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Transforming growth-interacting factor (*TGIF*) regulates proliferation and differentiation of human myeloid leukemia cells

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ABSTRACT

Transforming growth-interacting factor (*TGIF*) is a homeobox transcriptional repressor that has been implicated in holoprosencephaly and various types of cancer. *TGIF* is expressed in hematopoietic stem cells and modulates TGF-β and retinoic acid (RA) signaling, both of which play an important role in hematopoiesis. We recently reported that *TGIF*'s levels correlate inversely with survival in patients with acute myelogenous leukemia. Here we present the first direct evidence of a role for *TGIF* in myelopoiesis. We used short hairpin RNA interference to define the effects of *TGIF* knockdown on proliferation and differentiation of myeloid leukemia-derived cell lines. Decreased *TGIF* expression resulted in reduced proliferation and differentiation and lower expression of *CEBPβ*, *CEBPε*, *PU.1* and *RUNX1*, key myeloid transcription factors. Furthermore, TGF-β signaling was increased and RA signaling was decreased. Further insights into the molecular basis of *TGIF*'s effects were provided by a genome-wide chromatin immunoprecipitation-based elucidation of *TGIF* target genes. Together, these data suggest that *TGIF* has an important role myelopoiesis and may regulate the balance between proliferation and differentiation. Reduced *TGIF* expression could tip the balance toward quiescence thus providing progenitor as well as hematopoietic stem cells protection from anti-cycle agents.

1. Introduction

Transforming growth-interacting factor (*TGIF*) is a transcriptional repressor and a member of the three amino acid loop extension (TALE) class of homeodomain proteins. *TGIF* was initially identified as a nuclear protein that binds a retinoid X receptor (RXR) response element in the retinol binding protein II promoter (Bertolino et al., 1995). Transcriptional repression by *TGIF* likely involves multiple mechanisms, including competition with RXR for RXR response elements, interaction with the ligand-binding domain of RXR (Bartholin et al., 2006), and co-repression in conjunction with Smad2 (Wotton et al., 2001, 1999a, 1999b).

Mutations and deletions of the *TGIF* gene are associated with holoprosencephaly (HPE), which is the most common structural abnormality of the forebrain in humans. HPE is an

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autosomal dominant disorder, and *TGIF* is one of several genes, including *Sonic Hedgehog* (Belloni et al., 1996), *SIX3* (Wallis et al., 1999), *ZIC2* (Brown et al., 1998), *GLI2* (Roessler et al., 2003), *PATCHED-1* (Ming et al., 2002) and *TDGF-1* (de la Cruz et al., 2002), that have been associated with HPE. Although the role of *TGIF* has not been defined in specific hematopoietic lineages, *TGIF* transcripts have been detected in murine hematopoietic stem cells (HSCs) (Phillips et al., 2000). This transcription factor is also expressed in mouse and human embryonic stem cells and is represented on a short list of proteins proposed to mediate their ''stemness'' (Sato et al., 2003). Clinical relevance of a potential role for *TGIF* in hematopoiesis was provided by our observation that expression levels of *TGIF* are an independent predictor of overall survival in acute myelogenous leukemia (AML). AML patients with lower *TGIF* levels in their leukemia cells had a worse prognosis than patients with higher *TGIF* levels (Hamid et al., Submitted for publication).

While definitive evidence of *TGIF*'s role in hematopoiesis may be lacking, several studies over the past decade have suggested that both retinoic acid (RA) and TGF-β signaling play an important role in hematopoiesis and, in particular, myelopoiesis. TGF-β is generally regarded as a negative regulator of all stages of hematopoiesis, but, depending upon the context, it can be pro- or anti-proliferative, pro- or anti-apoptotic, and pro- or anti-differentiative (reviewed in (Kim and Letterio, 2003; Larsson and Karlsson, 2005)). Retinoids (all-*trans*-RA and 9-*cis*-RA) and their nuclear receptors, the retinoid alpha-receptor (RAR) and RXR, control the growth and development of various cell types, including hematopoietic cells. In hematopoiesis, the best-documented action of RA is the induction of differentiation in progenitor cells (reviewed in (Collins, 2002; Evans, 2005; Purton, 2007)).

Here, we provide the first direct evidence of a role for *TGIF* in hematopoiesis. We found that *TGIF* levels correlated in a linear fashion with myeloid cell proliferation. *TGIF* knockdown decreased and its over-expression increased proliferation and differentiation of myeloid cell lines. This phenotype was not due to a block at a specific stage of the cell cycle or a significant increase in apoptosis. Our data suggest that *TGIF* knockdown enhanced TGF-β and inhibited RA signaling. Furthermore, it also inhibited RA-induced differentiation and altered the myeloid transcriptional program. Identification and analysis of direct *TGIF* targets provided additional clues about the molecular basis of *TGIF*'s role in hematopoiesis.

2. Results

2.1. *TGIF levels affect myeloid cell proliferation in a linear fashion*

To determine a role for *TGIF* in myelopoiesis more directly, we used a lentiviral-based shRNA approach to knockdown *TGIF* transcripts and protein levels and the vector *pEFIRES-P* (P721) containing the strong elongation factor 1a promoter (Hobbs et al., 1998) to over-express *TGIF* in HL60 cells. We confirmed the degree of knockdown (~90%) and over-expression (3-fold) by Western blot and real-time PCR analysis (Figure 1A). We isolated several individual clones and subjected them to proliferation analysis by manual counting with trypan blue staining. Equal numbers of cells were plated in triplicate, and the number of viable cells was counted at 24-h intervals. As shown in Figure 1B, 120 h of *TGIF* knockdown resulted in a nearly 6-fold reduction in proliferation ($p < 0.0001$) of shRNA-transduced HL60 cells compared to cells transduced with a non-mammalian (GFP) control shRNA sequence. Interestingly, *TGIF* over-expression increased proliferation of HL60 cells by nearly 1.9-fold ($p < 0.0001$) (Figure 1C), although this effect was not as pronounced as that seen with *TGIF* knockdown. These data thus suggest that *TGIF* affects myeloid cells in a linear fashion, that is, knockdown results in decreased proliferation and over-expression results in increased proliferation. To confirm that these effects were not HL60-specific, we knocked down *TGIF* in two additional myeloid cell lines, TF-1 and AML-193, which showed a similar effect of *TGIF* depletion on cell proliferation (Supplementary Figures 1 and 2).

2.2. *TGIF knockdown did not affect the cell cycle of HL60 cells but did slightly increase apoptosis*

To determine if the observed effects of *TGIF* knockdown on cell proliferation were associated with alterations in the cell cycle, we evaluated the cell cycle distribution of HL60 clones by PI staining. As shown in Figure 2A, we did not observe a significant difference in the distribution of cells in cycle in HL60 cells transduced with a non-mammalian control shRNA (*GFP*) or with *TGIF* shRNA. We did notice, however, that there was a small ($p < 0.0001$) increase in the subG0 population in the *TGIF* knockdown clones. Since this population contains apoptotic cells, we further characterized this population with an Annexin V binding assay, a more sensitive method of detecting apoptosis. As shown in Figure 2B, *TGIF* knockdown resulted in a small increase in apoptosis, consistent with the cell cycle results. Whereas the proliferation analysis presented in Figure 1B showed that the *TGIF* knockdown resulted in a 6-fold decrease in proliferation, the differences in early (Annexin V staining) and late stages of apoptosis (Annexin V and PI staining) between the clones and control were much smaller. This suggests that the proliferation inhibition observed in the *TGIF* knockdown clones was not solely due to increased apoptosis.

2.3. *TGIF knockdown inhibits differentiation of myeloid cells lines*

Since *TGIF* levels have a linear effect on HL60 cell proliferation, we investigated whether *TGIF* levels would also affect the differentiation potential of these cells. As shown in Figure 3A, after five days of exposure to all-*trans*-retinoic acid (ATRA), 88.0% of control HL60 cells (transduced with a non-mammalian shRNA) differentiated into CD11b-expressing terminal myeloid cells compared to 11.1% of HL60 cells with *TGIF* knockdown. Just as *TGIF* over-expression increased HL60 proliferation, *TGIF* over-expression also resulted in the increased differentiation of HL60 cells into terminal myeloid cells (Figure 3B). These data thus support the conclusion that *TGIF* knockdown inhibits terminal myeloid differentiation of HL60 cells. *TGIF*'s effects on differentiation were not ATRA-specific, since similar decreased differentiation was also seen with TPA

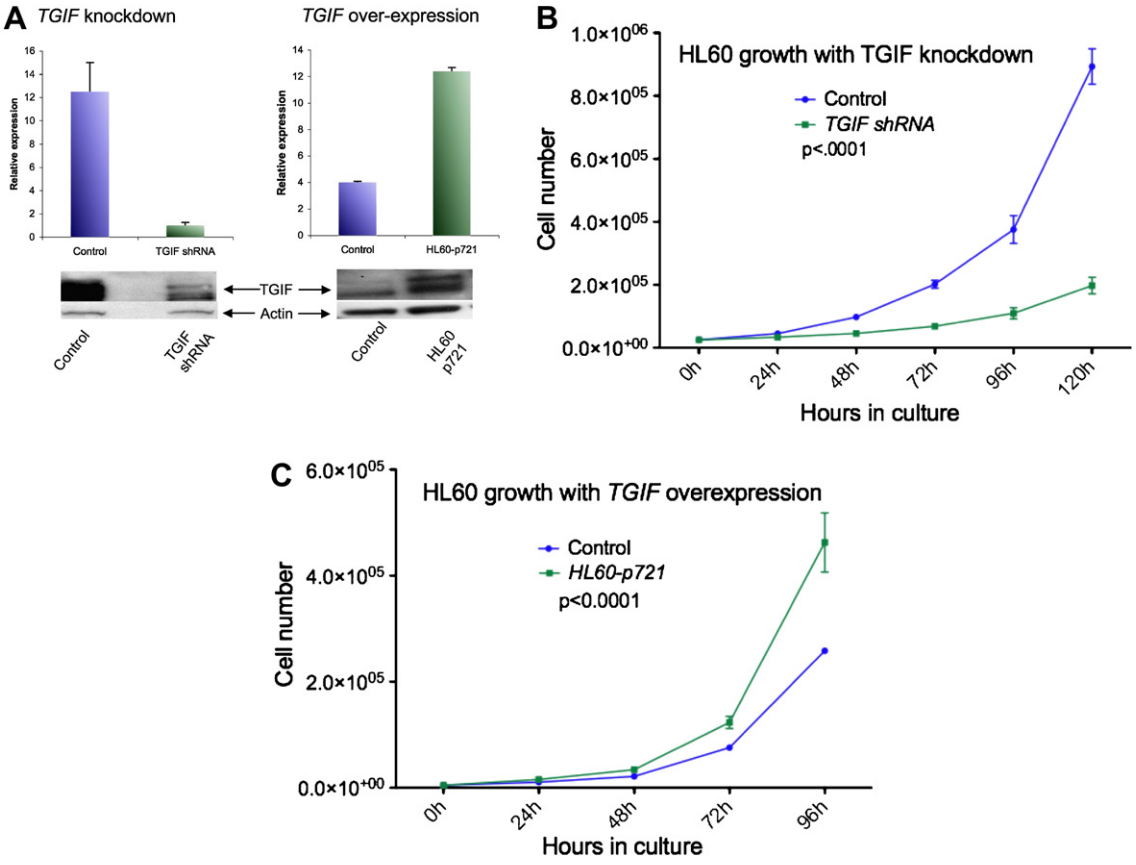

Figure 1 – *TGIF* has a linear effect on HL60 cell proliferation. (A) Lentiviral shRNA-mediated *TGIF* knockdown in HL60 cells. Several clones were analyzed and the degree of knockdown was confirmed with both relative real-time PCR and Western blot analysis (*TGIF* knockdown Western blot was exposure time was ~60 s while the *TGIF* over-expression Western blot time was ~10 s; unequal times were used for better visualization of the respective bands). Data from one such clone is shown. The real-time analysis was repeated at least 3 times and the results of one representative experiment are shown. Error bars represent SEM. Proliferation inhibition of HL60 cells with *TGIF* knockdown. (B) *TGIF* over-expression (with plasmid p721) resulted in increased proliferation. (C) Proliferation analysis was done in triplicate with manual counting with trypan blue exclusion. Each sample of the triplicate was counted at least 3 times at each time point. *X*-axis represents time in hours, *Y*-axis shows the number of cells per ml. Several clones were analyzed and compared with HL60 cells and cells transduced with non-mammalian shRNA. Proliferation analysis was repeated twice and the results of one such analysis is shown. Error bars represent SEM. *P* values were calculated using students t-test with the help of Prism 5 statistical suite.

stimulation (data not shown) as well as cell line specific since *TGIF* knockdown also inhibited differentiation of TF-1 and AML-193 cells (Supplementary Figures 1 and 2). Furthermore, the block in differentiation was not absolute and could be overcome by a 5-fold higher dose of TPA or ATRA (data not shown).

2.4. *TGIF knockdown results in increased TGF-β and decreased RA signaling*

Since *TGIF* has been shown to modulate TGF-β and RA signaling, both of which have pivotal roles in hematopoiesis, we initially hypothesized that the observed effects of *TGIF* knockdown on HL60 cell growth and differentiation were mediated by an effect on endogenous TGF-β or RA signaling or both. We analyzed the expression levels of several TGF-β and RA pathway genes, by real-time PCR, to provide a read-out of pathway activity. The results showed that TGF-β target gene expression, in particular the expression of the prototypic target gene *PAI1*, was higher in *TGIF* knockdown HL60 cells compared to controls (Figure 4A). On the other hand, reduced expression of *RARα* and a number of RA target genes, *HOXB1* and *STRA6* in particular, indicated that RA signaling was less active in *TGIF* knockdown clones compared to the controls (Figure 4B). Since increased TGF-β signaling can inhibit myeloid cell proliferation (Kim and Letterio, 2003) and RA signaling can lead to their differentiation (Gaines and Berliner, 2003; Kastner and Chan, 2001), our data suggest that *TGIF* may affect myeloid cell proliferation and differentiation through modulation of both TGF-β and RA signaling.

2.5. *TGIF knockdown disrupts the myeloid transcription program*

The transcriptional program associated with myeloid differentiation is well described and includes a limited number of transcription factors that are expressed at specific

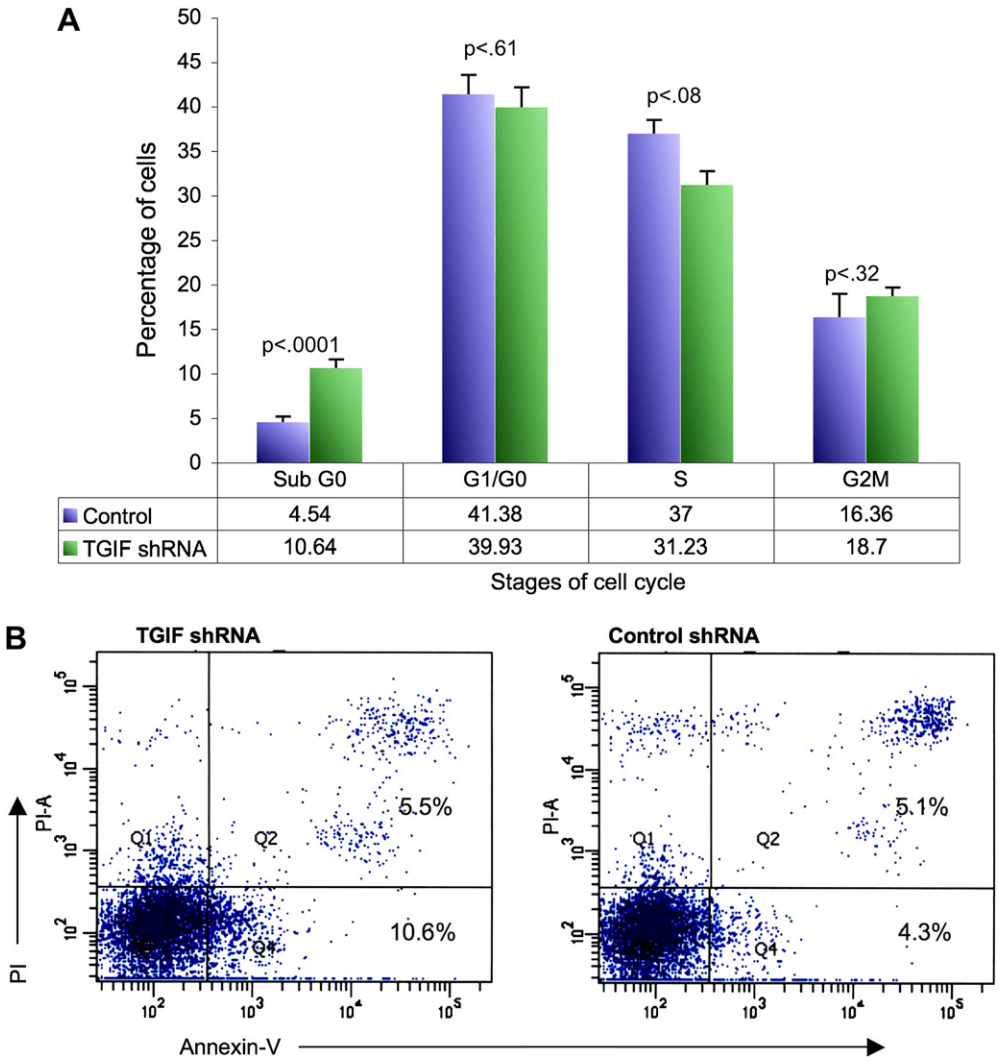

Figure 2 – (A) Cell cycle analysis of exponentially growing *TGIF* knockdown HL60 cells. Cells were stained with PI and analyzed by flow-cytometry. The histograms represent the mean of three separate experiments showing the cell cycle percentage of the isolated HL60 clones. SubG0 percentages likely represent apoptotic cells. Control cells were HL60 cells transduced with non-mammalian shRNA. Error bars represent SEM. *P* values were calculated using students t-test with the help of Prism 5 statistical suite. (B) Annexin V and PI staining of the *TGIF* knockdown HL60 cells. The *x*-axis shows Annexin V-FITC binding and the *y*-axis staining of the vital dye PI. Cells in the lower left quadrant are viable, cells in the lower right are early apoptotic and those in the upper right are late stage apoptotic/dead cells. The numbers represent the percentage of cells present in each quadrant. Results from one typical experiment are shown. Each experiment was repeated three times.

developmental stages to facilitate proper myeloid differentiation (Iwasaki and Akashi, 2007; Rosenbauer and Tenen, 2007). Since *TGIF* knockdown resulted in decreased differentiation of HL60 cells, we hypothesized that *TGIF* knockdown disrupted the myeloid transcription program. To that end, we determined the abundance of mRNA encoding 16 transcription factors, by real-time PCR, before and after induction of differentiation of the *TGIF* knockdown clonal HL60 cells. At baseline, the levels of only three transcription factors, *CEBPβ*, *EFNA2*, and *c-MYB*, were significantly different between *TGIF* knockdown and control HL60 cells (data not shown). We then compared the expression levels of these transcription factors by real-time PCR analysis after induction of differentiation with ATRA. As shown in Figure 5, upon induction of differentiation, HL60 cells with *TGIF* knockdown had lower transcript levels of five key myeloid transcription factors, *CEBPβ*, *CEBPε*, *PU.1*, *RUNX1*, *ZFPM1* and increased levels of *c-MYB* as compared to the control cells. Since increased expression of *CEBPβ*, *CEBPε*, *PU.1*, *RUNX1* and *ZFPM1* and decreased expression of *c-MYB* are required for proper myeloid differentiation (Rosenbauer and Tenen, 2007), these data suggest that alteration in this transcriptional program may be, in part, responsible for the decreased differentiation seen in the *TGIF* knockdown clones. Additionally, since *CEBPε*, *PU.1* and *ZFPM1 (FOG1)* have all been shown to be positively regulated by RA signaling, these data provide additional support for our hypothesis that reduced RA-directed transcription may play a role in the observed decrease in differentiation (Evans, 2005; Kastner and Chan, 2001; Lawson and Berliner, 1999; Mueller et al., 2006; Verbeek et al., 1999; Zhuang et al., 2003).

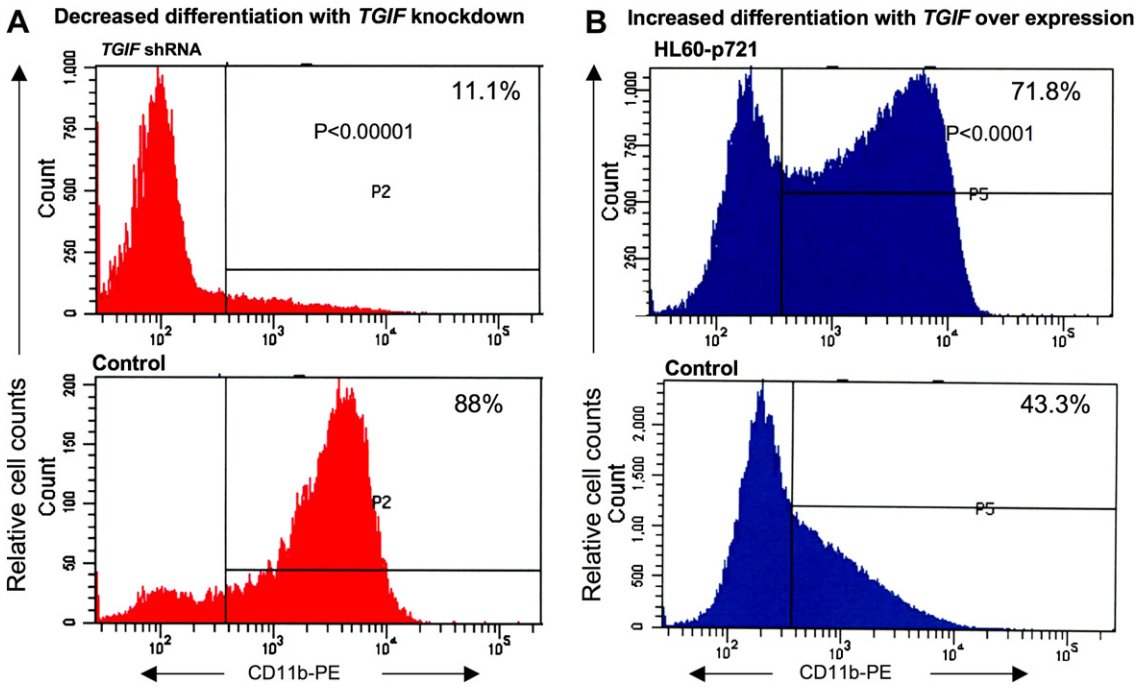

Figure 3 – *TGIF* knockdown resulted in decreased differentiation (A) and its over-expression (with plasmid p721) resulted in increased differentiation (B). For HL60 cells with *TGIF* knockdown, ATRA stimulation continued for 5 days while for cells with *TGIF* over-expression ATRA stimulation was stopped after 2 days. Cells were assayed for the expression of terminal myeloid marker CD11b with a PE-conjugated antibody. The numbers represent the percentage of cells that differentiated following induction with ATRA. Experiment was repeated three times and the results of one representative experiment are shown. *P* values were calculated using students t-test with the help of Prism 5 statistical suite.

2.6. *TGIF target genes have key functions in hematopoiesis*

Our data show that *TGIF* knockdown alters TGF-β and RA signaling, suggesting that its effects on HL60 proliferation and differentiation may be secondary to its modulation of these pathways. However, transcriptional repression by *TGIF* also involves its binding to target genes, and thus a thorough understanding of the molecular mechanisms that mediate its actions requires that the role of these target genes should also be taken into account. In practice this proved difficult, since no information existed as to the identity of direct *TGIF* target genes.

We therefore decided to use a genome-wide chromatin immunoprecipitation (ChIP) sequencing approach (Brynczka et al., 2007) to identify potential *TGIF* target genes. This analysis identified nearly 1655 direct targets. We then applied Ingenuity Pathway Analysis (IPA) to these data to obtain further insight into potential cellular pathways that may be modified/regulated by *TGIF* and its direct targets. This analysis showed that 35% of *TGIF* target genes regulate cellular proliferation, differentiation and apoptosis, 18% had a previously documented role in hematopoiesis and 15% were involved in cancer (Figure 6). We then used IPA to generate networks of connectivity based on the identified *TGIF* target genes. Seventy-two networks were generated; out of these, 34 were considered statistically significant insofar as they exceeded the IPA algorithm cutoff. The top eight are listed in Table 1. The most common functions associated with these networks were related to cancer, hematopoietic development, differentiation, apoptosis and the cell cycle. This was not surprising since, as noted above, 18% of the genes identified as *TGIF* targets have a defined role in hematopoiesis. Further analyses showed that *TGIF* binding sites were present in the upstream regions (between −1 and −1000 bp) of a majority of genes shown in Table 2.

We then reasoned that if *TGIF* directly binds to the proximal regulatory regions of these genes then its knockdown would affect their transcript levels positively and vice versa. We thus analyzed the transcripts' levels of a number of the genes listed in Table 2 by real-time PCR, in the HL60 cells with *TGIF* knockdown and over-expression. As shown in Table 3, *TGIF* knockdown resulted in increased transcription and its over-expression in decreased transcription of 13 of the 23 genes analyzed. These data represent the first experimental identification of downstream TGIF targets. It is interesting to note that several of these genes have key functions in hematopoiesis and hematopoietic stem cell biology, including *MLL*, *MLLT10* and the Forkhead transcription factor family members.

3. Discussion

In this study, we present the first *in vitro* evidence of a role for *TGIF* in myelopoiesis. Based on *TGIF*'s membership in a small

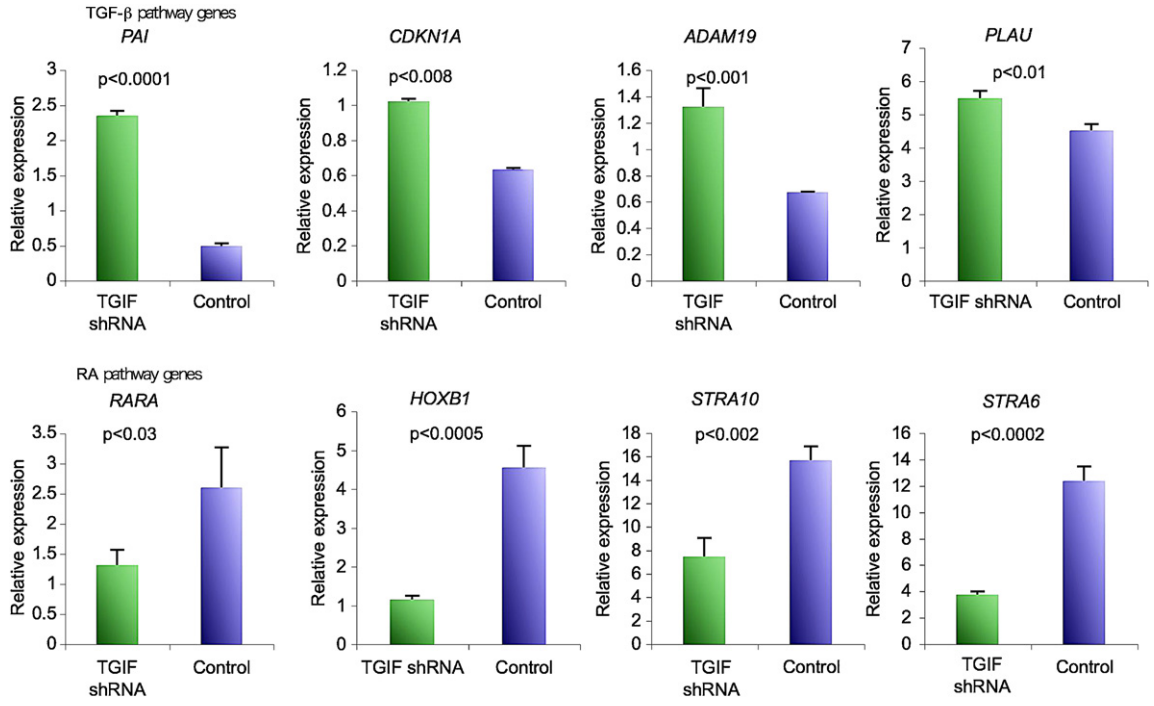

Figure 4 – Expression levels of TGF-β and RA target genes in *TGIF* knockdown HL60 cells as measured by relative real-time PCR. TGF-β target genes are up-regulated while RA target genes are down regulated as compared to control shRNA-transduced HL60 cells. Experiment was done in triplicate and repeated twice; the results of one representative experiment are shown. Error bars represent SEM. *P* values were calculated using students t-test with the help of Prism 5 statistical suite.

group of genes whose expression constitutes a molecular signature for HSC proliferation and our finding that its abundance correlated with survival in AML, we proceeded to determine whether *TGIF* has a direct role in myelopoiesis. We used HL60 cells, a well-characterized cell line model of leukemic myeloid cell differentiation, to study the effects of *TGIF* knockdown on hematopoietic myeloid cell function. We show that *TGIF* has a linear effect on HL60 growth and differentiation, that is, its knockdown resulted in decreased proliferation and differentiation and its over-expression had the opposite effect. Our data also suggest that the molecular mechanisms behind the observed effects are complex and likely mediated through modulations of both RA and TGF-β signaling and further complemented by TGIF's interactions with its direct downstream target genes.

Since *TGIF* is so intimately involved in the regulation of TGF-β and RA signaling pathways and may play a role in cross-talk between these pathways (Zhang et al., 2009), we first sought to determine whether our findings could be explained by altered activity of either of these pathways. TGF-β has well described inhibitory actions on cell proliferation, including hematopoietic cells (Batard et al., 2000; Garbe et al., 1997; Sitnicka et al., 1996), and can also function as an inducer of apoptosis (Lee and Bae, 2002). TGF-β can also affect cell cycle progression through alterations in expression of cytokine receptors and/or certain cyclin-dependent kinase (cdk) inhibitors, such as *CDKN1A* (p21). Our data showed that *TGIF* knockdown increased TGF-β signaling, providing support to our hypothesis that the observed growth inhibition may be due to increased TGF-β signaling. It is unlikely that the concomitantly decreased RA signaling is responsible for the observed slow proliferation, since it is increased RA activity that leads to growth inhibition, not decreased activity. These data are also consistent with the previous report that knockdown of *TGIF* results in increased TGF-β-induced expression of *PAI1* in Mv1Lu cells (Seo et al., 2006), increased TGF-β activity in mesenchyme micomass cultures (Zhang et al., 2009) and that murine embryonic fibroblasts isolated from *Tgif*−/− mice do not proliferate as well as those isolated from wild-type mice *in vitro* (Mar and Hoodless, 2006). However, there remain caveats to this hypothesis, one of the most important being that HL60 cells over-express *c-Myc* due to extra chromosomal amplification, and *c-Myc* over-expression has been shown to block the growth inhibitory responses of TGF-β (Ruegemer et al., 1990). In addition, even though a TGF-β-mediated reduction in overall progression through cell cycle has been reported (Stoeck et al., 1989), the most common effect of TGF-β on the cell cycle is G1 arrest (Ewen et al., 1993). Thus, significant growth inhibition in the presence of *c-Myc* gene amplification and overall slow progression through the cell cycle without an obvious block at a particular stage (specifically G1) suggest that other pathways or genes may mediate *TGIF*'s effects. Our *TGIF* target data provide intriguing clues to this end. For example, *TGIF* binds to several Forkhead and Forkhead binding factor genes. *TGIF* knockdown led to increased *FOXH1* expression, which has been shown have an inhibitory effect on the cell cycle through its interactions with p21 (van der Vos and Coffer, 2008). Similarly, *TGIF* binds

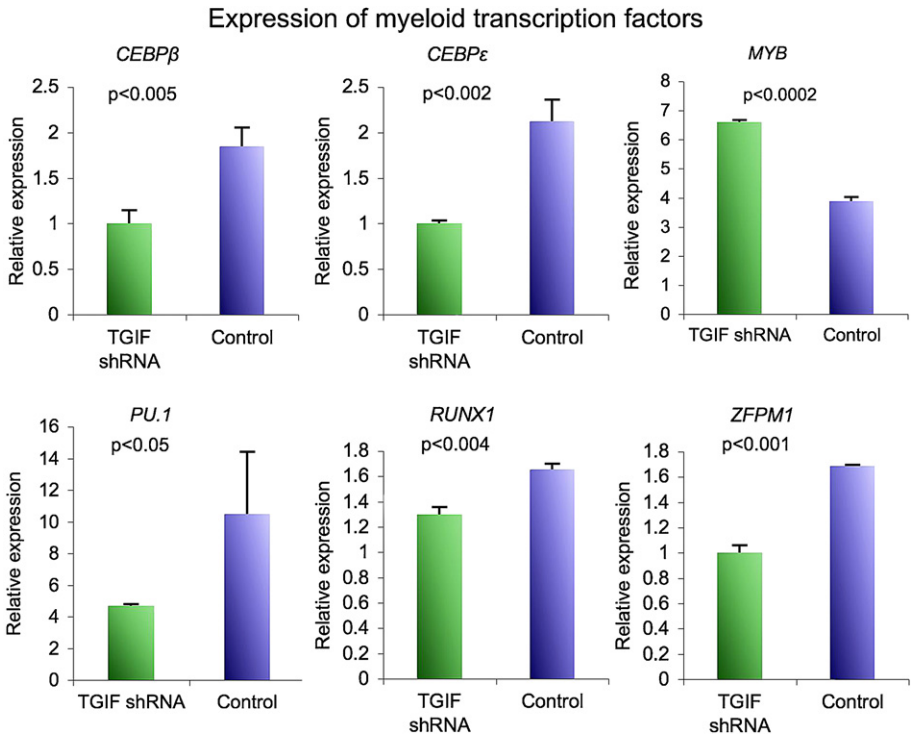

Figure 5 – Comparison of expression levels of myeloid specific transcription factors in HL60 cells with *TGIF* knockdown after the induction of differentiation as measured by real-time PCR. Out of the 16 transcription factors analyzed, six (*CEBPε*, *CEBPβ*, *RUNX1*, *PU.1*, *ZFPM1*, *MYB*) showed significant differences in expression, between HL60 cells with *TGIF* knockout and the control HL60cells. Experiment was done in triplicate and repeated twice; the results of one representative experiment are shown. Error bars represent SEM. *P* values were calculated using students t-test with the help of Prism 5 statistical suite.

to *MLL* regulatory regions and its knockdown increases *MLL* expression, which can decrease cell cycle progression (Milne et al., 2005).

The effects of *TGIF* on differentiation functions of HL60 cells, on the other hand, are likely secondary to altered RA signaling. In hematopoiesis, the best-documented action of RA is the induction of differentiation of progenitor cells (reviewed in (Collins, 2002; Evans, 2005; Purton, 2007)). Our data show that *TGIF* knockdown resulted not only in down-regulation of several known RA target genes, but also in altered expression of several RA-regulated myeloid transcription factors, including *CEBPβ*, *CEBPε*, *PU.1*, *RUNX1*, *c-MYB* and *ZFPM1*, supporting the notion that the decreased differentiation observed with *TGIF* depletion resulted in part from down-regulation of RA

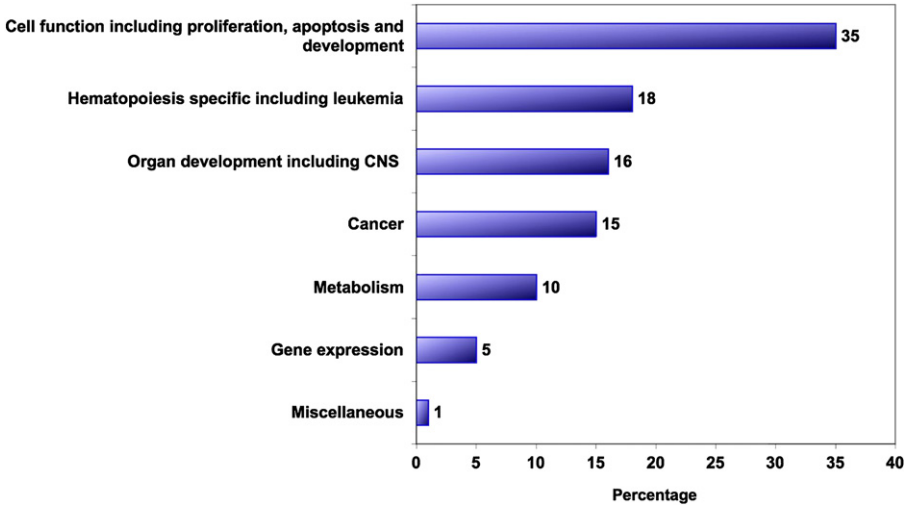

Figure 6 – Ingenuity Pathways Analysis (IPA) of the downstream *TGIF* targets. Numbers represents the percentage of genes in each category.

Table 1 – Biological networks generated by Ingenuity Pathways Analysis. Direct *TGIF* target genes in bold.

Molecules in networks	Functions
Ap1, **BCL10**, *CD3*, **CD38**, **CDX1**, **CREB3**, **FKBP5**, **FPRL1 (includes EG:2358)**, **GAB3**, **GNA15**, **HCLS1**, **IFNAR2**, *Ikb*, **IKBKG**, *IKK*, *IL1*, **LTA**, **MAP3K14**, **MAP4K1**, *NF-κB*, *Nfat*, *NFkB*, **NOD2**, **PTAFR**, **PTPN11**, **RELT**, **RHOH**, **SDC4**, **SIGLEC7**, **SIGLEC9**, *TCR*, *Tnf receptor*, **TNFRSF1A**, **TRAF3**, **TREML1**	Hematopoietic System Development and Function, Organ Morphology, Cancer
Ahr-aryl hydrocarbon-Arnt, **BAX**, **BBC3**, *Caspase*, **CBX5**, **CCL2**, **CCL5**, **CD44**, **CDKN1A**, *Creb*, *Cyclin A*, **DTL**, *E2f*, **E2F3**, **ETV5**, **FGF2**, *Histone h3*, **HNRNPA1**, *JAK*, *LDL*, *Mek*, **MLL**, *Mmp*, **NFE2**, *P38 MAPK*, *PI3K*, **PIK3CD**, *Rb*, *RNA polymerase II*, *TASP1*, *Tgf beta*, **TGFBR2**, **TRIP11**, **UCN2**, *Vegf*	Cellular Growth and Proliferation, Cancer, Cell Death
AMN, *beta-estradiol*, *BICD1*, *BMI1*, *CBX6*, **CDC14B**, **CHMP4A**, *COL4A5*, *CUBN*, *FUCA1*, *GLIPR1*, *HDAC1 (includes EG:3065)*, **HIST2H2BE**, *HOXA9*, **HOXC5**, **HOXC12**, *HS3ST1*, **IFNA14**, **MLLT10**, *MYOG*, **PBXIP1**, *PDCD6IP*, **PHC3**, **PTPRU**, *RAD54B*, *RCBTB2*, *RECQL4*, *retinoic acid*, *SCMH1*, **SIX5**, *SMARCB1*, **TGIF2**, *THSD4*, *TP53*, **TSC22D1**	Hematological Disease, Cell Cycle, Cancer
Akt, **ANXA11**, *ATYPICAL PROTEIN KINASE C*, **CBLB**, **COL13A1**, *Cyclin D*, *Gsk3*, **IL8RB**, *Insulin*, *Mapk*, *Mek1/2*, **MYB**, *N-cor*, *Pdgf*, *PDGF BB*, *Pdgfr*, **PDGFRB**, **PDPK1**, *Pkc(s)*, *Pld*, *PP2A*, **PRKCD**, **RAB2A**, **RARA**, **RARG**, *Ras*, *Rsk*, *Rxr*, **S100A8**, *Sos*, *STAT*, *STAT5a/b*, **TBL1XR1**, *VitaminD3-VDR-RXR*	Embryonic Development, Nervous System Development and Function
ARL6IP5, *BAG3*, **BMP1**, *C5*, **CAPRIN1**, **CD46**, *EGF*, **ETV3**, **FOXH1**, *G3BP1*, *GNPAT*, **GPR77**, *HMMR*, *HOXB5*, **HRH2**, *JARID1B*, *MBOAT5*, *MIA3*, *MTSS1*, *MYC*, *NOP5/NOP58*, *PDE6H*, **PHLDA2**, *PLC gamma*, *PLS3*, *PRPS1*, **PTPRD**, *RALGPS1*, *SRC*, *SRM*, **STOML2**, *TGFB1*, *TOM1L1*, *UNC119*, *ZFP161*	Cellular Movement, Cell Morphology, Cellular Assembly and Organization
ARHGAP26, *ASGR1*, **CABIN1**, *Calmodulin*, *Calmodulin-Hsp90-Nos3*, *Calpain*, *CDC37L1*, *Dynein*, **EIF3K**, *F Actin*, *FGD4*, **FKBP4**, *FKBP51-TEBP-GR-HSP90-HSP70*, *FKBP52-Dyenin-Glucocorticoid-GR-HSP90*, *Hsp70*, *Hsp90*, *IFNZ*, *IL29*, *IL27RA*, *Jnk*, *MAP6*, **MAP4K3**, *Pka*, *PLC*, *RAB19*, *Rac*, *Ras homolog*, **RASGRP2**, **SMC3**, *SOCS4*, *SRP9*, **STAT3**, **STAT5A**, **STAT5B**, *USP6*	Hematological Disease, Cell Death, Cellular Growth and Proliferation
B4GALT1, **BCL10**, **CASP8AP2**, *CD3*, **CD38**, **CDX1**, **CREB3**, **DDX58**, **ECSIT**, **FKBP4**, **FKBP5**, **FPRL1 (includes EG:2358)**, **GNA15**, *Ikb*, **IKBKG**, **IKK**, **ITCH**, **LTA**, **MAP3K14**, **MUC2 (includes EG:4583)**, *NF-kappa;B*, *NFkB*, **NIBP**, **NKIRAS1 (includes EG:28512)**, **NOD2**, *Peptidylprolyl isomerase*, **PPIF**, **PRKDC**, **PTAFR**, **RHOH**, **SDC4**, **SLC11A2**, **SLIT2**, *Tnf receptor*, **TXLNA**	Cell signaling, Cellular Movement
CBLB, **CNN1**, **DDEF1**, **DUSP3**, *ENaC*, **FCHSD2**, **GAB3**, **IL7**, *JAK*, **KLHL21**, **LYN**, **NEDD4L**, *Pdgf*, *PDGF BB*, *Pdgfr*, **PDGFRB**, *PI3K*, **PIK3CD**, **PIK3R5**, *PLC gamma*, **PLK2**, **PTPN11**, **SCNN1A**, **SND1**, *Sos*, *STAT*, **STAT5A**, *STAT5a/b*, **STAT5B**, **SV2A**, **TP53I11**, **TREML1**, *VAV*, **VAV3**, **WNK4**	Gene Expression, Hematological System Development and Function, Immune and Lymphatic System Development and Function

signaling. The inability of the *TGIF* knockdown HL60 cells to up-regulate *CEBPε* is particularly intriguing in that it is consistent with a recent report demonstrating that shRNA-mediated *TGIF* knockdown in pre-adipocytes inhibited their differentiation into mature adipocytes through a decrease in *CEBPε* expression (Horie et al., 2008). *CEBPϵ* is known to be important in myeloid differentiation (Yamanaka et al., 1997).

Our data thus indicate that *TGIF*'s regulation of myelopoiesis is complex, involving modulation of TGF-β and RA signaling, alteration of the myeloid transcription program, and interaction with downstream *TGIF* targets. Based on the data reported in this study and our recent finding that *TGIF* levels predict survival in AML, it was not surprising that a significant number of *TGIF* targets have a documented role in hematopoiesis. These hematopoiesis-specific targets include master regulators of transcriptional programs such as Forkhead box (FOX) proteins; *STAT5B* and *MLL*, which regulate histone methylation; cell cycle regulators such as p21; and important receptors such as *TGFBR2*.

The data presented in this report are only the first steps toward understanding how *TGIF* regulates myelopoiesis. Further studies will require the elucidation of the complete *TGIF* transcriptional network, which would include careful validation of all of the candidate target genes as well as identification of genes that regulate *TGIF* – both active areas of research in our laboratory. One caveat to our findings is that our data were collected using an *in vitro* model of myelopoiesis and thus would require detailed analyses using an *in vivo* model system. Preliminary studies from a knockout model, under validation in our laboratory, show that bone marrow cells from *Tgif*-null mice produce fewer myeloid colonies in colony forming units assays suggesting that, as was the case with the myeloid cell lines, *Tgif* knockout may also produce proliferation inhibition in primary bone marrow cells (Supplementary Figure 3).

Lastly, if *TGIF* knockdown results in growth inhibition and a relative differentiation block, as our data clearly indicate, how might this explain our previously reported finding that *TGIF* levels correlate inversely with clinical outcome in AML? One explanation may be that TGIF's effect on leukemic cells work within the framework of the ''two-hit'' hypothesis of leukemogenesis. In this scenario, decreased TGIF would provide the first hit, leading to a differentiation block; an additional hit would endow the cell with a proliferative/anti-apoptotic advantage, thus leading to accumulation of immature blasts. Another possible explanation could be that *TGIF*'s actions in myeloid cells are quite different than in hematopoietic or leukemic stem cells. Clinically, growth inhibitory effects of *TGIF* knockdown may result in decreased HSC cycling, offering them added protection against chemotherapeutic agents, since these agents primarily affect more rapidly growing cells. These growth inhibitory effects are likely due to increased TGF-β signaling, based on the data presented here and the known effects of TGF-β signaling in HSC function. TGIF's known interactions with cPML provide further support for this hypothesis, since they result in the nuclear sequestration of cPML and the disruption of the cPML-SARA complex with resulting decreased TGF-β signaling. Knockdown of *TGIF* by siRNA caused increased association of cPML with

Table 2 – Selected direct *TGIF* target genes with functions in hematopoiesis and myelopoiesis.

Gene ID	Gene symbol	Gene name
GeneID:5914	*RARA*	Retinoic acid receptor, alpha
GeneID:6776	*STAT5A*	Signal transducer and activator of transcription 5A
GeneID:6777	*STAT5B*	Signal transducer and activator of transcription 5B
GeneID:6774	*STAT3*	Signal transducer and activator of transcription 3 (acute-phase response factor)
GeneID:5914	*RARA*	Retinoic acid receptor, alpha
GeneID:8915	*BCL10*	B-cell CLL/lymphoma 10
GeneID:10488	*CREB3*	cAMP responsive element binding protein 3
GeneID:4297	*MLL*	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)
GeneID:649	*BMP1*	Bone morphogenetic protein 1
GeneID:4602	*MYB*	v-myb myeloblastosis viral oncogene homolog (avian)
GeneID:8928	*FOXH1*	Forkhead box H1
GeneID:8028	*MLLT10*	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 10
GeneID:3228	*HOXC12*	Homeobox C12
GeneID:60436	*TGIF2*	TGFB-induced factor 2 (TALE family homeobox)
GeneID:2299	*FOXI1*	Forkhead box I1
GeneID:9020	*MAP3K14*	Mitogen-activated protein kinase kinase kinase 14
GeneID:147912	*SIX5*	Sine oculis homeobox homolog 5 (Drosophila)
GeneID:55193	*PB1*	Polybromo 1
GeneID:50485	*SMARCAL1*	SWI/SNF related, matrix-associated, actin dependent regulator of chromatin, subfamily a-like 1
GeneID:11244	*ZHX1*	Zinc fingers and homeoboxes 1
GeneID:51130	*ASB3*	Ankyrin repeat and SOCS box-containing 3
GeneID:114907	*FBXO32*	F-box protein 32
GeneID:142	*PARP1*	Poly (ADP-ribose) polymerase 1
GeneID:29109	*FHOD1*	Formin homology 2 domain containing 1
GeneID:3228	*HOXC12*	Homeobox C12
GeneID:144699	*FBXL14*	F-box and leucine-rich repeat protein 14
GeneID:114907	*FBXO32*	F-box protein 32
GeneID:3274	*HRH2*	Histamine receptor H2
GeneID:7048	*TGFBR2*	Transforming growth factor, beta receptor II (70/80 kDa)
GeneID:80790	*CMIP*	c-Maf-inducing protein
GeneID:23523	*CABIN1*	Calcineurin binding protein 1
GeneID:81693	*AMN*	Amnionless homolog (mouse)
GeneID:5159	*PDGFRB*	Platelet-derived growth factor receptor, beta polypeptide
GeneID:3274	*HRH2*	Histamine receptor H2
GeneID:6352	*CCL5*	Chemokine (C–C motif) ligand 5
GeneID:3695	*ITGB7*	Integrin, beta 7
GeneID:868	*CBLB*	Cas-Br-M (murine) ecotropic retroviral transforming sequence b
GeneID:4049	*LTA*	Lymphotoxin alpha (TNF superfamily, member 1)
GeneID:56899	*EB-1*	E2a-Pbx1-associated protein
GeneID:8928	*FOXH1*	Forkhead box H1
GeneID:5170	*PDPK1*	3-Phosphoinositide dependent protein kinase-1
GeneID:10550	*JWA*	Cytoskeleton related vitamin A responsive protein
GeneID:27335	*eIF3k*	Eukaryotic translation initiation factor 3 subunit k
GeneID:148423	*gm117*	gm117
GeneID:4602	*MYB*	v-myb myeloblastosis viral oncogene homolog (avian)
GeneID:79718	*IRA1*	Likely ortholog of mouse IRA1 protein
GeneID:952	*CD38*	CD38 antigen (p45)
GeneID:285852	*TLT4*	Triggering receptor expressed on myeloid cells-like 4
GeneID:5781	*PTPN11*	Protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)
GeneID:7187	*TRAF3*	TNF receptor-associated factor 3
GeneID:399	*RHOH*	ras homolog gene family, member H
GeneID:9321	*TRIP11*	Thyroid hormone receptor interactor 11
GeneID:5293	*PIK3CD*	Phosphoinositide-3-kinase, catalytic, delta polypeptide
GeneID:5580	*PRKCD*	Protein kinase C, delta
GeneID:4297	*MLL*	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila)
GeneID:8778	*SIGLEC5*	Sialic acid binding Ig-like lectin 5
GeneID:6385	*SDC4*	Syndecan 4 (amphiglycan, ryudocan)
GeneID:2247	*FGF2*	Fibroblast growth factor 2 (basic)
GeneID:51514	*RAMP*	RA-regulated nuclear matrix-associated protein
GeneID:6347	*CCL2*	Chemokine (C–C motif) ligand 2

Table 3 – Real-time PCR confirmation of the ChIP identified *TGIF* targets. Plus (+) signifies genes that were up-regulated with *TGIF* knockdown and down regulated with *TGIF* over-expression. Negative (−) represent genes whose expression did not increase with *TGIF* knockdown and decrease with *TGIF* over-expression **HOXC12* and *BCL10* were down regulated by *TGIF* knockdown and unaffected by *TGIF* over-expression.

Gene	Up-regulated by *TGIF* knockdown & down regulated by *TGIF* over-expression	P value
FOXI1	+	0.0004
MLL	+	0.04
MLLT10	+	0.0024
FHOD1	+	0.014
FOXH1	+	0.0001
FBXL14	+	0.0008
TGFBR2	+	0.0001
PARP1	+	0.0011
ASB3	+	0.0001
PBRM1	+	0.034
TGIF2	+	0.02
STAT5B	+	0.0107
MYB	+	0.02
BMP	−	0.39
CREB	−	0.36
SMARCAL1	−	0.66
*HOXC12**	−	0.0001
*BCL10**	−	0.0002
FBOX32	−	0.34
RARA	−	0.2
STAT5A	−	0.07
MAP3K14	−	0.3228
SIX5	−	0.879

SARA and cytoplasmic accumulation of cPML with resultant increased TGF-β signaling (Faresse et al., 2008; Seo et al., 2006).

The association of *TGIF* with cPML is particularly intriguing since PML is the target of reciprocal translocation with the RAR locus in acute promyelocytic leukemia. PML likely regulates the activity of key tumor suppressive pathways such as p53 and retinoblastoma and has been shown regulate cell cycle, apoptosis and induction of quiescence (Bernardi et al., 2008). However, *TGIF* interacts with the cytoplasmic rather than the nuclear isoforms of PML, and the function of these isoforms in leukemia or other types of cancer remains to be fully elucidated. Thus, based on *TGIF*'s role in TGF-β signaling through its cPML-mediated and cPML-independent interactions with Smad proteins, low *TGIF*-expressing leukemic HSCs from an AML patient would be expected to survive induction chemotherapy, increasing the likelihood of relapse. We plan to test this hypothesis with the help of a *Tgif* knockout mouse model under validation.

In summary, this is the first report that identifies a role for the stem cell-expressed homeobox gene, *TGIF*, in myelopoiesis. In light of our earlier observation that the level of *TGIF* expression in blast cells at diagnosis appears to be a powerful predictor of disease relapse and patient survival in AML, our findings indicate that *TGIF* may also have a role in myeloid leukemia. Further *in vivo* work is necessary to confirm these findings and fully elucidate *TGIF*'s role in normal and abnormal myelopoiesis.

4. Experimental procedures

4.1. *shRNA-mediated silencing of TGIF expression*

Four pLKO.1-based lentiviral vectors that contain stem-loop cassettes encoding shRNAs targeted to human *TGIF* mRNA (GenBank accession number NM_003244) were obtained from Open Biosystems, cat number RHS4533-NM_003244 (Huntsville, AL). pLKO.1 TRC control (Addgene plasmid 10879) (Moffat et al., 2006), a non-mammalian silencing control against GFP (Addgene plasmid 12273) (Orimo et al., 2005), and packaging plasmids pMD2.G (Addgene plasmid 12259) (Klages et al., 2000) and pCMV-dR8.2 dvpr (Addgene plasmid 8455) (Stewart et al., 2003) were obtained from Addgene Inc. (Cambridge, MA). Lentiviral particles were produced by co-transfection of 293-T cells with pLKO.1 constructs and the compatible packaging plasmids as previously described (Naldini et al., 1996; Zufferey et al., 1997).

1 ml (high multiplicity of infection) of the harvested lentiviral supernatants were used to transduce HL60 cells at a density of 0.3×10^3 in a 10 ml volume containing 8 μg/ml Polybrene (American Bioanalytical) using the RNAi Consortium lentiviral protocol (http://www.broad.mit.edu/node/563). Infected cells were selected with 0.4 μg/ml puromycin (Sigma-Aldrich). After the selection was complete, the *TGIF* shRNA-lentiviral infected cells, as well as the cells infected with the three control lentiviral-shRNA constructs, GFP, TRC and Scramble, were tested for *TGIF* knockdown by real-time PCR and Western blot analysis (see below) to determine the degree of knockdown for each of the four lentiviruses tested. We then used limiting dilution in 96-well plates to isolate individual clones and quantify the extent of *TGIF* knockdown by real-time PCR and Western blot analysis.

4.2. *Western blot analysis*

Cell lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidenefluoride (PVDF) membranes and probed with a rabbit anti-*TGIF* polyclonal antibody, sc-9084, and an anti-actin polyclonal antibody, sc-1615-R (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with horseradish peroxidase-labeled secondary antibodies and the Immobilon Chemiluminescent HRP substrate (Millipore, Billerica, Massachusetts).

4.3. *Proliferation assays*

Logarithmically growing HL60 cells were counted with a hematocytometer and seeded in triplicate at a density of 2×10^4 cells/ml in full growth media without puromycin. The cell number and viability were determined every 24 h by manual counting with a hematocytometer using trypan blue (0.03%).

4.4. *TGIF over-expression*

TGIF over-expression vector was prepared by inserting *TGIF* cDNA into the vector *pEFIRES-P* at its *EcoR1* cloning site. The vector contains the strong elongation factor 1a promoter

(Hobbs et al., 1998). This plasmid is referred to as p721 in the text.

4.5. Apoptosis assay and cell cycle analysis

In order to measure apoptosis we used an Annexin-V/Propidium iodide (PI) double staining according to the manufacturer's instructions (Invitrogen Carlsbad, CA). For cell cycle analysis, exponentially growing cells were fixed with 70% ethanol for 24 h and then treated with 300 μl of DNA staining solution (10 ml of 0.1% Triton X-100 in PBS, 2 mg DNAse-free RNAse A and 0.40 ml of 500 μg/ml PI) for 15 min. Cells were analyzed by flow-cytometry (BD Bioscience, San Jose CA) using FACSDiva software (BD Bioscience, San Jose CA). Sample size was set at 20,000 events.

4.6. Differentiation assay

HL60 cells were induced to differentiate as previously described (Gaines and Berliner, 2005; Le Cabec et al., 1997). In brief, exponentially growing cells were seeded at a density of 0.5×10^6 per ml and then induced to differentiate with 0.5 μM all-*trans*-retinoic acid (ATRA) or 50 nM TPA for 2 days. Differentiation was monitored by the expression of the antigen marker CD11b, which is only expressed on the terminally differentiated granulocytes/monocytes, by a PE-conjugated anti-CD11b antibody. Data were acquired by flow-cytometry (BD Bioscience, San Jose CA) using FACSDiva software (BD Bioscience, San Jose CA). Sample size was set at 20,000 events.

4.7. Real-time PCR analysis

Real-time PCR analysis for *TGIF* abundance was done as previously described (Hamid et al., 2008). For analysis of RNA encoded by the TGF-β pathway and myeloid transcription factor genes and *TGIF* target genes we used predesigned QuantiTect Primer assays (Qiagen, Valencia, CA). Real-time PCR was carried out with SybrGreen (Applied Biosystems, Foster City, CA) and consisted of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s for 40 cycles.

4.8. Chromatin immunoprecipitation (ChIP) sequencing analysis

This analysis was done using Genpathway's FactorPath Discovery approach, as described previously (Brynczka et al., 2007). Briefly, 10 million HL60 cells were cross-linked by 1% formaldehyde and lysed by Dounce homogenization. Chromatin was sheared to an average length of 500 bp and *TGIF*–DNA complexes were immunoprecipitated using an anti-*TGIF* polyclonal antibody (sc-9084, Santa Cruz Biotech, Santa Cruz, CA). To assay for *TGIF* binding sites in purified ChIP DNA, target-specific primers were used to measure amounts of target sequence in immunoprecipitated samples by real-time PCR.

Enriched ChIP DNA was ligated to a DNA adapter and amplified by PCR. The amplified ChIP DNA was cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced. Individual tag sequences were aligned to NCBI human genome build v35.1 using Megablast 2.2.13. Tags producing low scoring or multiple alignments were eliminated from consideration. Clusters were generated by grouping of tags mapping to within 2 kb of each other. Alignments were associated with annotated or predicted genes mapping to within 10 kb of each tag. To confirm candidate *TGIF* binding sites, PCR primers targeting a region within 200 bp of each selected alignment or cluster were used to measure the amount of sequence in immunoprecipitated samples by real-time PCR.

4.9. In silico functional analysis of the TGIF targets using ingenuity pathways analysis

We used Ingenuity Pathways Analysis (IPA) (Ingenuity® Systems, www.ingenuity.com) to generate networks and conduct *TGIF* target functional analyses. A data set containing gene identifiers was uploaded into the application. These genes, called focus genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge Base. IPA generates models of gene interactions called networks that are presented graphically to show relationships between genes and the pathways they regulate. These networks have a maximum of 35 genes enriched for the input proteins and are ranked according to a score calculated via a right-tailed Fisher's exact test. In the network representations, genes are represented as nodes and their relationships as edges (lines). All edges are supported by references from the literature. The functional analysis of a network identified the biological functions and/or diseases that were most common to the genes in the network. The network genes associated with biological functions and/or diseases in the Ingenuity Pathways Knowledge Base were considered for the analysis. Fischer's exact test was used to calculate a *p*-value determining the probability that each biological function and/or disease assigned to that network is due to chance alone.

4.10. Cell culture

Human HL60, TF-1, AML-193 and 293-T cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). HL60, AML-193 and TF-1 are human acute myeloid leukemia cell lines and were maintained in 80% IMDM + 20% FBS, 70% IMDM + 10% FBS + 2 ηg/ml recombinant GM-CSF, and 70% RPMI 1640 + 20% FBS + 5 ηg/ml recombinant GM-CSF, respectively. 293-T cells were maintained in 90% DMEM and 10% FBS.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.molonc.2009.07.004.

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