RED CELLS, IRON, AND ERYTHROPOIESIS

Mi2β-mediated silencing of the fetal γ-*globin* gene in adult erythroid cells

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Key Points

- Mi2β exerts a major part of its silencing effect on embryonic and fetal globin genes by positively regulating the *BCL11A* and *KLF1* genes.
- Partial depletion of Mi2β induces increased γ-*globin* gene expression in primary human erythroid cells without impairing differentiation.

An understanding of the human fetal to adult hemoglobin switch offers the potential to ameliorate β-type globin gene disorders such as sickle cell anemia and β-thalassemia through activation of the fetal γ-*globin* gene. Chromatin modifying complexes, including MBD2-NuRD and GATA-1/FOG-1/NuRD, play a role in γ-*globin* gene silencing, and Mi2β (CHD4) is a critical component of NuRD complexes. We observed that knockdown of Mi2β relieves γ-*globin* gene silencing in β-YAC transgenic murine chemical inducer of dimerization hematopoietic cells and in CD34$^+$ progenitor-derived human primary adult erythroid cells. We show that independent of MBD2-NuRD and GATA-1/FOG-1/NuRD, Mi2β binds directly to and positively regulates both the *KLF1* and *BCL11A* genes, which encode transcription factors critical for γ-*globin* gene silencing during β-type globin gene switching. Remarkably, <50% knockdown of Mi2β is sufficient to significantly induce γ-*globin* gene expression without disrupting erythroid differentiation of primary human CD34$^+$ progenitors. These results indicate that Mi2β is a potential target for therapeutic induction of fetal hemoglobin. (*Blood*. 2013;121(17):3493-3501)

Introduction

Hemoglobinopathies such as sickle cell anemia and β-thalassemia result from among the most common single gene defects worldwide. A promising approach for the treatment of these conditions is through the induction of increased fetal hemoglobin (HbF) expression. Hydroxyurea, which is currently part of the standard treatment of sickle cell anemia, causes increased expression of HbF. However, the level of HbF induced in patients is variable, and hydroxyurea is not effective in the treatment of β-thalassemia. Development of effective and potentially less toxic targeted strategies to induce HbF production will require full understanding of the molecular basis of developmental repression of the fetal γ-*globin* gene. The γ-*globin* gene is located on chromosome 11 within the β-globin gene locus, which consists of a group of 5 β-type globin genes positioned in the order in which they are expressed during development and preceded by a locus control region (5′-LCR-ε-$^{A}\gamma^{G}$γ-δ-β-3′).[1-3] During the embryonic stage of development, the ε-*globin* gene is expressed in the yolk sac, followed by expression of the γ-*globin* gene in the fetal liver during most of gestation. At birth, γ-globin expression declines as the expression of adult β-globin in bone marrow–derived erythroid cells predominates.[4,5] There are numerous *trans*-acting factors and associated complexes involved in γ-*globin* gene silencing. These include BCL11A, KLF1/EKLF, MBD2/NuRD, TR2/TR4, and GATA-1/FOG-1/NuRD.[1,3,6] Among these, KLF1 (EKLF), a member of the Krüppel family of transcription factors, is critical in the expression of many erythroid-specific genes.[7-9] KLF1/EKLF binds directly to, and positively regulates, the β-*globin* gene in adult erythroid cells. It also negatively regulates the γ-*globin* gene indirectly through its role in mediating competition between the γ- and β-globin promoters for the LCR and by binding to and positively regulating expression of BCL11A, an important γ-*globin* gene silencing factor.[10,11] Originally identified in a Genome-wide Association Study (GWAS) study,[12] BCL11A is a zinc finger transcription factor that acts as a dominant negative regulator of the embryonic to adult hemoglobin switch during murine development.[13] It binds to the locus control region and to an intergenic region located between the γ-*globin* and δ-*globin* genes.[14] Knockout of BCL11A in a humanized sickle cell transgenic mouse model greatly ameliorates the sickle cell disease phenotype.[15]

Epigenetic mechanisms, including DNA methylation and histone modifications, also play an important role in developmental globin gene silencing,[6,16-20] and inhibitors of DNA methylation induce increased HbF levels in baboons and in humans.[21-23] The MBD2/NuRD complex, which selectively binds to methylated CpG-rich DNA, has been shown to play an important role in the silencing of the human embryonic ε- and fetal γ-*globin* genes.[24-26] NuRD corepressor complexes include ⩾1 copy of each of the proteins Mi2α and -β, HDAC-1 and -2, MTA-1 and -2, RbAp46/48, and p66α and p66β.[27,28] MBD2/NuRD does not appear to interact directly with promoters of human β-type globin genes, suggesting that its silencing effects occur through an indirect pathway.[24]

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The MBD3/NuRD complex, which is distinct from MBD2/NuRD,[29] directly interacts with and regulates genes within the β-globin locus through its association with the transcription factors GATA-1 and Friend of GATA-1 (FOG-1).[30,31] In βYAC transgenic mice, the GATA-1/FOG-1/NuRD complex negatively regulates the *γ-globin* gene by binding to its distal promoter.[32] This complex is associated with positive regulation of the adult *β-globin* gene,[33] suggesting that GATA-1/FOG-1/NuRD can act as either an activator or repressor complex.

Mi2β (CHD4), the largest protein of the NuRD complex, is a chromatin organization modifier (Chd), and a member of the SNF2 family of helicases.[34] It confers the chromatin remodeling function of the NuRD complex. Mi2β is highly expressed in multiple tissues including hematopoietic stem cells and early lymphoid, myeloid, and erythroid precursors.[35] In addition to its negative regulatory role as a part of the NuRD complex, Mi2β also acts as a coactivator in $CD4^+$ lymphocytes through interactions with p300 and the E box binding protein at the CD4 gene enhancer.[36]

Herein we describe a new role for Mi2β in hemoglobin switching. We show that Mi2β has an important role in the repression of *γ-globin* gene expression in mouse hematopoietic cells containing a transgenic human β-globin locus and in adult human primary erythroid cells. Although this repression is mediated in part by known negative regulatory activities of Mi2β-containing NuRD complexes, a major part of the *γ-globin* gene silencing effect of Mi2β is through positive regulation of BCL11A and KLF1/EKLF.

Methods

Cell lines

Chemical inducer of dimerization (CID) cells were a kind gift from Dr Kenneth Peterson. These cells contain a 248-kb yeast artificial chromosome carrying sequences of the human β-globin locus extending from ~40 kb 5′ upstream of hypersensitive site 4 (HS4) to ~130 kb 3′ downstream of the *β-globin* gene. Cells were grown in Iscove modified Dulbecco medium supplemented with 10% fetal bovine serum and 2% penicillin/streptomycin. The B/B homodimerizer (AP20187; Clontech, Mountainview, CA) at a concentration of 0.1 μM and G418 at a concentration of 100 to 200 μg/mL were added in every passage.

siRNA knockdown

Cells (5×10^6) were transfected with 1 μM of siRNA (QIAGEN, Valencia, CA) via nucleofection (Lonza, Walkersville, MA) according to the vendor's protocol. RNA was isolated 72 hours after transfection by phenol-chloroform extractions (TRIzol; Life Technologies, Grand Island, NY), and gene expression was determined through quantitative polymerase chain reaction (qPCR; Taqman, Roche Diagnostics, Indianapolis, IN) by the $2^{-\Delta\Delta Ct}$ method as previously described in our laboratory[25] using primers listed in supplemental Table 1 on the *Blood* Web site. Western blots were performed as previously described.[25]

$CD34^+$ hematopoietic progenitor cell isolation

$CD34^+$ cells were isolated from 10 mL deidentified apheresis packs discarded by the Virginia Commonweath University Bone Marrow Transplant unit. The EasySep kit (StemCell Technologies, Vancouver, BC, Canada) was used for positive selection of $CD34^+$ cells according to the vendor's protocol as previously described by our laboratory.[25] Cells were maintained in growth medium consisting of StemSpan SFEM medium with 1× CC100 cytokine mix (StemCell Technologies) and 2% penicillin/streptomycin.

Lentiviral constructs and infections

shMBD2 (GGGTAAACCAGACTTGAA) and shMi2β (1: CGGTGAGA TCATCCTGTGTGATA; 2: GGACCTGAATGATGAGAAACAGA) sequences were cloned into a pRRL.H1.shRNA vector and packaged into a lentivirus through calcium phosphate transfections in 293T cells. The packaged virus was used for infection of $CD34^+$ cells; green fluorescent protein–positive cells were selected by flow cytometry on a BD FACSAria II High-Speed Cell Sorter and grown in a well-characterized erythroid differentiation medium described in the supplemental Materials.

Fluorescence activated cell sorting profiling and Giemsa staining

Human primary erythroid cells (1×10^6) were collected at day 10 of differentiation and washed in 1× phosphate-buffered saline/10% fetal bovine serum buffer and incubated with 0.06 μg of CD71 and 0.015 μg CD235a per 100-μL reaction for 20 minutes. Samples were then analyzed and sorted by flow cytometry in a BD FACSCanto II machine. Antibodies used are included in the supplemental Materials. Differentiated cells were spun in a cytocentrifuge for 10 minutes at 1000 rpm and subsequently stained with Giemsa.

Chromatin immunoprecipitation assays

CID cells (10^7) or $CD34^+$ hematopoietic progenitor cells, the latter collected at day 2 of erythroid differentiation, were crosslinked by incubation with 2.0 mM ethylene glycol-bis(succinimidyl succinate) at room temperature for 30 minutes followed by 1% formaldehyde for 10 minutes. Chromatin immunoprecipitation (ChIP) assays were then performed using the EZ-Magna ChIP kit (Millipore, Billerica, MA) per the vendor's protocol, and enrichment of immunoprecipitated sequences was assayed by qPCR using primers described in supplemental Table 1.

Results

Mi2β is an important developmental regulator of human β-type globin genes and acts partially independently from the MBD2/NuRD complex

To study the role of Mi2β in MBD2/NuRD-mediated *γ-globin* gene silencing in adult erythroid cells, gene knockdowns were carried out in CID cells. They display characteristics of adult erythroid cells and express mainly the *β-globin* gene, with minimal *γ-globin* gene expression.[37] Transient siRNA-mediated knockdown of Mi2β in CID cells resulted in a very large increase in expression of the *γ-globin* gene at 72 hours after transfection (Figure 1A). This effect is ~900-fold greater than with knockdown of MBD2 (Figure 1B), which contrasts with the equivalent induction of *γ-globin* gene expression after knockdown of Mi2β, MBD2 and p66α for 24 hours.[25] Knockdown of Mi2β also resulted in a significant increase in human embryonic *ε-globin* gene expression in CID cells (Figure 1C), although the increase is ~8- to 10-fold less than for *γ-globin* RNA. As shown in Figure 1D, simultaneous knockdown of Mi2β and MBD2 did not result in greater *γ-globin* gene expression than knockdown of Mi2β alone. Knockdown of MBD3 does not affect the expression of the *γ-globin* gene in CID cells.[25] These data suggest that Mi2β silences the *γ-globin* gene in this cell line partially independently of MBD2/NuRD and MBD3/NuRD complexes.

Although *β-globin* gene expression is also induced by 30-fold following Mi2β knockdown, this effect is relatively small compared with the effect seen for the *ε-* or *γ-globin* genes, which are induced 350- and >3000-fold, respectively, when normalized to

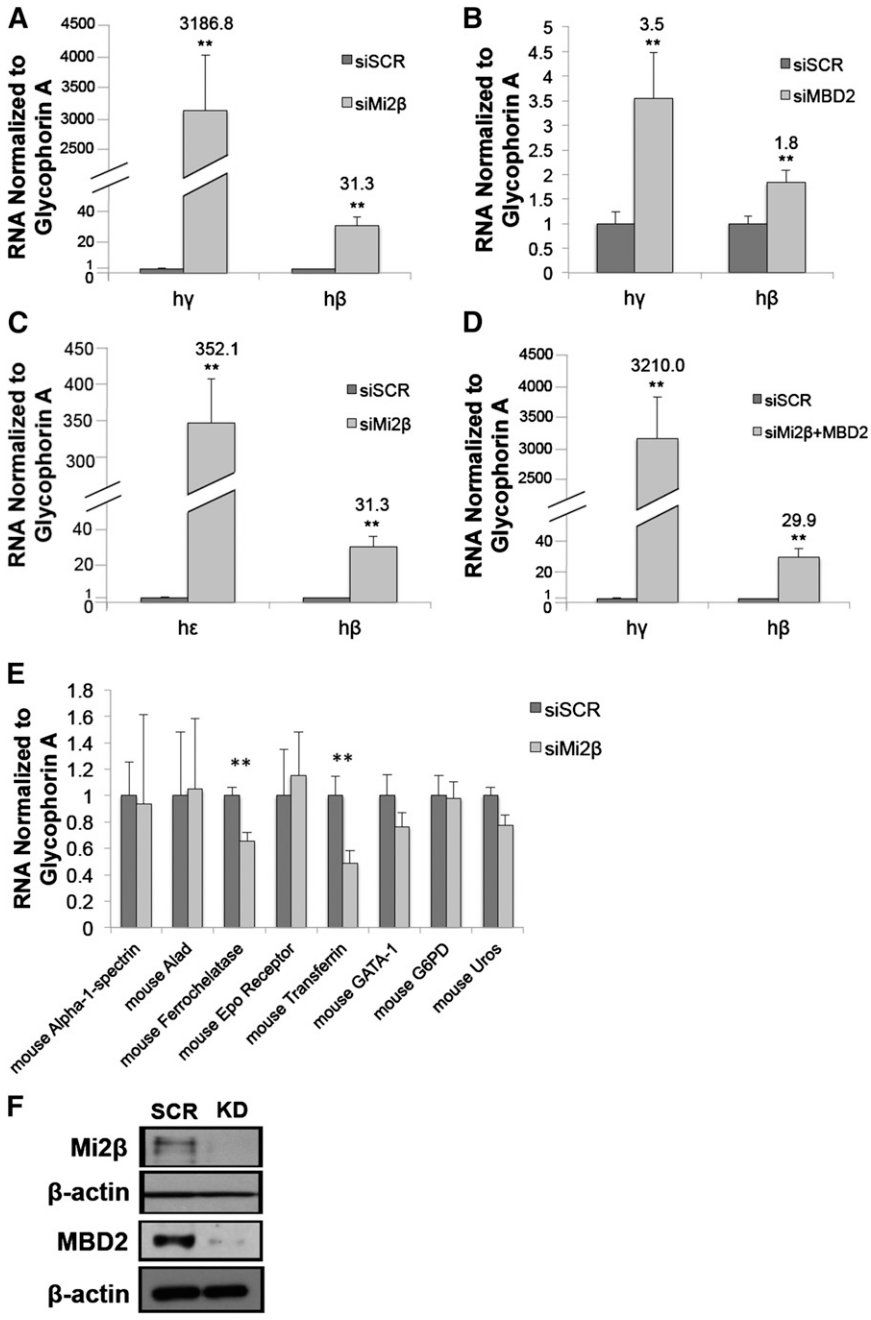

Figure 1. Mi2β preferentially regulates human β-globin locus gene expression in CID cells. CID cells were transiently transfected with siRNA for Mi2β, MBD2, or scramble control (siSCR) as indicated. (A) Transient knockdown of Mi2β by siRNA leads to a 3186.8-fold increase in the expression of *γ-globin* (hγ) gene expression in CID cells and a 31.3-fold increase in β-globin RNA (hβ) determined by qPCR. (B) Transient knockdown of MBD2 by siRNA leads to a 3.5-fold induction of *γ-globin* gene expression in CID cells and a 1.8-fold induction of β-globin RNA. (C) A 352.1-fold increase is seen in the expression of human ε-globin (hε) on knocking down Mi2β and a 31.3-fold increase in β-globin. (D) Combined knockdown of Mi2β and MBD2 leads to a 3210-fold increase in *γ-globin* gene expression and a 29.9-fold increase in β-globin RNA, similar to Mi2β knockdown alone. Data are expressed as human γ-, β-, or ε-globin RNA normalized to glycophorin A RNA. (E) qPCR analysis showing the expression of six murine genes (α-1-spectrin, aminolevulinate dehydratase [Alad], erythropoietin [Epo] receptor, GATA-1, glucose-6-phosphate dehydrogenase [G6PD], and uroporphynogen III synthase [Uros]) was not upregulated on Mi2β knockdown, whereas the mouse ferrochelatase and transferrin genes are slightly significantly down-regulated. (F) Western blot showing the degree of Mi2β and MBD2 protein knockdown in the CID cells used for the globin gene expression studies shown in Figures 1 and 2, respectively. Error bars represent the standard deviation of 3 independent experiments. $^{*}P < .05$, and $^{**}P < .02$, according to the Student *t* test.

glycophorin A expression (Figure 1A,C). To determine whether Mi2β knockdown induces expression of other erythroid-specific genes, we measured expression of the mouse *α-1 spectrin, Ferrochelatase, Epo receptor*, and *Alad* genes, as well as the *transferrin, GATA-1, G6PD*, and *Uros* genes. There was no significant increase in expression of any of these genes (Figure 1E), nor of the mouse *α-globin* gene (Figure 2C) after Mi2β knockdown. These results suggest that Mi2β knockdown preferentially induces expression of the human *γ-* and *ε-globin* genes rather than promoting further erythroid differentiation of CID cells.

Expression of the endogenous mouse *εy* and *βh1 globin* genes was measured after Mi2β knockdown. As shown in Figure 2A-B, both genes are significantly upregulated, suggesting that Mi2β plays a silencing role in both the murine and human β-globin loci. Interestingly, on Mi2β knockdown, the murine *εy* gene is derepressed to a much greater extent than βh1 (>100- vs ~3-fold), an observation similar to the relative effect on the human fetal and embryonic β-type globin genes, in which the *γ-globin* gene is up-regulated much more than the *ε-globin* gene (>3000- vs ~350-fold). These data are also consistent with the finding that the *εy* gene is activated later in murine erythroid development than βh1[38] and thus is more analogous to the human *γ-globin* gene than is *βh1*.

Because both Mi2β and its isoform Mi2α (CHD3) have been shown to be incorporated into NuRD complexes,[39-41] we examined the role of Mi2α in *γ-globin* gene silencing. The level of Mi2α RNA was found to be ~10- to 15-fold lower than Mi2β RNA by qPCR (data not shown). Knockdown of Mi2α, to the same degree as for Mi2β knockdown, had only a minor (~1.5-fold) effect on *γ-globin* gene expression in CID cells (supplemental Figure 1A-B),

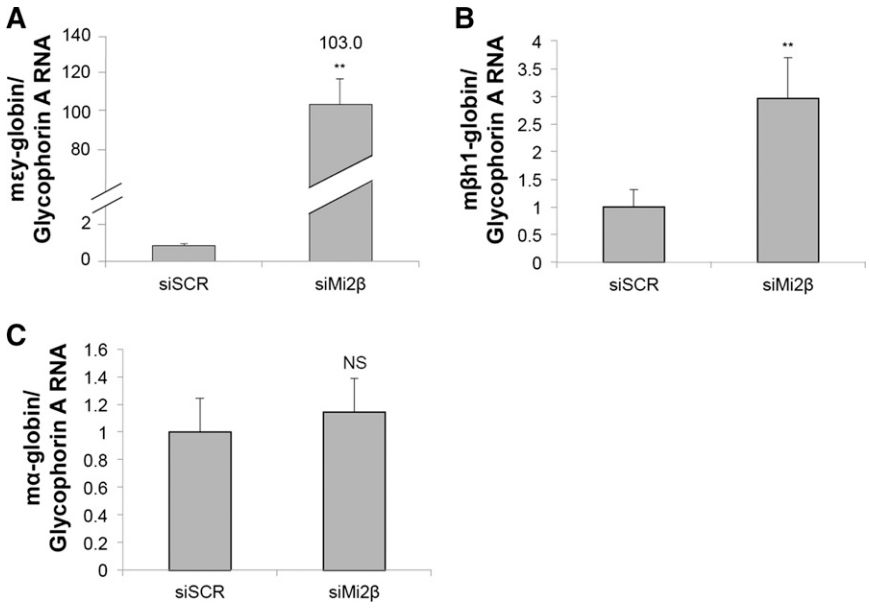

Figure 2. Mi2β regulates the expression of endogenous mouse β-type globin genes. CID cells that were transiently transfected as described in Figure 1 were then assayed for endogenous εγ-, βh1-, and α-globin RNA levels by qPCR. (A) Transient knockdown of Mi2β in CID cells leads to increased expression of the murine εγ (mεγ) gene by 103-fold normalized to glycophorin A. (B) Mi2β knockdown leads to a threefold increase in murine βh1 (mβh1) gene expression. (C) Murine α-globin (mα) RNA level is unchanged on Mi2β knockdown. These data are expressed as εγ-, βh1-, and α-globin RNA normalized to glycophorin A, a murine erythroid-specific housekeeping gene. Error bars represent the standard deviation of 3 independent experiments. $*P < .05$ and $**P < .02$ according to the Student *t* test. NS = not statistically significant.

suggesting that the great majority of *γ-globin* gene silencing requires only the Mi2β (CHD4) isoform.

On the basis of the observation that Mi2β knockdown had a larger effect on γ-globin expression than did MBD2 knockdown, we explored the possibility that loss of Mi2β, the largest component of the MBD2/NuRD complex, could lead to destabilization of the complex and reduce the levels of other components, thereby creating a larger effect than loss of an individual component. Western blot and MBD2 coprecipitation assays in cells in which Mi2β was knocked down by ~80% to 90%, showed that other components of the MBD2/NuRD complex are present in normal abundance and able to interact despite depletion of Mi2β (supplemental Figure 2).

Mi2β silences the *γ-globin* gene in human primary erythroid cells

To determine the role of Mi2β on *γ-globin* gene silencing in the context of primary adult human erythroid cells, we stably knocked down Mi2β in $CD34^+$ human hematopoietic progenitor-derived erythroid cells via lentivirus-mediated shRNA infection. Two different Mi2β shRNA constructs were tested. Both resulted in increased *γ-globin* gene expression at day 10 of erythroid differentiation, and the amount of increased γ-globin expression obtained was proportional to the degree of knockdown of Mi2β (Figure 3A). Mi2β knockdown with construct 2 resulted in a ~20-fold increase in γ/γ+β-globin mRNA expression compared with ~9-fold in the case of MBD2 knockdown (Figure 3B-C). At day 10 of differentiation, the level of *γ/γ+β-globin* gene expression is <1% in both untreated and scramble shRNA controls (supplemental Figure 3B). High-performance liquid chromatography assay in a representative experiment showed an HbF level of 13.2% of total hemoglobin when Mi2β was knocked down by ~25% to 30% (supplemental Table 2).

Because complete absence of Mi2β in the bone marrow of mice results in a block in erythroid differentiation at the proerythroblast stage,[42] we sought to determine whether a partial knockdown of this protein would interfere with erythroid differentiation. Fluorescence-activated cell sorting using the transferrin receptor (CD71) and glycophorin A (CD235a) surface markers showed that, after Mi2β knockdown, erythroid differentiation proceeds similarly to scramble shRNA controls, as shown in Figure 3D. Interestingly, as erythroid differentiation proceeds, 2 distinct populations of cells were observed by flow cytometry of both control and Mi2β knockdown cell populations. The major population was differentiated down the erythroid pathway and expressed both transferrin receptor and glycophorin A (Figure 3D, quadrant 2), whereas the minor population did not stain for either (Figure 3D, quadrant 3). When analyzed by morphology, the minor population was found to consist of normally differentiated myeloid cells (Figure 3G-I). The size of this myeloid cell population varied among different patient-specific $CD34^+$ progenitor batches, suggesting that a variable fraction of $CD34^+$ cells in each batch assayed was committed to myeloid differentiation before exposure to erythroid differentiation medium. The percentage of myeloid cells was generally higher in Mi2β knockdown samples, but this was highly variable across experiments and there was overlap with controls (Figure 3D).

The differentiated Mi2β knockdown erythroid cells in quadrant 2 were found to have ~40% Mi2β knockdown (Figure 3E) and a very high *γ/γ+β-globin* gene expression level (15-45% γ-globin RNA; Figure 3B). In contrast, the myeloid cells in quadrant 3 showed a much higher Mi2β knockdown (~90%; Figure 3F) and higher green fluorescent protein expression from the Lentiviral vector (supplemental Figure 4). Thus, the difference may reflect more Lentiviral Mi2β shRNA expression in the myeloid compartment, which could impart a slight growth advantage. Overall, these results show that partial Mi2β knockdown does not inhibit terminal erythroid differentiation. These results differ sharply from the effect of conditional knockout of Mi2β in murine hematopoietic cells in which there is a complete block in erythroid differentiation at the proerythroblast stage.[42]

Mi2β affects the levels of 2 important transcription factors involved in silencing embryonic and fetal β-type globin gene expression: BCL11A and KLF1/EKLF

To further investigate the mechanism(s) through which Mi2β exerts such a large effect on *γ-globin* gene silencing, we studied its effect on KLF1/EKLF and BCL11A, 2 important regulators of *γ-globin* gene silencing. Knockdown of Mi2β in CID cells

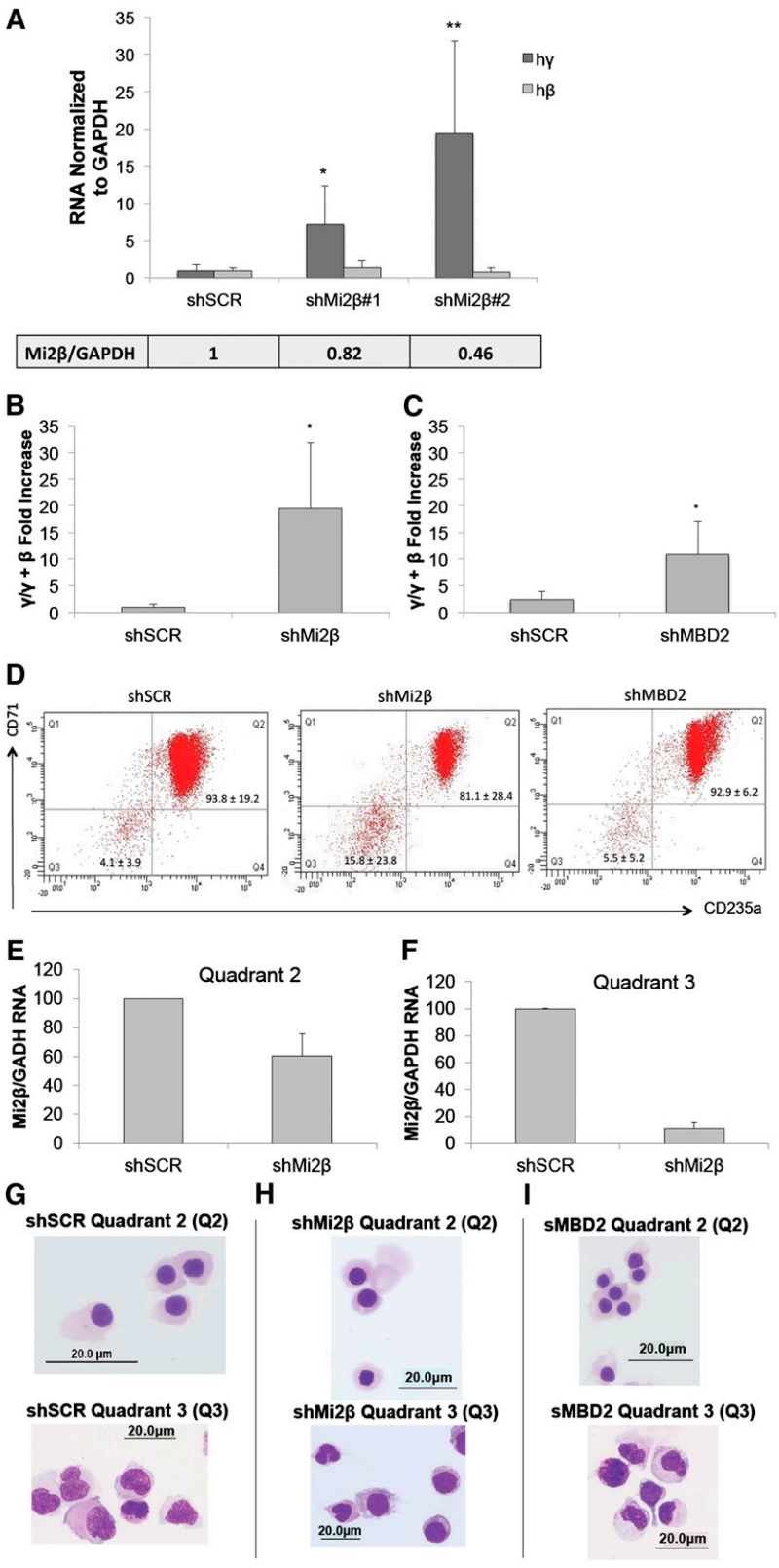

Figure 3. Mi2β regulates the expression of the *γ-globin* gene in human primary erythroid cells. CD34$^+$ human hematopoietic progenitor cells were infected with lentivirus vectors harboring shRNA either for scramble control, 2 different Mi2β constructs, or MBD2. (A) Knockdown with shMi2β 1 leads to a 7.2-fold induction of *γ-globin* gene expression determined by qPCR. Knockdown with shMi2β 2 leads to a ~20-fold increase in γ-globin expression and a slight decrease in *β-globin* gene expression. Shown below the graph are RNA levels in cells infected with scramble control or knockdown shRNA vectors. (B) Partial knockdown of Mi2β (construct 2) leads to a 20-fold increase in *γ/γ+β-globin* gene expression. (C) Knockdown of MBD2 leads to a ninefold increase in expression of *γ/γ+β-globin* gene expression. (D) Fluorescence activated cell sorting analysis showing erythroid differentiation of 81.1% of CD34$^+$ progenitor cells in which Mi2β is knocked down compared with 93.8% of scramble shRNA control cells and 92.9% of cells in which MBD2 is knocked down. Values signify standard deviations for ≥3 independent experiments. (E) qPCR analysis showing a ~40% knockdown level of Mi2β RNA in double-positive cells taken at the end of differentiation (quadrant 2). (F) qPCR analysis showing a 90% knockdown of Mi2β RNA in double-negative cells taken at the end of differentiation (quadrant 3). (G-I) Wright-Giemsa stain of scramble control, Mi2β knockdown, and MBD2 knockdown cell populations. Photomicrographs were generated using an Olympus (Center Valley, PA) BX41 compound microscope and Olympus DP71 digital camera at ×100 magnification. Images were acquired with Olympus DP Controller software. Error bars represent the standard deviation of ≥3 independent experiments. *$P < .05$ and **$P < .02$ according to the Student *t* test.

decreases both KLF1/EKLF and BCL11A protein levels as shown by western blot (Figure 4A). Consistent with the hypothesis that the silencing effect of Mi2β is at least partially independent from the MBD2/NuRD complex, loss of MBD2 does not result in any decrease of KLF1 or BCL11A in either CID cells (Figure 4B) or in primary erythroid cells of βYAC-containing MBD2 knockout mice (Figure 4C). Rather, cells with MBD2 knockdown or knockout appear to express slightly increased levels of BCL11A and KLF1 (Figure 4B-C). The same effect of Mi2β knockdown was observed in primary human erythroid cells, in which Mi2β knockdown

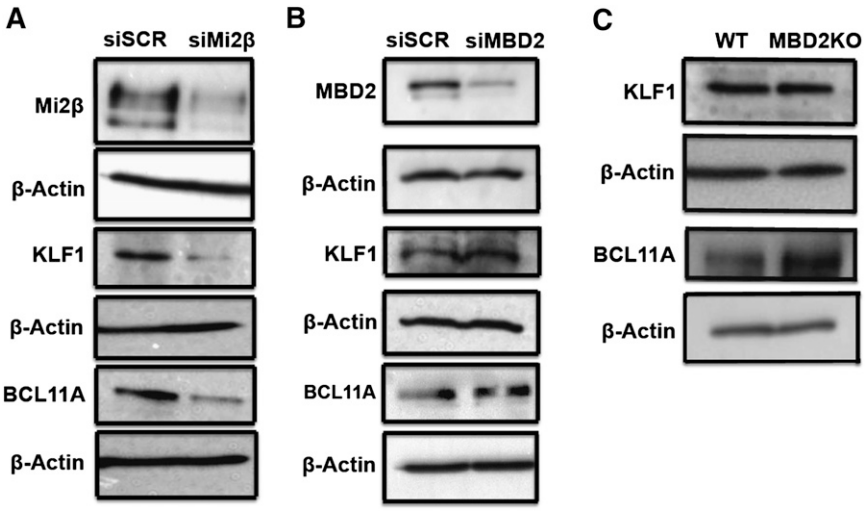

Figure 4. Mi2β positively regulates the expression of KLF1 and BCL11A in CID cells. (A) Western blot showing a decrease in murine KLF1 and murine BCL11A protein levels after Mi2β knockdown in CID cells. (B) Western blot showing no change in murine KLF1 and murine BCL11A protein levels after MBD2 knockdown in CID cells. (C) Western blot showing no change in murine KLF1 or murine BCL11A in primary adult mouse erythroblasts from MBD2 knockout mice.

downregulates both BCL11A and KLF1 mRNA and protein levels (Figure 5A,C). In CID cells, forced expression of KLF1 partially blocks the increased *γ-globin* gene expression induced by Mi2β knockdown (supplemental Figure 5). MBD2 knockdown did not decrease expression of either KLF1 or BCL11A in human primary erythroid cells (Figure 5B). These results suggest that Mi2β acts as a positive regulator of BCL11A and KLF1 in contrast to its negative regulatory role as part of the MBD2/NuRD complex.[25] To investigate whether or not Mi2β might directly activate the *BCL11A* and/or *KLF1* genes through interactions at their promoter regions, we carried out ChIP assays. As shown in Figure 5E, Mi2β is significantly enriched at the proximal promoter region of both the *BCL11A* and *KLF1* genes in primary erythroid cells. Thus, Mi2β appears to interact directly with and lead to increased expression of both the *BCL11A* and *KLF1* genes.

Mi2β affects *γ-globin* gene expression largely independently of the FOG-1/GATA-1/NuRD complex

Because GATA-1 and FOG-1 occupy the distal promoter region of the *γ-globin* gene and play a repressive role on its expression through the NuRD complex in βYAC mice,[32] we explored the extent to which Mi2β acts through the FOG-1/GATA-1/NuRD complex to exert its silencing effect on the *γ-globin* gene. ChIP

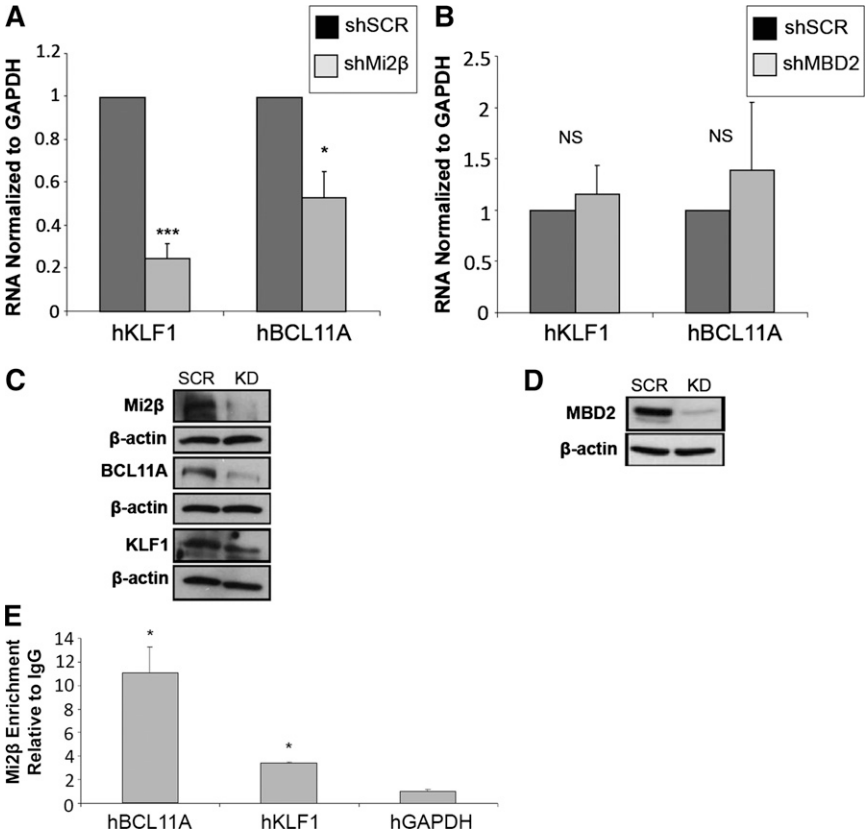

Figure 5. Mi2β positively regulates the expression of KLF1 and BCL11A in human CD34$^+$ hematopoietic progenitor-derived primary erythroid cells. (A) qPCR analysis showing mRNA levels of KLF1 and BCL11A following Mi2β knockdown are decreased by 70% and 40%, respectively. (B) mRNA levels of KLF1 and BCL11A following MBD2 knockdown are not affected. (C) Western blot showing a decrease in the levels of BCL11A and KLF1 protein after Mi2β knockdown in human primary erythroid cells. (D) Western blot showing the level of MBD2 protein knockdown in human primary erythroid cells. (E) ChIP assay showing significant enrichment of Mi2β at the BCL11A and KLF1 promoter regions. Glyceraldehyde-3-phosphate dehydrogenase was used as a negative control, and enrichment values are normalized to IgG controls. Error bars represent the standard deviation of ≥3 independent experiments. $^*P < .05$, $^{**}P < .02$, and $^{***}P < .001$ according to the Students *t* test. NS = not statistically significant.

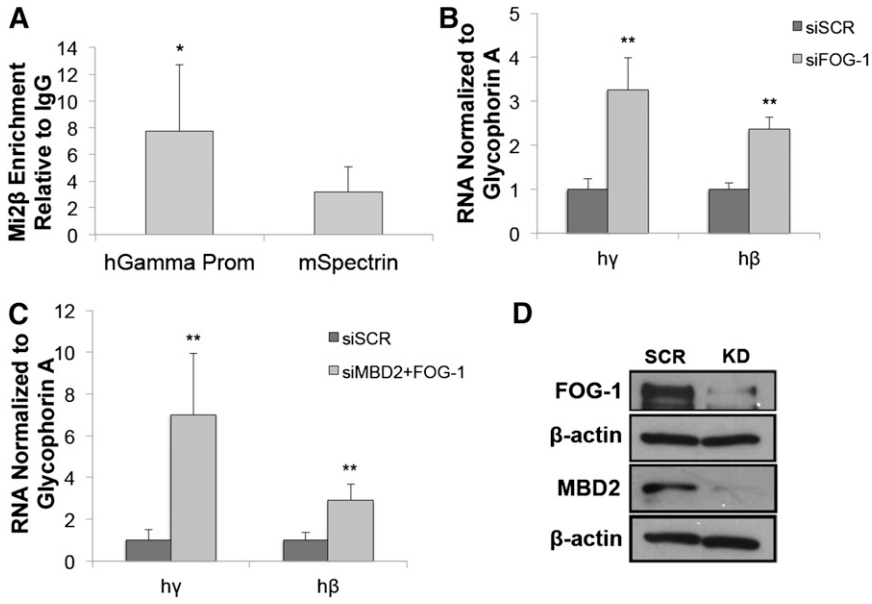

Figure 6. Mi2β occupies the γ-globin gene promoter and acts in a partially independent manner from GATA-1/FOG-1/NuRD. (A) ChIP assays showing significant Mi2β enrichment at the γ-globin promoter region in CID cells. The mouse *α-spectrin* gene was used as a negative control. (B) qPCR results showing knockdown of FOG-1 leads to a ~3-fold induction of the *γ-globin* (hγ) and an ~2.5-fold induction of the *β-globin* (hβ) gene. (C) Simultaneous knockdown of MBD2 and FOG-1 leads to an approximate sevenfold induction of γ-globin and an approximate threefold induction of β-globin. (D) Western blot showing both FOG-1 and MBD2 knockdown in CID cells. Error bars represent the standard deviation of ≥3 experiments. $^*P < .05$ and $^{**}P < .02$ according to the Student *t* test.

assays in CID cells showed that Mi2β occupies the *γ-globin* gene promoter region in CID cells as shown in Figure 6A, consistent with reported results in βYAC transgenic mice.[43] To determine the relative extent to which the silencing effect of Mi2β is mediated through the FOG-1/GATA-1/NuRD complex, FOG-1 was knocked down in CID cells. In contrast to the effect of Mi2β knockdown, this resulted in only a very small (approximately threefold) increase in *γ-globin* gene expression (compare Figure 6B to Figure 1A). This suggests that the strong silencing effect of Mi2β is mediated only in small part by the FOG-1/GATA-1/NuRD complex. To determine whether knockdown of Mi2β results in an additive or synergistic disruption of some type of cooperative effect mediated by interaction between the MBD2/NuRD and GATA-1/FOG-1/NuRD complexes, we simultaneously knocked down MBD2 and FOG-1 in CID cells. Combined knockdown of these 2 proteins resulted in an approximately sevenfold induction of γ-globin mRNA, which is much lower than with Mi2β knockdown and only slightly different than the effect of MBD2 knockdown alone[25] (compare Figure 6C to Figure 1B).

Discussion

The developmental switch from the fetal γ-globin chain to the adult β-globin chain is regulated by multiple factors including DNA methylation and transcription factors such as KLF1, BCL11A, GATA-1, TR2/TR4, and NF-E2,[1,3,6] and the MBD2/NuRD complex.[44]

As one of the major components of the MBD2/NuRD complex, Mi2β plays a critical functional role in this repression.[25] Disruption of the interaction between the MBD2 and p66α coiled coil domains results in a displacement of both p66α and Mi2β from the NuRD complex and leads to a significant derepression of the *γ-globin* gene in CID cells.[25] In this study, we found a novel role for Mi2β, which appears to be independent of its function in MBD2/NuRD and MBD3/NuRD complexes. We observed that knockdown of Mi2β has a greater effect in derepressing *γ-globin* gene expression than does knockdown of MBD2. Although the GATA-1/FOG-1/NuRD complex is not essential for silencing human γ-globin expression in transgenic βYAC bearing adult erythroid cells,[45] it contributes to the silencing of the *γ-globin* gene through a direct interaction of GATA-1 in the distal promoter region.[32] Here we show that knockdown of FOG-1 in CID cells induces a small increase in *γ-globin* gene expression in contrast to Mi2β knockdown. Further, combined MBD2 and FOG-1 knockdown results in much less *γ-globin* gene induction than Mi2β knockdown alone. Given that knockdown of MBD3 in CID cells does not lead to a significant increase in *γ-globin* gene expression,[25] a major part of the silencing effect of Mi2β appears to be independent of both the MBD2/NuRD and MBD3/NuRD complexes.

In this report we show that knockdown of Mi2β results in down-regulation of both BCL11A and KLF1/EKLF, which exert strong *γ-globin* gene silencing effects in transgenic βYAC mice and primary human erythroid cells.[10,11,13-15,46,47] The similar effect of Mi2β knockdown in both murine CID cells and human primary erythroid cells shows that Mi2β acts as an activator of the *BCL11A* and *KLF1* genes in both murine and human hematopoietic cells. As MBD2 knockdown results in slightly increased levels of BCL11A expression, these results confirm that Mi2β regulates the *BCL11A* and *KLF1/EKLF* genes independently of the MBD2/NuRD complex. Mi2β occupies the proximal promoter regions of both *BCL11A* and *KLF1*, a result consistent with a direct positive regulatory effect.

The concept of Mi2β as a gene activator is supported by previous reports for both the *CD4* gene in T cells[36] and the adult *β-globin* gene in mice.[45] We have shown previously that a partial knockdown of MBD3 in CID cells has little effect on the expression of the *γ-globin* gene.[25] Therefore, it seems unlikely that the role of Mi2β as an activator of the *BCL11A* and *KLF1/EKLF* genes is mediated through the GATA-1/FOG-1/NuRD or the MBD3/NuRD complexes.

Interestingly, in addition to a marked increase in γ-globin and ε-globin expression after Mi2β knockdown in CID cells, a small increase in β-globin RNA was observed, but expression of no other tested erythroid-specific genes was increased (Figures 1E and 2C). Together these results suggest that the silencing effect of Mi2β on erythroid-specific genes in CID cells may be restricted to the β-globin locus. In contrast to the results in CID cells, we observed a decrease in *β-globin* gene expression in primary human erythroid

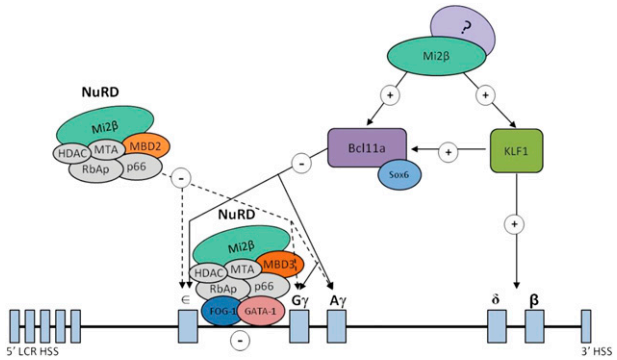

Figure 7. Working model of Mi2β-mediated developmental globin gene silencing through multiple mechanisms. Mi2β is a critical component of the MBD2/NuRD complex that regulates developmental globin gene silencing independently of BCL11A and KLF1-EKLF in an indirect manner. Mi2β binds to the distal promoter region of the *γ-globin* gene as part of the MBD3/NuRD/GATA-1/FOG-1 silencing complex. Mi2β binds to and activates expression of BCL11A and KLF1/EKLF, which in turn silence *γ-globin* gene expression. Solid arrows represent direct interactions, and dotted arrows represent indirect interactions.

cells with ~50% Mi2β knockdown, consistent with the predicted effect of decreased KLF1 expression.

On the basis of the results presented in this report in conjunction with the literature, we propose a testable working model in which Mi2β acts through multiple pathways to silence *γ-globin* gene expression, as shown in Figure 7. Mi2β is an important component of the MBD2/NuRD complex that acts as a repressor of γ-globin expression in adult erythroid cells. It also is a component of the GATA-1/FOG-1/MBD3/NuRD complex that binds upstream of the γ-globin promoter region and exerts a negative regulatory effect. A third and previously unreported function of Mi2β described here is through its positive regulation of the *KLF1* and *BCL11A* genes. In murine erythroid cells, the latter appears to be responsible for the great majority of the silencing effect of Mi2β, whereas in the primary human erythroid model, this effect is less pronounced. An intriguing observation is that knockdown of Mi2β by <50% in primary erythroid cells exerts a large effect on *γ-globin* gene expression. This suggests that full *γ-globin* gene silencing is dependent on maintaining a tightly controlled level of Mi2β.

There are a large number of potential molecular targets for therapeutically increasing fetal hemoglobin levels in patients with *β-globin* gene disorders. In this report, we focus on the chromatin remodeling complex component, Mi2β, as a potential target. Chromatin remodeling complexes could prove to be good targets for therapeutic induction of fetal hemoglobin expression, because even partial disruption of a complex component could affect expression of multiple genes that are involved in *γ-globin* gene silencing. Indeed, Mi2β appears to silence *γ-globin* gene expression through multiple pathways, and as shown in this report, its partial depletion relieves silencing in human erythroid cells. Complete depletion of Mi2β might have catastrophic consequences through blocking erythroid differentiation, as shown in conditional knockout mice.[42] However, in a very recent report, partial deficiency of Mi2β in erythroid cells of βYAC transgenic mice resulted in increased *γ-globin* gene expression without adversely affecting erythropoiesis,[48] consistent with our results in primary human erythroid cells. As Mi2β exerts its chromatin remodeling and helicase functions through its ATPase enzymatic activity, it potentially could be targeted by therapeutic agents.

In summary, identifying specific epigenetic mechanisms of *γ-globin* gene silencing as potential therapeutic targets seems promising. Indeed DNA methylation inhibitors and histone deacetylase inhibitors induce embryonic/fetal globin gene expression and have shown clinical efficacy.[21,22,49,50] We extended previous work that identified the chromatin remodeling protein, Mi2β, as an important factor in developmental β-type globin gene silencing through its role in NuRD complexes. Importantly, we show here that a major mechanism for its silencing effect is through a direct positive regulation of KLF1/EKLF and BCL11A. The striking finding that as little as 50% reduction in Mi2β expression results in a large increase in *γ-globin* gene expression in primary human erythroid cells suggests that it may serve as a useful molecular target for therapeutic induction of HbF in patients with *β-globin* gene disorders.

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Authorship

Contribution: M.A. and G.D.G. planned the experiments; M.A., M.D., M.N.G., S.Z.W., and S.Z.Z. executed the experiments; M.A., M.D., M.N.G., S.Z.W., D.C.W., and G.D.G. analyzed the experiments; and M.A. and G.D.G. wrote the paper.

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