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The Leukemia Inhibitory Factor Receptor Gene Is a Direct Target of RUNX1

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ABSTRACT

Activation of cytokine signaling via the leukemia inhibitory factor receptor (LIFR) plays an integral role in hematopoiesis, osteogenesis, and placental development, along with mediating neurotrophic mechanisms. However, the regulatory control of the LIFR gene has remained largely unexplored. Here, we characterize the *LIFR* gene as a novel target of the RUNX1 transcription factor. The RUNX1 transcription factor is an essential regulator of hematopoiesis and is a frequent target of point mutations and chromosomal alterations in leukemia. RUNX1 regulates hematopoiesis through its control of genes important for hematopoietic cell growth, proliferation, and differentiation, including a number of cytokines and cytokine receptors. LIFR is regulated by two alternate promoters: a placental-specific and a ubiquitously active general promoter. We show that both of these promoters are regulated by RUNX1. However, in myeloid cells LIFR expression is driven solely by the general LIFR promoter with our data indicating that the placental promoter is epigenetically silenced in these cells. While RUNX1 activates the LIFR general promoter, the oncogenic RUNX1-ETO fusion protein generated by the t(8;21) translocation commonly associated with acute myeloid leukemia represses promoter activity. The data presented here establish LIFR as a transcriptional target of RUNX1 and suggest that disruption of RUNX1 activity in myeloid cells may result in altered LIFR signaling in these cells. J. Cell. Biochem. 117: 49–58, 2016.

KEY WORDS: LIFR; RUNX1; GENE REGULATION

Leukemia inhibitory factor (LIF) is a polyfunctional cytokine, involved in the differentiation, survival, and proliferation of a wide range of cell types [Mathieu et al., 2012]. LIF is a member of the IL-6 family of cytokines, originally identified for its ability to induce differentiation of the M1 murine myeloid leukemia cell line [Gearing et al., 1987]. It plays an integral role in hematopoiesis [Metcalf, 2003], including the maintenance of stem cell populations [Escary et al., 1993; Schraml et al., 2008]. It also has roles in osteogenesis, cardiac hypertrophy, and neurogenesis [Metcalf, 2003; Mathieu et al., 2012] as well as in embryogenesis, implantation, and placental function [Dimitriadis et al., 2010]. Given its diverse range of biological functions LIF dysregulation has been linked to numerous disease states. It has recently been identified as a suppressor of metastasis in breast cancer [Chen et al., 2012] and is involved in the progression of melanoma [Kuphal et al., 2013]. This is in addition to its links with infertility [Wu et al., 2013], self-renewal of neural stem cells, remyelination, and axonal regeneration [Deverman and Patterson, 2012]. In addition, stromal cells from individuals with various types of leukemia and myelodysplastic syndrome have been shown to have elevated levels of LIF [Wetzler et al., 1994; Medyouf et al., 2014].

LIF signals via a heterodimeric receptor consisting of the low affinity LIFR subunit and the high affinity gp130 (glycoprotein 130) subunit. The LIFR:gp130 complex signals for the diverse range of biological activities relating to LIF along with other ligands including oncostatin M and cardiotrophin [Gearing, 1993]. gp130 is a shared receptor subunit for the IL-6 family of cytokines and its expression is therefore widespread likely explaining some of the

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biological redundancy of IL-6 family cytokines [Kishimoto et al., 1995]. Not surprisingly given its role in IL-6 signaling, gp130 knockout in mice is embryonic lethal [Yoshida et al., 1996]. There is evidence that other IL-6 family members besides LIF can also use the LIFR:gp130 receptor complex [Di Marco et al., 1996; Robledo et al., 1997; Nakashima and Taga, 1998]. However, LIFR knockout is not embryonic lethal, although the mice die shortly after birth and display a range of defects, particularly related to placental, bone, and neural development [Ware et al., 1995]. In contrast, LIF knockout mice appear relatively normal, although they have defects in hematopoiesis and neurogenesis, and female mice fail to become pregnant due to defects in blastocyst implantation [Escary et al., 1993; Bugga et al., 1998].

Given the widespread biological functions of LIF and the dependence on the expression of LIFR in target cell types in order for them to respond to LIF, there is surprisingly little known regarding the regulation of LIFR expression. The human *LIFR* gene is found on chromosome 5 and spans more than 20 kb with 20 exons. The identification of two 5′ non-coding exons and two transcripts was suggestive of alternae promoter usage, and two LIFR promoters have subsequently been identified [Wang and Melmed, 1997; Blanchard et al., 2002]. One of these promoters appears to be placental-specific while the other is active in a range of cell types. Both promoters are associated with CpG islands and have been found to be subject to epigenetic regulation [Blanchard et al., 2002]. To date, both of these promoters have remained relatively uncharacterized as far as their mechanism of regulation and the transcription factors that regulate their activity are largely unknown. Here, we show that both of the LIFR promoters are regulated by the RUNX1 transcription factor.

RUNX1 is a member of the RUNX family of transcription factors [Levanon and Groner, 2004], and is expressed in a range of cell types, most notably hematopoietic cell lineages. RUNX1 contains an N-terminal DNA binding region, called the Runt homology domain (RHD) and binds to DNA as a heterodimer with CBFβ, which does not bind to DNA, but increases the affinity of RUNX1 for DNA [Levanon and Groner, 2004]. Disruptions to RUNX1 or CBFβ occur in a significant proportion of leukemias [Speck and Gilliland, 2002]. RUNX1 was originally identified following characterization of the t(8;21) chromosomal translocation found in approximately 5% of acute myeloid leukemias (AMLs), that gives rise to the RUNX1-ETO fusion protein in which the N-terminal region of RUNX1 is fused to almost the entire ETO protein [Miyoshi et al., 1993]. The resultant protein retains the RHD and therefore binds to RUNX1 target genes, but has altered function, commonly acting as a transcriptional repressor as opposed to a transcriptional activator [Peterson and Zhang, 2004]. More than a dozen translocations have subsequently been found to disrupt RUNX1 in a range of leukemias [Speck and Gilliland, 2002].

Here, we show that both the general and placental LIFR promoters are regulated by RUNX1. However, only the general LIFR promoter is active in myeloid cells. Our data suggest that RUNX1 binds to and activates the general LIFR promoter in myeloid cell lines, while the placental promoter is epigenetically silenced in these cells. Disruption of RUNX1 activity in myeloid cells may therefore result in altered LIFR signaling in these cells.

MATERIALS AND METHODS

PLASMIDS

LIFR promoter reporter plasmids were constructed in the pXPG vector [Bert et al., 2000], provided by Dr. P. Cockerill (Institute of Biomedical Research, University of Birmingham), and contained a region of the LIFR general promoter from −305 to +24 [Blanchard et al., 2002] or a region of the LIFR placental promoter from −608 to +107 [Wang and Melmed, 1998]. RUNX1 binding sites were mutated using the QuickChange II XL kit (Stratagene, USA) to generate the following mutations: general LIFR promoter, TGCGGA to TGCCCA, placental LIFR promoter site 2 ACCACA to AGGTCA, site 3 TGCCAC to TGGGTC. Site 1 of the placental promoter was removed by generating a deletion construct in pXPG from −303 to +107 of the placental promoter. RUNX1 and RUNX1-ETO plasmids obtained from Addgene have been described previously [Meyers et al., 1995].

CELL CULTURE

KG-1, KG-1a [Furley et al., 1986], and K562 [Lozzio and Lozzio, 1975] myeloid cell lines, the human placental choriocarcinoma

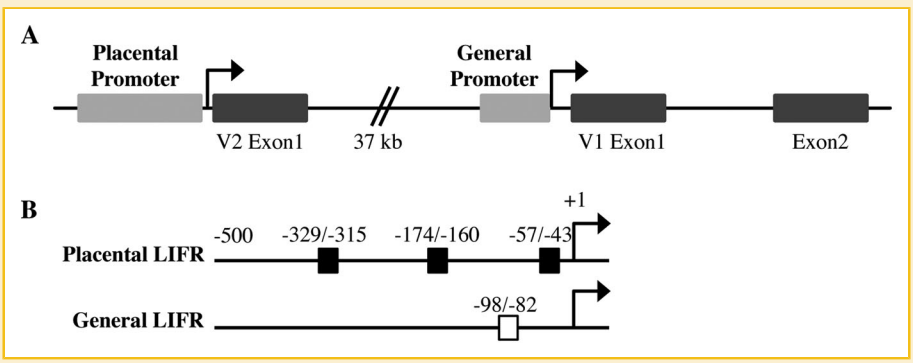

Fig. 1. (A) Schematic representation of the LIFR gene depicting the alternate promoters, the first exons specific to the variant 1 (V1) and variant 2 (V2) transcripts, and the shared second exon. (B) Schematic representation of the LIFR promoters (−510 to +50) relative to the transcription start site (arrow), showing potential RUNX1 binding sites, as determined using Gene2Promoter software (Genomatix). Sites with a core similarity of 1 (black boxes) or 0.909 (white boxes) are shown.

cell line JAR [Pattillo et al., 1972] and HeLa cells were cultured in RPMI 1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, JRH biosciences), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37°C and 5% CO_2. Kasumi-1 myeloid cells [Asou et al., 1991] were cultured similarly but supplemented with 20% FBS. Placental JEG-3 cells [Kohler and Bridson, 1971] were cultured in αMEM (GibcoBRL) supplemented with antibiotics and 10% FBS. All cell lines were obtained from ATCC, USA. Cells were stimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA; Boehringer-Mannheim) and 1 μM calcium ionophore A23187 (I; Sigma–Aldrich, USA).

TRANSFECTION AND REPORTER ASSAYS

Cells (2×10^6) were transfected with 5 μg of reporter plasmid and varying amounts of expression constructs equalized by addition of pSG5 or Rc-CMV, using a Bio-Rad Gene Pulser X Cell at 270 V and 975 μF, as previously described [Oakford et al., 2010]. Cell lysates were prepared 24 h post-transfection for myeloid cells, or 48 h post-transfection for JEG-3 cells, protein quantitated by Bradford protein assay (Bio-Rad), and 30 μg of protein analyzed for luciferase activity (Luciferase assay kit, Promega) using the Turner Biosystems Veritas Microplate Luminometer (Promega).

GENE EXPRESSION ANALYSIS

RNA was extracted from cell lines using Tri-reagent (Sigma–Aldrich). RNA was treated with DNase I (Sigma–Aldrich) and cDNA synthesized using Superscript III reverse transcriptase (Invitrogen), as described previously [Brettingham-Moore et al., 2008]. Quantitative RT-PCR (qRT-PCR) was performed using the QuantiTect SYBR Green PCR kit (Qiagen, USA) in a final volume of 25 μl, including 50 ng cDNA and 0.3 μM of each primer on a Rotor-Gene 6000 real-time cycler (Corbett Research, Australia). Cycling conditions were: 95°C 15 min; 40 cycles of 94°C 15 s, 60°C 60 s, using the following primers: RUNX1 (For 5′-CACCTACCACAGAGCCATCA-3′, Rev 5′-CTCGGAAAAGGACAAGCTCC-3′), LIFR variant1 (For 5′-GCAGGGGATGGCAAGATA-3′, Rev 5′-ATCCAGGATGGTCGTTTCAA-3′), and LIFR variant 2 (For 5′-AGCCTCTGCGACTCATTCAT-3′, Rev 5′-ATCCAGGATGGTCGTTTCAA-3′). PCR conducted in parallel using GAPDH primers (forward 5′-AAGTATGATGACATCAAGAAGGTGGT-3′; Rev 5′-AGCCCAGGATGCCCTTTAGT-3′) was used to normalize for differences in cDNA synthesis and RNA input. To correlate the threshold (Ct) values from the amplification plots to copy number a standard curve was generated for each primer set with PCR product. Melt curves were analyzed for a single peak and PCR products visualized by agarose gel electrophoresis to ensure that a single product was generated.

SIRNA ANALYSIS

KG-1 cells were transfected with 100 nM siRNAs (Ambion) targeting RUNX1b (RUNX1 #2904, Sense 5′-GGGAAACUGUGAAUGCUUCTT-3′; Antisense 5′-GAAGCAUUCACAGUUUCCCTC-3′) and Ambion siRNA control #1 by electroporation as described previously [Oakford et al., 2010]. At 48 h post-transfection, cells were stimulated with PMA and calcium ionophore (PI) for 8 h, RNA isolated and analyzed by qRT-PCR. JEG-3 cells were transfected with 100 nM RUNX1 ON-TARGET plus SMART pool (Dharmacon) or ON-TARGET plus Non-targeting Pool, using Attractene Transfection reagent (Qiagen). RNA was isolated 48 h post-transfection and analyzed by qRT-PCR.

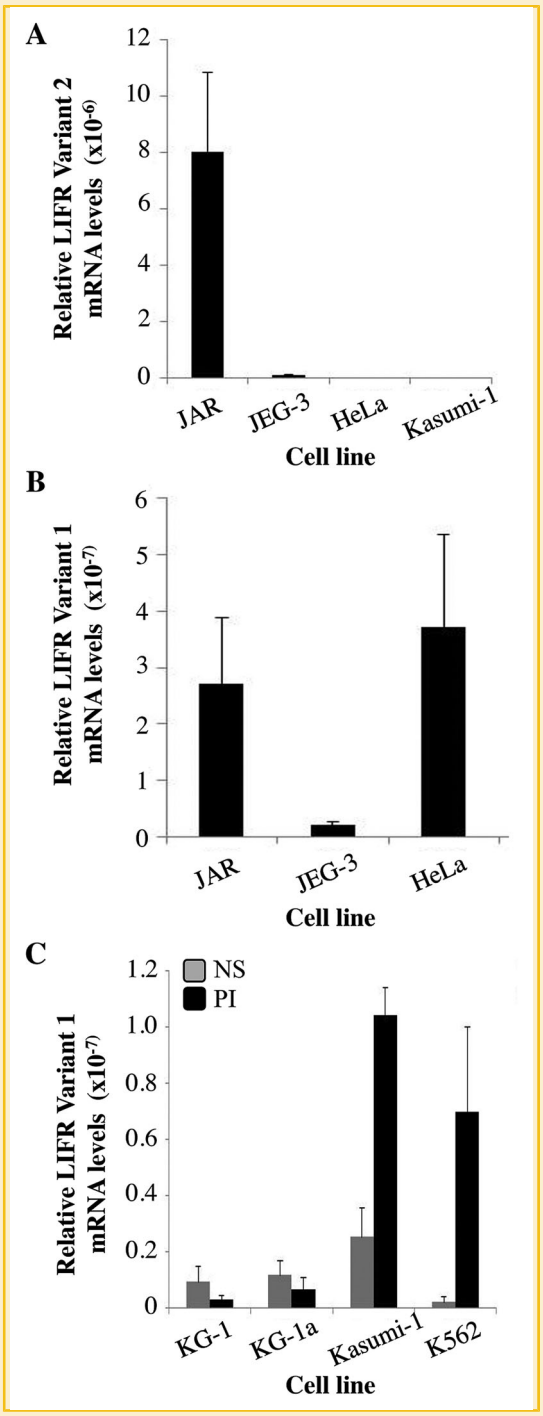

Fig. 2. LIFR expression across different cell types. (A, B) LIFR variant 2 (A) and variant 1 (B) mRNA levels relative to GAPDH were determined by qRT-PCR in a range of cell lines as indicated. (C) LIFR variant 1 mRNA levels were determined as in (A) in a range of myeloid cell lines that were either unstimulated (NS) or stimulated with PMA and calcium ionophore (PI) for 8 h. The mean and standard error of three independent experiments are shown in each case.

CHROMATIN IMMUNOPRECIPITATION (CHIP) ANALYSIS

DNA-protein interactions were examined by ChIP analysis, as described previously [Oakford et al., 2010]. Solubilized chromatin was immunoprecipitated with anti-H3 (1791 Abcam, USA), anti-acetyl H3 (06-599 Millipore, USA), anti-RUNX1 (C-19, Santa Cruz) or anti-ETO (C-20, Santa Cruz) antibodies. Immunoprecipitated DNA was amplified using qPCR with primers designed to the general LIFR promoter (For 5′-TAGAAAACCGAGGCCAAGTG-3′, Rev 5′-GGCTTATTTGTGCGGAGAAG-3′) and placental LIFR promoter (For 5′-CCTAACCTGGGTTGGACTCA-3′, Rev 5′-TGACTGAATGCAT-CAGCAGTGC-3′). Data were analyzed taking into account no antibody control immunoprecipitates and normalized to total input samples.

WESTERN ANALYSIS

Nuclear extracts were prepared as described previously [Brettingham-Moore et al., 2008]. Nuclear proteins were separated by SDS-PAGE, transferred to nitrocellulose membrane and subjected to western blot analysis using anti-RUNX1 (H-65, Santa Cruz), anti-ETO (C-20, Santa Cruz), and anti-H3 (1791, Abcam, UK) antibodies and the corresponding peroxidase-conjugated secondary antibodies (DAKO, Denmark). Proteins were visualized using the Supersignal West Pico Chemiluminescent kit (Pierce, USA).

BISULFITE SEQUENCING

Genomic DNA was isolated from Kasumi-1 cells using the QIAamp Blood Mini Kit (Qiagen) and 2 mg was subjected to bisulfite modification using the MethylEasy DNA Bisulphite Modification Kit (Human Genetic Signatures), following the manufacturers' instructions. Converted DNA was amplified by PCR, utilizing primer sets specific for the general (For 5′-AGGTGTGTTTGTAGAGTTTT-GATT-3′; Rev 5′-ATTACCTAAACAACCCAAAACC-3′) and placental (For 5′-TGATTTGGGTGTAATTGTTTA-3′, Rev 5′-TACCATTCTCC-TACCTCAACCT-3′) LIFR promoters. Purified PCR products (Illustra Gel Band Purification kit, GE Healthcare) were ligated into pGEM-T Easy (Promega). Individual clones were sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences were analyzed using the CpG Bubble Chart Generator, Version 20061209 Alpha, created by Mark A. Miranda.

RESULTS

THE GENERAL LIFR PROMOTER IS REGULATED BY RUNX1 IN MYELOID CELLS

Despite the important roles played by LIFR in a range of biological systems, including the hematopoietic system, surprisingly little is known about its regulation at a transcriptional level. The human LIFR gene is regulated by two alternate promoters (Fig. 1A): the so-called general and placental promoters [Wang and Melmed, 1997; Blanchard et al., 2002], which drive expression of different mRNA transcripts; variant 1 and variant 2, respectively. These transcripts contain different non-coding first exons, but produce the same protein. To date, the variant 2 transcript has only been detected in placental cell lines, while the variant 1 transcript has been detected in a range of cell types [Wang and Melmed, 1997; Blanchard et al., 2002]. However, promoter usage in the hematopoietic system has not been examined. To determine which of the LIFR promoters is active in myeloid cells, expression of the LIFR transcripts was examined by qRT-PCR, using transcript specific primers. Variant 2 transcript was detected in the placental JAR and JEG-3 cell lines (Fig. 2A), but not in HeLa cells, nor any myeloid cell line tested, including Kasumi-1 cells (Fig. 2A), and KG-1, KG-1a, or K562 cells (data not shown). In contrast, variant 1 transcript was detected in both placental cell lines, HeLa cells (Fig. 2B) and all of the myeloid cell lines examined (Fig. 2C). Further, variant 1 transcript expression was inducible in Kasumi-1 and K562 myeloid cells, upon stimulation with the differentiating agents PMA and calcium ionophore.

The transcription factors responsible for regulation of the LIFR promoters are largely unknown. Computational analysis of both LIFR promoters using the Gene2Promoter (release 4.2) tool of the Genomatix software suite identified AML1 consensus motifs indicating potential RUNX1 binding sites in both promoters (Fig. 1B); a single site in the general LIFR promoter and three potential sites in the placental LIFR promoter (Table I).

To determine whether the general LIFR promoter is regulated by RUNX1, a luciferase reporter construct containing the general LIFR promoter (pXPG-gLIFR) was transfected into KG-1 myeloid cells and assayed for luciferase activity. Co-transfection with a RUNX1 expression construct resulted in an approximately threefold increase in luciferase activity (Fig. 3A, $P < 0.05$, Student t-test). To determine whether the RUNX1-ETO fusion protein generated by the t(8;21) translocation also influences general LIFR promoter activity, cells were also transfected with the pXPG-gLIFR construct and RUNX1-ETO expression plasmid, which resulted in an approximately 2.9-old repression of promoter activity (Fig. 3B; $P < 0.05$, Student t-test). Western blotting following transfection of the RUNX1 expression plasmid into both COS-7 cells and K562 cells confirmed RUNX1 expression (Fig. 3C and D). Similarly, RUNX1-ETO expression was confirmed by Western blotting using an anti-ETO antibody in both COS-7 cells and K562 cells transfected with the RUNX1-ETO expression plasmid, which produced a protein of the same size as detected in Kasumi-1 cells (Fig. 3C and D). Overexpression of RUNX1 in K562 myeloid cells resulted in an approximately fivefold increase in activity of the general LIFR promoter (Fig. 3E; $P < 0.01$, one-way

TABLE I. RUNX1 Binding Sites in the LIFR Promoters as Predicted Using Gene2Promoter of the Genomatix Software Suite

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	tattGTGGttttcag
LIFR; placental	2	−174 to −160	(−)	1	0.809	gattGTGGttcaagg
LIFR; placental	3	−57 to −43	(−)	1	0.767	atgtGTGGcattgcc

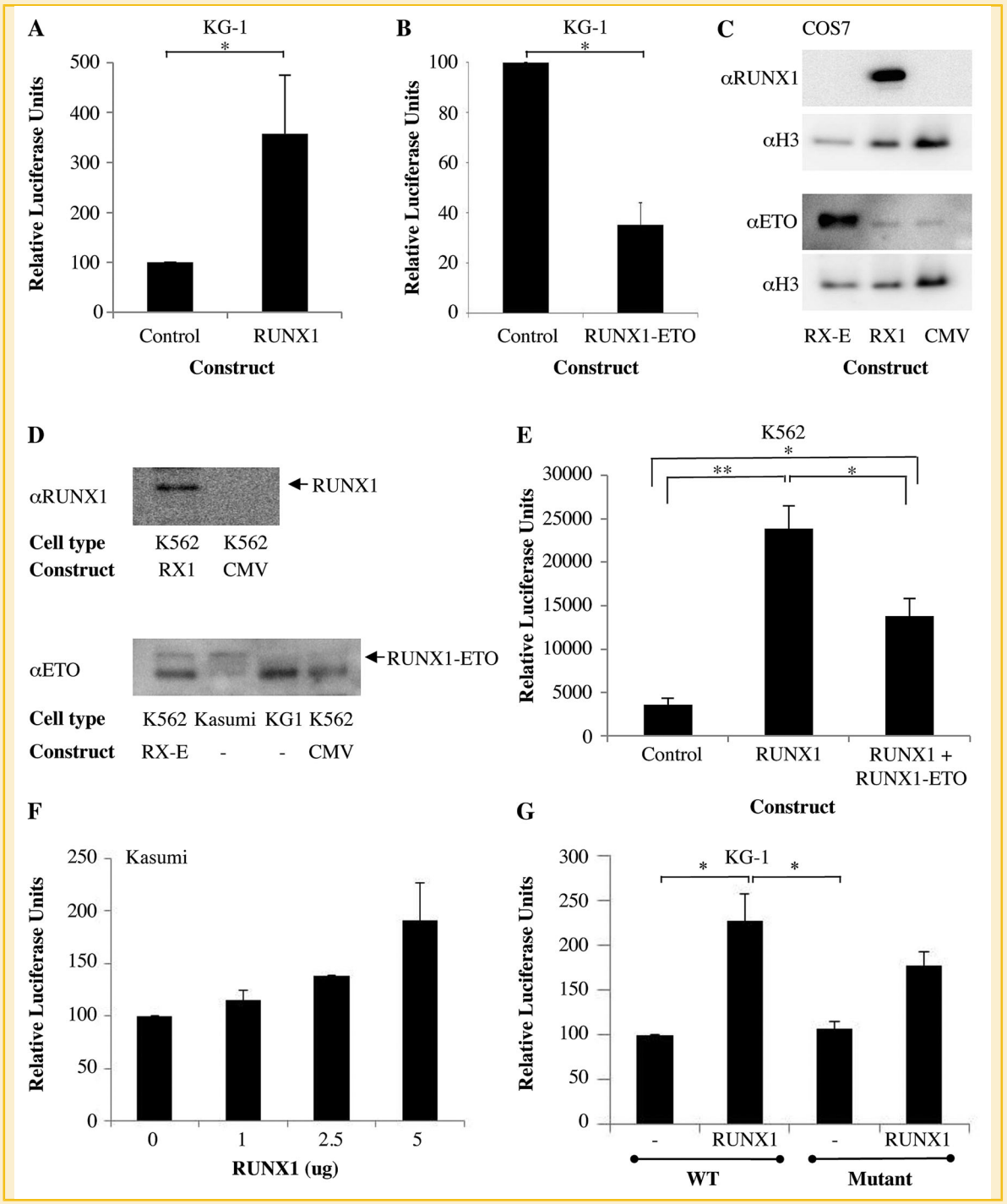

Fig. 3. The general LIFR promoter is regulated by RUNX1. (A) KG-1 myeloid cells were transfected with a LIFR general promoter luciferase reporter construct (pXPG-gLIFR) along with a construct expressing RUNX1. After 24 h protein was extracted and luciferase activity measured and depicted relative to the control transfected sample. The mean and standard error of three independent experiments are shown, $^{*}P < 0.05$, Student *t*-test. (B) KG-1 cells were transfected with pXPG-gLIFR with or without a RUNX1-ETO expression construct. Luciferase activity was measured as in (A). (C-D) Nuclear extracts were prepared from either COS cells (C) or the indicated myeloid cell lines (D) either left untransfected or transfected with RUNX1 (RX1) or RUNX1-ETO (RX-E) expressing plasmids, as indicated. Nuclear proteins were subjected to western analysis using the indicated antibodies. Bands representing RUNX1 and RUNX1-ETO proteins are indicated by arrows. (E) K562 cells were transfected with pXPG-gLIFR along with constructs expressing RUNX1 and/or RUNX1-ETO. Luciferase activity was measured as in (A). The mean and standard error of three independent experiments is shown, $^{**}P < 0.01$ $^{*}P < 0.05$, one-way ANOVA, Tukey's multiple comparison test. (F) Kasumi-1 cells were transfected with pXPG-gLIFR and increasing amounts of RUNX1 expressing plasmid, as indicated. Luciferase activity was measured as in (A). The mean and standard error of three independent experiments is shown, $P < 0.01$, one-way ANOVA, post-test for linear trend. (G) pXPG-gLIFR (WT) or a construct in which the RUNX1 binding site was mutated (mutant) were transfected into KG-1 cells with or without a RUNX1 expression construct, as indicated. Luciferase activity was measured as in (A). The mean and standard error of three independent experiments is shown, $^{*}P < 0.05$, two-way ANOVA.

ANOVA, Tukey's multiple comparison test), and this activity was reduced by co-expression of RUNX1-ETO (Fig. 3E; $P < 0.05$, one-way ANOVA, Tukey's multiple comparison test), demonstrating that RUNX1-ETO can compete with RUNX1 to repress the LIFR promoter. Similarly, overexpression of RUNX1 in the Kasumi-1 cell line containing RUNX1-ETO activated the LIFR promoter in a dose-dependent manner (Fig. 3F, $P < 0.01$, one-way ANOVA, post-test for linear trend), suggesting that RUNX1 overexpression can relieve the repressive effect of RUNX1-ETO on the LIFR promoter.

We next determined whether RUNX1 regulates the general LIFR promoter via the potential RUNX1 binding site in the promoter by examining RUNX1 activation of a reporter containing a mutated RUNX1 binding site. Basal activity of the mutated promoter was similar to the wild-type promoter (Fig. 3G). RUNX1 activated the wild-type promoter as expected ($P < 0.05$, two-way ANOVA) and while RUNX1 was still able to activate the mutant promoter (Fig. 3G), this was to a lesser degree than the wild-type promoter and not statistically different from basal levels of either the wild-type or mutant promoter (two-way ANOVA). This suggests that RUNX1 was acting at least partially through the bioinformatically identified site in the promoter (Fig. 1B). Given that the mutated promoter construct retains some responsiveness to high levels of RUNX1, it does not rule out the presence of another yet unidentified binding site within the promoter.

THE PLACENTAL LIFR PROMOTER IS ALSO REGULATED BY RUNX1

Our identification of three putative RUNX1 binding sites in the placental LIFR promoter (Fig. 1B) suggests that this promoter is also potentially regulated by RUNX1. RUNX1 plays a critical role in hematopoiesis and its expression has been documented in all tissues which support definitive hematopoiesis, including the placenta [Bee et al., 2009; Ottersbach and Dzierzak, 2010]. In keeping with this, RUNX1 expression was detected in the placental cell lines JAR and JEG-3 by qRT-PCR, although at lower levels than in the Kasumi-1 myeloid cell line (Fig. 4A). To determine whether RUNX1 regulates the placental LIFR promoter, JEG-3 cells were co-transfected with a RUNX1 expression plasmid and placental LIFR promoter reporter (pXPG-pLIFR), containing the three potential RUNX1 binding sites (Fig. 4B). RUNX1 overexpression increased activity of the LIFR promoter (Fig. 4C, $^{*}P < 0.05$, two-way ANOVA). JEG-3 cells were then transfected with reporter constructs in which each site was independently deleted or mutated (Fig. 4B), to determine which of the potential RUNX1 sites contribute to promoter activity. Basal activity of the placental LIFR promoter was not altered by deletion or mutation of any of the RUNX1 binding sites (Fig. 4C). RUNX1 activated the promoter in which site 1 was deleted or site 3 was mutated ($P < 0.05$, two-way ANOVA). In contrast to the mutation of site 3, mutation of site 2 dramatically reduced RUNX1 activation of the promoter (Fig. 4C), suggesting that RUNX1 activates the placental promoter through this site.

ACTIVITY OF THE LIFR GENE IS RUNX1-DEPENDENT IN MYELOID CELLS

The data presented above suggest that the LIFR promoters are regulated by RUNX1. To test this further, KG-1 cells were treated with either control or RUNX1 siRNA, as described previously [Oakford et al., 2010]. Transfection of KG-1 cells with RUNX1 siRNA resulted in reduction in RUNX1 mRNA levels by more than 60% (Fig. 5A) which lead to reduced expression of LIFR variant 1 mRNA (Fig. 5B, $P < 0.05$ Student t-test). These data suggest that the endogenous LIFR general promoter is regulated by RUNX1 in myeloid cells.

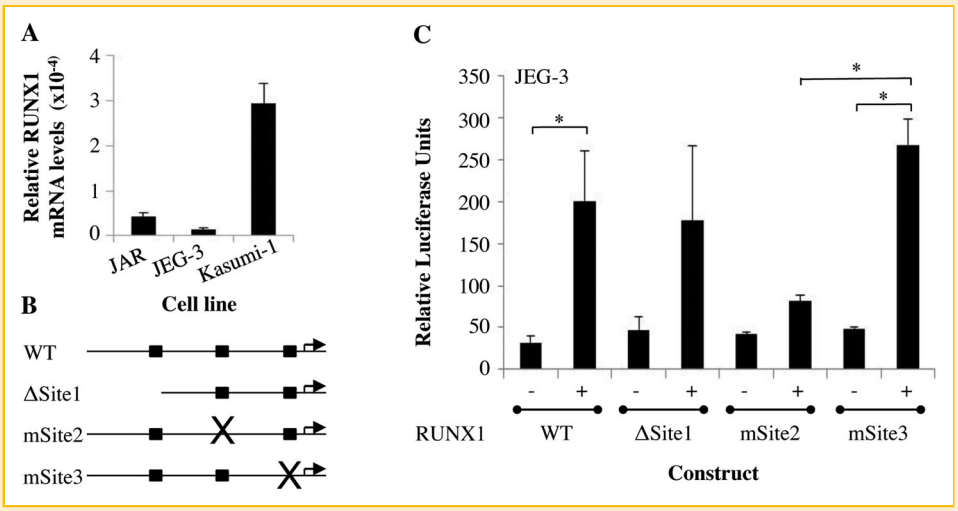

Fig. 4. RUNX1 activates the placental LIFR promoter (A) RUNX1 levels relative to GAPDH were determined by qRT-PCR in a range of cell lines as indicated. The mean and standard error of three independent experiments are shown. (B) Schematic representation of the LIFR placental promoter (−510 to +50) relative to the transcription start site (arrow), showing potential RUNX1 binding sites present in the LIFR placental promoter luciferase reporter construct, pXPG-gLIFR (WT), and the various deletion and mutant constructs examined. (C) JEG-3 cells were transfected with the LIFR placental promoter luciferase reporter construct (WT) along with constructs in which each of the RUNX1 binding sites was deleted (Δ) or mutated (m), either with or without a RUNX1 expression construct, as indicated. After 48 h protein was extracted and luciferase activity measured. The mean and standard error of three independent experiments are shown, $^{*}P < 0.05$, two-way ANOVA.

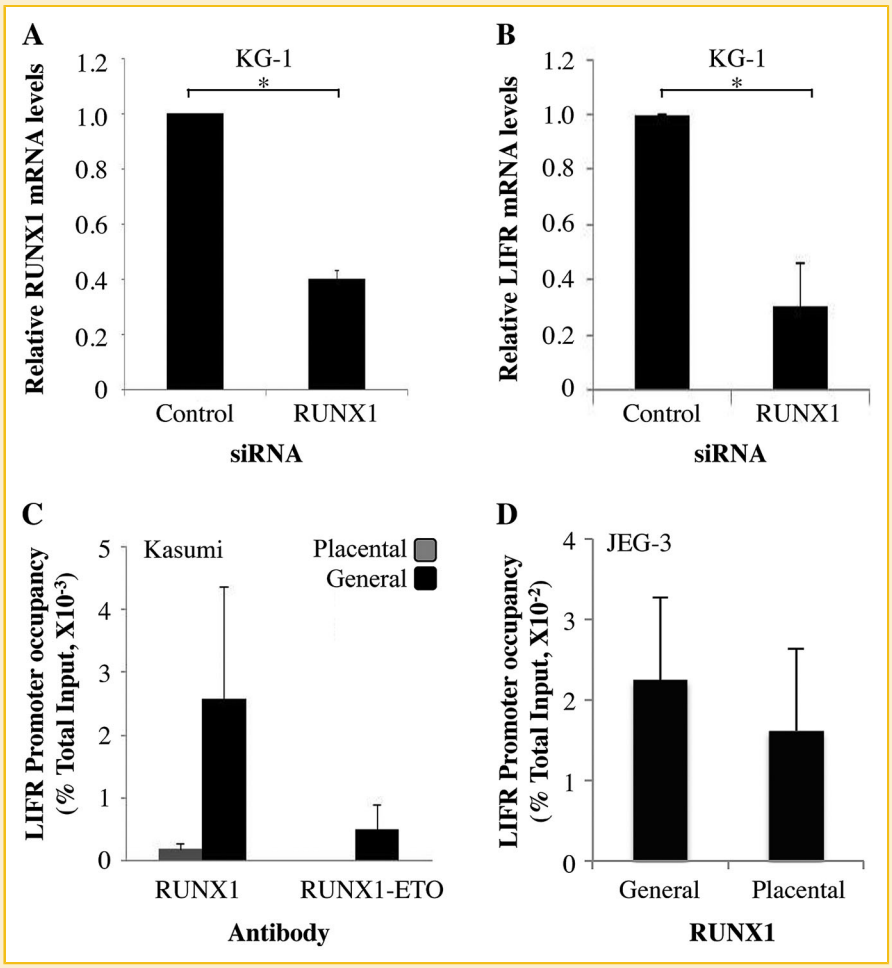

Fig. 5. RUNX1 regulates the LIFR gene in myeloid cells. (A–B) RNA was isolated from KG-1 myeloid cells transfected with control or RUNX1 siRNA and 48 h post-transfection were stimulated with PMA and calcium ionophore for 8 h. RUNX1 (A) and LIFR variant 1 (B) mRNA levels relative to GAPDH were determined by qRT-PCR, and are depicted relative to the control transfected cells. The mean and standard error of three independent experiments are shown, $^{*}P < 0.05$, Student *t*-test. (C) RUNX1 and ETO association with the general and placental LIFR promoters were determined by ChIP analysis in Kasumi-1 cells. Immunoprecipitated DNA was measured by qPCR and is shown relative to total input DNA. The mean and standard error of three independent experiments is shown. (D) RUNX1 association with the general and placental LIFR promoters was determined in JEG-3 cells, as in (C).

Kasumi-1 cells which contain the t(8:21) translocation and therefore endogenously express both RUNX1 and RUNX1-ETO [Asou et al., 1991], express LIFR variant 1 transcript (Fig. 2C). However, the RUNX1-ETO fusion protein was found to repress the general LIFR promoter in reporter assays (Fig. 3B and D). To investigate this further, ChIP analysis was used to examine RUNX1 and RUNX1-ETO binding at the LIFR promoters in Kasumi-1 cells. Enrichment of both RUNX1 and ETO was detected at the general LIFR promoter in Kasumi-1 cells (Fig. 5C). RUNX1 and ETO were also detected at the placental promoter, although at lower levels. Given that both RUNX1 and RUNX1-ETO bind to the general LIFR promoter, competition between RUNX1 and RUNX1-ETO at the general LIFR promoter may explain its expression in Kasumi-1 cells (Fig. 2), despite the presence of the repressive RUNX1-ETO protein in these cells. RUNX1 was detected at approximately equal levels at the general and placental LIFR promoters in JEG-3 cells (Fig 5D), in which both promoters are active.

To determine possible reasons for the differential LIFR promoter activity in myeloid cells, the chromatin environment of the promoters was examined. Accessible promoters are often associated with particular chromatin features, including acetylated histones and demethylated DNA. Therefore, histone H3 levels at the two LIFR promoters was examined by chromatin immunoprecipitation (ChIP) assay in Kasumi-1 myeloid and JEG-3 placental cells. In myeloid cells, while histone H3 occupancy was similar at both the general and placental LIFR promoters (Fig. 6A), acetylated H3 levels were significantly higher at the general compared to placental promoter (Fig. 6B, $P < 0.05$, Student *t*-test), indicative of a more open chromatin environment. This remained true when acetylated H3 levels were determined relative to H3 levels. (Fig. 6C). These data indicate that the general LIFR promoter is assembled into a more accessible chromatin state in myeloid cells. The chromatin environment was also examined in JEG-3 cells which express LIFR from both the general and placental promoters. While acetyl H3levels were

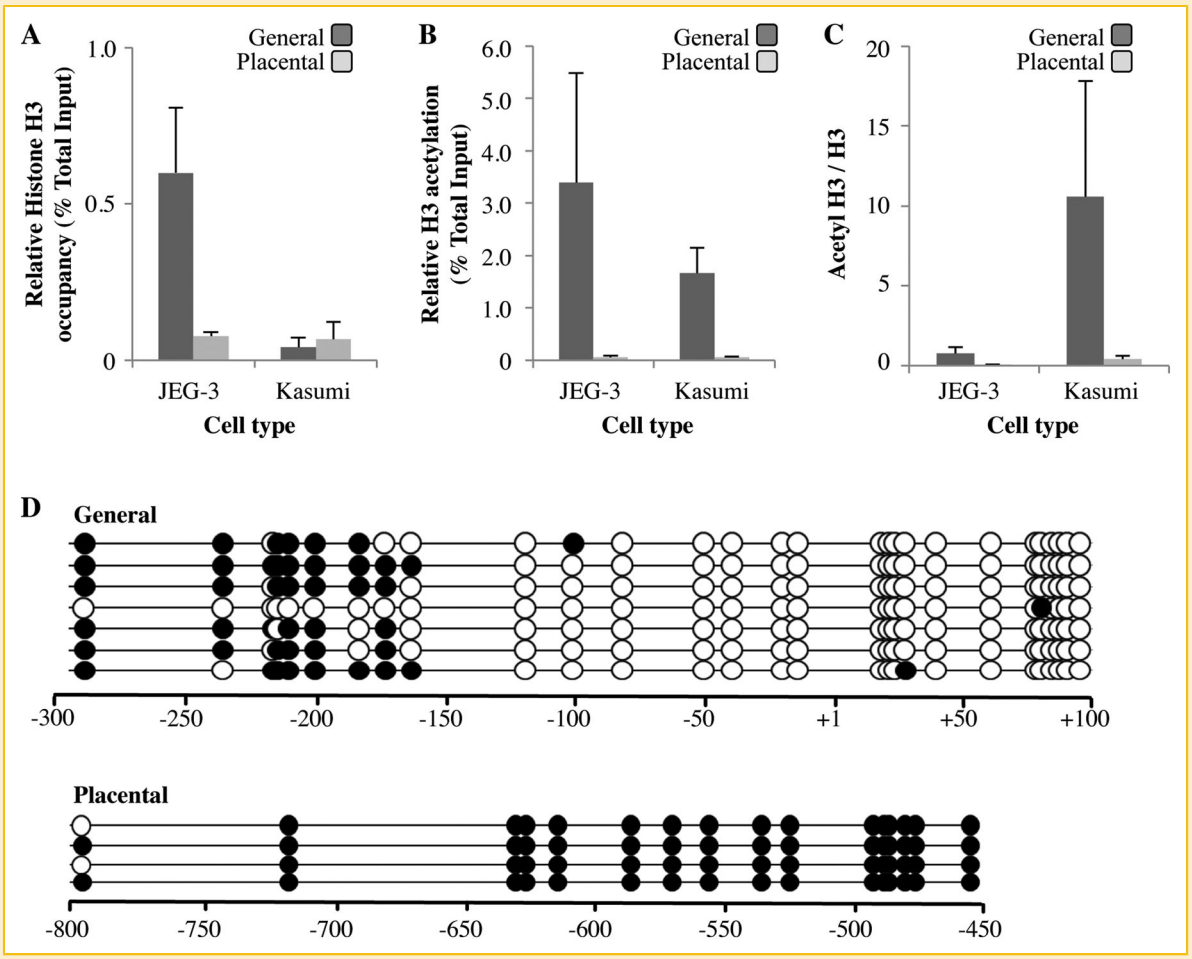

Fig. 6. The general but not placental LIFR promoter is associated with active chromatin in myeloid cells. (A–B) Histone H3 (A) and acetylated H3 (B) levels were determined by ChIP analysis at the general and placental LIFR promoters in Kasumi-1 cells and JEG-3 cells, as indicated. Immunoprecipitated DNA was measured by qPCR and is shown relative to total input DNA. The mean and standard error of three independent experiments is shown, $^{*}P < 0.05$, Student *t*-test. (C) The ratio of acetylated H3 to total H3 as determined in (B) and (A) is depicted. (D) Methylation of a region of the CpG islands associated with the general and placental LIFR promoters, as determined by bisulfite sequencing is shown. Regions analyzed are indicated relative to the transcription start site (+1). Each line represents an individual clone with methylated CpG and unmethylated CpG represented by filled and unfilled circles, respectively.

higher at the general compared to the placental promoter, this reflected higher H3 levels, such that acetylH3/H3 levels were not different at the two promoters (Fig. 6A–C).

The general LIFR promoter is associated with a CpG island and a GC-rich region is also found at the 5′ end of the placental promoter. DNA methylation status of these regions has previously been demonstrated to reflect their activity in different cell types [Blanchard et al., 2002]. Bisulfite sequencing was therefore used to determine whether DNA methylation status correlated with the differences in chromatin status and promoter activity in myeloid cells. Bisulfite sequencing detected considerably lower levels of DNA methylation associated with the general compared to the placental LIFR promoter in Kasumi-1 cells (Fig. 6D). Taken together, these data suggest that the general LIFR promoter is assembled in a more accessible chromatin environment than the placental promoter in Kasumi-1 myeloid cells, which is consistent with the data suggesting that the general but not placental promoter is active in myeloid cells.

Put together these data suggest that expression of the LIFR gene is regulated by RUNX1, with promoter usage in myeloid cells dependent on epigenetic factors.

DISCUSSION

LIFR:gp130 signaling is involved in a diverse array of biological processes ranging from hematopoiesis to blastocyst implantation to neural regeneration [Mathieu et al., 2012]. Despite this, transcriptional regulation of this receptor complex has remained largely unstudied. In this study, the gene encoding LIFR was identified as a novel target of RUNX1. The LIFR gene is regulated by alternate promoters, referred to as the general and placental promoters [Wang and Melmed, 1997; Blanchard et al., 2002]. In agreement with previous characterization, activity of the placental promoter was highly cell-type restricted driving expression only in placentally

derived cell lines, while the general promoter was ubiquitously active, with transcription from this promoter detected in all cell lines examined, including both myeloid and placental cell lines. Bioinformatic analysis identified potential RUNX1 binding sites in both the general and placental LIFR promoters and a number of lines of evidence demonstrate that these promoters are direct targets of RUNX1. Firstly, RUNX1 activates the general LIFR promoter in reporter assays. Secondly, depletion of RUNX1 by siRNA decreases expression from the general promoter, and finally RUNX1 binding was detected at the promoter in a myeloid cell line. While the placental LIFR promoter was also regulated by RUNX1 in placental cells, it was found to be inactive in myeloid cells. While these data support previous studies suggesting that the LIFR gene is regulated by alternate promoters, with activity of the distal promoter restricted to placental cell-types, interactions between the two regulatory elements has not been explored.

In leukemia, particularly AML, RUNX1 activity is commonly altered by point mutations and chromosomal rearrangements, the most frequent being the t(8;21) translocation that produces a RUNX1-ETO fusion protein [Peterson and Zhang, 2004]. While RUNX1 generally functions as a transcriptional activator, RUNX1-ETO most commonly acts as a transcriptional repressor of RUNX1 target genes [Peterson and Zhang, 2004]. In keeping with this, here RUNX1-ETO was demonstrated to have a repressive effect on the general LIFR promoter. Despite this, LIFR expression was detected in Kasumi-1 cells containing RUNX1-ETO, which may be explained by the binding of both RUNX1 and RUNX1-ETO at the promoter.

Known RUNX1 targets include a number of cytokines and cytokine receptor genes important in hemopoiesis, including GM-CSF [Cockerill et al., 1996; Oakford et al., 2010], IL-3 [Taylor et al., 1996; Uchida et al., 1997], and the M-CSF receptor gene [Zhang et al., 1994; Follows et al., 2003]. The data presented here suggest that the *LIFR* gene can now also be added to this list. The LIFR:gp130 receptor complex acts mainly as a signal transducer for the LIF cytokine. LIF is a pleiotropic cytokine with wide ranging action but was originally identified as a factor that prevented blast formation of the highly clonogenic murine myeloid leukaemic M1 cell line. Subsequent studies have described growth stimulating effects of LIF on haemopoietic stem and progenitor cells. Interestingly, synergistic effects of LIF with other RUNX1-dependent multi-lineage cytokines, such as IL-3 and GM-CSF, have been described [Verfaillie and McGlave, 1991]. RUNX1 therefore may influence hemopoetic function through regulation of a network of polyfunctional cytokines and cytokine receptors.

LIF has wide ranging biological effects, with important roles in a number of biological systems. It is therefore likely that LIFR expression is regulated by RUNX1 in a number of tissues, not just the hemopoietic system. In support of this RUNX1 was also shown here to regulate activity of the placental-specific promoter. In addition, the RUNX family includes three proteins: RUNX1, 2, and 3, with each of these proteins binding to similar DNA sequences [Cohen, 2009]. While RUNX1 has an indispensable role in hematopoiesis [Okuda et al., 1996], RUNX3 is important in the hemopoietic and nervous systems [Inoue et al., 2008], and RUNX2 plays a critical role in osteogenesis [Stein et al., 2004]. It is therefore plausible that these family members are involved in regulation of LIFR activity in other cellular systems in which LIFR plays important roles. Further, it is possible that RUNX3, in addition to RUNX1, is an important regulator of LIFR in the haematopoietic cells, and this warrants further investigation. While the focus of this study was on myeloid and placental regulation of LIFR by RUNX1, the findings have implications for other biological systems in which LIF and LIFR function and in other diseases in which RUNX1 dysreulation has been implicated. For example, RUNX1 is highly expressed in breast epithelial cells [Janes, 2011] and appears to have a tumor suppressor role in breast cancer [Chimge and Frenkel, 2013]. Interestingly, the RUNX1 target, LIFR suppresses metastasis in breast cancer [Chen et al., 2012] and therefore further analysis of RUNX1 regulation of LIFR in these and other tumor models is warranted.

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