FOXO3a Induces Differentiation of Bcr-Abl-transformed Cells through Transcriptional Down-regulation of Id1*

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Leukemia transformation often requires activation of protein kinase B (PKB/c-Akt) and is characterized by increased proliferation, decreased apoptosis, and a differentiation block. PKB phosphorylates and inactivates members of the FOXO subfamily of Forkhead transcription factors. It has been suggested that hyperactivation of PKB maintains the leukemic phenotype through actively repressing FOXO-mediated regulation of specific genes. We have found expression of the transcriptional repressor Id1 (inhibitor of DNA binding 1) to be abrogated by FOXO3a activation. Inhibition of PKB activation or growth factor deprivation also resulted in strong down-regulation of Id1 promoter activity, Id1 mRNA, and protein expression. Id1 is highly expressed in Bcr-Abl-transformed K562 cells, correlating with high PKB activation and FOXO3a phosphorylation. Inhibition of Bcr-Abl by the chemical inhibitor STI571 resulted in activation of FOXO3a and down-regulation of Id1 expression. By performing chromatin immunoprecipitation assays and promoter-mutation analysis, we demonstrate that FOXO3a acts as a transcriptional repressor by directly binding to the Id1 promoter. STI571 treatment, or expression of constitutively active FOXO3a, resulted in erythroid differentiation of K562 cells, which was inhibited by ectopic expression of Id1. Taken together our data strongly suggest that high expression of Id1, through PKB-mediated inhibition of FOXO3a, is critical for maintenance of the leukemic phenotype.

Interference with this highly regulated process can lead to the development of hematopoietic malignancies. Myeloid transformation is often associated with chromosomal translocations and somatic mutations, affecting gene expression in ways that lead to defects in normal programs of cell proliferation, differentiation, and survival (7–12). For instance, chronic myeloid leukemia (CML)9 is a lethal hematopoietic stem cell malignancy characterized by the t(9;22) chromosomal translocation, a translocation between the long arms of chromosomes 9 and 22, resulting in the formation of the Philadelphia (ph) chromosome and the fusion of a truncated bcr gene to the 5′-upstream sequences of the second exon of c-abl (9, 13). The bcr-abl fusion gene is known to be essential to the pathogenesis of CML, and the Bcr-Abl protein demonstrates constitutively active kinase activity, which is essential and sufficient for malignant transformation (9, 13, 14). Bcr-Abl exerts diverse actions on hematopoietic cells in terms of cellular transforming activity, inhibition of apoptosis, cell cycle progression, altered cell migration, and adhesion to extracellular matrix (9, 13, 14). Expression of Bcr-Abl results in growth factor independence of cells and activates multiple signaling cascades, including the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB/c-Akt) signaling pathway (15, 16).

In non-malignant cells activation of the PI3K/PKB-signaling module is stimulated by growth factors and cytokines and has been linked to regulation of cellular proliferation and survival in a diverse variety of cell systems (17–19). Recently, it has been demonstrated that the members of the FOXO subfamily of transcription factors FOXO1, FOXO3a, and FOXO4 are directly phosphorylated by PKB (20, 21). In the absence of growth or survival factors FOXOs are unphosphorylated, localized in the nucleus, and transcriptionally active. Upon stimulation with growth factors or cytokines, PKB activity is induced, and it translocates to the nucleus and phosphorylates FOXOs, leading to inhibition of transcriptional activity and nuclear export (22). We and others have demonstrated that FOXO transcription factors can regulate a variety of genes that influence cellular proliferation (e.g. p27Kip1 and cyclin D), survival
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(e.g. FasL and Bim), metabolism (e.g. PEPCK and glucose-6-phosphatase), and responses to stress (e.g. MnSOD and catalase) (21, 22).

Myeloid leukemic cells are characterized not only by uncontrolled proliferation and resistance to apoptosis, but also by a block in differentiation (7, 10). Because the PI3K/PKB pathway is constitutively activated in leukemic cells (23, 24), this suggests that hyperactivation of the PI3K/PKB pathway maintains the leukemic phenotype not only by regulating proliferation and apoptosis but also by actively repressing a set of genes regulating hematopoiesis. Importantly, PI3K signaling is crucial for transformation of Bcr-Abl. Skorski et al. (25) demonstrated that inhibition of PI3K downstream signaling by ectopic expression of dominant-negative PKB inhibited Bcr-Abl-dependent transformation of murine bone marrow cells in vitro and suppressed leukemia development in severe combined immunodeficiency disease (SCID) mice. In addition, recently it has been shown that FOXO3a was constitutively phosphorylated and therefore inactive in cell lines expressing Bcr-Abl (26, 27). This suggests that inhibition of FOXO3a transcriptional activity may be required to maintain the leukemic phenotype. Therefore, we looked for novel target genes of FOXO3a that might indeed play a role in regulating hematopoiesis.

To identify novel transcriptional target genes of the FOXO3a transcription factor FOXO3a, cDNA microarray analysis was performed using a bone marrow-derived cell line stably expressing an inducible active FOXO3a mutant. We found the transcriptional repressor Id1 (inhibitor of differentiation) (28) to be a direct transcriptional target of FOXO3a. Here we show that the transcriptional down-regulation of Id1 by FOXO3a is required for the induction of differentiation of leukemic cells. We show that expression of a constitutively active FOXO3a mutant induced differentiation of Bcr-Abl-transformed cells. Conversely, constitutive expression of Id1 inhibited differentiation. Taken together, our data strongly suggest that the high expression of Id1, through a PI3K/PKB-mediated inhibition of FOXO3a, is critical for maintaining the leukemic phenotype.

EXPERIMENTAL PROCEDURES

Cell Culture—Ba/F3 cells were cultured in RPMI 1640 medium supplemented with 8% HyClone serum (Invitrogen) and recombinant mouse IL-3 produced in COS cells (29). BaF3-FOXO3a(A3):ER* cells were previously described (29).

For the generation of clonal Ba/F3 cells stably expressing FOXO3a(A3):ER*, the pcdNA3-FOXO3a(A3):ER* construct was electroporated into Ba/F3 cells and maintained in the presence of 500 μg/ml Puromycin (InvivoGen, UK), and clonal cell lines were generated. Single cell clones were obtained with serial dilution and tested by Western blotting after addition of 200 nm tamoxifen (Sigma) at 5 × 10⁶ cells/ml for 24 h (Invitrogen).

Transfection of psiRNA-h7Skzeo:FOXO3a into K562—To obtain stable cell lines, the mammalian expression vector psiRNA-h7Skzeo (InvivoGen) was used for gene silencing of FOXO3a in K562 cells. The psiRNA plasmid (InvivoGen) is specifically designed for the cloning of small synthetic oligonucleotides that encode two complementary sequences of 19 nucleotides, separated by the used hairpin sequence: TCACGTCAGTAGCGATTCA.

The cells were split 24 h prior to the transfection and were washed and resuspended in conditioned medium and left to grow overnight. 24 h post transfection, cells were checked and carefully washed, and then selection was started according to the siRNA manufacturer recommendation. During the first week, the cells were selected in growth medium supplemented with 5 μg/ml Zeocin, followed by single cell cloning and expansion for another 2 weeks. The cells were tested for the efficiency of siRNA silencing by Western blotting.

Antibodies and Reagents—Polyclonal antibodies against PKB and phospho-Ser-473 PKB were from Cell Signaling Technologies (Hitchin, UK). Anti-p27Kip1 was purchased from BD Transduction Laboratories (Lexington, KY). Polyclonal antibodies against Id1 and actin were from Santa Cruz Biotechnology (Santa Cruz, CA). Total and Phospho-Thr-32 FOXO3a were from Upstate Biotechnology (Lake Placid, NY). STI571 was a kind gift from Dr. S. Ebeling (Dept. Hematology, University Medical Center, Utrecht, The Netherlands).

The pLZRS-FOXO3a(A3) construct was generated as follows. First, the Xba/HindIII FOXO3a(A3) fragment from the pECE-FOXO3a(A3) vector was ligated into Xba/HindIII, cut and dephosphorylated pBluescript. Subsequently, pLZRS-FOXO3a(A3) was created by ligation a Xho/NotI fragment from pBluescript into Xho/NotI cut pLZRS.

The pGL3-Id1 promoter construct was obtained by amplifying a 1692-bp fragment of the Id1 promoter from human chromosomal DNA, which was ligated into pGL3 (pGL3-basic from Promega) cut with Smal and Xhol. The FOXO3a binding site 1 was mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol with primer 5’-GGGGGCGTTGCGTGGCTATAAAAGACACGC-3′ and its complementary sequence.

Viral Transduction—A bicistronic retroviral DNA construct was utilized, expressing Id1 (kindly provided by Dr. H. Spits, Amsterdam, The Netherlands) and an internal ribosomal entry site followed by the gene encoding eGFP (30). Retrovirus was produced by transient transfection of 293T cells by FuGENE-6 (Roche Applied Science). 0.6 × 10⁶ cells were seeded in 9-cm dishes. The following day, 20 min before transfection the medium of the cells was refreshed. 12 μl of FuGENE-6, 2 μg of pCL-ampho, and 2 μg of pLZRS-eGFP, pLZRS-Id1, or pLZRS-FOXO3a(A3) were added to 184 μl of Dulbecco’s modified...
Eagle’s medium, incubated at room temperature for 15 min, and subsequently added to the cells. Again after 24 h the medium was replaced with RPMI 1640 medium (supplemented with 8% HyClone). After again 24 h later the viral supernatants were collected, filtered through a 0.22-μm Acrodisc filter, and stored at −80 °C.

Before transduction K562 cells were cultured at a density of 2 × 10⁵ cells/ml. The next day K562 cells were transduced by resuspending 10⁶ cells in 0.5 ml of RPMI. After addition of 8 μg/ml Polybrene (Sigma-Aldrich) and 5 ml of viral supernatant, the cells were centrifuged at 2500 rpm for 1.5 h at room temperature. After 24 h the cells were washed once and transduced for a second time. Two days after transduction, eGFP-positive cells were sorted by fluorescence-activated cell sorting and treated with or without STI571.

Western Blotting—For the detection of Id1, p27, phospho-PKB, and phospho-FOXO3a, cells were lysed in Laemmli sample buffer, and the protein concentration was determined. Equal amounts of each protein sample were analyzed by SDS-PAGE, electrophoretically transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with the respective antibodies. Immune complexes were detected using enhanced chemiluminescence (ECL, Amersham Biosciences).

Luciferase Assays—For transient transfections Ba/F3 cells were electroporated (0.28 kV, capacitance 950 microfarads) with 16 μg of a luciferase reporter plasmid containing the Id1 promoter. Cells were co-transfected with 50 ng of a Renilla luciferase plasmid (pRL-TK, Promega, Madison, WI) to normalize for transfection efficiency. After transfection cells were cultured with or without IL-3 or in the presence of IL-3 with or without 4-hydroxytamoxifen (100 nm) for 24 h. Cells were then harvested and lysed in commercially available luciferase lysis buffer, and luciferase activity was determined as in a previous study (29).

COS cells were transiently transfected with the pGL3-Id1 luciferase promoter construct, together with pECE-FOXO3a(A3) (20), or control vectors and the internal transfection control (pRL-TK) by calcium phosphate precipitation. Values were corrected for transfection efficiency and represent the mean of at least three independent experiments (± S.E.).

RNA Isolation and cDNA Synthesis—Cells were stimulated as indicated and at the respective times harvested. 5 × 10⁶ cells were harvested, washed twice with phosphate-buffered saline, lysed in 1 ml of TRIzol (Invitrogen), and stored at −20 °C. Total cellular RNA was isolated, and cDNA was generated as previously described (31) according to the manufacturer’s protocol (Invitrogen). 10 μg of RNA was treated with DNase according to the manufacturer’s protocol (DNAfree, Ambion Inc., Austin, TX). cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers. Samples containing 1 μg of total RNA in a total volume of 12.5 μl were heated for 3 min at 65 °C and quickly chilled on ice. A mixture of 12.5 μl containing 20 μg/ml oligo(dT) primers, 2.5 μl 5× iScript Reaction Mix (BioRad), 20 mM dithiothreitol, 2 mM dNTPs, 0.8 unit/μl of RNase inhibitor, and 200 units of Moloney murine leukemia virus reverse transcriptase was added. The total mixture was incubated for 90 min at 37 °C, followed by inactivation of the reverse transcriptase for 10 min at 65 °C. cDNA was stored at −20 °C before further use. All reagents used for cDNA synthesis were obtained from Invitrogen.

Real-time PCR—Id1, β-actin, and GAPDH mRNA were analyzed by real-time PCR using SYBR green 1 (Nieuwekerk a/d IJsse1, The Netherlands). Primers were designed using Primer 3 software from the Whitehead Institute/ Massachusetts Institute of Technology Center for Genome Research (www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). For mouse Id1 a 86-bp fragment was amplified using the FW primer 5′-ACGACATGACGCTGTCTAC-3′ and the reverse primer 5′-CAGGATCTCTACACCTTGGCTAC-3′. For human Id1 a 68-bp fragment was amplified using the FW primer 5′-CTGGACGAGCAGCTGTTAAA-3′ and the reverse primer 5′-AGCTCTTTGGGCGTGAGTAA-3′. For mouse GAPDH as a control a 191-bp fragment was amplified using the FW primer 5′-AACGACCTCTTACCTGAC-3′ and RV primer 5′-TCCACGACATACATCGCAC-3′ were used. For human GAPDH a 135-bp fragment was amplified using the FW primer 5′-AGAAAGCTGGCGCTCAATT-3′ and RV primer 5′-GAGGCAATTGGCTATGATCCTTG-3′, and a 174-bp fragment of β-actin was amplified using primer 5′-AGCTCTGCGTTGGCGA-3′ and RV primer 5′-CTGGTGCCCTTGGGCG-3′ (32). The real-time PCR was performed as previously described (31). Results were normalized for the housekeeping genes β-actin and GAPDH, and results were expressed as -fold regulation.

Chromatin Immunoprecipitation Assay—K562 cells cultured at 10⁶ cells/ml were either untreated or stimulated with 5 μM STI571 for 4 or 8 h, and then collected by centrifugation and resuspended in 10 ml of cold phosphate-buffered saline. Chromatin immunoprecipitation assays were performed as previously described by Fernandez de Mattos et al. (33). Protein-DNA complexes were formaldehyde-cross-linked and immunoprecipitated with either the FOXO3a (Upstate, Lake Placid, NY) or an isotype control antibody (BabCO). PCRs were then performed on the purified DNA, according to the manufacturer’s protocol, in the presence of 2.5 mM MgCl₂, at 55 °C, for 28 cycles, using the FOXY3a primers sense (Primer1: 5′-CTTTGAGGCGT-3′) and RV primer 5′-TCCACGACATACATCGCAC-3′ and primer 5′-CTGGTGCCCTTGGGCG-3′ (32). The real-time PCR was performed as previously described (31). Results were normalized for the housekeeping genes β-actin and GAPDH, and results were expressed as -fold regulation.

Measurement of Hemoglobin Expression—The benzidine oxidation test was performed as described (34). In short, cells (0.2–3 × 10⁶ cells/ml) were incubated with STI571 for 24, 48, or 72 h, then washed twice in phosphate-buffered saline at low speeds for 10 min, and finally resuspended in 0.9% NaCl-benzidine reagent solution (to 1 ml of 0.2% tetramethylbenzidine (Sigma) in 0.5 M acetic acid, 20 ml of 30% H₂O₂ was added just prior to use), which was added to start the reaction. After incubation for 30 min in darkness at room temperature, 200 cells were counted in a Burker chamber. The number of cells containing blue crystals indicative of oxidized tetramethylbenzidine and reflecting hemoglobin production was determined.

Extraction of DNA-binding Proteins—Briefly, 25 × 10⁶ cells were centrifuged at 4000 × g for 10 min at 4 °C, washed with
ice-cold phosphate-buffered saline, and resuspended in 400 μl of cold low salt buffer A (10 mM HEPES-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, and 0.2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride) by gently flicking the tube. The cells were allowed to swell on ice for 10 min. After a brief vortex, samples were centrifuged for 2 min at 4 °C, and the supernatant fraction was discarded. The pellet was then resuspended in 30–40 μl of cold high salt buffer C (20 mM HEPES-KOH, pH 7.9, 25% v/v glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation (13,000 g, 2 min, 4 °C). The supernatant fraction contains the DNA-binding proteins. Protein yield was quantified by using a Dc protein assay kit (Bio-Rad).

Oligonucleotide “Pull-down” Assay—Nuclear extracts were prepared from cultured cells using the high salt buffer as described. After diluting with 2 volumes of low salt lysis buffer, 50 μg of the cell extracts was incubated at 30 °C for 10 min with either 0.5 nmol of the 5′-biotinylated double-stranded wild-type (5′-TGGCGTGTTTATAAAAAGACAAGCTGTGGCT-3′) or mutant (5′-TGGCGATTTATAAAGGGGCGCTGTGGCT-3′) oligonucleotides (Invitrogen) previously coupled to streptavidin-agarose beads (Sigma). All the pull-down experiments were performed with exactly equal aliquots from the same sample of either untreated or STI571-treated nuclear extracts. The wild-type oligonucleotide corresponded to the region of -114 to -144 of the human Id1 promoter. After incubation, the biotinylated oligonucleotide-coupled streptavidin beads were washed at least six times with low salt buffer containing 150 mM NaCl and denatured in SDS-sample buffer before being run on an SDS-acrylamide gel. The separated proteins were then Western-blotted for FOXO3a using specific antibodies. For competition experiments, the extracts were incubated with the wild-type oligonucleotide-coupled beads in the presence of 1, 5, or 50 molar excess of either the wild-type or mutant non-biotinylated oligonucleotide.

RESULTS
Identification of Id1 as a Novel Transcriptional Target of FOXO3a—We and others have found that enforced expression of FOXO activity can induce either cell cycle arrest or apopto-
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Id1 Protein Levels Are Down-regulated by FOXO3a Activation—Next we examined whether the effect of FOXO3a on Id1 mRNA was also reflected at the protein level. Ba/F3-FOXO3a(A3):ER* cells were stimulated with 4-OHT as indicated, and protein was isolated and subsequently analyzed for Id1 expression. As shown in Fig. 2A, addition of 4-OHT resulted in a striking and rapid down-regulation of Id1 protein levels. In contrast, another target gene of FOXO3a, the cell cycle inhibitor p27, was strongly up-regulated upon 4-OHT treatment. Furthermore, other treatments resulting in FOXO3a activation, such as cytokine deprivation (Fig. 2B), or inhibition of PI3K by the chemical inhibitor LY294002 (Fig. 2C), had similar effects on Id1 protein expression, but with distinct kinetics reflecting activation of FOXO3a.

These data demonstrate that FOXO3a-induced inhibition of Id1 mRNA is also reflected at the protein level. The very rapid down-regulation of Id1 protein is to be expected, because it has been described to be very unstable, with Id1 having a half-life of ~30 min (40).

Activation of FOXO3a Inhibits Transcriptional Activity of the Id1 Promoter—Although we have demonstrated regulation of Id1 mRNA by FOXO3a, we wished to determine if FOXO3a can also modulate Id1 promoter activity. COS cells were transiently transfected with a luciferase reporter construct under the control of the Id1 promoter, together with increasing concentrations of a constitutively active FOXO3a mutant. As shown in Fig. 3A, ectopic expression of the active FOXO3a(A3) mutant strongly inhibited Id1 promoter activity in a dose-dependent manner. Similarly, in Ba/F3 cells stably expressing FOXO3a(A3):ER*, addition of 4-OHT resulted in a strong down-regulation of Id1 promoter activity (Fig. 3B). Deprivation of IL-3 also resulted in down-regulation of Id1 promoter activity in Ba/F3 wild-type cells (Fig. 3B). Together these data strongly suggest that the FOXO3a-induced inhibition of Id1 expression is through direct inhibition of Id1 promoter activity.

Constitutive Id1 Expression Contributes to the Leukemic Phenotype—The Id transcriptional repressors (Id1–Id4) have been suggested to act at the checkpoint at which undifferentiated progenitor cells make the commitment to terminal differentiation (28, 41, 42). Indeed, we have recently observed that the down-regulation of Id1 is required during myelopoiesis (30). It is now well established that members of the Id family are overexpressed in a range of human tumors, and Id1 is the family member most widely overexpressed in human cancers. How-

FIGURE 4. Id1 expression is down-regulated upon STI571 treatment. A and B, K562 cells were cultured with or without STI571 (5 μM) for the indicated times. Cells were lysed, and equal amounts of protein were analyzed by SDS-PAGE and Western blotting. C, K562 cells were cultured with or without STI571 (5 μM) for the indicated times, RNA was isolated, cDNA was synthesized, and real-time PCR was performed using specific primers for Id1. Results were normalized for the housekeeping genes β-actin and GAPDH. The results reflect the relative change of Id1 from three independent experiments ± S.E. D, K562 cells were generated expressing FOXO3A(A3):ER or control vector (pBABE). Cells were treated with 4-OHT (100 nM) for the times indicated, and cell lysates were prepared. Samples were analyzed for FOXO3a, Id1, and p27 expression by SDS-PAGE and Western blotting.

sis, depending on the cell type (22, 35–37). However, recently a role for FOXOs in regulating differentiation of several cell types has been described (36, 38). To identify novel transcriptional targets of FOXO3a that might be involved in hematopoiesis, we made use of a bone marrow-derived cell line (Ba/F3 cells) stably expressing an inducible form of active FOXO3a, in which all three PKB-phosphorylation sites were mutated to alanine, FOXO3a(A3):ER* (29). Addition of 4-hydroxytamoxifen (4-OHT) to these cells results in the rapid induction of FOXO3a transcriptional activity, promoting induction of FOXO target genes. To identify novel transcriptional target genes of FOXO3a, cDNA derived from the FOXO3a(A3):ER* expressing cells were stimulated for 0 or 2 h with 100 nM 4-OHT were hybridized to custom-made DNA microarrays containing 15,000 cDNAs (data not shown) (39). Use of this short time point increases the chance of identifying direct FOXO targets. Interestingly, we identified the transcriptional repressor Id1 (inhibitor of DNA binding) as one of the genes that was most prominently down-regulated (11.6-fold, data not shown). Id1 transcript levels were confirmed by real-time PCR. We analyzed the kinetics of FOXO3a-mediated Id1 down-regulation by analyzing FOXO3a(A3):ER* cells that were treated with 4-OHT for the times indicated (Fig. 1). FOXO3a activation resulted in a very rapid down-regulation of Id1 mRNA, which was maintained for at least 24 h.
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A

Site 4
-1692
-1630

Site 3
-1506
-1320

Site 2
-576
-390

Site 1
-142
-18

B

-1892 Id1 promoter -353 Id1 promoter

C

FOXO3: 3′UTR

anti-IgG IP
anti-FOXO3a IP

STI571 (hours)

Primers 1
Primers 2
Primers 3

D

WT Id1 promoter
(mut 1146/444)

mut Id1 promoter
(mut 1146/444)

Comp. oligo
STI571

pull-down oligo

mut
WT

E

Luciferase activity

C 4 6 8 24

Mutant Id1 promoter

WT Id1 promoter

Time (hours)
ever, a direct link between Id1 expression and chronic myeloid leukemia has not yet been clearly proven. Therefore, we examined whether hyperactivation of the PI3K/PKB signaling module might contribute to the CML phenotype. We questioned whether the constitutive inhibition of FOXO3a, which would result in high expression of Id1, might be responsible for maintaining cells in an undifferentiated state. In accordance with recently published data (26, 27), we found that in the human Bcr-Abl-expressing leukemic cell line K562, PKB was strongly activated as shown by its high phosphorylation status, whereas its downstream target FOXO3a was inactive (Fig. 4A). Treatment of cells with the chemical inhibitor STI571 (43), which specifically inhibits Bcr-Abl kinase activity, resulted in dephosphorylation and therefore inhibition of PKB (Fig. 4A). In addition, upon STI571 treatment FOXO3a was dephosphorylated and therefore activated (Fig. 4A), as shown by up-regulation of p27, a direct target gene (Fig. 4B). Importantly, activation of FOXO3a was accompanied by a dramatic down-regulation of Id1 protein levels (Fig. 4B), which was also reflected at mRNA (Fig. 4C). In addition, stimulation of K562 cells stably expressing FOXO3a(A3);ER* (29) with 4-OHT resulted in strong down-regulation of Id1 mRNA (data not shown) and protein expression (Fig. 4D), whereas in cells expressing the empty vector no effect on Id1 expression was observed, demonstrating that sole activation of FOXO3a is enough to down-regulate Id1 expression. These data demonstrate that indeed in leukemic Bcr-Abl-expressing cells high Id1 expression is observed correlating with high PI3K/PKB activity.

**FOXO3a Directly Regulates Expression of Id1**—To investigate whether FOXO3a regulates Id1 expression through direct binding to its promoter, we analyzed the Id1 promoter region for putative FOXO binding sites. We found four potential sites at positions −134 to −128 bp upstream of the ATG (site 1), one at position −565 to −559 bp (site 2), and one at position −1509 to −1503 bp (site 3). One nearly perfect sequence at position −1627 to −1621 bp (site 4) was found allowing a 2-bp mismatch compared with the consensus sequence TTGTTTAC (37) (Fig. 5A).

To determine whether addition of STI571 was also sufficient to regulate Id1 promoter activity, K562 cells were transfected with either a full-length, or a truncated, Id1 promoter reporter construct. Addition of STI571 resulted in inhibition of both full-length and truncated Id1 promoter activity (Fig. 5B). The shorter promoter construct (-353) only contained a single FOXO binding element (site 1). This suggests that site 1 is the critical binding site for FOXO3A-mediated Id1 repression. To examine whether FOXO3a directly associates with the Id1 promoter we investigated whether STI571 treatment of K562 cells influences occupation of the four potential FOXO3a binding sites of the Id1 promoter (Fig. 5A) by chromatin immunoprecipitation assay. Protein-DNA complexes were formaldehyde-cross-linked, and sites bound by FOXO3a were immunoprecipitated with the appropriate antibodies. PCR primer pairs were designed to detect selectively the four different potential FOXO3a binding sites in the human Id1 gene. In untreated cells, no FOXO3a binding to the Id1 promoter was detected (Fig. 5C). STI571 treatment resulted in a strong association of FOXO3a only to the binding site most proximal to the ATG of the Id1 promoter (site 1). In contrast, the other three sites did not demonstrate any association with FOXO3a (Fig. 5B, Primers 2 and 3). This is in support of the promoter deletion analysis (Fig. 5B).

Although the chromatin immunoprecipitation analysis confirms that FOXO3a is associated with the Id1 promoter in vivo, it does not distinguish between direct DNA binding or indirect association with promoter-bound complexes. To confirm that direct FOXO3A DNA binding to the Id1 promoter (site 1) occurs, we performed an oligonucleotide "pull-down assay." As shown in Fig. 5D, FOXO3A only associated with the Id1 promoter site 1 when the forkhead responsive element (FHRE) binding site was intact. Mutation of the FHRE resulted in abrogation of FOXO3A binding. To demonstrate that binding of FOXO3a to site 1 (−134 to −128 bp) is required for down-regulation of the Id1 promoter, the TTT core sequence was mutated to CGT in the Id1 promoter sequence. As shown in Fig. 5E, STI571 treatment of K562 cells resulted in down-regulation of Id1 promoter activity, whereas no significant effect was observed on the mutated Id1 promoter. Taken together, these experiments demonstrate that, upon STI571-induced FOXO3a activation, FOXO3a directly associates with the Id1 promoter resulting in inhibition of Id1 expression.

**FOXO3a Is Required for STI571-mediated Inhibition of Id1 Expression**—To prove that FOXO3a activity is required for STI571-induced down-regulation of Id1, K562 cells were stably transfected with the full-length Id1 promoter sequence. As shown in Fig. 6, STI571 treatment of K562 cells resulted in down-regulation of Id1 expression. These data provide evidence that FOXO3a activity is required for down-regulation of Id1 expression. FOXO3a directly associates with the Id1 promoter resulting in inhibition of Id1 expression. FOXO3a-induced Differentiation by Id1 Down-regulation

**FIGURE 5. Id1 is a direct transcriptional target of FOXO3a.** A, sequence of the Id1 promoter region. The potential FOXO binding sites are indicated in bold and underlined. B, K562 cells were electroporated with 16 μg of pGL3-Id1 −1692 to pGL3-Id1 −333 together with 500 ng of pRl-TK plasmid as an internal transfection control. Cells were cultured with or without STI571 (5 μM) for 4 or 8 h. Protein-DNA complexes were formaldehyde-cross-linked in vivo. Chromatin fragments from these cells were subjected to immunoprecipitation with a control antibody or antibodies to FOXO3a as indicated. After cross-link reversal, the co-immunoprecipitated DNA was amplified by PCR (resolved in 2% agarose gel). D, nuclear extracts were prepared from K562 cells treated with STI571 and analyzed by pull-down assay using either wild-type or mutated Id1 forkhead responsive element (FHRE) oligonucleotide probes as described under "Experimental Procedures." Samples were analyzed for FOXO3a binding by Western blot. E, K562 cells were electroporated with 16 μg of either the wild-type or the mutated Id1 promoter. Cells were cultured with or without STI571 (5 μM) for the indicated times, before analyzing luciferase activity as described under "Experimental Procedures." The results reflect the relative luciferase activity from three independent experiments ± S.E.
transfected with a vector expressing FOXO3a RNA interference duplexes, and the effect on Id1 expression was examined. As shown in Fig. 6, transfection of cells with FOXO3a RNA interference resulted in complete abrogation of FOXO3a protein expression. Interestingly, STI571 treatment had no effect on Id1 protein expression in these K562 FOXO3a knock-down cells. This in contrast to the control cells where Id1 protein expression was strongly down-regulated after STI571 treatment. These data clearly demonstrate that Id1 is indeed a direct target of FOXO3a and that FOXO3a activity is required for STI571-mediated Id1 down-regulation.

FOXO3a Activation Induces Differentiation of Bcr-Abl-transformed Cells—Next we questioned whether the Bcr-Abl-mediated inhibition of FOXO3a maintains cells in an undifferentiated state and therefore contributes to the leukemic phenotype, or conversely, whether the activation of FOXO3a and the subsequent down-regulation of Id1 can induce differentiation of K562 cells. Treatment with STI571 resulted in FOXO3a activation and Id1 down-regulation (Fig. 4). K562 cells can be differentiated into the erythrocytic lineage by treatment with STI571, characterized by the expression of hemoglobin. Using K562 cells expressing a 4-OHT-inducible FOXO3A(A3):ER mutant, addition of 4-OHT resulted in a dramatic increase in the number of cells expressing hemoglobin (Fig. 7A). Furthermore, activation of FOXO3A also resulted in down-regulation of Id1 levels in K562 cells (Fig. 7B). To determine whether FOXO3A activity is required for STI571-induced K562 differentiation, we utilized the K562 cells stably transfected with a vector expressing FOXO3a siRNA and subsequently treated with STI571 (Fig. 6). These data demonstrate that FOXO3A is both necessary and sufficient for STI571-induced differentiation of K562 cells.

Constitutive Id1 Expression Maintains Bcr-Abl-transformed Cells in an Undifferentiated State—To determine whether expression of Id1 indeed plays a critical role in maintaining the undifferentiated state of K562 cells, a bicistronic retroviral DNA construct co-expressing eGFP and Id1 was utilized to generate retrovirus and subsequently to infect K562 cells. Two days after infection the eGFP-positive cells were selected by fluorescence-activated cell sorting. As shown in Fig. 8, treatment with STI571 resulted in induction of hemoglobin expression. However, in K562 cells transduced with Id1, hemoglobin expression was reduced. These data demonstrate that the expression of Id1 indeed plays a critical role in maintaining the undifferentiated state of K562 cells.
DISCUSSION

Myeloid malignancies are often associated with mutations in upstream signaling components, such as for instance Bcr-Abl in CML, which result in constitutive activation of the PI3K/PKB pathway (7–9, 15). Leukemic cells are, in addition to uncontrolled proliferation and resistance to apoptosis, also characterized by a block in differentiation. The PI3K/PKB-signaling pathway plays a crucial role in proliferation and apoptosis and mediates these effects through modulation of the activity of FOXO transcription factors (22, 25). Hyperactivation of PKB results in inhibition of FOXO, which we suggest is critical in mediating the transforming activity of PI3K/PKB. We suggest that PKB maintains the leukemic phenotype through FOXO3a-mediated regulation of differentiation-specific genes. In the present study we aimed to identify novel transcriptional targets of the FOXO3a transcription factor that could play a role in differentiation of hematopoietic cells. Our hypothesis was that inhibition of such genes is likely to play a critical role in the maintenance of the leukemic phenotype. We identified the inhibitor of differentiation Id1 to be a direct target of FOXO3a and could demonstrate that, upon activation of FOXO3a, Id1 expression is strongly reduced, both at the transcriptional and protein levels. The fact that in FOXO3a knockdown cells Id1 was not down-regulated anymore clearly demonstrates that FOXO3a is indeed required for Id1 down-regulation. Furthermore, by performing chromatin immunoprecipitation assays and promoter mutations we also provide evidence that FOXO3a directly binds to the Id1 promoter. Our findings are in contrast to the hypothesis of Ramsawamy et al. (44), which suggested that FOXOs down-regulate genes not through direct binding to the DNA but through modulation of cofactors. However, we provide evidence that the FOXO3a-mediated down-regulation of Id1 is indeed through direct binding to the promoter. Of course, we cannot rule out the possibility that FOXO3a in addition also regulates the activity of additional transcription factors or cofactors. In accordance with our data are the results of Shi et al. (45) who demonstrated that the forkhead transcription factor Foxp1 directly binds to the c-fms promoter and functions as transcriptional repressor, thereby controlling monocyte differentiation. Treatment of K562 cells with the Bcr-Abl inhibitor STI571 resulted in dephosphorylation and, therefore, activation of FOXO3a. This correlated with down-regulation of Id1 and induction of erythroid differentiation (Figs. 4 and 7). Specific activation of a constitutively active FOXO3a was sufficient to reverse the leukemic phenotype and to induce differentiation (Fig. 7A). We provide evidence that Id1 expression is a critical factor in maintaining the undifferentiated state of K562 cells. Ectopic expression of Id1 resulted in inhibition of STI571-induced differentiation, which suggests that the down-regulation of Id1 is a pre-requisite for STI571-induced differentiation (Fig. 8).

Although involvement of the PI3K/PKB-signaling module in Bcr-Abl-mediated transformation has been demonstrated, this has previously been placed in the context of apoptosis and proliferation, but a direct link with differentiation has not been demonstrated (23, 25, 47, 48). Skorski et al. demonstrated that inhibition of PI3K signaling by ectopic expression of dominant-negative PKB suppressed Bcr-Abl-dependent colony formation in vitro. Similarly, dominant negative PKB was shown to inhibit leukemia development in SCID (severe combined immunodeficiency disease) mice that were injected with bone marrow cells expressing Bcr-Abl. Our data strongly suggest that constitutive expression of Id1 is critical for maintenance of the myeloid phenotype and ectopic expression of FOXO3a can reverse this phenotype.

Recently it has been shown that FOXO3a can play a role in erythroid differentiation through up-regulation of the B-cell translocation gene 1 protein (38). Bakker et al. demonstrated that B-cell translocation gene 1 can modulate protein arginine methylation activity, which they propose is a novel mechanism regulating erythroid differentiation. The authors suggest that B-cell translocation gene 1-mediated activation of enzymes, which induce methylation, could contribute to epigenetic gene regulation, including condensation of the nucleus and enucleation late in erythroid differentiation. However, we demonstrate regulation of differentiation through FOXO3a-mediated modulation of Id1 expression. This directly regulates the activity of basic helix-loop-helix (bHLH) transcription factors that specifically induce expression of differentiation-linked genes (28, 41, 42). The Id transcriptional repressors have been suggested to act at the checkpoint at which undifferentiated progenitor cells make the commitment to terminal differentiation. Id1 expression is high in proliferating, undifferentiated cells, whereas its expression is down-regulated as cells differentiate (38, 49, 50). For example, ectopic expression of Id1 inhibits B-cell development and differentiation of muscle and mammary epithelial cells (42). More recently in vivo studies using targeted expression of Id1 to thymocytes (51), intestinal epithelia (52), and B-lymphocytes (53) of mice have demonstrated inhibition of cellular differentiation in these systems (41, 42). In addition, very recently it was demonstrated that Id1 plays a role in myelopoiesis (30, 49). Down-regulation of Id1 was required for normal myelopoiesis, and the current study suggests a model whereby high expression of Id1 maintains leukemic cells in an undifferentiated state.

Id proteins do not possess a DNA binding domain and thereby function as dominant-negative regulators of bHLH proteins (28, 41, 42). They can dimerize with bHLH transcription factors and inhibit bHLH-dependent expression of differentiation-linked genes. However, relatively little is known concerning the specific molecular mechanisms by which Id1 regulates hematopoiesis. In other cell types bHLH proteins have been identified that can bind to Id1. Id1 inhibits Ets-mediated transcription of p16INK4a, a tumor suppressor (50). In addition, the Id1 target MyoD activates p21Waft/Cip1 gene expression in myoblasts and its partner E2A positively regulates p21Waft/Cip1 transcription in fibroblasts (54). This regulation of p21 by E2A is antagonized by Id1, suggesting that Id1 may stimulate proliferation through antagonism of E2A-dependent p21 expression. The bHLH that is primarily expressed in hematopoietic cells is SCL/TAL1 (38). However, ectopic expression of SCL/TAL1 in the HL-60 granulocytic cell line resulted in enhanced proliferation, not differentiation (55). It remains to be seen which bHLH Id1-binding partners are inhibited in Bcr-Abl-transformed cells.
FOXO3a-induced Differentiation by Id1 Down-regulation

Although it has recently been shown by Kuzelova et al. (56) that STI571 can induce, in addition to cell cycle arrest and apoptosis, erythroid differentiation of K562 cells, they do not provide evidence concerning which signal-transduction pathways are mediating this effect. Here we clearly show that STI571-induced erythroid differentiation is mediated through the FOXO3a-mediated down-regulation of Id1. Treatment of CML patients with STI571 is now a common treatment strategy. Although complete remissions are observed upon treatment with STI571 in patients with CML blast crisis, most patients enjoy only a short duration of response, with eventual emergence of STI571-resistant leukemic cells and a clinical relapse (43, 57). Therefore, new treatment strategies are required. Because the choice of drug targets must take into account the adverse effects resulting from the inhibition of other general PI3K/PKB-dependent cellular processes, it would be desirable to target downstream components of this signaling module, such as Id1. In conclusion, our data demonstrate that high expression of Id1, through PI3K/PKB-mediated inhibition of FOXO3a, is critical for maintenance of the leukemic phenotype.

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