Expression of p89c-Mybex9b, an alternatively spliced form of c-Myb, is required for proliferation and survival of p210BCR/ABL-expressing cells

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INTRODUCTION

The c-Myb gene encodes the p75c-Myb isoform and less-abundant proteins generated by alternatively spliced transcripts. Among these, the best known is p89c-Mybex9b, which contains 121 additional amino acids between exon 9 and 10, in a domain involved in protein–protein interactions and negative regulation. In hematopoietic cells, expression of p89c-Mybex9b accounts for 10–15% of total c-Myb; these levels may be biologically relevant because modest changes in c-Myb expression affects proliferation and survival of leukemic cells and lineage choice and frequency of normal hematopoietic progenitors. In this study, we assessed biochemical activities of p89c-Mybex9b and the consequences of perturbing its expression in K562 and primary chronic myeloid leukemia (CML) progenitor cells. Compared with p75c-Myb, p89c-Mybex9b is more stable and more effective in transactivating Myb-regulated promoters. Ectopic expression of p89c-Mybex9b enhanced proliferation and colony formation and reduced imatinib (IM) sensitivity of K562 cells; conversely, specific downregulation of p89c-Mybex9b reduced proliferation and colony formation, enhanced IM sensitivity of K562 cells and markedly suppressed colony formation of CML CD34+ cells, without affecting the levels of p75c-Myb. Together, these studies indicate that expression of the low-abundance p89c-Mybex9b isoform has an important role for the overall biological effects of c-Myb in BCR/ABL-transformed cells.

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The proto-oncogene c-myb encodes a transcription factor that is expressed predominantly in immature hematopoietic, epithelial and endothelial cells and in many tumor types1,2 and is downregulated as cells become more differentiated.3

In hematopoietic progenitor cells, c-Myb has an important role in the control of cell proliferation, survival and differentiation;4 in vitro, downregulation of c-Myb expression leads to decreased proliferation and colony formation of myeloid progenitors,5 whereas loss of c-Myb, in vivo, is embryonically lethal due to failure of fetal hematopoiesis.6

Conditional knockout of c-Myb expression in adult hematopoietic stem cells causes loss of self-renewal due to impaired proliferation and accelerated differentiation,7 suggesting that c-Myb has an essential role also in bone marrow primitive hematopoiesis. Constitutive overexpression of c-Myb in myeloid and erythroid cell lines blocks differentiation and prevents maturation-associated growth arrest.8,9 aberrant c-Myb expression has been detected in several malignancies including T-cell leukemia,10 chronic myeloid leukemia (CML), acute myeloid leukemia,11,12 colorectal cancer,13 breast cancer14 and, more recently, adenoid cystic carcinomas.15 In CML, the increased expression of c-Myb is, in part, due to enhanced protein stability via BCR/ABL-regulated activation of PI3K/Akt/GSK3β-dependent pathways;16,17 this altered regulatory mechanism may explain the requirement of c-Myb for in vitro proliferation and survival of leukemic progenitor cells18,19 and for leukemogenesis in mice.20

Leukemic blast cells appear to rely on c-Myb expression more than their normal counterpart,21 suggesting that this differential requirement for c-Myb may be exploited therapeutically.

The requirement of c-Myb may depend on its ability to modulate the expression of genes (that is, CD34, c-kit, c-myc, flt-3 and Bcl-2) with important roles for the proliferation and survival of hematopoietic cells.22–26 c-Myb also regulates the expression of cyclin B1, contributing to the control of the G2/M phase of the cell cycle, in addition to its role during G1/S-phase transition.27

More recently, we found that c-Myb activates, in a DNA-binding manner, the expression of Slug and it promotes, via Slug, the homing of K562 cells to the bone marrow.28

The main product of the c-myb gene is a 75KDa nuclear protein that contains three functional domains: (i) an N-terminal DNA-binding domain that specifically binds to the sequence PyAAGC/TG; (ii) a centrally-located transcription activation domain; and (iii) a C-terminal negative regulatory domain (NRD), which includes a leucine zipper and an EVS motif that modulate the activity of c-Myb via inter-and intra-molecular interactions.29,30

Alternatively, spliced c-myb transcripts have been detected in hematopoietic cells of several species, including humans.31–33 The best-characterized of these transcripts includes a 363 bp segment between exons 9 and 10 (designated as exon 9b in humans), which is translated into 121 additional amino acids that disrupt
the NRD domain.\textsuperscript{34,35} p89\textsuperscript{c-Mybex9b} represents 10–15% of total c-Myb protein in hematopoietic cells and has, apparently, the same properties of the predominant p75\textsuperscript{c-Myb} species: it localizes into the nucleus and binds to the same DNA-binding sequence.\textsuperscript{36,37} However, the 121 amino acids added by exon 9b could, in principle, enhance and/or modify the activity of the p89\textsuperscript{c-Mybex9b} isoform by disruption of the intra- and/or inter-molecular interactions that may regulate stability and transactivation activity of c-Myb.\textsuperscript{38}

The function and requirement of the p89\textsuperscript{c-Mybex9b} isoform is understood only in part: it appears to transactivate the expression of certain c-Myb targets more effectively than the predominant p75\textsuperscript{c-Myb} isoform,\textsuperscript{39} and yet the specific knockout of p89\textsuperscript{c-Myb} expression has no deleterious consequences on mammalian hematopoiesis and development,\textsuperscript{40} suggesting that its loss is compensated by expression of p75\textsuperscript{c-Myb}.

However, it is unknown whether expression of p89\textsuperscript{c-Mybex9b} is required for the proliferation and survival of transformed hematopoietic cells independently of p75\textsuperscript{c-Myb} expression.

We show here that p89\textsuperscript{c-Mybex9b} is more stable and has higher transactivation activity than p75\textsuperscript{c-Myb}, moreover, its specific downregulation impairs proliferation and colony formation and enhances the imatinib (IM) sensitivity of BCR/ABL-expressing cells, in spite of unperturbed expression of p75\textsuperscript{c-Myb}.

\section*{MATERIALS AND METHODS}

\subsection*{Plasmids}

Mig-Ri-c-Myb-PA and Mig-Ri-\Delta358-452c-Myb were obtained as described.\textsuperscript{17} Cyclin B1-Luc-pG3L was a gift from the late Dr AM Gewirtz (University of Pennsylvania, Philadelphia, PA, USA). SLUG-Luc-pG3L was recently described.\textsuperscript{28} MSCV-p89\textsuperscript{c-Mybex9b} was amplified from human PH\textsuperscript{K562} cells by reverse-transcription PCR using exon 9b-specific primers (Fw 5'-GCCCTTGGAGTGGACCAAAGGTGAGTACGGG-3' and Rv 5'-ACTGCCTGGACGGAGAATAATGGA-3').

MSCV-p89\textsuperscript{c-Mybex9b-MUT} was generated by mutating four nucleotides of MSCV-p89\textsuperscript{c-Mybex9b} by two subsequent site-directed mutagenesis according to the manufacturer's instructions (QuickChange II site-directed mutagenesis kit, Stratagene, Santa Clara, CA). Mutated primers were: 5'-CCACCTGGTCATCTCGGGAAAAGGCGGCGGA-3' and 5'-CAACAGCACAATCTAATGGCTCTCCCGGAAA-3' and its reverse complement (second mutagenesis).

Mig-Ri-p89\textsuperscript{c-Mybex9b} and Mig-Ri-p75\textsuperscript{c-Myb} were generated by digesting MSCV-p89\textsuperscript{c-Mybex9b} with Xhol/AatII and EcoRl/DigI and ligating the two fragments into Xhol/EcoRl-digested Mig-Ri.

MSCV-p89\textsuperscript{c-Mybex9b}_{SH} and MSCV-p75\textsuperscript{c-Myb}_{SH}MUT, the mutant forms of p89\textsuperscript{c-Mybex9b} and p75\textsuperscript{c-Myb}, not inhibitable by the doxycycline (DOX)-regulated c-Myb-sh short hairpin RNA (shRNA) plVLTSh lentivirus (gift of Dr TJ Gonda, Brisbane University, Australia),\textsuperscript{41} were used in the experiments. The c-Myb-sh coding region was amplified by qMig-Ri-p89\textsuperscript{c-Mybex9b} and MSCV-p75\textsuperscript{c-Myb} used as templates with primer set A (Fw 5'-CGCCCGGATTAGCTCATTAGAAGATGAGGACA-3', Rv 5'-AACTTCAACTAGATCTTCAATTTCCAG-3') and primer set B (Fw 5'-CGCCCGGGATTAGCTCATTAGAAGATGAGGACA-3', Rv 5'-AACTTCAACTAGATCTTCAATTTCCAG-3') (which amplify a region of 1221 bp common to both c-Myb isoforms) and to its reverse complement (first mutagenesis); 5'-CACAGCACAATCTAATGGCTCTCCCGGAAA-3' and its reverse complement (second mutagenesis).

\subsection*{Chromatin immunoprecipitation and co-immunoprecipitation}

For chromatin immunoprecipitation experiments performed in Mig-Ri-p89\textsuperscript{c-Mybex9b} or FLAG K562 cells, samples were prepared as described.\textsuperscript{44} IPS were performed with anti-c-Myb antibody (30\,\mu g; clone 1–1, Upstate Biotechnology, Billerica, MA, USA) or anti-FLAG antibody (30\,\mu g; clone M2, Sigma-Aldrich, St Louis, MO, USA) or anti-rabbit IgG or without antibody at 4°C for 1 h with rotation. Immune complexes were collected with 45\,\mu l of protein G-agarose beads at 4°C overnight with rotation. Recovered DNA (2\,\mu l) was used as a template for Real-Time PCR using GoTaq Real-Time PCR Master Mix (Promega) and primers on the 5'-flanking region of the human cyclin B1\textsuperscript{17} or SLUG\textsuperscript{32} promoter. Data were analyzed with the percent input method: percentage of input was calculated by 100 × 2^{\text{-ΔΔCt}}.

For co-immunoprecipitations, 293T cells were transfected with plasmids expressing tagged c-Myb and lysates were immunoprecipitated with Anti-FLAG M2-Afinity gel (Sigma-Aldrich) following the manufacturer's instructions. Tagged proteins were detected by anti-FLAG M2-peroxidase-HRP (Sigma-Aldrich) and anti-α-pheromone High Affinity 3F10 (Roche, Nutley, NJ, USA).

\subsection*{Measurement of c-Myb half-life}

To assess the half-life of 75\textsuperscript{c-Myb} and 89\textsuperscript{c-Myb}, cells (pre-treated or not with 2\,\mu M IM for 16 h) were treated with cycloheximide (25\,\mu g/ml) for 2 h to suppress protein synthesis. Samples were collected every 30 min and lysates (100\,\mu g each) were analyzed by anti-c-Myb western blotting. Later, bands corresponding to the c-Myb isoforms and to β-actin as loading control were scanned and densitometric analysis was carried out with ImageJ Software (National Institutes of Health). The half-lives of 75\textsuperscript{c-Myb} and 89\textsuperscript{c-Myb} were calculated using the formula: $t_{1/2} = \frac{0.693t}{\ln(No/No)}$ as described.\textsuperscript{17}

Small interfering RNA (siRNA) transfection and real-time PCR

Human c-Myb siRNA pool (catalog no. L-003910-00-0005) and control siRNAs (catalog no. D-001810-10-05) were purchased from Dharmaco, Thermo Fisher Scientific (Lafayette, CO, USA).

p89\textsuperscript{c-Mybex9b}_{shRNA-1} and p89\textsuperscript{c-Mybex9b}_{shRNA-2} were customized through the Dharmaco web site and purchased from Sigma-Aldrich. A total of 10\,\times\,10^6 K562 cells were resuspended in Ingenio Electroporation Solution (Mirus Bio LLC, Madison, WI, USA) and mixed with 5\,\mu g of each siRNA. The solution was added to Ingenio Cuvettes (Mirus Bio LLC) and electroporated according to Amaxa Nucleofector II using program T-16. Then, cells were diluted in 2\,ml of Iscove’s modified Dulbecco’s medium supplemented with 20% fetal bovine serum, penicillin/streptomycin (100\,\mu g/ml each) and
in p89c-Mybex9b and in the c-Myb mutant, suggesting that was detected with the following primers: Fw 5'-AACATCTACACAGCAAGCAGAGG-3' and Rev 5'-TGAATCTTCATCAACATAG-3'. p75c-Myb-specific transcript: Fw 5'-TAGATCTTCTTTCAAACATCTCC-3' and Rev 5'-GTCTCTATGAATGTTTGTTAAC-3'; for normalization, expression of hypoxanthine-guanine phosphoribosyltransferase (LC Laboratories, Woburn, MA, USA). Cells were counted every 24 h by trypan blue exclusion.

For induction of apoptosis, parental or derivative K562 cells were seeded at 2 x 10^5 cells/ml and treated with 2 μM IM (1 μM added every 12 h) (LC Laboratories, Woburn, MA, USA). Cells were counted every 24 h by trypan blue exclusion and percentage of apoptosis evaluated by the hypotonic propidium iodide method through Coulter Epics XL-MCL (Beckman Coulter Inc., Indianapolis, IN, USA).

G2/M-phase cells were also evaluated by propidium iodide staining. Phospho-histone-H3 positivity was assessed by Phospho-(Ser10)-histone-H3 (Cell Signalling Technology, Danvers, MA, USA) staining according to the manufacturer’s instructions.

For colony-formation assays, K562 cells were plated in methylcellulose (Stem Cell Technologies) with or without IM (1 μM pre-treatment in liquid culture for 24 h and added to the plates) and with or without doxycycline (5 μg/ml; pre-treatment in liquid culture for 24 h and added to the plates); colonies were counted 6 days later. CD34^+ cells were plated in methylcellulose supplemented with Cytokine Cocktail CC100 (Stem Cell Technologies) and colonies were counted 9 days later.

Statistical analyses

Data (presented as the means ± s.d. of two or three experiments) were analyzed for statistical significance by the unpaired, two-tailed Student’s t-test. P-values of < 0.05 were considered statistically significant.

RESULTS

Increased transactivation of c-Myb-regulated promoters by p89c-Mybex9b

Two c-Myb mutants, Δ(358–452)c-Myb and Δ(389–418)c-Myb, which lack 30 and 30 amino acids, respectively, in the leucine-zipper-NRD (LZ-NRD) are more potent than wild-type p75c-Myb in enhancing proliferation and blocking apoptosis of normal and transformed hematopoietic cells.17

p89c-Mybex9b, a naturally occurring alternatively spliced form of c-Myb, which accounts for approximately 10–15% of total c-Myb levels, contains an insertion of 121 amino acids, respectively, in the leucine-zipper-NRD (LZ-NRD) and in the c-Myb mutant, suggesting that p89c-Mybex9b may function like the artificial LZ-NRD Δ(358–452)c-Myb.

The transactivation potential of p89c-Mybex9b, Δ(358–452)c-Myb, and p75c-Myb was compared by dual-luciferase assays using reporter plasmids consisting of a fragment of the Myb-regulated cyclin B1 or SLUG promoter driving the luciferase gene (cyclin B1-Luc or SLUG-Luc).

Following co-transfection of reporter and effector plasmids in 293T cells, expression and transcription of each c-Myb protein were analyzed.

Expression of the three c-Myb isoforms was essentially identical in 293T cells (Figure 1b). p89c-Mybex9b, Δ(358–452)c-Myb were slightly more effective than p75c-Myb in transactivation of the cyclin B1 promoter but not of the SLUG promoter (Figure 1e). The transactivating ability of the three c-Myb proteins was also tested in Ph1 K562 cells; because K562 cells express high levels of endogenous c-Myb, the increase in expression of p75c-Myb in transfected cells (Figure 1c) was quantitated by densitometry (Figure 1d), whereas expression of p89c-Mybex9b and Δ(358–452) c-Myb was detected because of size differences and quantitated by densitometry (Figures 1c and d).

Compared with 293T cells, the effects of the c-Myb isoforms in K562 cells were markedly different: p75c-Myb induced approximately a three-fold increase in transactivation of the cyclin B1 promoter, whereas p89c-Mybex9b and Δ(358–452) c-Myb were much more potent (≈ five- and twelve-fold increase, respectively) (Figure 1f, upper panel); likewise, p89c-Mybex9b and Δ(358–452) c-Myb transactivated the SLUG promoter more effectively than p75c-Myb (≈ five- and six- versus three-fold, respectively) (Figure 1f, lower panel).

Enhanced stability of p89c-Mybex9b in BCR/ABL-positive cells

In addition to the transactivation ability, we also tested the stability of p89c-Mybex9b because the insertion of ex9b disrupts the LZ domain and c-Myb LZ-domain mutant proteins are more stable than wild-type p75c-Myb.17

Thus, parental and p89c-Mybex9b-expressing K562 cells were treated with cycloheximide for different times and cell lysates blotted with an anti-c-Myb antibody to assess half-life of endogenous p75c-Myb and ectopic p89c-Mybex9b using either an anti-FLAG antibody (which binds only the ectopically expressed protein) or an anti-c-Myb antibody (which binds the endogenous and the ectopically expressed protein). The ability of p89c-Mybex9b to bind the two promoters was assessed by real-time PCR amplification of a 5′-flanking region segment, which includes putative c-Myb-binding sites (Figures 2a and b, right). The ~122 to +177 nucleotide segment of the cyclin B1 promoter and the ~77 to +175 segment of the SLUG promoter were amplified above background in both the anti-c-Myb and anti-FLAG chromatin IPs, indicating that p89c-Mybex9b binds to these c-Myb-regulated promoters (Figures 2a and b, left).

Because p210BCR/ABL enhances the stability of c-Myb,17 we also assessed the half-life of p89c-Mybex9b expression in K562 cells treated with cycloheximide. Inhibition of BCR/ABL tyrosine kinase activity decreased the half-life of p89c-Mybex9b (~35 min in IM-treated versus 60 min in untreated cells); in contrast, the half-life of p89c-Mybex9b was unaffected by treatment with IM (Figure 3c). By densitometry analysis, the half-life of p89c-Mybex9b in IM-treated cells was ~75 min longer than that of p75c-Myb (110 versus 35 min, Figure 3d).

Inhibition of p89c-Mybex9b expression suppresses proliferation and colony formation of K562 cells.

We investigated the role of p89c-Mybex9b expression in transformed cells by assessing whether its specific knockdown has any effect on the proliferation and clonogenic potential of Ph1 K562 cells.

Two siRNAs targeting two different regions of human exon 9b c-Myb transcript (siRNA-1 and siRNA-2, Figure 4a) suppressed very effectively p89c-Mybex9b expression in K562 cells (Figure 4b, left).
with no effects on levels of the p75 c-Myb isoform. As control, we also used a pool of siRNAs targeting both p75 c-Myb and the p89 c-Mybex9b transcripts; transfection with this c-Myb siRNA pool led to the disappearance of both c-Myb isoforms (Figure 4b, right). Real-Time PCR using primers specific for p75 c-Myb or p89 c-Mybex9b transcripts further demonstrated that silencing of p89 c-Mybex9b...
expression had no effect on levels of p75 c-Myb transcripts (Supplementary Figure 1).

K562 cells transfected with the c-Myb siRNA pool or with p89 c-Mybex9b-specific siRNAs proliferated less than control siRNA-transfected cells (Figure 4c); at 48 h, the c-Myb siRNA pool was more effective than the p89 c-Mybex9b-specific siRNAs (54% versus 30% inhibition, respectively), but at 72 h, the effects were comparable.

We also assessed the effect of p89 c-Mybex9b-specific down-regulation on colony formation of K562 cells. Thus, cells were transfected with control or specific siRNAs and 24 h after the second transfection were plated in methylcellulose (500 cells per plate) and colonies were counted 6 days later. Cells treated with control siRNA formed 350 ± 36 colonies (Figure 4d), whereas K562 cells transfected with the c-Myb siRNA pool formed 178 ± 27 colonies; a significant decrease in colony formation was also noted by plating cells transfected with p89 c-Mybex9b-siRNA-2 (230 ± 14 colonies) or p89 c-Mybex9b-siRNA-1 (230 ± 11) (Figure 4d).

To assess the specificity of the biological effects induced by p89 c-Mybex9b-siRNA-2, we generated K562-derivative lines

**Figure 2.** p89 c-Mybex9b binds the cyclin B1 and SLUG promoter. Chromatin immunoprecipitation assays show binding (detected by real-time PCR) of p75 c-Myb and p89 c-Mybex9b to a segment of the cyclin B1 promoter (a, left) and SLUG promoter (b, left). Error bars denote s.d. of the means of two experiments performed in triplicate. (a and b, right): partial sequence of the human promoter of cyclin B1 (GenBank: U22364.1) and SLUG (GenBank: AB300659.1). Putative c-Myb-binding sites located in the 5′-untranslated region (italics) are underlined; primers used for real-time PCR are in bold.

**Figure 3.** Half-life of p89 c-Mybex9b in K562 cells. Representative western blot shows levels of p75 c-Myb and p89 c-Mybex9b in: (a) cells treated with cycloheximide only and (c) cells pre-treated for 16 h with 2 μM IM before cycloheximide treatment. Levels of β-actin were measured as loading control. (b–d) Densitometric analysis from two combined experiments assessing the half-life of c-Myb isoforms.
Figure 4. Effects of p89c-Mybex9b downregulation in K562 cells. (a) Schematic diagram representing the siRNAs target sequences of p89c-Mybex9b and the mutations in p89c-Mybex9b MUT. (b) Western blot shows levels of p75 c-Myb and p89 c-Mybex9b in K562 cells transfected with the c-Myb siRNA pool, c-Myb ex9b-specific siRNAs (sequence 1 and 2) or their relative controls. β-actin levels were measured as control of equal loading. (c) Cell counts and (d) colony formation of K562 cells transfected with c-Myb siRNA pool or its control (upper) or c-Myb ex9b siRNA 1 or siRNA2 or their scramble control (lower). (e) Levels of p89c-Mybex9b in MSCV-p89c-Mybex9b and MSCV-p89c-Mybex9bMUT K562 cells transfected with a control or the p89c-Mybex9b-siRNA 2. Expression of β-actin detected as loading control. (f) Methylcellulose colony formation of MSCV-p89c-Mybex9b and MSCV-p89c-Mybex9bMUT K562 cells transfected with a control or the p89c-Mybex9b-siRNA 2 (values of cell counts and number of colonies are given as mean ± s.d. of three independent experiments performed in triplicate; * denotes statistical significance of the differences between scrambled- and siRNA-transfected cells). (g) Levels of p75 c-Myb and p89c-Mybex9b in untreated and DOX-treated (24 h) K562 cells expressing a DOX-inducible c-Myb shRNA and transduced with p75c-Myb_shMUT or p89c-Mybex9b_shMUT. Expression of β-actin is detected as loading control. (h) Colony formation of untreated and DOX-treated cells; values are expressed as % of colonies formed by treated cells, compared with untreated cells taken as 100% (data are given as mean ± s.d. of two independent experiments performed in triplicate; * denotes statistical significance of the differences between K562 cells with shMUT c-Mybs and cells with only c-Myb shRNA).
ectopically expressing wild-type p89c-Mybex9b and a mutant, p89c-Mybex9bMUT, with four nucleotide substitutions in the sequence targeted by p89c-Mybex9b-siRNA-2; these mutations were designed to prevent interaction with siRNA-2 while preserving the amino-acid sequence of the protein (Figure 4a).

MSCV-p89c-Mybex9b and MSCV-p89c-Mybex9bMUT K562 cells were then transfected with a control or the p89c-Mybex9b-siRNA-2 and levels of p89c-Mybex9b were tested by western blotting. Levels of p89c-Mybex9b were undetectable in MSCV-p89c-Mybex9b K562 cells transfected with p89c-Mybex9b siRNA-2 (Figure 4e); in contrast, expression of p89c-Mybex9bMUT was detected in siRNA-2-transfected MSCV-p89c-Mybex9bMUT K562 cells, reflecting downregulation of the endogenous protein but not of the p89c-Mybex9b protein derived from p89c-Mybex9bMUT, which is not targeted by p89c-Mybex9b siRNA-2. As expected, MSCV-p89c-Mybex9bMUT K562 cells transfected with p89c-Mybex9bMUT-siRNA-2 formed fewer colonies (~50% inhibition) than the scramble siRNA-transfected counterpart (Figure 4f); in contrast, there were no significant differences in colony formation of scrambled- or p89c-Mybex9bMUT-siRNA-2 transfected MSCV-p89c-Mybex9bMUT K562 cells (Figure 4f), further confirming that the inhibition of K562 colony formation by p89c-Mybex9b-siRNA-2 is due to specific downregulation of p89c-Mybex9b expression.

The importance of p89c-Mybex9b expression for K562 colony formation was also assessed in K562 cells expressing a DOX-inducible c-Myb shRNA, which downregulates the p75c-Myb and p89c-Mybex9b isoforms, and carrying the p75c-Myb or p89c-Mybex9b expression vector non-targetable by the DOX-inducible c-Myb shRNA (p75c-Myb_shMUT and p89c-Mybex9b_shMUT, respectively) (Figure 4g). As expected, colony formation of DOX-treated K562-c-Myb shRNA cells was markedly suppressed (~80% inhibition); in contrast, expression of p89c-Mybex9b_shMUT or p75c-Myb_shMUT rescued, in part, the inhibitory effect of the DOX-inducible c-Myb shRNA (Figure 4h), consistent with redundant and yet non-overlapping effects of the p75c-Myb and the p89c-Mybex9b-Myb isoforms.

Levels of cyclin B1 are downregulated in p89c-Mybex9b-silenced K562 cells

The reduced colony formation of c-Myb-silenced (p75c-Myb and/or p89c-Mybex9b) K562 cells may be caused by changes in the expression of c-Myb-regulated genes with a role in cell proliferation. Cyclin B1, a gene with an essential role in the G2/M transition, is one of the c-Myb targets whose change in expression may explain, in part, the effects of p89c-Mybex9b downregulation.

Indeed, compared with control siRNA-transfected cells, cyclin B1 expression was reduced in K562 cells transfected with the c-Myb siRNA pool (~45% decrease of cyclin B1 levels); although expression of p75c-Myb was unaffected, the specific downregulation of p89c-Mybex9b led to a similar decrease (~42%) of cyclin B1 expression (Figures 5a and b).
We also analyzed the fraction of G2/M-phase cells in c-Myb- or p89c-Myb-ex9b-silenced K562 cells by evaluating DNA content of propidium iodide-stained cells and percentage of cells positive for phospho-histone-H3, which is a marker of M phase. As expected, the decrease in the cyclin B1 levels was accompanied by a reduction in the number of cells in G2/M phase (Figure 5c).

Although not statistically significant, in three different experiments, the number of M-phase cells was lower in c-Myb- or p89c-Myb-ex9b-siRNA-2 than in control siRNA-transfected K562 cells (Figure 5d).

p89c-Myb-ex9b expression affects the response of K562 cells to treatment with IM

Because expression of p89c-Myb-ex9b appears to be important for proliferation and colony formation of K562 cells, we speculated that it may also influence the response to treatment with IM. Parental, p89c-Myb-ex9b- or Δ(358–452)c-Myb-expressing K562 cells were treated with IM, and cell counts and DNA content analyses were performed every 24 h.

As expected, expression of Δ(358–452)c-Myb reduced the IM sensitivity of K562 cells (Figure 6a); expression of p89c-Myb-ex9b had a similar, although less potent, effect as IM-treated p89c-Myb-ex9b-expressing K562 cells exhibited a less pronounced decrease in cell number than parental cells (Figure 6a). Similar effects were observed upon assessing apoptosis of IM-treated K562 cells; as expected, IM-treated parental K562 cells showed a high percentage of apoptosis, which increased from 24 to 72 h. In contrast, apoptosis of IM-treated K562 cells expressing p89c-Myb-ex9b or Δ(358–452)c-Myb was approximately 50% lower than that of parental cells (Figure 6b). In normal growth conditions, overexpression of p89c-Myb-ex9b or Δ(358–452)c-Myb has no effect on the proliferation and basal apoptosis of K562 cells (Supplementary Figure 2).

We also assessed the effect of c-Myb downregulation on IM-treated K562 cells. Parental cells transfected with the c-Myb siRNA pool, p89c-Myb-ex9b isoform-specific or control siRNAs were counted and analyzed by flow cytometry 24, 48 and 72 h after IM treatment. Downregulation of c-Myb or p89c-Myb-ex9b enhanced the proliferation inhibitory effects of IM, compared with cells transfected with the control siRNA (at 72 h, ~60% fewer cells than in control siRNA-transfected K562 cells) (Figure 6c). Evaluation of apoptosis by DNA content analysis showed a similar pattern: c-Myb-silenced cells exhibited increased apoptosis compared with cells transfected with control siRNAs. Apoptotic cells were slightly more numerous after transfection with c-Myb siRNAs than with the p89c-Myb-ex9b-specific siRNA but the differences were not significant (Figure 6d).

We also assessed the relationship between levels of p89c-Myb-ex9b and colony formation after IM treatment. Thus, parental or
ectopically expressing \( p89c\)-Mybex9b or \( \Delta (358–452) \)c-Myb K562 cells were treated for 24 h with IM (1 \( \mu \)M) and plated in methylcellulose (500 cells per plate, in the presence of 1 \( \mu \)M IM); colonies were counted after 6 days.

Consistent with the effects on cell proliferation, expression of the \( p89c\)-Mybex9b isoform enhanced the number of clonogenic cells after IM treatment (13.8% and 6.5% of residual clonogenic cells in \( p89c\)-Mybex9b-expressing versus parental K562 cells). Residual colonies formed by \( p89c\)-Mybex9b- or \( \Delta (358–452) \)c-Myb-expressing K562 cells were nearly identical (Figure 6e).

Colony assays were also performed after c-Myb or \( p89c\)-Mybex9b-specific siRNA tranfection and IM treatment of K562 cells. As expected, the combination of Myb downregulation and IM treatment caused a marked decrease in the clonogenic potential of K562 cells. Residual colony formation of cells transfected with the c-Myb siRNA pool or \( p89c\)-Mybex9b-siRNA was similar (1.1% and 1.9%, respectively) and lower of that from cells transfected with control siRNAs (3.1%) (Figure 6f). Together, these data suggest that expression of the \( p89c\)-Mybex9b isoform can modulate the effects of IM in K562 cells.

Inhibition of \( p89c\)-Mybex9b suppresses colony formation of normal and CML CD34\(^+\) progenitors

In Figure 6c, the role of \( p89c\)-Mybex9b expression was also assessed in colony-formation assays of CD34\(^+\) cells from healthy donors and CML patients.

Normal human CD34\(^+\) cells (\( n = 3 \)) were transfected with the c-Myb siRNA pool or a \( p89c\)-Mybex9b-specific siRNA or the control siRNA. 24 h after transfection, levels of c-Myb proteins were tested and cells were plated for colony-formation assays. A representative western blot shows that levels of p75c-Myb and \( p89c\)-Mybex9b were completely downregulated in cells transfected with the c-Myb siRNA pool, whereas transfection with \( p89c\)-Mybex9b-siRNA reduced only the levels of the \( p89c\)-Mybex9b isoform (Figure 7a). Normal CD34\(^+\) cells transfected with the c-Myb siRNA pool or the \( p89c\)-Mybex9b-specific siRNA formed fewer colonies than control siRNA-transfected cells (~33% and 28% inhibition, respectively; range: 23–43% and 22–37%, respectively) (Figure 7b). Peripheral blood CD34\(^+\) cells from CML chronic-phase patients (\( n = 5 \)) were also transfected with c-Myb or control siRNAs. Like in normal CD34\(^+\) cells, expression of p75c-Myb and \( p89c\)-Mybex9b was markedly downregulated in CML CD34\(^+\) cells transfected with the c-Myb siRNA pool, whereas only \( p89c\)-Mybex9b levels decreased in cells transfected with \( p89c\)-Mybex9b-siRNA (Figure 7c). Transfected CML CD34\(^+\) cells were seeded in cytokine-supplemented methylcellulose and colonies were counted 9 days later. Colonies arising from cells transfected with the c-Myb siRNA pool or \( p89c\)-Mybex9b-siRNA were fewer (63% and 41% decrease, respectively; range: 50–90% and 28–58%, respectively) than those from the scramble siRNA-transfected counterpart (Figure 7d).

Together, these results suggest that expression of \( p89c\)-Mybex9b is more important for colony formation of CML than normal CD34\(^+\) progenitors.

DISCUSSION

The c-Myb gene generates multiple transcripts that encode the predominant p75c-Myb isoform and, potentially, several less-abundant species.\(^4\) However, only the \( p89c\)-Mybex9b isoform is readily detectable in hematopoietic cells, suggesting that other alternatively spliced transcripts are too rare to generate detectable proteins or that the encoded gene products are unstable and rapidly degraded. \( p89c\)-Mybex9b accounts for 10–15% of total c-Myb protein, an amount that could be biologically relevant because...
small changes in the levels of c-Myb can affect lineage choice and progenitor cell frequencies in normal hematopoiesis, but the long-term proliferative potential, survival and differentiation of specific progenitor subsets was not investigated in detail. Although this study suggests that, in normal cells, loss of p89c-Myb expression is compensated by expression of the more abundant p75c-Myb isoform, it is conceivable that expression of p89c-Myb in K562 cells is reminiscent of ectopic expression or by specific downregulation modulates the predominant p75c-Myb isoform and the less-abundant p89c-Mybex9b isoform. Further studies are necessary to address whether such a mechanism is also involved in leukemia.

We show here that perturbation of p89c-Mybex9b levels by ectopic expression or by specific downregulation modulates the proliferation, survival and IM sensitivity of BCR/ABL-transformed cells. The increased proliferation, survival and IM resistance of p89c-Mybex9b ectopically expressing K562 cells is reminiscent of similar effects observed in experiments carried out with artificial degradation-resistant mutants of the c-Myb LZ-NRD. The similarity of the effects is likely to reflect the comparable increase in protein stability and transactivation ability of the artificial c-Myb mutants and the p89c-Mybex9b isoform. Of greater interest, downregulation of the p89c-Mybex9b isoform was associated with reduced proliferation and colony formation and with enhanced IM sensitivity of BCR/ABL-transformed cells comparable to that observed after transfection with the c-Myb siRNA pool, which caused the downregulation of both the predominant p75c-Myb isoform and the less-abundant p89c-Mybex9b species. An explanation for these surprising findings could be that downregulation of p89c-Mybex9b expression suppresses an autoregulatory loop, which may maintain elevated levels of the p75c-Myb isoform. However, this putative mechanism does not appear to be involved because p75c-Myb levels were not reduced in p89c-Mybex9b silenced cells.

Another possibility is that disruption of the LZ-NRD in the p89c-Mybex9b isoform prevents protein–protein interactions with p75c-Myb or p75c-Myb-interacting proteins, allowing p89c-Mybex9b to escape the negative regulation imposed by p75c-Myb and/or its interacting proteins. Consistent with this interpretation, co-immunoprecipitation assays showed that p75c-Myb interacted with p89c-Mybex9b less efficiently than it did with itself (Supplementary Figure 3).

Thus, the biological effects of c-Myb in p210BCR/ABL-transformed cells might be exerted, to a large extent, by the less-abundant but more potent p89c-Mybex9b isoform. The fact that p89c-Mybex9b is a more potent transactivator of p75c-Myb and downregulation of p89c-Mybex9b is almost as effective as the combined downregulation of p75c-Myb and p89c-Mybex9b in inducing a decrease in the expression of the c-Myb-target cyclin B1 supports this interpretation, although the entire catalogue of c-Myb-target genes should be examined.

Lastly, the preferential requirement of the p89c-Mybex9b isoform by BCR/ABL-transformed cells may depend on the regulation of a specific gene subset, which may be distinct from that regulated by p75c-Myb. This possibility is supported by the observation that restoring the expression of either the p75c-Myb or p89c-Mybex9b isoform rescued only in part the inhibition of colony formation induced by downregulation of both, consistent with non-overlapping effects of the two isoforms.

Regardless of the mechanisms involved, the observation that the effects of c-Myb in leukemic cells are, in a significant part, due to minute amounts of an alternatively spliced isoform raises new questions regarding the elusive oncogenic potential of c-Myb.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

GM performed most of the work and wrote parts of the paper. SAM performed colony assays of normal umbilical cord blood and primary CML cells. FC generated some plasmid, established K562-derivative cell lines and performed some proliferation and colony assays. RB designed p88c-Mybex9b-specific siRNAs and performed initial nucleofection experiments. VC generated some plasmids. JV established some K562-derivative cell lines. GF-A and VF helped with flow cytometry analyses and luciferase assays. ARS and SC helped in chromatin immunoprecipitation and real-time PCR analyses, GR provided reagents and critically reviewed the manuscript. TLH provided primary CML samples. BC designed experiments and wrote manuscript.

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