

BP1 is a negative modulator of definitive erythropoiesis

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ABSTRACT

Beta protein 1 (BP1), a human homeotic transcription factor, is expressed during hematopoiesis in the erythroid lineage. To determine the *in vivo* role of BP1 in erythropoiesis, we have undertaken two complementary approaches using enforced BP1 expression in both transgenic mice and embryonic stem (ES) cells. Despite repeated attempts, only one adult transgenic BP1 founder mouse among 121 mice was obtained. This mouse presumably survived due to transgene mosaicism because the transgene could not be transmitted. This mouse expressed BP1 and displayed splenomegaly, extramedullary erythropoiesis and severe amyloidosis A in the kidney, a phenotype compatible with thalassemia. Consistently, the presence of BP1 transgene in fetuses was associated with paleness and lethality. In ES cells, BP1 expression in primary differentiation appeared to antagonize adult β -globin expression. In secondary differentiation, BP1 expression reduced significantly β -globin gene expression in both primitive and definitive erythroid cells, whereas it impaired only the definitive erythroid cell differentiation. These studies showed that BP1 can negatively modulate adult β -globin gene expression and definitive erythroid cell differentiation, and suggest that BP1 could play a role in thalassemia.

INTRODUCTION

The transcription factor beta protein 1 (BP1) is a member of the homeobox gene family and the distal-less subfamily. The expression of BP1 is highly tissue-specific and developmentally restricted. Among 50 human tissues, BP1 was highly

expressed in placenta, kidney and at lower levels in fetal liver (1). Such restricted pattern of expression is compatible with a specific gene function in development and/or differentiation.

In transient transfection studies, BP1 protein appears to act as a repressor of the human adult β -globin gene, through two silencers upstream of the β -globin gene (1–3). In addition, BP1 protein was shown to bind to negative control regions upstream of the adult β -globin gene and the murine β -major globin gene (4). The clinical relevance of BP1 is supported by the inverse correlation between the binding affinity of BP1 protein to polymorphic DNA sequences associated with sickle cell disease (5). We found that DNA with stronger BP1 binding in the Indian-Arabo haplotype, or weaker BP1 binding, as in the Bantu haplotype, have respectively, reduced or increased clinical evidence for red blood cell sickling. Taken together, these studies support the hypothesis that BP1 is a physiologic repressor of β -globin gene expression.

A role for BP1 in bone marrow hematopoiesis is also emerging. BP1 is expressed in purified CD34⁺ hematopoietic bone marrow cells, a primitive subpopulation of human hematopoietic cells, and its expression decreases to undetectable levels during terminal erythropoiesis (1,6). Enforced expression of BP1 in embryonic/fetal erythroid K562 cells leads to decreased erythroid differentiation potential, while ectopic expression of BP1 increases clonogenicity, suggesting a role for BP1 in leukemogenesis. In fact, expression of BP1 was detected in 63% of primary leukemia blasts from AML patients and in 32% of leukemic blasts from T-ALL patients (6).

The aim of this study was to determine the role of BP1 in erythroid differentiation of transgenic mice and in totipotent embryonic stem (ES) cells. In ES cells constitutively expressing BP1, we demonstrate marked repression in the generation of definitive erythroid (E^d) cells with no effect on growth of the ES-derived embryoid bodies (EB) or in primitive erythroid (E^p) cells. Moreover, expression of BP1 in definitive erythroid cells in transgenic mice appears incompatible with survival.

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MATERIALS AND METHODS

Constructs

The LCRBP1 construct was produced in p-Bluescript vector by joining the human β -globin promoter to the 1.1 kb BP1 cDNA (7). Subsequently, the human β -globin 8 kb midi LCR (8) was linked upstream and the human 2.8 kb β -globin 3' sequences consisting of 18 bp of exon 2, all of exon 3 and 3' flanking sequences were adjoined downstream. The LCR Δ BP1 construct was identical to LCRBP1 but without the BP1 cDNA. A 12 kb Sal I–Not I fragment was purified from LCRBP1 to generate transgenic mice in (C57Bl/6J X CBA/J)F2 mouse strains as described previously (8).

Cell culture and differentiation

CCE-ES cells (gift from Dr G. Keller, NY) were maintained as described previously (9). ES cells were co-electroporated (10) using equivalent molar ratios of the LCRBP1 or LCR Δ BP1 and the pSV3neo vector and selected with G418 (400 μ g/ml). DNA from several independent subclones was analyzed by Southern blotting with two restriction digests, Sall/EcoRI and Sall/BamHI, and a BP1– β -globin probe to verify contiguous integrity. Conditions for erythroid differentiation consist of SCF (100 ng/ml), IL-3 (5 ng/ml) and Epo (2 U/ml) (11).

RNA extraction and expression analysis

Total RNA was isolated from ES cells using Trizol reagent (Invitrogen). cDNA synthesis was performed for E^p and E^d samples by two successive series of oligo(dT)-primers and PCR amplification; for EB samples, 1 μ g of RNA was used as described previously (12).

Semi-quantitative expression analysis for the BP1 transgene and β -globin genes were carried out with the following primers: for BP1, forward 5'-TGGAGCCTGGTATCAGC-ATC-3' and reverse 5'-TGGACAGCAAGAAAGCGAGC-3' (in exon 3 β -globin gene) producing an amplicon of 417 bp; for β -globin, forward 5'-GGACCCAGCGGTACTTTGATAG-3' (exon 2) and reverse 5'-GTGGCCAGCACAAT-CACGAT-3' (in exon 3) generating a 239 bp product. S16 ribosomal primers were described previously (13). Conditions for BP1 amplification were 94°C, 30 s, 57°C, 30 s, 72°C, 30 s for 30 cycles; conditions for β -globin and S16 were 94°C, 30 s, 60°C, 30 s, 72°C, 30 s for 14 and 20 cycles and a final elongation of 10 min at 72°C. Radioactive PCR products were analyzed on 8% polyacrylamide/TBE gel and quantified by phosphorimager. For real-time quantitative analysis, the same primers were used, except the BP1 primers were replaced with forward 5'-CCCCAATTTGTCCTACTCCA-3' and reverse 5'-GGTTGCTGGCAGGACAGGTA-3', and produced an amplicon of 100 bp. Four series of ES primary and secondary differentiation experiments were performed with duplicate of each clone in each experiment. All reactions were performed in triplicate in a master mix (Qiagen, Mississauga, Canada), containing 0.3 μ M of each primer. Amplification conditions were 95°C, 15 min followed by 44 cycles of 94°C, 30 s, 60°C, 35 s, 72°C, 30 s, in a MX3500pro multiplex quantitative PCR analyzer.

Histological analysis

Age-matched controls and transgenic tissues were formalin-fixed and embedded in paraffin. Tissue sections (5 μ m) were stained with hematoxylin/eosin and Congo red for light microscopy. Immunofluorescence for IgG, IgM, IgA, Kappa and Lambda light chains was performed following pronase digestion of paraffin sections. Staining for serum amyloid A (SAA) protein was performed by immunoperoxidase using avidin–biotin labeling. Tissues for ultrastructural analysis were fixed in 2.5% glutaraldehyde-buffered with 0.01 M cacodylate (pH 7.4) and postfixated in 2% osmium tetroxide and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate (14).

RESULTS AND DISCUSSION

BP1 expression *in vivo* appears to be embryonic lethal

Characterization of the *in vivo* role of BP1 in erythroid cells was undertaken by introducing a LCRBP1 construct into mice. LCRBP1 was produced by linking the human β -globin regulatory sequences without the two BP1 silencer regions to the human BP1 cDNA (Figure 1a). Only one adult founder transgenic mouse with an intact transgene was generated from 121 mice (P28 in Table 1). This female founder mouse appeared to have one copy of the transgene and was extensively mated with wild-type (C57Bl/6J X CBA/J)F1 mice. From 11 litters, all 76 mice were negative for the

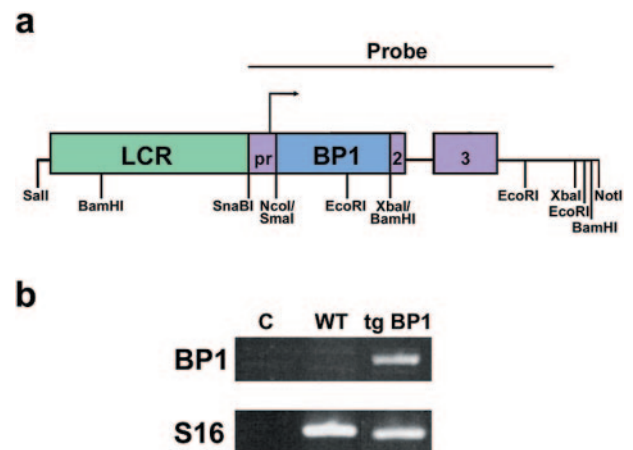


Figure 1. LCRBP1 transgene structure and expression in transgenic mouse. (a) The LCRBP1 construct was produced in a bluescript vector by joining the human β -globin promoter (pr) lacking the two silencer sequences (–264/SnaBI to +48/NcoI) to the 1.1 kb BP1 cDNA (SmaI–XbaI). The human β -globin 8 kb midi LCR was linked to this DNA upstream, and downstream 2.8 kb of the β -globin gene including 3' sequences of exon 2, all of exon 3, and 3' flanking sequences [BamHI (nt 62613) to XbaI (nt 65421), GenBank accession no. U01317], containing the two globin 3' enhancers (18). The probe BP1– β -globin (SnaBI–XbaI, 3.2 kb) used for transgenic mouse screening by Southern blot analysis is indicated above the LCRBP1 construct. (b) Expression analysis in peripheral blood using RT–PCR. Three RNA blood samples from control H₂O (C), transgenic mouse (tg BP1) and control identical mouse strain (WT) were monitored for BP1 and S16 expression. Human BP1 expression was readily detectable in the blood of the transgenic founder mouse and undetectable in the blood of the control identical mouse strain. Expression of the ribosomal protein S16 served as internal control.

Table 1. Genotype analysis of fetuses/pups from LCRBP1 microinjected oocytes

Stage (age)	Total n (fetuses or pups)	BP1 Genotype	
		-	+
E15.5	2	1	1
E16.5	27	23	4 ^a
E17.5	41	40	1
E18.5	39	38	1 ^b
P28	121	120	1 ^c

^aAmong 4 fetuses: 1 resorbed and 1 pale.^bFetus completely resorbed.^cMouse does not transmit the transgene.

transgene. The average litter size was 7 pups, well below the 10–12 pups on average for these strains. The lack of transmission can be explained by high mosaicism and/or embryonic lethality resulting from BP1 transgene expression. The very low frequency of adult transgenic mice obtained compared to the frequency of ~10–15% for other transgenes (data not shown), also indicated that the expression of BP1 in differentiating and mature erythroid cells could be lethal during gestation. To test this hypothesis, microinjection of additional oocytes of the LCRBP1 construct was carried out and fetuses were analyzed for the presence of the transgene at various stages of gestation. As shown in Table 1, one of two embryos at E15.5 and four of 27 embryos at E16.5 were LCRBP1

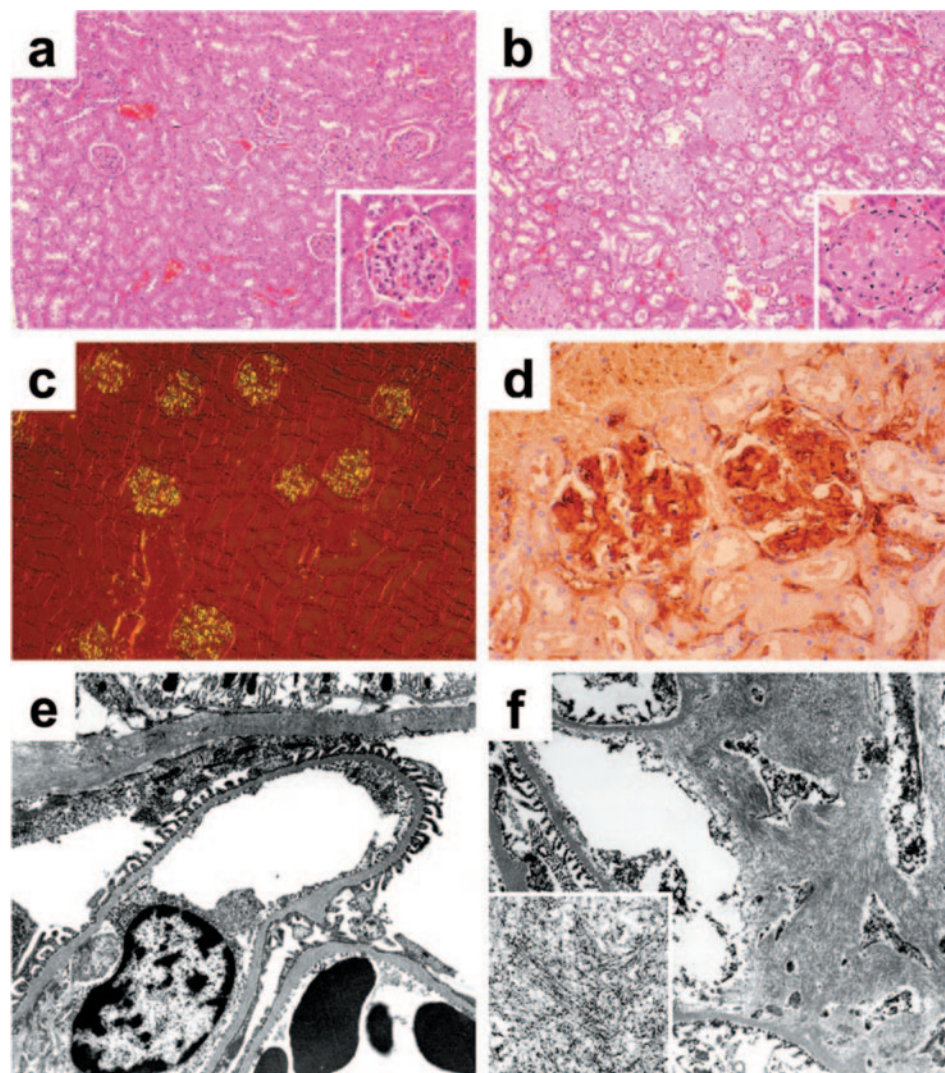


Figure 2. Histopathological analysis of the LCRBP1 transgenic founder mouse. (a) Normal histology of the kidney displaying small regular glomeruli and surrounding tubulo-Interstitial compartment (H&E, ×100). Inset shows glomerulus of normal size and cellularity with patent capillaries and basement membranes of normal thickness (H&E, ×400). (b) Renal histology of the transgenic LCRBP1 mouse showed diffuse and uniform enlargement of the glomeruli due to expansion of the glomerular tuft by amorphous pale eosinophilic material. The outlying tubules, interstitium and blood vessels are uninvolved (H&E, ×100). Inset shows a representative glomerulus of the transgenic LCRBP1 mouse with expansion of the mesangium and narrowing of the glomerular capillary lumina by voluminous deposits of amorphous material typical of amyloid. The glomerular cellularity appeared reduced (H&E, ×400). (c) Kidney of the transgenic LCRBP1 mouse viewed under polarized light displayed apple green birefringence diagnostic of amyloid deposits in a diffuse and global glomerular distribution (Congo red, polarized light, ×100). (d) Kidney of the transgenic LCRBP1 mouse showed strong diffuse and global glomerular positivity for serum amyloid A protein (SAA immunostain, ×250). (e) Ultrastructural analysis of a normal renal glomerular capillary showed the normal glomerular architecture, without fibrillar deposits (EM, ×6000). (f) Ultrastructure of a renal glomerulus from the transgenic LCRBP1 mouse showed abundant randomly oriented 8–10 nm fibrils expanding the mesangial regions and narrowing the adjacent glomerular capillary lumen (EM, ×6000). Inset shows fibrils at higher magnification (EM, ×20 000).

positive. Among those four transgenic fetuses, one was resorbed and one was pale (anemic). The frequency of transgenic fetuses decreased thereafter. Since the maximal expression of the endogenous murine adult globin gene occurs at E16.5 for definitive erythroid cells, it would be expected that repression by BP1 would be most detrimental from this stage onwards.

The only adult female transgenic founder expressed BP1 in peripheral blood, shown by RT-PCR and Rnase protection (Figure 1b and data not shown). Pathological analysis upon the unexpected death of this transgenic founder mouse showed splenic enlargement by ~2-fold compared to the age-matched control mouse strain. The bone marrow cellularity was increased by 1.8-fold in the femur of the founder mouse (8.4×10^7 cells) relative to controls ($4.6 \times 10^7 \pm 0.9$ cells; $n = 4$) which is consistent with the ~1.4-fold increase in cellularity observed in hemizygous β -globin knock-out mice, a model of β -thalassemia (15). Both these features are indicative of stimulated hematopoiesis and consistent with the observation of numerous foci of extramedullary hematopoiesis in organs such as spleen and kidney. While renal tissues had normal tubules, interstitium and blood vessels, the glomeruli showed expansion of the mesangium compared to controls (Figure 2a and b), with narrowing of the capillaries due to large Congo red positive amyloid deposits (Figure 2c and d). By specific staining for identification of amyloid precursor proteins, these amyloid deposits were negative for immunoglobulins, kappa and lambda light chains but positive for SAA (Figure 2c and d). Electron microscopy revealed glomerular deposits of randomly oriented 8–10 nm fibrils typical of amyloid (Figure 2e and f). Presence of increased SAA is observed in conditions of chronic inflammation as in thalassemia (16). The composite of these pathologic findings, increase in erythropoiesis/hematopoiesis and amyloid deposits, are consistent with a thalassemic condition in the BP1 transgenic mouse.

BP1 expression impairs definitive erythropoiesis

To gain further insight into the *in vivo* role of BP1 in primitive and definitive hematopoiesis, the LCRBP1 and empty vector control LCR Δ BP1 constructs were targeted to totipotent ES cells by co-electroporation with the pSV3neo vector. Several independent clones were obtained for both constructs and were analyzed by Southern blot for integrity of the transgenes (Figure 3). Among the clones with an intact transgene, three clones for LCRBP1 (1, 2 and 5 transgene copies) and three clones for LCR Δ BP1 (1 and 2 copies) were selected for subsequent analysis.

Parental and electroporated ES cells were differentiated *in vitro* to form EBs (which contain a variety of cell types) and assessed for BP1 expression. The potential of these electroporated ES cells to generate EBs was not significantly affected, as the primary plating efficiency was $17.5 \pm 3.9\%$ for parental ES cells, $17.1 \pm 4.3\%$ for LCR Δ BP1 cells and $14.1 \pm 3.5\%$ for LCRBP1 cells (mean \pm SD). In addition, LCRBP1 electroporated ES cells yielded similar-sized EBs as the parental and empty vector ES cells (data not shown), suggesting that the number of cells per EB was not altered. During this primary differentiation process, the percentage of hemoglobinized EBs was comparable between the parental

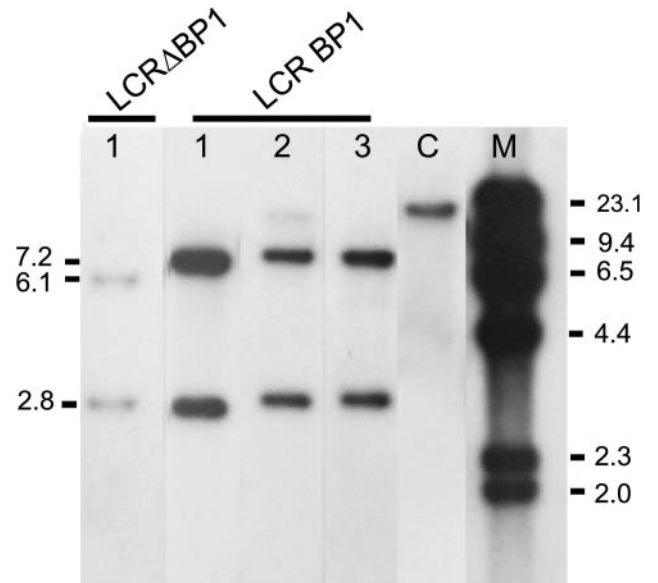


Figure 3. Integrity of the BP1 transgene in ES cells. Southern blot analysis of electroporated ES cells with LCR Δ BP1 and LCRBP1 constructs. Restriction enzyme digestion was carried out for LCR Δ BP1 and LCRBP1 clones using SaliI/BamHI. Probe used is depicted in Figure 1a and spanned the adult β -globin and BP1 sequences. Expected fragments are 6.1 and 2.8 kb for the LCR Δ BP1 clones, and 7.2 and 2.8 kb for the LCRBP1 clones. The intensity of the 6.1 kb band of LCR Δ BP1 clones is weaker than the 2.8 kb or the 7.2 kb of the LCRBP1 clones since only ~0.3 kb of the probe is homologous to globin. Extra bands correspond to junction of the constructs with genomic sequences. M, molecular weight marker λ digested with HindIII and C, copy of LCRBP1 unit length.

Table 2. Quantification of erythroid progenitors

	EBs d9 % Hb EBs/total EBs	E ^p d3 No. E ^p /10 ⁵ cells	E ^d d7 % E ^d /total colonies
WT	53.97 \pm 1.45	0.17 \pm 0.01	27.00 \pm 0.50
LCR Δ BP1	53.49 \pm 3.39	0.18 \pm 0.01	25.00 \pm 0.35
LCRBP1-1	55.48 \pm 8.77	0.17 \pm 0.02	17.16 \pm 1.75****
LCRBP1-2	54.00 \pm 2.82	0.19 \pm 0.00	17.50 \pm 0.00****
LCRBP1-3	51.35 \pm 3.32	0.15 \pm 0.02	17.66 \pm 0.76****

Analysis was carried out on optimal culture days for EBs (day 9), primitive erythroid progenitors E^p(day 3), definitive erythroid progenitors E^d (day 7) from parental or electroporated ES cells as described (9). The proportion of erythroid CFC was determined in the presence of BP1 expression for EBs (% hemoglobinized Hb/ EBs), E^p (No. E^p per 10⁵ cells) and E^d (% E^d/total colonies). The data from wild type WT, LCR Δ BP1 (mean of two clones), or LCRBP1 from three clones (1–3) are shown as mean \pm standard deviation. *P*-value: student's *t*-test.

Values are compared to WT-CCE: **P* \leq 0.01, ***P* \leq 0.001, and to LCR Δ BP1: ****P* \leq 0.01.

and electroporated cells at day 9 (Table 2). To quantify expression levels, we first established the RT-PCR conditions to be within the linear range (Figure 4a). The expression time course of the endogenous adult β -globin gene was similar between wild-type and empty vector control (Figure 4b and data not shown). Noticeably, wild-type ES cells or EBs with empty LCR Δ BP1 control did not express BP1 (Figure 4). In two independent experiments, the expression of BP1 in the LCRBP1 clones was highest at days 6 and 9 and decreased from day 12 onwards whereas the β -globin

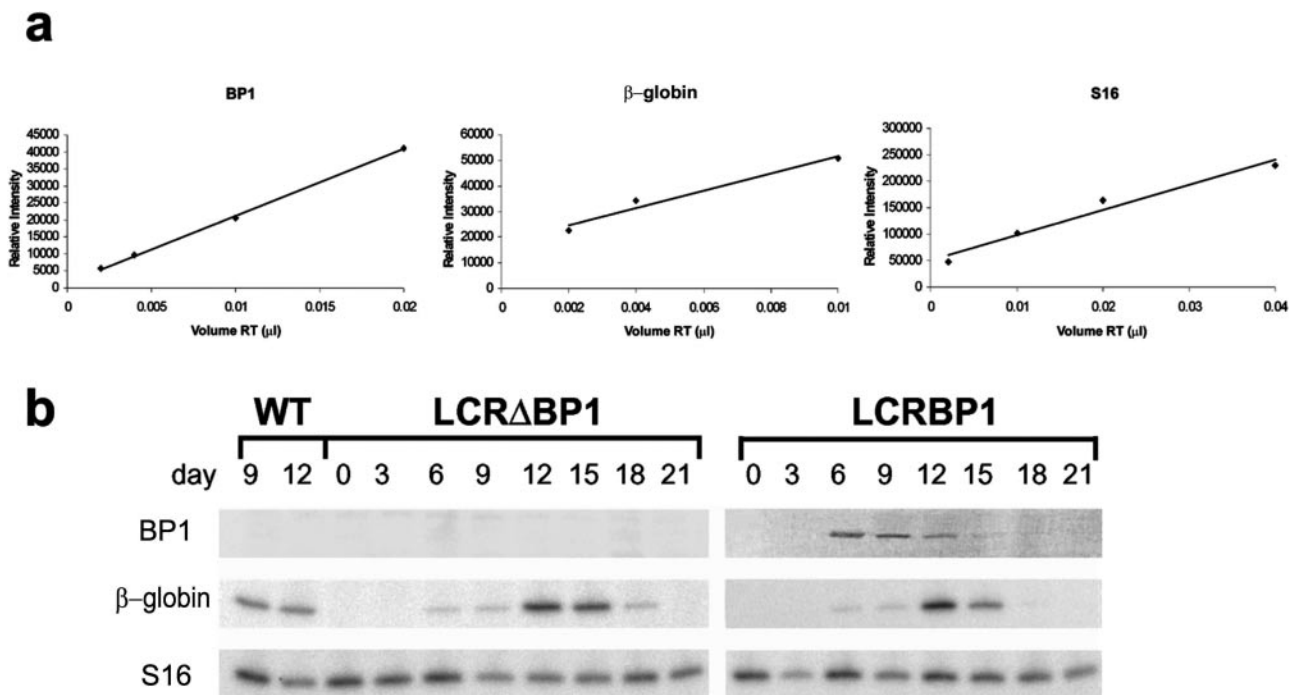


Figure 4. Expression analysis of BP1 and β -globin genes in EBs at various stages of primary differentiation. **(a)** Demonstration of linearity of RT-PCR amplification. Varying quantities of total cDNA aliquots were amplified with primers specific for BP1, β -globin and S16 as internal control to ensure that conditions were within linear range of amplification. The reactions were evaluated semiquantitatively on polyacrylamide gels scanned by phosphorimager and values plotted on linear graphs. **(b)** Parental ES cells (WT), ES cells containing the empty vector (LCR Δ BP1) or ES cells containing BP1 (LCRBP1) were differentiated into embryoid bodies (EBs). Expression analysis of BP1 and of the endogenous β -globin gene was carried out by RT-PCR on the indicated days of differentiation, using S16 expression as an internal control. Days indicated were selected for analysis of the kinetics of erythroid differentiation since primitive erythroid expression is maximum on day 7 and definitive erythroid lineage expression is high from day 5 to day 12 (11). Semi-quantitative evaluation was carried out for each sample run on the same gel and normalized to the ribosomal RNA control.

Table 3. Quantitative expression of BP1 and adult β -globin genes in erythroid progenitors

	EBs d9		E ^p d3		E ^d d7	
	BP1/S16	β -globin(10^{-3})/S16	BP1/S16	β -globin/S16	BP1/S16	β -globin/S16
WT	N.D.	28 \pm 2.6	0.03 \pm 0.0	10.0 \pm 2.2	N.D.	300.5 \pm 7.3
LCR Δ BP1	N.D.	14 \pm 8.5	0.05 \pm 0.0	10.3 \pm 1.5	N.D.	250.4 \pm 4.2
LCRBP1-1	2.7 \pm 0.6	2 \pm 0.6*	73.8 \pm 1.7	3.9 \pm 0.3**	60.0 \pm 2.8	150.3 \pm 14.7**
LCRBP1-2	25.4 \pm 4.5	3 \pm 0.4*	672.4 \pm 19.4	5.1 \pm 1.9*	702.3 \pm 99.5	225.9 \pm 19.2*
LCRBP1-3	5.2 \pm 1.8	1 \pm 0.1*	126.3 \pm 2.0	7.5 \pm 2.1*	117.7 \pm 22.0	180.8 \pm 24.5*

The data from wild type (WT), LCR Δ BP1 (mean of three clones), or LCRBP1 from three clones (1 to 3) are shown. Values of real-time PCR represent relative units normalized to control S16 expression \pm standard deviation. N.D.: Not detected.

Statistical significance: * $P \leq 0.05$, ** $P \leq 0.005$, compared to WT.

gene showed strongest expression on days 12 and 15. Semi-quantitative analysis of the LCRBP1 clones indicated a \sim 1.5- to 2-fold reduction in β -globin expression normalized to S16 on days 12 and 15 relative to empty vector control. Consistently, quantification by real-time PCR of EBs at day 9 showed a decrease in β -globin expression levels by at least 4-fold, a time when BP1 is strongest (Table 3 and Figure 4). Hence the LCRBP1 clones indicated that BP1 might antagonize expression of the murine β -globin gene. Conversely, repression of BP1 in the erythroleukemic K562 cell line was shown to enhance endogenous human β -globin gene expression (3).

To investigate more precisely the role of BP1, we carried out secondary differentiation under conditions favorable for erythroid/hematopoietic colony forming cells (CFCs). In four

series of experiments, the number of primitive erythroid E^p progenitors detected at day 3 was unchanged upon differentiation of parental, LCRBP1 and control LCR Δ BP1 ES cells (Table 2). Quantitative expression analysis from pools of LCRBP1 E^p normalized to S16 showed a significant increase in BP1 expression levels (\sim 20- to 25-fold) compared to pools of LCRBP1 EBs (Table 3). As shown in Table 2, this increase in BP1 expression appeared to exert no significant negative effect on production of primitive erythroid cells. However, the number of definitive erythroid-CFC (E^d-CFC) at day 7, as identified by hemoglobinized colonies, was significantly decreased in LCRBP1 relative to empty vector and parental ES cells. Pools of individual hemoglobinized definitive erythroid E^d LCRBP1 progenitors showed high BP1 expression levels similar to those observed for primitive

erythroid E^P LCRBP1 progenitors by real-time PCR (Table 3). These results indicated that the elevated BP1 expression led to a specific decrease of definitive erythropoiesis compared to primitive erythropoiesis. Importantly, BP1 expression caused a significant decrease in adult β -globin gene expression for both E^P and E^d cells relative to wild-type and empty vector control, reaching 2- to 2.5-fold (Table 3). In contrast to definitive erythropoiesis, the adult β -globin chain is not the most prevalent β -like globin chain in primitive erythropoiesis and consistently, decreased expression had no apparent impact on primitive differentiation. Of note, pools of hemoglobinized erythroid E^d LCRBP1 progenitors were picked for expression analysis since non-hemoglobinized colonies include other cell types. However, pooling hemoglobinized E^d would be expected to select for higher β -globin levels, possibly skewing the data toward less repression. Thus, the actual decrease in β -globin may be much greater than the one observed. Nevertheless, such a decrease in expression would be likely to have an important impact in the mouse, since the hemizygous β -globin knock-out mice with only a 25% reduction in β -globin gene expression resulted in a severe β -thalassemic phenotype with half the levels of hemoglobin and hematocrit levels (15,17). Further, expression of BP1 did not significantly alter the number of mac-CFC (macrophages), mix-CFC (mixed lineages: erythroid/granulocyte/macrophage/megakaryocyte) or E^d/mac-CFC (data not shown). Therefore, BP1 expression in the erythroid lineage targeted specifically definitive erythroid progenitors.

In summary, our findings demonstrate that high expression of BP1 leads to decreased adult β -globin gene expression and inhibition of definitive erythroid differentiation. Together, previous *in vitro* studies and the data presented here support the concept that sustained BP1 expression, through repression of the adult β -globin gene, may cause globin chain imbalance resulting in thalassemia. Future studies should be directed to determining the role of BP1 in human thalassemia.

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