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The Transcription Factor KLF11 Can Induce γ-Globin Gene Expression in the Setting of In Vivo Adult Erythropoiesis

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Abstract Previous studies in a fetal erythroid cell line demonstrated that the transcription factor, Krüppel-like factor 11 (KLF11), could specifically induce transcription from a γ-globin gene promoter, and that this induction was mediated through a specific canonical CACCC *cis*-DNA binding motif. We report here that ectopic expression of KLF11 can also induce fetal γ-globin gene expression in the setting of adult erythropoiesis both in vitro and in vivo. Studies in an adult-stage murine erythroleukemia (MEL) cell line demonstrated that retrovirus vector-mediated transduction of KLF11 could increase both the amount of expression from a basally active, but not from a overtly silenced, recombinant γ-globin transgene, as well as the frequency of cells expressing this transgene. A similar pattern of γ-globin gene induction was also observed both in vitro and in vivo following KLF11 transduction of bone marrow from mice containing a basally active γ-globin transgene. These studies provide the first evidence that ectopic expression of a transcription factor can induce γ-globin gene expression in vivo during adult erythropoiesis. J. Cell. Biochem. 100: 1045–1055, 2007.

Key words: transcription factor; KLF11; gamma globin; murine model; gene therapy

It has long been recognized that patients with sickle cell disease or β-thalassemia can benefit from elevated levels of fetal hemoglobin (HbF) [Bunn, 2001; Weatherall, 2001]. In the case of sickle cell disease, HbF interferes directly with the polymerization of sickle hemoglobin and can render patients asymptomatic at relatively modest levels [Bunn, 2001]. In the case of β-thalassemia, the synthesis of γ-globin chains compensates for the decreased level of β-globin chains and reduces the excess of α-globin chains that is responsible for several pathological consequences of the disease [Loukopoulos and Fessas, 1965; Sofroniadou et al., 1975]. As recently reviewed [Stamatoyannopoulos, 2005], a large effort has been made to identify the *cis*-acting sequences and *trans*-acting factors that participate in the regulation of γ-globin gene expression throughout development and differentiation, with the goal of identifying potential targets for the therapeutic induction of γ-globin gene expression in adults suffering from the β-chain hemoglobinopathies.

At least five separate transcription factors have been proposed to play key roles in γ-globin gene regulation: GATA-binding transcription factor-2 (GATA-2), nuclear factor-erythroid 4 (NF-E4), fetal globin increasing factor (FGIF), Krüppel-like factor 13 (KLF13, also known as fetal Krüppel-like factor 2), and KLF11 [Persons et al., 1999; Asano et al., 2000; Ikonomi et al., 2000; Yang et al., 2001; Zhou et al., 2004; Blau et al., 2005; Zhang et al., 2005]. Expression levels for all of these factors are elevated in fetal erythroid tissues and decline during the switch to adult erythropoiesis. They have also been shown to increase the activity of γ-globin gene promoters in fetal erythroid cell line models, and, with the exception of FGIF, to bind either directly or indirectly to specific target sequences within γ-globin gene promoters. Initial studies

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with GATA-2 were carried out in the human fetal erythroid cell line K562 and demonstrated that elevated levels of GATA-2 can increase γ-globin gene expression [Ikonomi et al., 2000]. However, other studies with retrovirus vector-mediated transduction in mouse bone marrow demonstrated that ectopic over-expression of GATA-2 blocks normal hematopoietic development, indicating that this transcription factor has pleiotropic effects well beyond its role in γ-globin gene regulation [Persons et al., 1999]. Studies in transgenic mice demonstrated that ectopic NF-E4 over-expression is capable of increasing γ-globin gene expression from a β-globin yeast artificial chromosome (YAC) within fetal liver without other significant pleiotropic effects [Zhou et al., 2004]. However, in this setting ectopic NF-E4 over-expression was proven incapable of inducing γ-globin gene expression in adult transgenic animals. Ectopic over-expression of FGIF has been demonstrated to increase γ-globin gene expression and delay the switch from a fetal to adult pattern of globin gene expression in hybrid cell lines generated by fusing human fetal erythrocytes and an adult-stage murine erythroleukemia (MEL) cell line [Yang et al., 2001]. Over-expression of FGIF has also been shown to activate γ-globin gene expression in long-term cultures of immortalized mouse bone marrow cells containing a β-globin YAC [Blau et al., 2005]. However, the degree to which this model recapitulates normal erythropoiesis, as well as the level of γ-globin induction obtained in this model, remains unclear. The γ-globin inducing activity of KLF13 has only been assessed in the primitive erythroid cell line K562 [Asano et al., 2000; Zhang et al., 2005]. To date, none of these transcription factors have been shown to activate γ-globin gene expression in the setting of in vivo adult erythropoiesis.

The transcription factor KLF11 was originally identified as fetal Krüppel-like factor 1 in a screen for transcription factors from human fetal liver cells expressing fetal globin genes [Asano et al., 1999]. This screen involved cloning factors which bound the essential, evolutionarily conserved CACCC box in the γ-globin gene promoter. Transient transfection studies in the human fetal erythroid cell line K562 showed that KLF11 preferentially up-regulated transcription from γ-globin and ε-globin gene promoters, and that it was not a general transcriptional activator for several other genes containing the CACCC motif in their promoters. This initial characterization also indicated that KLF11 expression was mainly restricted to fetal liver cells, although subsequent studies showed that it is also expressed at basal levels throughout the body, with higher levels seen in selected hematopoietic tissues, fetal brain, pancreas, and testes. The ability of KLF11 to induce γ-globin gene expression was later confirmed in the long-term mouse bone marrow culture model described above for FGIF [Blau et al., 2005]. Targeted KLF11 knockout studies failed to reveal an obvious phenotype [Song et al., 2005].

These initial studies left unresolved several important questions, including whether KLF11 is able to induce γ-globin gene expression in the setting of adult erythropoiesis, and whether KLF11 plays other roles in primary hematopoiesis.

In order to address these questions, KLF11 cDNA was introduced into a recombinant retrovirus expression vector, and this vector was tested for the ability to induce the expression of γ-globin transgenes and for other pleiotropic effects (such as effects on cell growth or differentiation) in a combination of adult-stage MEL cell lines and mouse bone marrow culture and bone marrow transplantation models. This experimental approach of expressing a candidate transcription factor ectopically and assessing the effects on target gene expression has been used extensively as a tool in biomedical research, including studies into the regulation of globin gene expression [e.g., see Li et al., 1997]. These studies showed that KLF11 induces γ-globin gene expression in adult erythropoiesis both in vitro and in vivo, but only when the γ-globin transgene was basally active. They also suggest that KLF11 may also play other roles in hematopoietic cell development and differentiation.

MATERIALS AND METHODS

Cell Lines

All cell lines were derived using the adult-stage MEL cell line MEL585 [Enver et al., 1991], and were maintained at 37°C and 7.5% CO_2 in Dulbecco's Modified Eagle's media supplemented with 10% heat-inactivated characterized fetal bovine serum. One cell line was stably transfected with a YAC containing 144 kb from the human β-globin locus, including all of the human β-globin like genes (embryonic

ε-globin, fetal γ-globin, and adult β-globin), as well as native regulatory sequences from the β-globin locus control region (LCR) [Vassilopoulos et al., 1999]. The other cell line was stably transfected with an expression cassette for human $^{A}\gamma$-globin with a −159 promoter linked to truncated portions of the LCR (termed a μLCR) similar to that previously described [Skarpidi et al., 1998]. MEL cells were induced to differentiate by culture in 3 mM *N*,*N*^{1}-hexamethylene bisacetamide (Aldrich) and 10 μM hemin (Sigma) for 4 days as previously described [Enver et al., 1991].

Retrovirus Vectors

As shown in Figure 1, the KLF11 vector was generated by isolating a 1.5 kb *Eco*RI-*Not*I fragment containing the KLF11 cDNA from the plasmid pSG5DD/FKLF-1 [Asano et al., 1999], and inserting this fragment into the *Eco*RI-*Not*I site of the gammaretrovirus vector pMX-IRES-GFP [Nosaka et al., 1999]. The previously described green-fluorescence protein (GFP) reporter vector MGIN was used as a mock control [Cheng et al., 1997]. Vector producer lines were generated by transduction of the ecotropic packaging line GP + E86 [Markowitz et al., 1988], and isolation of clones by fluorescence-activated cell sorting (FACS). Virus titers were determined by serial dilution and transfer of GFP expression to naive NIH3T3 cells. Producer clones were tested for the absence of replication-competent virus using a marker-rescue assay [Miller and Rosman, 1989]. Southern blot analysis of producer and transduced cells was performed using standard techniques [Sambrook et al., 1989].

MEL Cell Transductions

The MEL cell lines were transduced by 24 h culture in filtered virus-containing supernatant and 8 μg/ml hexadimethrine bromide (polybrene). The cells were then washed, expanded for a few days, and GFP(+) cells were isolated by sorting on a FACS Vantage (Beckton Dickinson, San Jose, CA). Individual clones were subsequently isolated by limiting dilution.

Fig. 1. KLF11 vector design. The KLF11 cDNA was inserted upstream of the internal ribosomal entry site (IRES) and enhanced green-fluorescence protein (GFP) reporter gene in the gammaretrovirus vector pMX-IRES-EGFP [Nosaka et al., 1999]. This places the bi-cistronic KLF11/GFP cassette under the transcriptional control of the 5′ viral long terminal repeat (LTR). Arrow, start of transcription; sd and sa, splice donor and acceptor sites; pA, poly-adenylation signal.

Mouse Bone Marrow Transductions

The transgenic mouse line 1279 used for these studies has been previously described [Constantoulakis et al., 1991]. This line contains three copies in *cis* of a transgene consisting of a 2.5 kb μLCR linked to a coding cassette for human $^{A}\gamma$-globin including a −1,348 bp promoter. Bone marrow cells were collected from 5-FU treated donors and transduced by 2-day preinduction and 2-day co-cultivation on irradiated producer cells in a cytokine cocktail as previously described [Emery et al., 2002]. For erythroid progenitor colony assays, transduced cells were plated at 4,000 cells/ml in complete murine progenitor methylcellulose culture medium containing Epo (StemGenix, Amherst, NY). For bone marrow transplantation studies, cells were transplanted into irradiated (1025 cGy) syngeneic recipients at a dose of 5–10×10^5 cells per animal. Blood cell analysis was performed on a Cell-DYN 3500 hematology analyzer (Abbott Diagnostics, Santa Clara, CA).

Immunofluorescent Staining and Flow Cytometry Analysis

As previously described [Emery et al., 2002, 2005], 1×10^6 induced MEL cells, pooled cells from erythroid progenitor colonies, or 3 μl peripheral blood, were treated for 30 min in HBSS with 4% formaldehyde in order to fix the intracellular globin and GFP proteins, permeablized by serial washes in cold acetone to allow intracellular access, and stained with a mAb specific for human γ-globin conjugated to the red fluorochrome phycoerythrin (BD Bioscience, San Jose, CA). The cells were again washed and analyzed by two-color flow cytometry on a FACScan flow cytometer (BD Bioscience) using CellQuest software. The fraction of cells expressing γ-globin, GFP, or both, was determined by measuring the fraction of cells expressing the red or green fluorescence, respectively, above background. The relative level of expression for γ-globin, GFP, or both, was likewise determined by measuring the intensity of red or green fluorescence. An example of this analysis is presented in Figure 5.

RESULTS

Retrovirus Vector for KLF11

In order to facilitate the assessment of ectopic KLF11 expression in a variety of settings, we transferred the KLF11 cDNA into the retrovirus vector pMX-IRES-GFP [Nosaka et al., 1999]. As shown in Figure 1, the KLF11 cDNA was placed under the transcriptional control of the vector LTR promoter, and was linked to a bi-cistronic expression cassette for GFP. This configuration allows for a ubiquitous pattern of expression, and the ability to identify cells expressing vector-derived KLF11 by the co-expression of GFP. Titer analysis showed this vector can be generated from a stable ecotropic producer line at about 10^6 infectious units per milliliter. Southern analysis demonstrated that this vector is genetically stable (data not shown). No deficits were observed in the rate of growth following transduction of the packing line or NIH3T3 cells used in the titer assays.

KLF11 Transduction Induces Expression of Basally Active But Not Overtly Silenced γ-Globin Transgenes in MEL Cells

The ability of KLF11 transduction to induce γ-globin gene expression in the setting of adult erythropoiesis was first assessed in MEL cell lines containing either an intact β-globin YAC or a recombinant ^{A}γ-globin expression cassette. The intact β-globin YAC was chosen because the γ-globin genes are transcriptionally silent in adult transgenic mice [Peterson et al., 1993], and in transfected MEL cells [Vassilopoulos et al., 1999]. The recombinant ^{A}γ-globin expression cassette, which includes a truncated (−159 bp) promoter and composite μLCR regulatory element, was chosen because it is expressed at a low but nearly pan-cellular level [Skarpidi et al., 1998]. As such, this construct provided an opportunity to assess the ability of KLF11 to induce the expression of a γ-globin gene that is already partially active, a situation often observed in a small but distinct fraction of developing erythrocytes during normal human adult erythropoiesis.

In the first series of studies, MEL cells containing the intact human β-globin YAC were transduced with the KLF11 vector, GFP(+) transduced cells were isolated by FACS, and a total of seven independent clones were isolated by limiting dilution in the absence of further selection. These clones were then analyzed for the expression of both GFP (as a surrogate for KLF11) and γ-globin by immunofluorescent staining (for hybrid human/mouse HbF) and two-color flow cytometry. The GFP cassette was expressed in $98.6 \pm 1.1\%$ of cells at an average level of 782 ± 455 mean fluorescence units (mfu). As shown in Figure 2A, KLF11 transduction resulted in a small, dose-dependent increase in the fraction of cells expressing γ-globin, ranging from 0.2% to 2.1% ($R^2 = 0.77$, $P = 0.001$). However, this range was not significantly different than the level measured for the parental cell line (0.9%) or the cells transduced with the GFP-only control vector (0.4%). As shown in Figure 2B, KLF11 transduction also had no effect on the level of γ-globin expression within γ-positive cells. In this case, the level of γ-globin expression, as measured by the intensity of the immunofluorescent staining of the few γ-globin positive cells, averaged 39.3 ± 8.1 mfu for the KLF11-transduced clones, versus 37.5 mfu for the parental cell line and 27.9 mfu for the GFP-only control. Moreover, there were no obvious deficits in the rate of growth for the transduced clones observed during routine passaging.

In contrast to the results with the full-length β-globin YAC transgene, a pattern of γ-globin

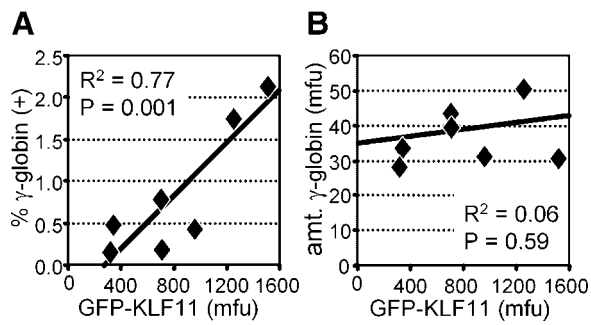

Fig. 2. Effects of KLF11 transduction in MEL cells containing a intact human β-globin YAC. MEL cells containing a β-globin YAC in which the γ-globin genes are predominantly silent were transduced with the KLF11 vector, and seven transduced clones were isolated by FACSorting for GFP and limiting dilution cloning. These clones were induced with HMBA and hemin, fixed and stained with a phycoerythrin-conjugated mAb for HbF, and analyzed by two-color flow cytometry for co-expression of GFP and γ-globin. **A**: Comparison between the amount of GFP expression in mean fluorescence units (mfu) and the frequency of cells expressing γ-globin. **B**: Comparison between the amount of GFP expression and the amount of γ-globin expression (in mfu) within the cells expressing γ-globin. The coefficient of determination (R^2) and resulting *P*-value determined using the F statistic are presented for each comparison.

gene induction was observed following KLF11 transduction of the MEL cells containing the basally active recombinant $^{A}\gamma$-globin coding cassette. In this case, a total of 12 independent transduced clones were generated and analyzed as described above. In this series, the GFP cassette was expressed in $74.4 \pm 33.5\%$ of cells at an average level of 203 ± 195 mfu. As shown in Figure 3A, there was modest evidence that KLF11 transduction increased the fraction of cells expressing γ-globin, which averaged $80.0 \pm 17.0\%$ for all 12 clones ($R^2 = 0.21$, $P = 0.014$). The lack of a more significant increase presumably reflects in part the fact that this recombinant γ-globin transgene is already expressed in 83.6% of cells in the parental cell line. However, as seen in Figure 3B, KLF11 transduction did result in a significant, dose-dependent increase in the level of γ-globin expression within γ-positive cells, ranging from an average 40.3 ± 14.4 mfu for cells expressing GFP at <250 mfu to 87.5 ± 24.0 mfu for cells expressing GFP at >250 mfu ($R^2 = 0.41$, $P = 0.025$). The level of γ-globin expression in this GFP-high subset was significantly greater than the 45.6 mfu level of γ-globin expression seen in the parental cell line ($P < 0.001$). Again, there were no obvious deficits in the rate of growth for the transduced clones observed during routine passaging.

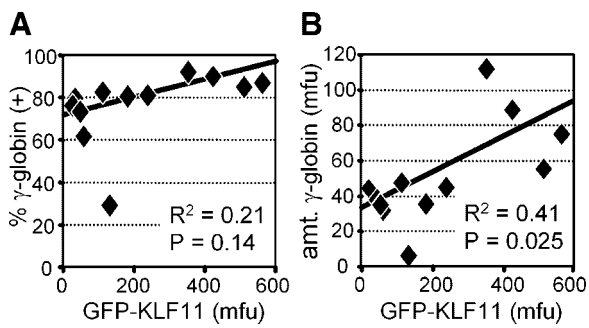

Fig. 3. Effects of KLF11 transduction in MEL cells containing a basally active recombinant γ-globin cassette. MEL cells containing a recombinant μLCR/$^{A}\gamma$-globin cassette expressed at a basal level were transduced with the KLF11 vector, and 12 transduced clones were isolated by FACSorting for GFP and limiting dilution cloning. These clones were then induced, stained for HbF, and analyzed as described in the legend of Figure 2. **A**: Comparison between the amount of GFP expression and the frequency of cells expressing γ-globin. **B**: Comparison between the amount of GFP expression and the amount of γ-globin expression within the cells expressing γ-globin. The coefficient of determination (R^2) and resulting *P*-value determined using the F statistic are presented for each comparison.

KLF11 Transduction Induces Expression of a Basally Active γ-globin Transgene in Primary Mouse Erythroid Progenitor Colonies

As a first approach toward assessing the ability of KLF11 to induce γ-globin gene expression in primary cells, we transduced bone marrow cells from mice containing a γ-globin transgene and analyzed vector GFP and γ-globin gene expression in erythroid progenitor cultures. In this case the transgene consisted of a recombinant $^{A}\gamma$-globin coding cassette containing an extended (-1348 bp) promoter and linked to a composite μLCR regulatory element [Constantoulakis et al., 1991]. This particular transgene was chosen because it contains all of the critical *cis*-acting elements involved in γ-globin gene regulation, including a putative stage-specific silencer, and is expressed in a low, heterocellular pattern similar to that often seen in patients with severe forms of the β-chain hemoglobinopathies, patients treated with fetal globin inducing agents such as hydroxyurea, and individuals with elevated levels of fetal hemoglobin due to specific genetic backgrounds.

In order to determine the effect of KLF11 transduction on the frequency of γ-globin transgene expression, we compared the frequency of HbF(+) cells in the GFP-negative (untransduced) and GFP-positive (transduce) cells within pools of erythroid colonies, and normalized this ratio to the ratio observed in the sample transduced with the GFP-only control vector. A similar approach was used to determine the effect of KLF11 transduction on the amount of γ-globin gene expression. In this case, we compared the amount of γ-globin expression, based on the intensity of immunofluorescent staining, in the GFP-positive (transduce) and GFP-negative (untransduced) cells within pools of erythroid colonies, and normalized this ratio to the ratio observed in the sample transduced with the GFP-only control vector. By comparing expression levels for transduced and untransduced cells within individual cultures, this approach allowed us to control for the wide degree of inter-culture variation in the frequency and amount of γ-globin gene expression often observed with this particular, heterocellularly expressed γ-globin transgene. Using this approach, we determined that transduction with KLF11 resulted in a 80% increase in the fraction of cells expressing the γ-globin transgene, and a 65% increase in the amount of

expression from the γ-globin transgene, compared to the samples transduced with the GFP-only control vector (Fig. 4). Moreover, ectopic KLF11 expression had no obvious effects on erythroid progenitor colony size or morphology compared to the mock and GFP-only vector controls. Taken together, these results demonstrate that ectopic KLF11 expression can induce both the frequency and the amount of γ-globin transgene expression in primary erythroid cells without major non-specific pleiotropic effects.

KLF11 Transduction Induces Expression of a Basally Active γ-Globin Transgene Following Mouse Bone Marrow Transduction and Transplantation

As a pilot study to confirm the results observed in vitro, bone marrow cells from the γ-globin transgenic line described above were transduced with either the KLF11 vector or the GFP-only control vector, and then transplanted into two congenic recipients each. At 1 month post-transplant, peripheral red blood cells (RBC) from these recipients were analyzed for GFP and γ-globin expression by two-color flow cytometry. The expression patterns for the recipients, as well as from a normal C57BL/6 control and a non-transduced transgenic control, are shown in Figure 5 and summarized in Figure 6. As seen in Figure 5, the frequency of vector transfer was very comparable in both groups, ranging from 34% to 35% for the GFP-only control group and 41% to 42% for the KLF11 vector group. The overall amount of vector GFP expression was also comparable in both groups, ranging from 232 to 348 mfu for the GFP-only control group and 276 to 487 mfu for the KLF11 vector group. The frequency and amount of γ-globin transgene expression in the GFP-positive (transduced) and GFP-negative (untransduced) was again analyzed using the ratio within individual animals to control for inter-animal variation, and normalized to the average of the GFP-only control vector. As seen in Figure 6A, KLF11 transduction resulted in a small but statistically significant 9–11% increase in the fraction of RBC expressing the γ-globin transgene compared to the GFP-only control vector. As seen in Figure 6B, KLF11 transduction also resulted in a small but statistically significant 12–20% increase in the amount of expression from the γ-globin transgene compared to the GFP-only control vector.

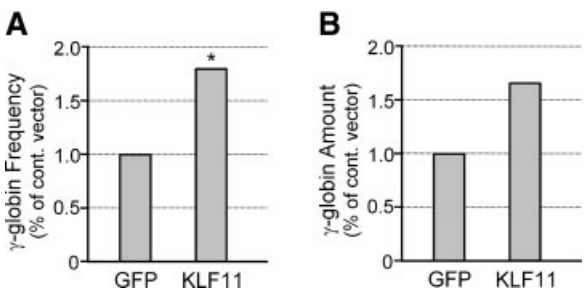

Fig. 4. Effects of KLF11 transduction in the progeny of primary erythroid progenitor cells containing a recombinant γ-globin cassette. Bone marrow cells from mice containing a recombinant μLCR/^{A}γ-globin transgene expressed at a basal level were transduced with the KLF11 vector and plated in erythroid progenitor colony cultures. Controls included mock-transduced cells or cells transduced with a GFP-only vector. Pools of approximately 20 erythroid burst-forming unit (BFU-E) colonies were collected, stained for HbF, and analyzed as described in the legend of Figure 2. **A**: The frequency of cells expressing the γ-globin transgene as a result of KLF11 transduction was determined by dividing the fraction of cells expressing γ-globin in the GFP(+) (transduced) population of cells by the fraction of cells expressing γ-globin in the GFP(−) (untransduced) population of cells, and normalizing this ratio to the ratio obtained with the GFP-only control vector. **B**: The amount of expression from the γ-globin transgene as a result of KLF11 transduction was determined by dividing the amount of immunofluorescent staining for γ-globin (in mfu) in the GFP(+) (transduced) population of cells by the amount of immunofluorescent staining for γ-globin in the GFP(−) (untransduced) population of cells, and again normalizing this ratio by the ratio obtained with the GFP-only control vector. *$P < 0.001$ versus GFP-only transduced sample by one-sided Z-test for two proportions.

KLF11 Transduction Has Modest Pleiotropic Effects on Adult Hematopoiesis

The fact that the mice transplanted with the KLF11-transduced marrow exhibited a high frequency of RBC expressing GFP, and by supposition KLF11, indicates that KLF11 expression does not interfere with HSC engraftment. However, further analysis did reveal some subtle effects of KLF11 transduction on several hematological parameters. As seen in Table I, mice transplanted with KLF11-transduced cells exhibited a 15% decrease in total hemoglobin, a 12% decrease in hematocrit, a 17% decrease in RBC, a 42% decrease in platelets, and a 179% increase in total white blood cells. This increase in WBC was due almost solely to a near doubling of lymphocytes, as well as 160% increase in basophiles. These differences point to a modest influence of ectopic KLF11 expression on hematopoietic cell fate and/or growth decisions in vivo.

DISCUSSION

In the studies presented here, we demonstrate that KLF11 can be stably transferred by retrovirus-mediated gene transfer, and that retrovirus vector-mediated KLF11 transduction can induce γ-globin gene expression in MEL cells, primary erythroid cultures, and mice transplanted with transduced marrow. As such, these studies provide the first evidence that KLF11, and indeed any transcription factor, can activate γ-globin gene expression in the setting of adult erythropoiesis. We also demonstrate that KLF11 transduction results in a modest increase in the fraction of cells expressing γ-globin in primary erythroid cultures and mice transplanted with transduced marrow. However, these activities were only observed in settings where the target γ-globin cassette was already expressed at a basal level. In contrast, KLF11 transduction had little if any effect on γ-globin gene expression in MEL cells containing a human β-globin YAC in which the γ-globin gene was predominantly silent. These results suggest that KLF11 can exert its effect on γ-globin gene expression only when the γ-globin gene chromatin is in an open configuration. Although we did not test the effects of KLF11 transduction on expression of the endogenous mouse globin genes, previous studies found that the effects of KLF11 are greatest on human γ-globin, and that other erythroid promoters containing the CACCC element are activated minimally, if at all, by KLF11 [Asano et al., 1999]. In addition, knocking out KLF11 expression in mice has no discernable effect on mouse globin gene expression [Song et al., 2005].

The results reported here for KLF11 are reminiscent of the findings reported by Zhou et al. [2000, 2004] for the transcription factor NF-E4 in gene transfer and transgenic mouse models. In particular, both series of studies revealed a specific ability to induce expression from γ-globin genes that are basally active, but the inability to activate or maintain expression

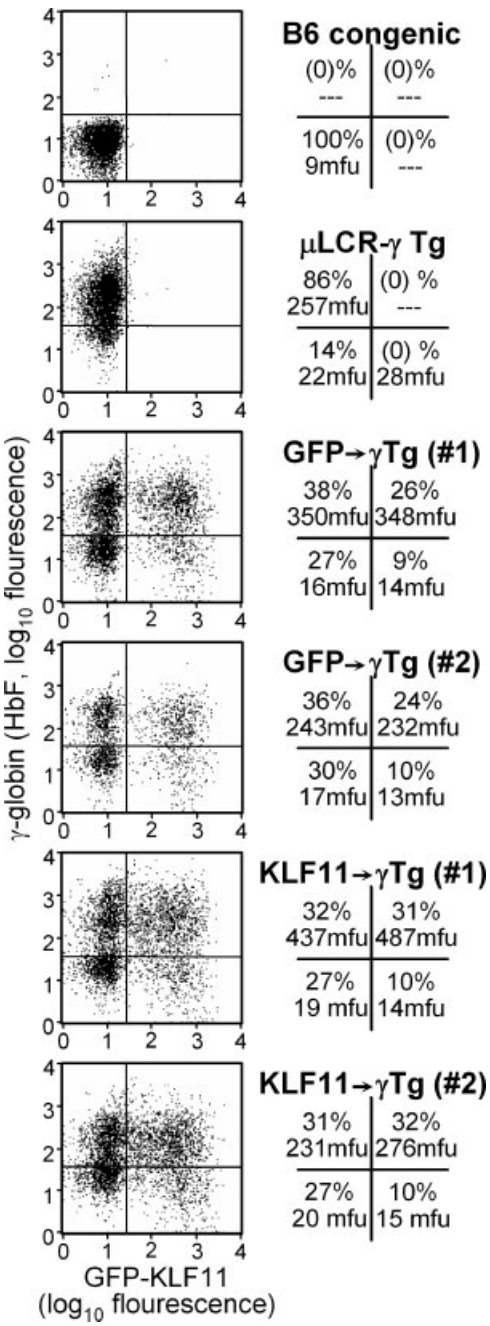

Fig. 5. Flow cytometric analysis of RBC from mice transplanted with KLF11-transduced bone marrow cells. Bone marrow cells from mice containing a recombinant μLCR/^{A}γ-globin transgene expressed at a basal level were transduced with the KLF11 vector and transplanted into two syngeneic recipients (labeled KLF11 → γTg #1 and #2). Controls included two animals transplanted with transgenic cells transduced with the GFP-only control vector (labeled GFP → γTg #1 and #2), a normal transgenic animal (labeled μLCR-γ Tg), and a normal non-transgenic C57BL/6 animal (labeled B6 congenic). At 1 month post-transplant, RBC were collected, fixed, and stained with a phycoerythrin-conjugated mAb for HbF, and analyzed by two-color flow cytometry for co-expression of GFP and γ-globin. Scatter plots from the analysis of individual animals are shown to the left, with the log fluorescence for GFP on the x-axis and the log fluorescence for γ-globin (in the form of Ab staining for hybrid mouse/human HbF) on the y-axis. To the right is shown the relative frequency of cells within the indicated quadrants, as well as the relative amount of γ-globin (in mfu) for the cells within these quadrants.

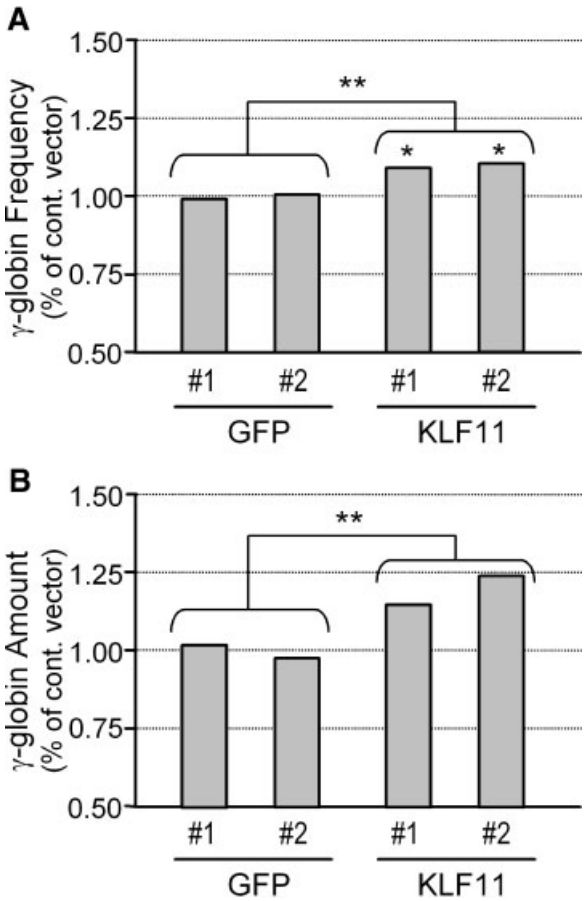

Fig. 6. Effects of KLF11 transduction in bone marrow of mice containing a basally active recombinant γ-globin cassette. **A**: Summary of results shown in Figure 5, showing the frequency of cells expressing the γ-globin transgene as a result of KLF11 transduction calculated as described in the legend of Figure 4. **B**: Summary of results shown in Figure 5, showing the amount of expression from the γ-globin transgene as a result of KLF11 transduction calculated as described in the legend of Figure 4. For both panels, results are shown for each of two animals (#1 and #2) transduced with either the GFP-only vector (GFP) or the KFL11 vector (KLF11). *$P < 0.001$ versus GFP-only transduced sample by one-sided Z-test for two proportions. **$P < 0.05$ by student's t-test.

from γ-globin genes that are silenced. Recent studies indicate that the ability of NF-E4 to interact with other key proteins in the stage-selector protein complex involved in γ-globin gene regulation can itself be regulated by post-translation acetylation of the NF-E4 protein, providing a possible mechanism for the lack of NF-E4 activity in the adult stage [Zhao et al., 2004]. This is in contrast to the findings with KLF11, where the in vivo studies presented here indicate that KLF11 is capable of inducing γ-globin gene expression in adult mice.

As recently reviewed [Kaczynski et al., 2003; Lomberk and Urrutia, 2005; Suske et al., 2005], KLF11 is a member of the Sp1/KLF family of highly conserved transcription regulators that characteristically contain three highly homologous Cys^2/His^2-type zinc fingers that bind the GC/CACCC DNA motif present in a broad range of promoters. Aside from this highly conserved zinc-finger motif, there is a significant degree of variability in the activation-repression domains of KLF family members, leading to a wide range of regulatory activities that are often cell- and gene-specific. Several studies suggest that these activities are often mediated through interactions with co-activators or repressors [Zhang et al., 2001; Fernandez-Zapico et al., 2003; Song et al., 2003]. KLF11 was also identified independently as TIEG2 (transforming growth factor (TGF) β-inducible early gene-2) in a screen for TGFβ-inducible genes involved in the regulation of cell growth [Cook et al., 1998]. Subsequent studies indicated that KLF11 inhibits cell growth in vivo when targeted to epithelial cells, and that this activity is associated in part with transcriptional repression of key oxidative stress scavenger genes (SOD2 and Catalase 1) mediated through an interaction

TABLE I. Hematological Analysis of Mice Transplanted With Transduced Marrow

Mouse	Hb (g/dl)	Hct (%)	RBC ($\times 10^6/\mu l$)	Plt ($\times 10^3/\mu l$)	WBC ($\times 10^3/\mu l$)	Lym ($\times 10^3/\mu l$)	Baso ($\times 10^3/\mu l$)
C57BL/6	17.4	53.2	11.1	1196	2.83	2.24	0.51
Transgenic	17.2	52.4	10.7	1408	3.51	2.81	0.49
GFP (a)	17.1	51.6	10.5	996	2.14	1.37	0.64
GFP (b)	17.0	52.8	11.0	1028	3.92	3.29	0.57
KLF11 (a)	15.0	45.0	9.0	692	8.88	7.17	1.68
FLF11 (b)	14.5	47.0	9.0	656	8.44	7.19	1.19
avg cont's	17.2	52.5	10.8	1157	3.10	2.43	0.55
avg KLF11	14.7	46.0	9.0	674	8.66	7.18	1.43
P	0.0002	0.001	0.001	0.027	0.0008	0.002	0.005

Mice containing a γ-globin transgene were transplanted with marrow transduced with a vector for KLF11 or a control vector for green-fluorescence protein (GFP) only, and analyzed for hemoglobin (Hb), hematocrit (Hct), red blood cells (RBC), platelets (Plt), total white blood cells (WBC), lymphocytes (Lym), and basophiles (Baso). Untransplanted controls included a normal transgenic mouse and a normal non-transgenic C57BL/6 mouse.

with the co-repressor mSin3A [Fernandez-Zapico et al., 2003]. Of note, these studies also found that KLF11 expression was downregulated in human cancer cells and that KLF11 over-expression suppressed oncogene-induced neoplastic transformation. KLF11 has also been shown to play a direct role as a glucose-inducible regulator of the insulin gene, with KLF11 variants associated with the development of diabetes [Neve et al., 2005]. However, KLF11 knockout studies in mice revealed no discernable phenotype, indicating either a high degree of redundancy in KLF11 activity, or a relatively minor role for KLF11 in development [Song et al., 2005].

It was with interest that we observed no effects of KLF11 transduction on cell growth in the culture studies with MEL cell lines and primary mouse erythroid progenitors, given the results from others documenting that ectopic KLF11 expression inhibits cell growth in vitro and in vivo [Fernandez-Zapico et al., 2003]. It is possible this discrepancy may simply reflect unique properties of the cell types studied. We did, however, observe subtle changes in multiple hematological parameters following engraftment with KLF11-transduced marrow, including a decrease in total Hb and platelets and an increase in lymphocytes and, to a lesser degree, basophiles. It is possible that the decrease in total Hb reflects a direct effect of KLF11 on transcriptional regulation of the globin gene loci, although other indirect models cannot be ruled out. It is likewise possible that the decrease in platelets reflects the same pathway of cell growth inhibition observed in pancreatic epithelial cells [Fernandez-Zapico, 2003], although other indirect models again cannot be ruled out.

Given the broad pattern of expression and variety of activities ascribed to KLF11 in the literature, including the fact that it has been proposed as a transcription repressor in at least one setting [Cook et al., 1998], how can we explain the fact that KLF11 transduction has a specific effect on γ-globin gene expression in the setting of adult hematopoiesis? It has been previously demonstrated that the activity of the closely related transcription factor KLF13 is regulated post-translationally through acetylation mediated by a functional interplay with the transcription co-activators CBP/p300 and PCAF [Song et al., 2003]. It is therefore likely that post-translational modifications of interactions with other transcription co-regulators account for the specific effect of KLF11 on γ-globin gene expression in adult erythropoiesis. Future studies will be needed to validate this model.

Taken together, these studies demonstrate that ectopic over-expression of KLF11 can induce γ-globin gene expression in adult-stage erythroid cells both in vitro and in vivo, and thus that retrovirus vector-mediated gene transfer of KLF11 may provide a viable alternative to pharmacological inducers of fetal hemoglobin. This is especially true for sickle cell disease, were even modest induction of HbF can render patients asymptomatic [Bunn, 2001]. These studies also point to several limitations of this approach, including the inability of KLF11 to reactivate transcriptionally silent γ-globin genes and the pliotropic effects of pan-hematopoietic expression of KLF11 on multiple hematological parameters. Future studies will be needed to determine whether these limitations can be overcome through modifications of this vector, and to determine whether KLF11 vector transduction results in sufficiently high levels of γ-globin gene induction in non-human primates and primary human erythroid cultures.

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REFERENCES

Asano H, Li XS, Stamatoyannopoulos G. 1999. FKLF, a novel Kruppel-like factor that activates human embryonic and fetal beta-like globin genes. Mol Cell Biol 19: 3571–3579.

Asano H, Li XS, Stamatoyannopoulos G. 2000. FKLF-2: A novel Kruppel-like transcriptional factor that activates globin and other erythroid lineage genes. Blood 95:3578–3584.

Blau CA, Barbas CF III, Bomhoff AL, Neades R, Yan J, Navas PA, Peterson KR. 2005. Gamma-globin gene expression in chemical inducer of dimerization (CID)-dependent multipotential cells established from human beta-globin locus yeast artificial chromosome (beta-YAC) transgenic mice. J Biol Chem 280:36642–36647.

Bunn FH. 2001. Human hemoglobins: Sickle hemoglobin and other mutants. In: Stamatoyannopoulos G, Majerus PW, Perlmutter RM, Varmus H, editors. Molecular basis of blood diseases, 3rd edition. Philadelphia, PA: WB Saunders. pp 227–262.

Cheng L, Du C, Murray D, Tong X, Zhang YA, Chen BP, Hawley RG. 1997. A GFP reporter system to assess gene

transfer and expression in human hematopoietic progenitor cells. Gene Ther 4:1013–1022.
Constantoulakis P, Josephson B, Mangahas L, Mangahas L, Papayannopoulou T, Enver T, Costantini F, Stamatoyannopoulos G. 1991. Locus control region-A gamma transgenic mice: A new model for studying the induction of fetal hemoglobin in the adult. Blood 77: 1326–1333.
Cook T, Gebelein B, Mesa K, Mladek A, Urrutia R. 1998. Molecular cloning and characterization of TIEG2 reveals a new subfamily of transforming growth factor-beta-inducible Sp1-like zinc finger-encoding genes involved in the regulation of cell growth. J Biol Chem 273:25929–25936.
Emery DW, Yannaki E, Tubb J, Nishino T, Li Q, Stamatoyannopoulos G. 2002. Development of virus vectors for gene therapy of β chain hemoglobinopathies: Flanking with a chromatin insulator reduces γ-globin gene silencing in vivo. Blood 100:2012–2021.
Emery DW, Tubb J, Nishino Y, Nishino T, Otto KG, Stamatoyannopoulos G, Blau CA. 2005. Selection with a regulated cell growth switch increases the likelihood of expression for a linked gamma-globin gene. Blood Cells Mol Dis 34:235–247.
Enver T, Brice M, Karlinsey J, Stamatoyannopoulos G, Papayannopoulou T. 1991. Developmental regulation of fetal to adult globin gene switching in human fetal erythroid x mouse erythroleukemia cell hybrids. Dev Biol 148:129–137.
Fernandez-Zapico ME, Mladek A, Ellenrieder V, Folch-Puy E, Miller L, Urrutia R. 2003. An mSin3A interaction domain links the transcriptional activity of KLF11 with its role in growth regulation. EMBO J 22:4748–4758.
Ikonomi P, Noguchi CT, Miller W, Kassahun H, Hardison R, Schechter AN. 2000. Levels of GATA-1/GATA-2 transcription factors modulate expression of embryonic and fetal hemoglobins. Gene 261:277–287.
Kaczynski J, Cook T, Urrutia R. 2003. Sp1- and Krupple-like transcription factors. Genome Biol 4:206.
Li Q, Clegg C, Peterson K, Shaw S, Raich N, Stamatoyannopoulos G. 1997. Binary transgenic mouse model for studying the trans control of globin gene switching: Evidence that GATA-1 is an in vivo repressor of human epsilon gene expression. Proc Natl Acad Sci USA 94: 2444–2448.
Lomberk G, Urrutia R. 2005. The Family feud: Turning off Sp1 by Sp1-like KLF proteins. Biochem J 392:1–11.
Loukopoulos D, Fessas P. 1965. The distribution of hemoglobin types in thalassemic erythrocytes. J Clin Invest 44:231–240.
Markowitz D, Goff S, Bank A. 1988. A safe packaging line for gene transfer: Separating viral genes on two different plasmids. J Virol 62:1120–1124.
Miller AD, Rosman GJ. 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques 7:980–990.
Neve B, Fernandez-Zapico ME, Ashkenazi-Katalan V, Dina C, Hamid YH, Joly E, Vaillant E, Benmezroua Y, Durand E, Bakaher N, Delannoy V, Vaxillaire M, Cook T, Dallinga-Thie GM, Jansen H, Charles MA, Clement K, Galan P, Hercberg S, Helbecque N, Charpentier G, Prentki M, Hansen T, Pedersen O, Urrutia R, Melloul D, Froguel P. 2005. Role of transcription factor KLF11 and its diabetes-associated gene variants in pancreatic beta cell function. Proc Natl Acad Sci USA 102:4807–4812.
Nosaka T, Kawashima T, Misawa K, Ikuta K, Mui AL, Kitamura T. 1999. STAT5 as a molecular regulator of proliferation, differentiation and apoptosis in hematopoietic cells. EMBO J 18:4754–4765.
Persons DA, Allay JA, Allay ER, Ashmun RA, Orlic D, Jane SM, Cunningham JM, Nienhuis AW. 1999. Enforced expression of the GATA-2 transcription factor blocks normal hematopoiesis. Blood 93:488–499.
Peterson KR, Clegg CH, Huxley C, Josephson BM, Haugen HS, Furukawa T, Stamatoyannopoulos G. 1993. Transgenic mice containing a 248-kb yeast artificial chromosome carrying the human beta-globin locus display proper developmental control of human globin genes. Proc Natl Acad Sci USA 90:7593–7597.
Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: A laboratory manual, 2nd edition. Cold Spring Harbor, NY: Cold Spring Harbor Press.
Skarpidi E, Vassilopoulos G, Stamatoyannopoulos G, Li Q. 1998. Comparison of expression of humn globin genes transferred into mouse erytholeukemia cells and in transgenic mice. Blood 92:3416–3421.
Sofroniadou K, Wood WG, Nute PE, Stamatoyannopoulos G. 1975. Globin chain synthesis in the greek type (A gamma) of hereditary persistanc eof fetal hemoglobin. Br J Hematol 29:137–148.
Song CZ, Keller K, Chen Y, Stamatoyannopoulos G. 2003. Functional interplay between CBP and PCAF in acetylation and regulation of transcription factor KLF13 activity. J Mol Biol 329:207–215.
Song CZ, Gavriilidis G, Asano H, Stamatoyannopoulos G. 2005. Functional study of transcription factor KLF11 by targeted gene inactivation. Blood Cells Mol Dis 34:53–59.
Stamatoyannopoulos G. 2005. Control of globin gene expression during development and erythroid differentiation. Exp Hematol 33:259–271.
Suske G, Bruford E, Philipsen S. 2005. Mammalian SP/KLF transcription factors: Bring in the family. Genomics 85:551–556.
Vassilopoulos G, Navas PA, Skarpidi E, Peterson KR, Lowrey CH, Papayannopoulou T, Stamatoyannopoulos G. 1999. Correct function of the locus control region may require passage through a nonerythroid cellular environment. Blood 93:703–712.
Weatherall DJ. 2001. The thalassemias. In: Stamatoyannopoulos G, Majerus PW, Perlmutter RM, Varmus H, editors. Molecular basis of blood diseases, 3rd edition. Philadelphia, PA: WB Saunders. pp 183–226.
Yang Y, Duan Z, Skarpidi E, Li Q, Papayannopoulou T, Stamatoyannopoulos G. 2001. Cloning and characterization of a potential transcriptional activator of human gamma-globin genes. Blood Cells Mol Dis 27:1–15.
Zhang JS, Moncrieffe MC, Kaczynski J, Ellenrieder V, Rrendergast FG, Urrutia R. 2001. A conserved alpha-helix motif mediates the interaction of Sp1-like transcriptional repressors with the corepressor mSin3A. Mol Cell Biol 21:5041–5049.
Zhang P, Basu P, Redmond LC, Morris PE, Rupon JW, Ginder GD, Lloyd JA. 2005. A functional screen for

Kruppel-like factors that regulate the human gamma-globin gene through the CACCC promoter element. Blood Cells Mol Dis 35:227–235.

Zhao Q, Cumming H, Cerruti L, Cunningham JM, Jane SM. 2004. Site-specific acetylation of the fetal globin activator NF-E4 prevents its ubiquitination and regulates its interaction with the histone deacetylase, HDAC1. J Biol Chem 279:41477–41486.

Zhou W, Clouston DR, Wang X, Cerruti L, Cunningham JM, Jane SM. 2000. Induction of human fetal globin expression by a novel erythroid factor, NF-E4. Mol Cell Biol 20:7662–7672.

Zhou W, Zhao Q, Sutton R, Cumming H, Wang X, Cerruti L, Hall M, Wu R, Cunningham JM, Jane SM. 2004. The role of p22 NF-E4 in human globin gene switching. J Biol Chem 279:26227–26232.