cell numbers primarily in the first 24 hr suggesting that LIF may not have been bioactive at later time points.

Data presented in this chapter indicate a regulation of the general and placental LIFR promoter by RUNX1, giving that gp130 is also regulated by RUNX1. Suggest that LIFR/gp130 receptor complex is tightly regulated by RUNX1.

Chapter 5

5.0 DISCUSSION AND FUTURE DIRECTIONS

The RUNX1 transcription factor is essential for myeloid cell differentiation and its role is well described during haematopoiesis (Okuda et al., 2001). In this study the LIFR/gp130 receptor complex was identified as a novel target of RUNX1 in the hematopoietic system. The LIFR/gp130 Receptor complex acts as a signal transducer for the LIF cytokine. LIF is a polyfunctional cytokine that has a pronounced role in hematopoietic stem cell survival and differentiation (Zandstra PW et al., 2000). The LIFR and gp130 gene products are essential components of LIF signaling and therefore control various cellular processes including; cellular development, differentiation and the immune response. Although LIF and LIFR/gp130 signaling is well described in the hematopoietic system (Auernhammer et al., 2000), regulation of the receptor expression has remained largely uncharacterized.

The LIFR gene is regulated by alternative promoters, the so called 'general' and 'placental' promoters, which give rise to two different transcripts (Blanchard et al., 2002). LIFR expression was described previously in a range of tissues (Metcalf et al., 2003) but not in the haematopoietic system. The data presented here demonstrate placental LIFR promoter activity restricted to placental cells and that the general LIFR promoter is active in all cell lines examined thus far. This is in agreement with previous characterisation of the LIFR alternative promoters, suggesting a highly cell-type restricted expression pattern driven by the placental promoter, and ubiquitous activity of the general promoter. As anticipated, gp130 promoter activity was demonstrated in all cell lines examined, including myeloid cells.

Bioinformatic analysis identified potential RUNX1 binding sites in the promoters of both the LIFR and gp130 genes. Reporter assays then demonstrated regulation of both the general LIFR and gp130 promoters by RUNX1 in myeloid cells. In addition the placental LIFR promoter was found to be regulated by RUNX1 in placental cells. This was confirmed by Chromatin immunoprecipitation, which demonstrated binding of RUNX1 to the general LIFR promoter and gp130 promoter in myeloid cells, and also to both LIFR promoters in a placental cell line. In addition knock down of RUNX1 decreased the expression of both the LIFR and gp130 genes in myeloid cells.

Mutation of the RUNX1 binding site in the general LIFR promoter decreased activation of the general LIFR promoter by RUNX1. In addition the placental LIFR promoter contains three RUNX1 binding sites with only disruption of the second RUNX1 binding site affecting activation of the promoter by RUNX1. Sequence analysis of the RUNX1 binding sites in the LIFR promoters indicate high conservation of the general LIFR promoter RUNX1 site in mammals and some conservation of the second site in the placental promoter, with no evolutionary conservation of the other placental sites. Analysis of the promoter conservation appears to be a better predictor of the functionality of the binding sites than similarity to the consensus sequence, as the general promoter site had lower homology to the consensus sequence than the three placental sites, which all have high similarity to the consensus sequence including 100% matches in the core region. This also highlights the need to experimentally verify the functionality of predicted transcription factor binding sites.

In leukemia, particularly acute myeloid leukemia, RUNX1 activity is commonly altered by point mutations and chromosomal rearrangements, most frequently producing a RUNX1-ETO fusion protein. While RUNX1 is generally associated with gene activation, RUNX1-ETO usually acts as a transcriptional repressor and is associated with gene silencing, blocking normal RUNX1 function in myeloid cells

(Tokito et al, 2007). In keeping with this, here RUNX1-ETO was demonstrated to have a repressive effect on the general LIFR promoter as well as the gp130 promoter, although overexpression of RUNX1 in Kasumi-1 cells was able to overcome the repressive effect of RUNX1-ETO.

Despite the repressive effect of RUNX1-ETO on the general LIFR promoter, an apparently contradictory result in this study was the relatively high LIFR mRNA expression levels observed in Kasumi-1 cells containing RUNX1-ETO. However, while RUNX1 and RUNX1-ETO were shown here to regulate the LIFR promoters, there are likely to be additional transcription factors, yet to be identified, which also contribute to LIFR expression. In addition, the data presented here, and previously (Blanchard et al., 2002) indicate that the general LIFR promoter is subject to epigenetic regulation. It has previously been shown that the general LIFR promoter is regulated by DNA methylation, with decreased methylation associated with increased expression (Blanchard et al., 2002). Further, the data presented in this thesis suggest that the general LIFR transcript expression is correlated with a hyperactylated promoter. While LIFR expression was found here to be expressed more highly in the Kasumi-1 cell line which contains RUNX1-ETO than the KG-1 and KG-1a cell lines which do not, analysis of the LIFR promoters identified two CpG islands associated with the LIFR gene: the placental promoter has a small CpG island located 5′ to the promoter, while a large CpG island encompasses the general LIFR promoter (Bowditch and Holloway, unpublished, 2010). Analysis of these promoters in the KG-1 and Kasumi-1 cell lines, suggest that the lower expression level observed in the KG-1 cell line is due to hypermethylation of the general promoter in this cell type, which may affect the ability of the RUNX1 transcription factor to activate the promoter. In support of this, genome wide analysis shows a strong influence of chromatin architect on transcription factors recruitment to DNA (Lam et al., 2008).

Inhibition of RUNX1 activity slows G1 to S phase cell cycle progression in Ba/F3 myeloid cells (Bernardin and Friedman, 2007), and alteration to RUNX1 can block 32Dcl3 murine myeloid cell differentiation (Tanaka et al., 1995). The molecular

mechanism by which RUNX1 regulates haematopoietic cell differentiation involves its regulation of the expression of cytokines and their receptors important for haematopoietic cell growth and differentiation. Among these genes are those encoding the TCR (Lauzurica et al., 1997), MCSFR (Zhang et al., 1994), GM-CSF, IL3 and MPO (Takahashi et al.,1995; Cameron et al., 1994; Nuchprayoon et al., 1994). The data presented in this thesis suggests that LIFR: gp130 can now also be added to this list. In addition, gp130 is a shared component of a number of other IL-6 family receptors (reviewed in Taga, 1997), suggesting that responses to other IL-6 cytokines may also be influenced by RUNX1. In addition, preliminary bioinformatics analysis predicted RUNX1 binding sites in a number of the other IL-6 cytokine family receptor genes (data not shown). Thus RUNX1 regulates a program of cytokine and cytokine receptor gene expression, and this program is likely to be even more expansive than currently thought, and warrants further investigation.

The RUNX family of transcription factors are developmental regulators and have been shown to have roles in a number of different tissues (reviewed in Hart, 2002). The RUNX family includes three proteins (RUNX1, 2 and 3), and each of these proteins appear to bind to the same consensus DNA sequence. While RUNX1 has an indispensable role in haematopoiesis (Okuda et al., 1996), RUNX3 has important roles in the haematopoietic system and nervous system, and RUNX2 plays a critical role in osteogenesis (Simth et al., 2005). While LIFR has a well described role in the hematopoietic system, it also has important roles in osteogenesis and neural development (Murphy et al 1997; Malaval et al., 2005). It is therefore likely that LIFR expression is regulated by RUNX1 in these tissues also. In support of this, in this study RUNX1 was shown to regulate LIFR promoter activity in placental cell lines as well as in myeloid cell lines. Alternatively other RUNX family members may regulate LIFR and gp130 gene activity in other systems.

Data in this thesis is the first to describe analysis of the LIFR promoters and their regulation in myeloid cells. In this study the LIFR and gp130 genes were characterized as novel targets of RUNX1 in myeloid cells. However the interactions of RUNX1 with its target genes are complex and therefore further analysis of LIFR/gp130 regulation by RUNX1 in the hematopoietic system is essential to understand the regulation of the receptor complex, and how disruption of RUNX1 in leukemia may affect LIF signaling, and the impact of this on myeloid cell behavior. While LIF was originally described as a differentiation factor for its ability to inhibit blast formation of a highly clonogenic cell line, there has been little follow up of this early observation (Metcalf et al., 2003). However, treatment of Kasumi-1 cells with LIF inhibited their growth, suggesting that alteration of LIFR/gp130 expression and signaling in leukemic cells may alter cell proliferation. These data suggest that altered expression of LIFR/gp130 may contribute to the development and/or progression of leukemia in which RUNX1 is disrupted, and this warrants further investigation. The logical next step would be to investigate the effect of RUNX1 disruption on LIF signaling in mouse models, which have proved invaluable in understanding the role of RUNX1 in leukaemiogenesis (Lund et al., 2002). However, such studies may not be straight forward as while as described earlier the general LIFR promoter is highly conserved in mammals including mouse, two transcripts are generated from this promoter in mice, one of which encodes a soluble form of LIFR which has the ability to neutralize LIF signaling (Chambers et al., 1997).

Here a model is presented in which RUNX1 regulates the LIFR gene. Understanding the mechanism and pathways involve in this interaction will contribute to the development of efficient treatment strategies for cancer. First, RUNX1 is described as an activator molecule which regulates gene expression by the recruitment of co-activator molecules. The p300 complex interacts with the c-terminal of RUNX1 and activates transcription through chromatin remodeling. RUNX1-ETO on the other hand is a repressor of transcription, with the ETO domain recruiting N-CoR /mSin3 and HDAC complexes to RUNX1 responsive promoters (Wang et al., 1998). In the case of the LIFR promoters, in myeloid cells the general LIFR promoter is preferentially occupied by higher levels of RUNX1

than RUNX1-ETO and RUNX1-ETO represses the active general LIFR promoter, suggesting the value of HDAC inhibitors' and chromatin remodeling therapy in the treatment of t (8, 21) AML. This has been explored by several studies; RUNX1-ETO undergoes degradation in response to depsipeptide (DEP, also known as FK228 and FR901228) an HDAC inhibitor (Yang et al., 2007; Yu et al., 2011). Phase II studies of DEP in combination with other anticancer drugs are ongoing to determine its effect on solid and heamatological cancers. DEP clinical trials showed significant clinical activity against cutaneous T cell lymphoma (Piekarz and Bates, 2004). Although HDAC inhibitors and other epigenetic modulators provided promising results in the treatment of t (8, 21) AML cases, a better understanding of the mechanism of RUNX1-ETO interaction with genes is essential. In vivo analysis of RUNX1-ETO might be useful in this case, and there are a number of animal models of the t (8; 21) leukemia which could be explored (Lict et al., 2001), however such analysis would be complicated in this case as the LIFR gene structure is not conserved between mouse and human.

In addition, the LIFR promoters contain CpG islands with different methylation patterns. For example; hypermethylation of the general LIFR promoter is correlated with low expression of the promoter in KG-1cells (Bowditch and Holloway, unpublished, 2010), Hypermethylation is associated with gene silencing and is a common mechanism of gene repression in cancer. Therefore the use of DNA methyltransferase inhibitors may represent another useful anti-cancer strategy in this case (reviewed by Poke and Holloway; 2010).

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