RUNX1/Core Binding Factor A2 Regulates Platelet 12-Lipoxygenase Gene (ALOX12): Studies in Human RUNX1 Haplodeficiency

Running Head: 12-Lipoxygenase is a direct RUNX1 target gene

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Abstract

Haploinsufficiency of RUNX1 (also known as CBFA2/ AML1) is associated with familial thrombocytopenia, platelet dysfunction and predisposition to acute leukemia. We have reported on a patient with thrombocytopenia and impaired agonist-induced aggregation, secretion and protein phosphorylation associated with a RUNX1 mutation. Expression profiling of platelets revealed ~5 fold decreased expression of 12-lipoxygenase (12-LO, gene ALOX12), which catalyzes 12-hydroxyeicosatetraenoic acid production from arachidonic acid. We hypothesized that ALOX12 is a direct transcriptional target gene of RUNX1. In present studies agonist-induced platelet 12-HETE production was decreased in the patient. Four RUNX1 consensus sites were identified in 2 kb promoter region of ALOX12 (at -1498, -1481, -708, -526 from ATG). In luciferase reporter studies in human erythroleukemia cells, mutation of each site decreased activity; over-expression of RUNX1 upregulated promoter activity, which was abolished by mutation of RUNX1 sites. Gel shift studies, including with recombinant protein, revealed RUNX1 binding to each site. Chromatin immunoprecipitation revealed in vivo RUNX1 binding in the region of interest. siRNA knockdown of RUNX1 decreased RUNX1 and 12-LO proteins.

Conclusions: ALOX12 is a direct transcriptional target of RUNX1. Our studies provide further proof of principle that platelet expression profiling can elucidate novel alterations in platelets with inherited dysfunction.
Introduction

RUNX1, also known as CBFA2 (core binding factor A2), is a member of a family of transcription factors that regulate the expression of several hematopoietic-specific genes through a highly conserved DNA binding region called the RUNT homology domain (RHD).\textsuperscript{1} The RHD dimerizes with CBFβ to form a stable complex. The complex acts as an anchor to recruit other cofactors that bind in cis to adjacent sites or interact directly with RUNX1. RUNX1 plays a critical role in normal fetal hematopoiesis.\textsuperscript{2,3} Homozygous deletion of RUNX1 results in embryonic lethality related to absence of definitive hematopoiesis.\textsuperscript{2-4} In humans, haploinsufficiency of RUNX1 is associated with familial thrombocytopenia, platelet dysfunction and predisposition to acute leukemia.\textsuperscript{5} Most of the point mutations identified in RUNX1 occur in the RHD leading to loss of DNA binding.\textsuperscript{6,7}

We have previously reported\textsuperscript{8,9} studies in a patient with mild thrombocytopenia, impaired agonist-induced platelet aggregation, secretion and protein phosphorylation (myosin light chain and pleckstrin), and decreased platelet PKC-θ, associated with a mutation (haplodeficiency) in the conserved region of RUNX1. Expression profiling of patient platelets revealed ~5 fold decreased mRNA expression of platelet-type 12-lipoxygenase (12-LO, gene ALOX12).\textsuperscript{10} Lipoxygenases are a family of non-heme iron-containing enzymes that catalyze the incorporation of molecular oxygen into polyunsaturated fatty acids, such as arachidonic acid (AA). The platelet 12-LO is expressed primarily in platelets, megakaryocytes, and epidermis; and is present in HEL cells.\textsuperscript{11,12} Activation of platelets results in the release of free arachidonic acid, which is metabolized by two major pathways – cyclooxygenase to thromboxane A2 and by 12-LO to 12-hydroperoxyeicosatetraenoic acid (12-HPETE) that is further reduced to hydroxyeicosatetraenoic
acid (12-S HETE). Thus, 12-LO mediates a major pathway in the metabolism of AA on platelet activation.

While the role of 12-LO in platelets is not as well understood as that of cyclooxygenase-1, LO products have been implicated in several aspects of platelet function. 12-LO has been reported to play a role in thrombin- and thromboxane-induced platelet aggregation and calcium signaling. 12-HETE potentiates thrombin-induced aggregation of bovine and human platelets. Addition of nanomolar concentration of 12-HPETE to platelets primed with non-aggregating concentration of AA or collagen potentiates platelet aggregation; this is associated with increased mobilization of cellular arachidonic acid and thromboxane formation. Inhibition of 12-LO has been reported to reduce GPIIb-IIIa activation and platelet aggregation in ADP, thrombin or U46619 stimulated platelets. Interestingly, mouse platelets deficient in 12-LO had normal aggregation and secretion responses upon activation with most agonists but enhanced aggregation on exposure to ADP, and increased mortality in a thrombosis model involving the injection of ADP. 12-HETE amplifies p-selectin induced tissue factor expression by monocytes. Lastly, 12-LO mediates the generation of peroxide and other reactive oxygen species in platelets by the NADPH oxidase pathway and is a major player in antibody-induced peroxide lysis of platelets. Currently, little is known regarding the transcriptional regulation of 12-LO in platelets. Based on the findings in our patient of decreased platelet ALOX-J2 mRNA expression and RUNX1 haplodeficiency, we postulated that ALOX-J2 is a direct target of this transcription factor, which is implicated in platelet production and function. We provide the first evidence for this in the present studies and show also that platelet 12-HETE production is indeed decreased in our patient with RUNX1 haplodeficiency. Equally important, our studies validate
the concept that platelet expression profiling in patients with inherited platelet dysfunction has
the potential to provide new insights into specific gene/protein abnormalities.

**Materials and Methods**

**Patient Information.** We have previously described\(^8,9\) the clinical presentation and detailed studies in this 24 year old white male, documenting decreased platelet aggregation, secretion, activation of GPIIb-IIIa, pleckstrin and myosin light chain (MLC) phosphorylation, and PKC-\(\theta\) level. This patient has a single point mutation in intron 3 at the splice acceptor site for exon 4 leading to a frameshift with premature termination in the conserved Runt homology domain of RUNX1.\(^9\) Platelet expression profiling studies have been described\(^10\) and show decreased expression of \(ALOX12\) (by 5 fold compared to normal platelets) and other genes. Control subjects used in the studies described here were healthy individuals, not on any medications. These studies were performed after approval by Temple University's Institutional Review Board.

**Platelet Preparation.** Blood was collected in one-tenth volume of acid-citrate-dextrose (ACD) from the patient and control subjects, and platelets isolated as described.\(^23\) Platelet-rich plasma (PRP) was prepared by centrifugation at 180 g for 20 min at room temperature. Platelets were washed twice by pelleting at 500 g for 20 min and resuspended in HEPES modified Tyrode’s buffer (HMTB, pH 7.4, containing 138 mM NaCl, 2.9 mM KCl, 12 mM NaHCO\(_3\), 5.5 mM glucose, and 10 mM HEPES) with 14 mM EDTA. Washed platelets were resuspended in HEPES-Tyrode’s buffer containing 1 mM CaCl\(_2\) and the platelet count was adjusted to a concentration of 3 X 10\(^8\)/ml.
Determination of Platelet 12-HETE Production. 1 ml aliquots of platelet suspensions were stimulated with arachidonic acid (1 mM) or thrombin (1 or 5 U/ml); both from Sigma Chemical, St. Louis, MO) at 37°C for 30 min. Methanol (2 ml) was added to the samples on ice and then acidified with 2N HCl to a pH 3.5 for 15 min. Lyophilized samples were resuspended in 250 μl of assay buffer and used for 12-HETE measurements using an enzyme-linked immunoassay kit (Assay Designs, Ann Arbor, MI).

Cell-line and Culture Conditions. Human erythroleukemia (HEL) cells (American Type Cell Culture, Rockville, MD) were grown at 37°C in 5% CO2. The culture media used was RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin. Cells were stimulated with 50 nM phorbol 12-myristate 13-acetate (PMA; Fisher Scientific, Pittsburgh, PA) to induce megakaryocytic transformation.24

Construction of Luciferase Reporter Vectors. To construct wildtype and deletion mutants, human ALOX-12 promoter regions of various lengths with common 3’-prime end were prepared by PCR amplification of human genomic DNA. The primers used for amplification are shown in Table 1 (Supplement). To facilitate directional cloning, two restriction sites Xho1 and Nhe1 were incorporated into the primer at the 5’ and 3’ end, respectively. The PCR products were verified by sequencing (Genewiz Inc; NJ) and cloned into promoterless luciferase reporter gene vector PGL3- Basic (Promega Corp, Madison, WI).

Mutagenesis in the RUNX1 consensus site (TGGGGT) was done by altering 3 nucleotides in the binding site by a PCR-based method using mutant primers (Supplement Table 1). The PCR
products were verified by sequencing and then cloned into PGL3-Basic (Promega) luciferase expression vector.

**Transfection of Plasmids in HEL Cells.** HEL cells (5X10^5/well) were cotransfected with firefly luciferase reporter plasmid (2 μg) and Renilla luciferase reporter plasmid (Promega, Madison, WI) in a ratio of 50:1 using Lipofectamine (Invitrogen) or FuGene 6 Transfection Reagent (Roche, Indianapolis, IN) as per manufacturer’s instructions. Transfected cells were incubated for 3 hr at 370 C in 5% CO2. At the end of incubation, 250 μl of growth media containing 2X concentration of serum and 50 nM of PMA was added to each well. Cells were harvested after 24 hr and analyzed for luciferase activity using Dual-Luciferase assay system (Promega). The promoter activity was calculated by dividing the luciferase activity of the constructs by the internal Renilla luciferase activity and expressed relative to that of the promoterless vector. All transfection studies were performed in triplicate. To study the effect of over-expression of RUNX1, HEL cells (1x10^6) were cotransfected using Turbofectin 8.0 (ORIGENE Technologies, Rockville, MD) with 2 μg each of luciferase reporter constructs with ALOX12 promoter region and RUNX1 expression plasmid, RUNX1-pCMV6-XL4 (SC106348, ORIGENE Technologies, Rockville, MD). In parallel, luciferase reporter vectors were cotransfected with empty vector, pCMV6-XL4, as a control. Renilla luciferase reporter plasmid was cotransfected as an internal standard, as described above. After 3 hours of transfection, medium containing 50 nM PMA was added to the cells. Cells were lysed at 48 hours and assayed for luciferase activities.

**Electrophoretic Mobility Shift Assay (EMSA).** Nuclear extracts were prepared from PMA-stimulated HEL cells using Pierce Kit (NE-PER Nuclear and Cytoplasmic Extraction kit,
Rockford, IL) as per manufacturer’s instructions. Cell extracts were stored at -80°C until use. A 33 bp wild type and five mutant IRDye 700 Infrared Dye Labeled oligonucleotides corresponding to each RUNX1 binding site were synthesized (Integrated DNA Technologies, Coralville IA) (Supplement Table 1). EMSA was performed using a commercially available kit (Light Shift™ EMSA Kit, Pierce) according to manufacturer’s instructions. Nuclear extracts, with or without 50-fold excess of unlabeled competitor oligo, were incubated for 15 min at room temperature in 1X binding buffer. IRDye 700-labeled, double stranded oligos were then added and the binding reactions were performed for 30 min at room temperature. For supershift assays, antibody against RUNX1 (Santa Cruz Biotechnology; sc-8563) or nonspecific IgG (Santa Cruz) was pre-incubated with nuclear extracts at room temperature for 30 min before addition of labeled probe. The DNA-protein complexes were resolved on native 4% polyacrylamide gels and the protein-DNA complexes were detected using Odyssey Infrared Imaging System (LiCor Biosciences).

Binding of recombinant RUNX1 (ABNOVA Taiwan Corporation) to ALOX12 was conducted in 1X LiCor binding buffer (LiCor Biosciences). 50 ng of recombinant protein, diluted in buffer containing 0.1 mM EDTA, 100 mM KCl, 20 mM HEPES pH 7.8, 5 μg/ml BSA and 20% glycerol was incubated with IRDye 700-labeled or unlabeled, double stranded oligos and 20 ng dI:dC along with 2 mM MgCl2 and 25 mM DTT/2.5% Tween-20. For supershift assay antibody against RUNX1 (Santa Cruz Biotechnology; sc-8564X) or nonspecific IgG (Santa Cruz) was pre-incubated for 30 min in cold before addition of labeled probe. Rest of the procedure was performed as described earlier.
**Immunoblotting Analysis.** Total cell lysates were prepared from co-transfected HEL cells using M-Per™ Mammalian Protein Extraction Reagent (Pierce Chemical) with proteinase inhibitors (1 mM EGTA, 1mM dithiothreitol [DTT], 1mM phenylmethylsulfonyl fluoride [PMSF], 10 mg/ml leupeptin), and protein concentrations were determined by the biocinchoninic acid method (BCA) using a commercial assay (Pierce Chemical, Rockford, IL); the aliquots were stored at –80°C. Samples were boiled, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% resolving gel) and electrophoretically transferred onto polyvinylidene fluoride (PVDF) nitrocellulose (Millipore, Bedford, MA). The membranes were washed in tris-buffered saline (TBS) with 0.1% Tween 20. The primary antibodies were used at a dilution 1:1000 for rabbit polyclonal anti-RUNX1, 1:400 for rabbit polyclonal anti-12-LO, and 1:7000 for anti-β-actin (Santa Cruz, CA). Antibodies bound to nitrocellulose were detected using a peroxidase-conjugated goat anti-rabbit IgG as the secondary antibody (1:10000) (Promega, Madison, WI). Blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore corporation, Bedford, MA) and detected using a Luminescent Image Analyzer (Fuji Film Medical Systems, Stamford, CT). For studies on the effect of over-expression of RUNX1, HEL cell lysates were subjected to immunoblotting using anti-RUNX1 (sc-8563) and anti-actin (I-19)-R (sc-1616-R) antibodies from Santa Cruz Biotechnology. Infrared labeled secondary antibodies were used to detect the expression on Odyssey Infrared Imaging System (Li-Cor Biosciences).

**siRNA Mediated Knockdown of RUNX1.** siRNA against RUNX1 and unrelated mock siRNA were purchased from Santa Cruz Biotechnologies. The RUNX1 siRNA used was a pool of 3 target-specific 19-25 nt siRNAs designed to knock down gene expression. For transfection 400
nM of siRNA and 2 μg of wildtype construct were dissolved in 100 μl of Optimem media (Invitrogen). In another tube transfection media was dissolved in 100 μl of Optimem media. The two mixtures were then combined and incubated for 45’ at room temperature. The mixture was added to 50,000 HEL cells resuspended in 400 μl of serum free media, plated in a 24 well plate and incubated for 5 hr at 37°C. After transfection, 2X fetal bovine serum medium containing 50 nM of PMA was added to the wells. On the following day, the 2X medium was replaced with regular medium containing 50 nM PMA. Cells were harvested at 48 hr after PMA stimulation for whole cell extract and luciferase assays as described.

**Chromatin Immunoprecipitation (ChIP) Analysis.** This was performed using ChIP-It kit (Active Motif). PMA-stimulated (24 h) HEL cells were cross-linked with 1% formaldehyde at room temperature for 10 min followed by addition of glycine to a final concentration of 0.125 M. Cell lysates were dounce homogenized and nuclear pellet was sheared enzymatically. The sheared chromatin was incubated with anti-RUNX1 antibody (sc-8564x; Santa Cruz, CA) or control IgG (Santa Cruz, CA) overnight at 4°C. This anti-RUNX1 antibody has been used previously in ChIP studies. The chromatin/antibody complex was eluted with 1% SDS/1M NaHCO₃ and reverse cross-linked by heating to 65°C. The DNA was treated with proteinase K and purified by spin columns as per kit instructions. The purified DNA samples were used as templates for PCR, with primer pairs spanning ~200 bp of ALOX12 gene promoter. As a negative control GAPDH was amplified with specific primers provided in the kit.

**Bioinformatics.** Potential binding sites for transcription factors were analyzed by computer program TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCH.html).
Results:

Activation-Induced Platelet 12-HETE Production is Decreased

As an index of 12-LO activity, 12-HETE production was measured in platelets activated with arachidonic acid and thrombin (Fig 1). These studies showed that platelet 12-HETE production was markedly diminished in the patient with both agonists.

Luciferase Reporter Studies in HEL Cells

Analysis of ALOX12 5' upstream region (2 kb) using ‘TFSEARCH’ program indicated the presence of four potential RUNX1 binding sites (Fig 2A): a palindromic sequence at -1498/-1486 with two RUNX1 consensus sites (labeled site 1A and site 1B) separated by a single nucleotide; at -708 (site 2), and -526 (site 3) bp upstream from ATG. None of the identified RUNX1 sites were the predominant consensus (5’-PuACCPuCA-3’) RUNX1 sequence but were a RUNX1 variant (TGGGGT) sequence that has previously been reported and shown to play a major role in regulation of αIIb promoter and megakaryocytic differentiation. A similar palindromic sequence with two RUNX1 sites has been reported in human urokinase plasminogen activator promoter.

Luciferase reporter studies were performed using the 5’ upstream region -1593/-1 and its serial truncations in HEL cells treated or not with PMA (Fig 2B). The luciferase activity was markedly greater in HEL cells treated with PMA than in its absence (Fig 2B) indicating that PMA-induced megakaryocyte transformation is associated with upregulation in ALOX12 promoter activity. In PMA-treated HEL cells, truncation to -871 bases showed an 85% decrease in promoter activity,
indicating presence of a positive regulatory region. Further deletion to -705 did not lead to additional decrease in activity. Deletion to -438 bp, which eliminates all 4 potential RUNX1 binding sites, showed a ~90% decrease in promoter activity compared to the -1593/-1 construct. The initial large decline in activity with truncation at -871 with no further loss with subsequent truncations suggested that -1593/-871 region may be particularly important for maximal promoter activity.

In subsequent reporter studies using construct -1593/-1 we mutated each of the putative RUNX1 binding sites (Fig 2C). Site 1A mutant construct contained 3 bp mutation (from TGGGGT to TGGATC) in the region of -1498 / -1493. Site 1B mutant construct had 3 bp mutation in -1491 / -1485 region (from ACCCCA to ACGTAT). Site 2 (-708) mutant construct was mutated from ACCCCA to ATCCAG; and Site 3 mutant (-526) was mutated (from ACCCCA to GATCCA). Compared to the wildtype sequence there was a decrease in luciferase activity on mutating each of the above four sites, indicating their individual functional relevance. Taken together, the findings in studies with serial deletions (Fig 2B) and mutations of the individual RUNX1 sites (Fig 2C) are consistent with the conclusion that multiple RUNX1 binding sites (including sites 2 and 3) are required for optimal promoter activity. This has been previously noted with respect to regulation of αIIb promoter by multiple RUNX1 sites.\textsuperscript{27} To determine that these sites are specific to RUNX1 we carried out EMSA.

**Binding of RUNX1 to Consensus Sites by Electrophoretic Mobility Shift Assay**

EMSA was performed with 30-mer probes (Supplemental Table 1) with the RUNX1 consensus sites and nuclear extracts from PMA-stimulated HEL cells. Fig 3A shows the results with the
DNA probe -1510/-1481 that has the palindromic sequence with two consensus (sites 1A and 1B). Protein binding was observed that was competed by excess unlabeled probe with loss of specific bands (Fig 3A, lanes 2 and 3). Competition with excess unlabeled probes with either site 1A (lane 4) or site 1B (lanes 5) mutated revealed inhibition of protein binding, consistent with the presence of the second RUNX1 binding site in each of these probes. In contrast, excess unlabeled probe with both sites mutated (lane 8) did not inhibit protein binding. The observed binding was inhibited by anti-RUNX1 antibody (lane 6), but not by a control IgG (lane 7).

Binding studies were also performed with probes with site 1A or site 1B mutated (Fig 3B). With both probes protein binding was observed (lanes 2 and 7) that was competed by excess unlabeled probe (lanes 3 and 8). Anti-RUNX1 antibody inhibited protein binding (lanes 5 and 10); control IgG had no effect (lanes 4 and 9). These findings indicate that RUNX1 can bind to both site 1A and site 1B.

EMSA with an oligo encompassing the RUNX1 site 2 at –721 bp also showed protein-binding (Fig 3C, lane 2), which was competed by unlabeled probe (lane 3) but not by a mutant probe (lane 4). It was blocked by RUNX1 antibody (lane 5) but not control IgG (lane 6). Similarly, studies with the probe with the potential RUNX1 site at -537 bp (site 3) showed protein-binding (Fig 3D, lane 2) that was competed by excess unlabeled probe (lane 3) and by RUNX1 antibody (lane 5), but not by a mutant probe (lane 4) or control IgG (lane 6). These studies suggest that RUNX1 binds to the probes with sites 2 and 3. Overall, the gel-shift studies provide evidence of RUNX1 binding to all of the 4 sites in the promoter region studied.
To obtain additional evidence that RUNX1 binds to the DNA elements we performed studies using recombinant RUNX1 (Fig 4). Fig 4A shows RUNX1 bindings to the probe (-1510/-1481) containing two consensus sites (lane 2). This binding was competed by excess unlabeled probe (lane 3). The observed band was abolished by anti-RUNX1 antibody and a supershifted band was noted (lane 4); this was not noted with control IgG (lane 5). Likewise, studies with the probes containing site 2 at -721 bp (Fig 4B, lanes 1-5) and site 3 at -537 bp (Fig 4B, lanes 6-10) also showed similar results with a loss of the observed band and a supershifted band observed with anti-RUNX1 antibody (lanes 4 and 9, respectively), but not IgG (lanes 5 and 10, respectively). These studies provide evidence that RUNX1 binds to the regions of ALOX12 promoter.

**Binding of RUNX1 in vivo using Chromatin Immunoprecipitation Studies**

To demonstrate that RUNX1 binds to the region of interest *in vivo* as well, ChIP analysis was performed using PMA stimulated HEL cells. The DNA fragment (-1679/-1376), which encompasses RUNX1 binding sites 1A and 1B, showed specific amplification by PCR of the RUNX1 precipitate (Fig 5, lane 2) whereas IgG control did not show any amplification. Fragments corresponding to region -840/-600 (containing site 2) and -597/-360 (containing site 3) showed no amplification in the DNA precipitated with the RUNX1 antibody or the control (IgG). These results indicate that RUNX1 binds *in vivo* to the region of -1498/-1485 bp with sites 1A and 1B, and suggest that the relevance *in vivo* of the other two sites may be less under the cellular conditions studied, but does not exclude such a role under a different cellular state.

**Effect of siRNA Knockdown of RUNX1**
To determine whether knockdown of endogenous RUNX1 would have an effect upon ALOX12 promoter activity, ALOX12 wildtype luciferase reporter construct (-1593/-1) was cotransfected with either RUNX1 siRNA or with mock siRNA. RUNX1 siRNA decreased promoter activity by ~50% compared to cells transfected with mock siRNA (Fig 6A). In parallel experiments immunoblotting also showed a decrease in RUNX1 as well as 12 LO protein levels in cells transfected with siRNA as compared to mock transfected cells (Fig 6B).

Effect of Over-Expression of RUNX1 on ALOX12 Promoter Activity in HEL Cells

The promoter activity of wildtype ALOX12 construct was markedly enhanced by RUNX1-over-expression (Fig 7). Promoter activity was markedly decreased with mutations in site 1A or site 1B, and over-expression of RUNX1 did not increase the activity. Mutations of site 2 or site 3 also decreased the activity relative to wildtype construct; with over-expression of RUNX1 there was a minimal increase, but the activity was markedly less than the effect noted with the wildtype construct. We interpret the minimal increases observed with sites 2 and 3 as indicating an effect of RUNX1 over-expression on intact sites 1A and 1B. Together, these results are also consistent with the conclusion the multiple RUNX1 sites participate in the overall regulation of ALOX12.

Discussion

Our studies provide evidence that ALOX-12 is a direct target of transcription factor RUNX1 in megakaryocytes/platelets. RUNX1 is a transcription factor that regulates the expression of hematopoietic-specific genes, and plays a major role in hematopoiesis. Our previous transcript profiling studies have shown that ALOX-12 expression is decreased in platelets from the
patient with RUNX1 haplodeficiency. Computer-based analysis identified four RUNX1 binding sites in the proximal 2 kb ALOX-12 promoter region. In luciferase reporter studies, mutations in the individual sites resulted in substantial loss of transcriptional activity (Fig 2C) suggesting that each site is functionally important and that multiple sites may interact to produce optimal promoter activity. Gel-shift assays using nuclear extracts revealed that each of the four sites binds RUNX1 (Fig 3). In additional EMSA studies recombinant RUNX1 also bound to the DNA regions of interest (Fig 4). Presence of multiple RUNX1 consensus sites is a frequent finding in RUNX1-regulated promoters, and they could be closed spaced or separated by hundreds of base pairs. RUNX1 can also form homodimers that modulate RUNX1 activity. ChIP analysis showed enrichment of the region -1676/-1376 that encompasses sites 1A and 1B (Fig 5) indicating in vivo binding of RUNX1 to this region. Consistent with this, the EMSA studies showed RUNX1 binding to these two sites (Fig 3) and in luciferase studies mutations inhibited promoter activity (Fig 2). Our studies using siRNA to downregulate RUNX1 showed a decrease in promoter activity of ALOX12 (Fig 6) along with a concomitant decrease in RUNX1 and 12-LO protein. In addition, over-expression of RUNX1 markedly enhanced promoter activity with a loss of this effect with mutation of the four RUNX1 sites (Fig 7A). Lastly, but most importantly from the perspective of physiological relevance, we show a decrease in agonist-induced 12-HETE production (Fig 1) in the platelets from the patient with the decreased ALOX-12 expression, providing strong evidence from the primary cell (platelet) and a direct functional validation of the decreased transcriptional regulation. Together, these studies constitute the first evidence that platelet ALOX-12 is regulated by RUNX1 at the transcriptional level.
Computer-based analysis also revealed the presence of multiple consensus sites for GATA-1 a proto-typic transcription factor that serves a major role in megakaryocytic differentiation\textsuperscript{32} and cooperates with RUNX1.\textsuperscript{27} Interestingly, the four RUNX1 sites present in the 2 kb upstream region are flanked by GATA1/GATA2 consensus sites located at -1926, -1538, -1408, -1328, -1152, -1068, -857, -729, -690, -531 and -421. The role of these flanking sites in transcription regulation of \textit{ALOX12} remains to be determined. Other significant elements such as AP2 (-558, and -707), NFkB (-539), SP1 (-158 and -594), C/EBP (-433), CACC (-289), Ets (-1479), p300 (-488), and cMyb (-891 and -278) binding sites have also been predicted in 2 kb upstream region. NFkB has been reported as negative regulator of 12-lipoxygenase.\textsuperscript{33} Full transcriptional activity of RUNX1 is dependent upon cofactors such as Ets, cMyb and p300 which can bind in cis to adjacent DNA sequences to form a complex. Clustering of these sites around RUNX1 binding sites suggest that these transcription factors may work in conjunction with RUNX1 to regulate the \textit{ALOX12}. These interactions remain to be elucidated.

Thrombin-stimulated platelet 12-HETE production is decreased in our patient (Fig 1). Although the anucleate platelets possess a functional spliceosome and the ability to synthesize some proteins on activation,\textsuperscript{34,35} the bulk of the proteins present in platelets come from megakaryocytes. Therefore, downregulation of \textit{ALOX12} at the megakaryocyte level as a result of the RUNX1 haplodeficiency and associated decreased enzyme levels provide a cogent explanation for the impaired 12-HETE production in platelets.

Deficiency of platelet 12-LO activity has been reported in patients with myeloproliferative diseases associated with thrombo-hemorrhagic symptoms\textsuperscript{36,37} and in one report mRNA levels
were also decreased. To our knowledge, an inherited deficiency of platelet 12-LO has hitherto not been described. A number of platelet abnormalities have been described in patients with RUNX1 haplodeficiency, including in platelet aggregation and secretion, dense and alpha granule contents, Mpl receptors, activation of GPIIb-IIIa, phosphorylation of myosin light chain and pleckstrin, and platelet PKC-θ. To this we now add decreased 12-LO activity and 12-HETE production (Fig 1). Platelet function defect in RUNX1 haplodeficiency is, therefore, complex and arises by multiple mechanisms mediated by distinct gene products presumably regulated by the transcription factor RUNX1. Additional studies on the targets of RUNX1 should provide insights into those specific genes.

Another major hallmark of RUNX1 haplodeficiency is familial thrombocytopenia and impaired megakaryopoiesis. Lipoxygenase products have been implicated in aspects of hematopoiesis. Lipoxygenase inhibitors block myelopoiesis induced by colony-stimulating factor and PMA, and erythropoietin-stimulated erythropoiesis. 15-LO has been implicated in maturation of reticulocytes to mature red cells. 12-LO is actively expressed in immature megakaryocytes. In human bone marrow cells, there is strong expression in megakaryocytes but not erythroblasts. PMA induces megakaryocytic transformation of HEL cells and our promoter studies show a striking upregulation of ALOX-12 expression during such a transformation (Fig 2B). These observations suggest a potential role of ALOX-12 in megakaryocyte biology. Moreover, RUNX1 interacts with GATA-1 and enforced RUNX1 expression in K562 cells enhances induction of megakaryocytic-specific integrin αIIb, suggesting an important RUNX1 role in megakaryocytic lineage commitment. Because of altered megakaryopoiesis associated
with RUNX1 mutations, the contribution of ALOX-12 to megakaryocyte biology needs to be explored.

Several studies have established the feasibility of platelet expression profiling despite the limited amount of mRNA present in these anucleate cells. Our present studies provide further proof of concept that this technology can indeed elucidate specific and novel molecular aberrations in patients with inherited platelet dysfunction - in the vast majority of whom we currently have no understanding of the molecular mechanisms.

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Authorship Contributions
G.K. performed the research, analyzed the data and wrote the paper. G.M. performed the research. G.J. performed the research. A.K.R. designed the research, analyzed the data and wrote the paper.

Conflicts of Interest
None of the authors have a conflict of interest.

References


Figure Legends

Figure 1. Arachidonic acid (AA) and thrombin-induced platelet HETE production in patient (filled bars, mean of two visits) and control subjects (open bars, mean ± SE).

Figure 2: Luciferase reporter studies on ALOX12 promoter in PMA-treated HEL cells.

A. Schema showing 5’ upstream region of ALOX12 gene with four RUNX1 consensus sites.

B. Effect of 5’ sequential truncation of ALOX12 promoter region on expression of luciferase reporter in untreated (closed bars) and PMA-treated (open bars) HEL cells. Fold increase in luciferase activity was measured as ratio of control vector to luciferase vectors carrying various lengths of promoter and normalized to renilla luciferase constructs. Shown values are mean (± SEM) of three experiments.

C. Effect of disruption of individual RUNX1 sites on ALOX12 expression. Each RUNX1 site was disrupted sequentially by introducing point mutations (open boxes) in RUNX1 binding sites. Shown are mean of three experiments.

Figure 3. A. EMSA using wildtype (WT) oligonucleotide probe with sites 1A and 1B, and nuclear extract from PMA-treated HEL cells. Lane 1: probe alone; lane 2: probe with nuclear extract; lane 3: competition with excess unlabeled probe; lane 4: competition with unlabeled site 1A mutant probe; lane 5: competition with unlabeled site 1B mutant probe; lane 6: effect of RUNX1 antibody; lane 7: effect of nonspecific IgG; lane 8: competition with unlabeled probe with sites 1A and 1B mutated.

B. EMSA using oligonucleotide probes with site 1A mutated (Mutant probe 1A, lanes 1-5) or site 1B mutated (Mutant probe 1B, lanes 6-10). Lanes 1 and 6: probe alone; lanes 2 and
7: protein binding with nuclear extract; lanes 3 and 8: competition with excess respective unlabeled probe; lanes 4 and 9: effect of non-specific IgG; lanes 5 and 10: inhibition of binding with RUNX1 antibody.

C. EMSA using oligonucleotide probe with site 2 (-708/-703). Lane 1: free probe; lane 2: protein binding with extract; lane 3: competition with excess unlabeled probe; lane 4: competition with unlabeled probe with site 2 mutated; lane 5: inhibition of binding with RUNX1 antibody; lane 6: effect of non-specific IgG.

D. EMSA using oligonucleotide probe with site 3 (-526/-521). Lane 1: free probe; lane 2: protein binding with extract; lanes 3 competition with excess unlabeled probe; lane 4: competition with unlabeled probe with site 3 mutated; lane 5: inhibition of binding with RUNX1 antibody; lane 6: effect of non-specific IgG.

Figure 4. A. EMSA using oligonucleotide probe with sites 1A and 1B and recombinant RUNX1. Lane 1: probe alone; lane 2: probe with recombinant RUNX1; lane 3: competition with excess unlabeled probe; lane 4: effect of RUNX1 antibody (arrow, supershift); lane 5: effect of nonspecific IgG.

B. EMSA using recombinant RUNX1 and oligonucleotide probes with site 2 (-708/-703) (lanes 1-5) and with site 3 (-526/-521) (lanes 6-10). Lane 1: free probe with site 2; lane 2: protein binding with recombinant RUNX1; lane 3: competition with excess unlabeled probe; lane 4: effect of RUNX1 antibody (arrow, supershift); lane 5: effect of non-specific IgG; lane 6: free probe with site 3; lane 7: protein binding with recombinant RUNX1; lanes 8 competition with excess unlabeled probe; lane 9: effect of RUNX1 antibody (arrow, supershift); lane 10: effect of non-specific IgG.
Figure 5. Binding of RUNX1 to ALOX12 promoter region in vivo. Chromatin immunoprecipitation (ChIP) using control IgG (column 1) and RUNX1 antibody (column 2). Column 3 shows PCR of total input DNA. Shown are PCR amplification of ALOX12 promoter regions encompassing sites 1A and 1B (-1679/-1376 nt), site 2 (-840/-600 nt) and site 3 (-597/-360 nt), and GAPDH.

Figure 6. A. Effect of siRNA RUNX1 knock down on ALOX12 promoter activity. HEL cells were cotransfected with RUNX1 or mock siRNA and ALOX12 luciferase-reporter construct (-1593/-1). Bar graphs show activity as mean (±SD) of three experiments.  
B. Western blotting analysis of RUNX1, 12-lipoxygenase (12-LO) and actin in HEL cells transfected with mock or RUNX1 siRNA. Shown representative of three experiments. 

Figure 7. A. Effect of transient over-expression of RUNX1 on ALOX12 promoter activity. HEL cells were cotransfected with RUNX1-pCMV6 expression vector (black bars), empty pCMV6 vector (stippled bars), or neither (open bars), along with ALOX12 luciferase-reporter construct (-1593/-1), wildtype or with mutations in sites 1A, 1B, 2 or 3. Reporter activity was measured at 48 h. Bar graphs show activity as mean (±SD) of three experiments.  
B. Western blotting analysis of RUNX1 and actin (control) in HEL cells transfected with pCMV6 vector alone or with RUNX1-pCMV6 vector.
Figure 1
Figure 2
**Figure 3**

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Figure 5
Figure 6
Figure 7
RUNX1/core binding factor A2 regulates platelet 12-Lipoxygenase gene (ALOX12): studies in human RUNX1 haplodeficiency

Gurpreet Kaur, Gauthami Jalagadugula, Guangfen Mao and A. Koneti Rao