High-mobility group protein HMGB2 regulates human erythroid differentiation through trans-activation of GFI1B

Benoit Laurent,1,2 Voahangy Randrianarison-Huetz,1,2 Vincent Maréchal,3-5 Patrick Mayeux,1,2 Isabella Dusantier-Fourt,1,2 and Dominique Duménil1,2

1Institut Cochin, Université Paris Descartes, Centre National de la Recherche Scientifique (CNRS; Unité Mixte de Recherche [UMR] 8104), Paris; 2Inserm, U657, Paris; 3Centre de Recherche des Cordeliers, Université Pierre et Marie Curie–Paris, Unité Mixte de Recherche en Santé (UMRS) 872, Paris; 4Université Paris Descartes, UMRS 872, Paris; and 5Inserm, UMRS 872, Paris, France

Gfi-1B is a transcriptional repressor that is crucial for erythroid differentiation: inactivation of the GFI1B gene in mice leads to embryonic death due to failure to produce differentiated red cells. Accordingly, GFI1B expression is tightly regulated during erythropoiesis, but the mechanisms involved in such regulation remain partially understood. We here identify HMGB2, a high-mobility group HMG protein, as a key regulator of GFI1B transcription. HMGB2 binds to the GFI1B promoter in vivo and up-regulates its trans-activation most likely by enhancing the binding of Oct-1 and, to a lesser extent, of GATA-1 and NF-Y to the GFI1B promoter. HMGB2 expression increases during erythroid differentiation concomitantly to the increase of GFI1B transcription. Importantly, knockdown of HMGB2 in immature hematopoietic progenitor cells leads to decreased Gfi-1B expression and impairs their erythroid differentiation. We propose that HMGB2 potentiates GATA-1–dependent transcription of GFI1B by Oct-1 and thereby controls erythroid differentiation. (Blood. 2010;115:687-695)

Introduction

Gfi-1 and Gfi-1B are members of the Gfi zinc-finger transcriptional repressor family, whose structure is characterized by an N-terminal repressor domain called SNAG and 6 C-terminal C2H2 zinc fingers.1 Gfi-1 and Gfi-1B are differentially expressed in hematopoietic cells. Gfi-1 is expressed in immature progenitors and highly expressed in granulocytes,2,3 whereas Gfi-1B expression is restricted to erythroid and megakaryocytic cells.4,5 Analysis of Gfi-1B-green fluorescent protein knock-in mice has shown that Gfi-1B expression is dynamically regulated during murine erythropoiesis.6 Deletion of the Gfi-1 gene in mice provokes a severe disturbance of hematopoietic stem cell function due to excessive cycling and severe neutropenia.7,8,9 GFI1B–deficient mice are not viable beyond embryonic day 14.5 and fail to produce definitive enucleated red cells.8 Accordingly, Gfi-1B overexpression in erythroid progenitors strongly disturbs erythroid maturation.5,9 Gfi-1 and Gfi-1B bind to the same consensus DNA sequence TAAATCAC(A/T)GCA,1,10,11 and knock-in mice in which the Gfi-1 coding region was replaced by GFI1B showed that Gfi-1B can replace Gfi-1 in the regulation of hematopoiesis.12

The mechanisms accounting for the GFI1B transcriptional regulation are not fully understood. The GFI1B promoter was cloned and an erythroblast-specific promoter region was characterized in K562 cells. GATA-1 and NF-YA cooperate to activate Gfi-1B transcription.13 Recently, chromatin regulatory proteins (Lysine-Specific Demethylase 1 [LSD1], Co-RE1-Silencing Transcription factor [CoREST], and Histone deacetylase [HDAC]) have been suggested to mediate transcriptional repression of Gfi-1B target genes.14 Overexpression of Gfi-1B in NIH3T3 or undifferentiated K562 cells,15 as well as in the spleen or thymus of vav-GFI1B transgenic mice,16 provided evidence of an autoregulation mechanism of GFI1B transcription. We have shown that Gfi-1B does not repress its own transcription during erythroid differentiation. Indeed, GFI1B promoter remains associated with a transcriptionally active chromatin configuration throughout erythroid differentiation as highlighted by an increase in histone H3 acetylation and concomitant release of the corepressors LSD1 and CoREST.17 Besides GATA-1, few activators of the GFI1B transcription have been identified.

In this report, we identify a high-mobility group box (HMGB) protein, HMGB2, associated with the GFI1B promoter and investigate the function of HMGB2 in the regulation of Gfi-1B expression during erythroid and megakaryocytic differentiation. HMGB proteins, 1 of the 3 classes of high-mobility group (HMG) proteins, are abundant nonsine nuclear proteins that associate with chromatin. HMGB proteins consist of an acidic C-terminal tail of variable length and of 2 tandem HMG boxes, the A and B domains, which bind to the minor groove of DNA. It has been proposed that HMGB proteins can act as architectural facilitators in the assembly of nucleoprotein complexes by bending DNA.18,19 In addition, HMGB proteins were also shown to interact with transcription factors such as p53,20 HoxD9,21 Oct-1/2,22 and NF-Y.23 These interactions eventually lead to the recruitment of HMGB to specific sites of the genome where it locally modulates the association of transcription factors to their cognate DNA-binding sites.

We found that Gfi-1B and HMGB2 follow the same kinetics of expression during erythroid differentiation. HMGB2 binds in vivo to the GFI1B promoter and up-regulates its activity by increasing Oct-1 and, to a lesser extent, GATA-1 and NF-Y binding. Finally, we show that knockdown of HMGB2 in CD34+ cells severely impedes their erythroid and megakaryocytic potential.

Methods

Cell culture

Human UT-7 cells were maintained in α-minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine and 2 U/mL erythropoietin (EPO). Human umbilical cord blood


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samples were collected from normal full-term deliveries, after informed consent of the mothers according to the approved institutional guidelines of Assistance Publique–Hôpitaux de Paris (AP-HP) following the Declaration of Helsinki. After isolation of mononuclear cells by density gradient separation, CD34+ cells were purified using magnetic bead separation (StemCell Technologies). CD34+ cells were used immediately or after storage in liquid nitrogen. CD34+ cells were maintained for 5 days in serum-free StemSpan medium (StemCell Technologies) supplemented with 25 ng/mL stem cell factor (SCF), 10 ng/mL interleukin-3 (IL-3), 10−6 M dexamethasone, and 2 μM EPO. Then, cells were induced to differentiate for 5 to 6 days in StemSpan medium supplemented with 25 ng/mL SCF and 2 μM EPO. Recombinant human EPO was a gift from Dr. M. Brandt (Roche Molecular Biochemicals). Ours cytokines were purchased from Promocell Biosience Alive GMBH.

**Cell transduction**

Five pLKO.1 lentiviral shRNAs against human HMGB2 (from Open Biosystems) were tested for their ability to deplete HMGB2 protein in erythroid cells. Two of 5 were very efficient at knocking down HMGB2. These 2 lentiviral vectors had the same effect on erythroid differentiation and were used indifferently. CD34+ cells were infected twice (D1 and D2 during the amplification step) with lentiviral vectors and, 48 hours after infection, puromycin (1 μg/mL) was added to the medium. UT-7 cells were infected only once and selected with puromycin. The effects of the shRNA on HMGB2 protein level was tested 48 hours after the beginning of the puromycin selection by Western blot. As control vectors we used a nontargeting scramble shRNA with a green fluorescent protein sequence or a vector containing only the puromycin-resistant gene. The 2 control vectors gave the same results. We showed only results with puromycin-resistant vector.

**Colony-forming unit assays for cell progenitor quantification**

For myeloid colony assays, CD34+ were plated in duplicate in Methocult H4100 methylcellulose medium (StemCell Technologies) supplemented with 125 ng/mL SCF, 50 ng/mL IL-3, 50 ng/mL IL-6, and 2 μL/mL EPO for 6 (erythroid colony-forming unit [CFU-E]) or 10 to 12 (erythroid bursting-forming unit [BFU-E]) days. Colonies were scored under microscope. To evaluate the differentiation status of the cells, 3 to 5 colonies were collected and pooled to cytospin and stained with May-Grünwald-Giemsa. For megakaryocytic progenitors, CD34+ cells were plated in H4230 Megacult-C medium (StemCell Technologies) containing 50 ng/mL recombinant human thrombopoietin (TPO), 10 ng/mL IL-6 and 10 ng/mL IL-3 for 10 to 12 days and then, dehydrated, fixed, stained with glycoprotein Ib/IIa antibodies, and revealed by alkaline phosphatase reaction using a staining detection kit (H-4962; StemCell Technologies). Colony score was also made under microscope.

**Plasmid constructs and site-directed mutagenesis**

The human wild-type GF11B erythroid-specific promoter (−145/+19)–luciferase construct was a gift from Dr. Chang (Taipei, Taiwan). Mutations on the GF11B promoter were generated using QuikChange Site-directed Mutagenesis (Stratagene). G6f-1/G6f-1-binding sites (AATC at −59/−56, −54/−51 and −47/−44 within site 2) that were also GATA-binding sites (GATT reverse orientation) were simultaneously mutated into GTGC. GATA-binding site (GATA at −132/−129 within site 1) was mutated into GACC. AAAT sites (Oct-1 homeodomain–binding site) at −125/−122 (site 1) and −63/−60 (site 2) were mutated simultaneously or independently into ACGG.

**Luciferase assays**

HeLa cells were transiently transfected using Lipofectamine 2000 (Invitrogen). The total amount of DNA in each transfection was kept constant at 625 ng (250 ng of promoter-luciferase construct and 125 ng of each expression plasmid) and 10 ng of internal control plasmid (pRL-TK; Promega). Expression plasmid encoding GATA-1 was described and constructed by Kadi et al.34 The expression plasmid encoding Oct-1 was already described35 and the expression plasmids encoding NF-YA were from M. L. Vignais (Montpellier, France) and R. Mantovani (Milan, Italy). The expression plasmid encoding HMGB2 was generated by polymerase chain reaction (PCR) from pEGP-N1-HMGB226 using primers 5′-GGACACATTGGTTAAGGAGA-3′ and 5′-TCTTTCATCTTCAT-CCTTCTCCTC-3′. The PCR product was inserted into pCDNA3.1/V5-His TOPO. This plasmid pCDNA3.1/V5-His-HMGB2 encodes human HMGB2 fused to the V5 and 6His Tag. After 24 to 30 hours, firefly luciferase activity was measured according to the manufacturer’s instructions (Dual Luciferase Assay System; Promega), and individual transfections were normalized by measurement of Renilla luciferase activity (pRL-TK; Promega) and pGL2-luciferase activity.

**Nuclear extract preparation and biotin-streptavidin oligonucleotide pull-down assays**

For nuclear extract preparation, cells were washed once with PBS and incubated for 10 minutes at 4°C in buffer A (10 mM N2-2-hydroxyethylpipera-

**Western blot analysis and antibodies**

Samples were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane (Schleicher & Schuell). Filters were blocked overnight in 5% skimmed milk Tris-buffered saline (TBS)–0.05% Tween 20 and incubated with the appropriate antibody. Membranes were washed 4 times in TBS-Tween 20 and incubated for 1 hour with the appropriate peroxidase-conjugated secondary antibody. The primary antibodies used were as follows: GATA-1 N1 (sc-266; Santa Cruz Biotechnology); HMGB2 (556529; BD PharMingen); Oct-1 C-21 (sc-232; Santa Cruz Biotechnology); NF-YA (sc-10779; Santa Cruz Biotechnology); and β-actin (A5451; Sigma). Senata-G6-1B was prepared in the laboratory.17 The horseradish peroxidase–conjugated secondary antibodies were as follows: anti–rat (sc-2006; Santa Cruz Biotechnology), anti–rabbit (7074; Cell Signaling) and anti–mouse (7076; Cell Signaling).

**ChIP assays**

Cells were fixed with 1% formaldehyde for 10 minutes at room temperature before termination with 0.125M glycine. Cells were then lysed in chromatin immunoprecipitation (ChIP) buffer (1% SDS, 10mM ethylenediaminetetraacetic acid, and 50mM Tris-HCl, pH 8.1) and cross-linked chromatin was sonicated to obtain DNA fragments of 300 to 800 bp. Immunoprecipitations were performed following the Upstate protocol.17 Antibodies against HMGB2, NF-YA, and Oct-1 used for ChIP assays were the same as for Western blot. GATA-1 ChIP was performed with the antibody GATA-1 C20 (sc-1233; Santa Cruz Biotechnology). For analysis of histone modifications, acetyl Lys9/14-H3 (06-599; Upstate), dimethyl Lys4-H3 (07-030; Upstate), and trimethyl Lys27-H3 (07-449; Upstate) were used. The corresponding normal rabbit, goat, or mouse immunoglobulins G (IgGs; Santa Cruz Biotechnology) were used as control. The immunoprecipitated DNA was used for PCR with the following thermal cycling program: 95°C for 5 minutes, 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 45 seconds at 72°C, followed by a 5-minute extension time at 72°C. The GF11B promoter sequence was amplified with the following primers: 5′-GAATTCAGAATTCGGATCC-3′ (sense) and 5′-GATCCGCGCCTCCGTTCGTTTC-3′ (antisense) and the β-microglobulin promoter, used as
control, with the following primers: 5'-CCAGTCTTAGCATGCGCTTCTGAC-3' (sense) and 5'-CAAGCCAGCGACCGACGT-3' (antisense).

**Mass spectrometry**

Proteins bound on oligonucleotides were separated by SDS-PAGE on a 10% polyacrylamide gel. After Coomassie staining, the protein band with a 25-kDa apparent molecular weight was cut and analyzed by mass spectrometry. Extracted peptides were analyzed by mass spectrometry using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager DEPro; Applied Biosystems). Monoisotopic mass list was used to search the Swiss-Prot database for human proteins using the Mascot search engine. Schematic representation of the GFI1B promoter. Two specific regulatory regions were indicated "site 1" (~138 to ~116) and "site 2" (~69 to ~37). The 2 putative Oct-1-binding sites identified in this study and the NF-Y-binding site were indicated. (D) GATA-1, Oct-1, NF-Y, and HMGB2 association with oligonucleotides corresponding to the GFI1B promoter. Oligo pull-down assays were performed with cell lysates from differentiated erythroid cells harvested 3 days after induction of erythroid differentiation (E3) of CD34+ cells. Cell lysates were immunoprecipitated with increasing amount of oligonucleotides representing wild-type (indicated above the lanes by black wedges) or mutated site 1 or site 2 of the GFI1B promoter. Proteins bound to the DNA template were separated by SDS-PAGE and analyzed by Western blotting using antibodies against GATA-1, Oct-1, NF-YA, or HMGB2. The results shown on this figure are representative of 4 experiments.

**Results**

**HMGB2 is associated with GFI1B promoter oligonucleotides in vitro**

We have shown that Gfi-1B remains highly expressed all along erythroid differentiation. To identify transcriptional regulators other than GATA proteins responsible for sustained GFI1B promoter activity in erythroid cells, we performed oligo pull-down experiments using oligonucleotides corresponding to the described transcriptionally active part of the GFI1B promoter (Figure 1A) followed by mass-spectroscopy analysis. We thereby identified HMGB2 as a major GFI1B promoter-binding protein. Analysis using the Mascot search program showed that 12 peptides matched with HMGB2 sequence (Figure 1B and supplemental Figure 1, available on the Blood website; see the Supplemental Materials link at the top of the online article). HMGB2 binding increased when enhanced amounts of oligonucleotides were used (Figure 1A). We thus investigated the function of HMGB2 in the regulation of Gfi-1B expression during erythroid differentiation.

Erythroid-specific Gfi-1B expression relies on 2 main sites on its promoter that are both targeted by the GATA-1 transcription activator (sites 1 and 2) and separated by an NF-Y–binding site (Figure 1C). Interestingly, sequence analysis showed the presence of 2 Oct putative binding sequences at both of these...
sites (Figure 1C). Indeed, HMGB2 was described as binding to DNA without any sequence specificity but potentiating the activity of other transcription factors such as Oct-1\(^{22}\) and NF-Y.\(^{23}\) We thus investigated whether Oct-1, NF-YA, and HMGB2 associated with sites 1 and 2 at the \textit{GFI1B} promoter in primary erythroid cells. CD34\(^+\) cells were isolated from cord blood and amplified in a 2-phase culture system: a 5-day amplification step (D1-D5) in the presence of SCF, IL-3, EPO, and dexamethasone followed by a 5-day differentiation step (E0-E5) in the presence of SCF and EPO. DNA affinity precipitation experiments were carried out using differentiated erythroid cells harvested 4 days after induction of erythroid differentiation (E4) and an increasing amount of biotinylated oligonucleotides that contain \textit{GFI1B} promoter site 1 or 2 (Figure 1D). We found that GATA-1 associated with similar affinities to site 1 and 2. By contrast, Oct-1 and NF-YA associated to site 1 with a higher affinity compared with site 2. HMGB2 bound similarly to both oligonucleotides (Figure 1D). Mutations on the GATA-binding sites contained within the site 1 and mutations on the 3 Gfi-1/Gfi-1B contained within the site 2 of the \textit{GFI1B} promoter abolished the association of GATA-1, NF-YA, and Oct-1. However, these mutations did not modify the binding of HMGB2 to both sites, confirming that GATA-1, NF-YA, and Oct-1 bindings are sequence specific, whereas HMGB2 binding is not. Furthermore, this result also shows that HMGB2 binds to sites 1 and 2 in the absence of Oct-1, NF-YA, or GATA-1. We conclude that HMGB2 together with GATA-1, NF-Y, and Oct-1 bind as a protein complex to the \textit{GFI1B} promoter in erythroid cells. The high affinity of HMGB2 for DNA was already described.\(^{29}\) However, we studied the binding of HMGB2 to DNA sequences of different promoters or intergenic regions by oligo pull-down and ChIP assays and confirmed that HMGB2 binds to DNA with no sequence specificity (supplementary Figure 2C).

**HMGB2 potentiates GATA-1–dependent \textit{GFI1B} transcription in the presence of Oct-1**

Having shown that HMGB2 binds to the \textit{GFI1B} promoter, we investigated whether it modifies its transcriptional activity by performing luciferase gene reporter experiments. Transfection of the \textit{GFI1B} promoter construct into HeLa cells together with a GATA-1 expression plasmid increased its activity by 4-fold, as previously described.\(^{13}\) This effect was amplified when cotransfecting both GATA-1 and Oct-1 (358 \(\pm\) 33 vs 503 \(\pm\) 130, \(P < .05\)), whereas transfection of Oct-1 alone or in association with HMGB2 did not have any impact on \textit{GFI1B} promoter activity. Interestingly, whereas cotransfection of HMGB2 with GATA-1 slightly affected \textit{GFI1B} transcription, cotransfection of HMGB2 together with GATA-1 and Oct-1 strongly increased the \textit{GFI1B} promoter activity (362 \(\pm\) 59 vs 895 \(\pm\) 144, \(P < .001\); Figure 2A). By contrast, cotransfection of HMGB2 together with GATA-1 and NF-YA did not significantly increase the \textit{GFI1B} promoter activity (GATA + HMGB2: 503 \(\pm\) 129 vs GATA + HMGB2 + NF-YA: 546 \(\pm\) 132). Thus, in vivo transient transfection experiments, HMGB2 increases the up-regulation of GATA-1–dependent \textit{GFI1B} transcription by Oct-1 but not by NF-Y.

To confirm the specificity of the GATA-1 plus Oct-1–mediated \textit{GFI1B} transactivation by HMGB2, we performed equivalent experiments in cells knocked down for HMGB2. Both endogenous and exogenous HMGB2 were efficiently depleted when transducing the cells with lentiviral vectors expressing HMGB2-specific shRNA (data not shown). Importantly, the potentiation of \textit{GFI1B} promoter activity observed when cotransfecting HMGB2 with GATA-1 and Oct-1 factors was lost upon HMGB2 depletion (Figure 2A). Therefore, HMGB2 strongly enhances the activation of \textit{GFI1B} transcription by GATA-1 when Oct-1 is present.

To identify the regions within the \textit{GFI1B} promoter required for potentiation of \textit{GFI1B} transcription by HMGB2, we mutated the GATA-binding sites (site 1 and 2) as well as the 2 putative Oct-binding sites (sites 1 and 2) at the \textit{GFI1B} promoter. Because simultaneous mutations of the 3 AATC sites at the site 2 of the \textit{GFI1B} promoter abolished completely the GATA-1–mediated transactivation of the \textit{GFI1B} promoter (data not shown and Huang et al\(^{15}\)), it is irrelevant to analyze the effect of HMGB2 on GATA-mediated transactivation of this mutated promoter. Mutation of the GATA site (site 1) abolished the potentiation effect of HMGB2 on \textit{GFI1B} promoter transactivation (Figure 2B). In contrast, mutation of only 1 of the 2 Oct-binding sites had slightly significant (930 \(\pm\) 180 vs 1580 \(\pm\) 260, \(P < .03\)) or no significant (1285 \(\pm\) 210 vs 1580 \(\pm\) 260, \(P = .2\)) effect. Strikingly, HMGB2 did not modify \textit{GFI1B} promoter activity when both Oct-binding sites were mutated. This result is in full agreement with our finding that Oct-1 is indeed required for the potentiation of GATA-1–dependent transactivation by HMGB2. We therefore conclude that HMGB2 potentiates the ability of Oct-1 to enhance GATA-1–dependent transcription of \textit{GFI1B}.

**HMGB2 promotes the binding of Oct-1 to the \textit{GFI1B} promoter in human erythroid cells**

So far, we have shown that HMGB2 increases the up-regulation of GATA-1–dependent \textit{GFI1B} transcription by Oct-1. We thus raised the hypothesis that HMGB2 increases the binding of Oct-1 to the \textit{GFI1B} promoter in erythroid cells. To test this hypothesis, we compared the ability of Oct-1 to associate with the oligonucleotide corresponding to the \textit{GFI1B} promoter site 1 and site 2 in the presence or in the absence of HMGB2. Cellular extracts from UT-7 cells transduced with a shControl or with a shHMGB2 were used for these experiments (Figure 3A). Western blot analysis of bound proteins showed that Oct-1 and NF-Y associate with site 1 of the \textit{GFI1B} promoter. Oct-1—and not NF-YA—binding to site 2 was observed only during overexposure (data not shown). Oct-1 and NF-YA did not bind to the \textit{GFI1B} promoter when mutating the GATA-binding site (Figure 3A). These results confirm our previous results described in Figure 1D showing that GATA is required for Oct-1 and NF-Y association. Interestingly, the association of Oct-1—but not the association of NF-YA—with site 1 slightly decreased in the absence of HMGB2. Thus, HMGB2 increases the binding of Oct-1 to the promoter of the \textit{GFI1B} gene in vitro.

We next investigated whether HMGB2 increases the binding of Oct-1 to the \textit{GFI1B} promoter in vivo. Chromatin immunoprecipitation experiments were performed in erythroid UT-7 cells knocked down or not for HMGB2. Chromatin was precipitated with HMGB2-, GATA-1–, NF-YA–, or Oct-1–specific antibodies and precipitated DNA was amplified using primers specific or not for the \textit{GFI1B} promoter. As expected from its ability to bind DNA nonspecifically, HMGB2 bound to the \(\beta_{2m}\) promoter (used as control) as well as to the \textit{GFI1B} promoter (Figure 3B). In contrast, GATA-1, NF-Y, and Oct-1 associated with the \textit{GFI1B} promoter exclusively. Remarkably, cells lacking HMGB2 showed decreased binding of Oct-1 to the \textit{GFI1B} promoter (80% of decrease). A smaller but significant decrease in GATA-1 and NF-Y binding was observed (37% and 40% of decrease, respectively). Thus, HMGB2 is detected at the \textit{GFI1B} promoter in erythroid cells where
it strongly enhances the binding of Oct-1 and, to a lesser extent, of NF-Y and GATA-1 transcription factors.

**HMGB2 is required for sustained Gfi-1B expression during erythroid differentiation**

Our results show that HMGB2 increases the binding of Oct-1, NF-Y, and GATA-1 to the GFI1B promoter in vitro. Gfi-1B luciferase reporter construct was transfected into HeLa cells together with GATA-1, Oct-1, NF-Y, or HMGB2 expression vectors. Thirty hours after transfection, luciferase activity was evaluated. Individual transfection was normalized by measurement of Renilla luciferase activity (pRL-TK; Promega) and pGL2-luciferase activity. Experiments were performed with shControl-transduced (shControl) or shHMGB2-transduced (shHMGB2) cells. Results are means ± SD of 4 independent experiments; *P < .05 and ***P < .001. (B) Effects of mutations at GATA or Oct-1–putative binding sites at the GFI1B promoter. The construct bearing the GFI1B promoter sequence was mutated at the putative GATA-binding (site 1) or Oct-1–binding (sites 1 and 2) sites (mutations were indicated at the bottom of the figure by a cross on the schematic representation of the GFI1B promoter). Wild-type or mutated reporter constructs were transfected into HeLa cells together with GATA-1 alone or with GATA-1, Oct-1, and HMGB2 expression vectors. Luciferase activity was measured 30 hours after transfection. Results are means ± SD of 4 independent experiments; *P < .02 and **P < .003.

Figure 2. HMGB2 stimulates GFI1B promoter activity through GATA-1 and Oct-1 binding to the GFI1B promoter in vitro. (A) Effects of HMGB2 on GFI1B promoter activity. Gfi-1B luciferase reporter construct was transfected into HeLa cells together with GATA-1, Oct-1, NF-Y, or HMGB2 expression vectors. Thirty hours after transfection, luciferase activity was evaluated. Individual transfection was normalized by measurement of Renilla luciferase activity (pRL-TK; Promega) and pGL2-luciferase activity. Experiments were performed with shControl-transduced (shControl) or shHMGB2-transduced (shHMGB2) cells. Results are means ± SD of 4 independent experiments; *P < .05 and ***P < .001. (B) Effects of mutations at GATA or Oct-1–putative binding sites at the GFI1B promoter. The construct bearing the GFI1B promoter sequence was mutated at the putative GATA-binding (site 1) or Oct-1–binding (sites 1 and 2) sites (mutations were indicated at the bottom of the figure by a cross on the schematic representation of the GFI1B promoter). Wild-type or mutated reporter constructs were transfected into HeLa cells together with GATA-1 alone or with GATA-1, Oct-1, and HMGB2 expression vectors. Luciferase activity was measured 30 hours after transfection. Results are means ± SD of 4 independent experiments; *P < .02 and **P < .003.
HMGB2 is required for sustained Gfi-1B expression but not that, although HMGB2 does not display sequence specificity for physiologic specificity of the HMGB2 binding on another promoter, the 2D). The 2 efficient shRNAs used to deplete HMGB2 had the same effect on Gfi-1B expression and did not modify HMGB1 expression (supplemental Figure 4), indicating that the shRNA used was indeed specific to HMGB2.

Furthermore, having shown that HMGB2 has no sequence specificity and that HMGB2 is necessary for sustained Gfi-1B expression during erythroid differentiation, we studied the physiologic specificity of the HMGB2 binding on another promoter, the GATA-1 promoter. We found that HMGB2 bound to the GATA-1 promoter, but did not enhance the GATA-1 transcription (supplemental Figure 3A-B). However, we observed a decrease in GATA-1 expression in the absence of HMGB2. This decrease was probably due to the absence of Gfi-1B that may stabilize the GATA-1 protein. Indeed, first, Gfi-1B depletion leads to the decrease of GATA-1 expression in erythroid cells (supplemental data 3C-D) and, second, it has been shown that Gfi-1 plays an important role in the stable differentiated erythroblast stages (Figure 3C-D). Furthermore, 82.2% (± 7.9%) of control cells were benzidine positive, whereas to E5; Figure 4C). Induction of Gfi-1B expression after induction of erythroid differentiation was significantly impaired in HMGB2-depleted cells (decrease of 60% at E5, Figure 4C). Inhibition of Gfi-1B expression in the absence of HMGB2 was not a consequence of chromatin status modification because the methylation of H3-K4 and H3-K27 and the acetylation of H3-K4/9 were not modified in HMGB2-depleted cell populations (Figure 4C). Induction of Gfi-1B expression after induction of erythroid differentiation was significantly impaired in HMGB2-depleted cell populations (Figure 5A). Furthermore, the BFU-E colonies that arose from shHMGB2-transduced cells were smaller than those generated from shControl-transduced cells (Figure 5A). Interestingly, HMGB2 and HMGB3 knockdown have opposite effect in immature erythroid progenitor cells.31 Importantly, no difference was observed in the number of granulomacrophagic colonies generated from the HMGB2 knocked-down population (Figure 5A), which does not rely on Gfi-1B for its differentiation. Thus, depletion of HMGB2 in immature progenitors profoundly impairs their erythroid development potential.

To study terminal erythroid differentiation, HMGB2-depleted CD34+ cells were plated in semisolid medium to study hematopoietic progenitors or in liquid culture to study terminal erythroid differentiation.

To define whether HMGB2 equally plays a role at early stages of differentiation, the puromycin-resistant cell populations were cultured in methyl cellulose in the presence of a cocktail of growth factors to allow erythroid and granulomacrophagic development or in Megacult medium in the presence of TPO to allow megakaryocytic differentiation. Significantly fewer BFU-E (35.4 vs 94.7 of 1000), CFU-E (56.4 vs 147.17 of 1000), and megakaryocytic (10 ± 1.4 vs 45.5 ± 0.7 of 1000) colonies were observed in HMGB2-depleted cell populations (Figure 5A). Furthermore, the BFU-E colonies that arose from shHMGB2-transduced cells were smaller than those generated from shControl-transduced cells (Figure 5A). Interestingly, HMGB2 and HMGB3 knockdown have opposite effect in immature erythroid progenitor cells.31 Importantly, no difference was observed in the number of granulomacrophagic colonies generated from the HMGB2 knocked-down population (Figure 5A), which does not rely on Gfi-1B for its differentiation. Thus, depletion of HMGB2 in immature progenitors profoundly impairs their erythroid development potential.

To study terminal erythroid differentiation, HMGB2-depleted CD34+ cells were plated in liquid culture in the presence of EPO. May-Grunwald-Giemsa staining and cytfluorimetry analysis showed that the cells remained immature (CD34+) with no phenotypical changes or morphologic features during the amplification step (D0 to D5-E0); in the presence or absence of HMGB2, the cells stayed immature and were CD34+ (data not shown). However, we observed a defect in terminal erythroid maturation in cells lacking HMGB2: at 5 days after induction of erythroid differentiation, 56.2% (± 15.0%) of control cells were differentiated erythroblasts (polychromatophilic or acidophilic erythroblasts), whereas only 18.0% (± 9.5%) of HMGB2-depleted cells had reached the differentiated erythroblast stages (Figure 5C-D). Furthermore, to E5; Figure 4C). Induction of Gfi-1B expression after induction of erythroid differentiation was significantly impaired in HMGB2-depleted cells (decrease of 60% at E5, Figure 4C). Inhibition of Gfi-1B expression in the absence of HMGB2 was not a consequence of chromatin status modification because the methylation of H3-K4 and H3-K27 and the acetylation of H3-K4/9 were not modified in HMGB2-depleted cell populations (Figure 4C). Induction of Gfi-1B expression after induction of erythroid differentiation was significantly impaired in HMGB2-depleted cell populations (Figure 5A). Furthermore, the BFU-E colonies that arose from shHMGB2-transduced cells were smaller than those generated from shControl-transduced cells (Figure 5A). Interestingly, HMGB2 and HMGB3 knockdown have opposite effect in immature erythroid progenitor cells.31 Importantly, no difference was observed in the number of granulomacrophagic colonies generated from the HMGB2 knocked-down population (Figure 5A), which does not rely on Gfi-1B for its differentiation. Thus, depletion of HMGB2 in immature progenitors profoundly impairs their erythroid development potential.

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Table 1. Cell numbers (×10^3) from the 2-phase cultures

<table>
<thead>
<tr>
<th></th>
<th>Amplification step</th>
<th>Differentiation step</th>
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<tbody>
<tr>
<td></td>
<td>D3</td>
<td>D5</td>
</tr>
<tr>
<td>shControl</td>
<td>100</td>
<td>215 ± 1.5</td>
</tr>
<tr>
<td>shHMGB2</td>
<td>100</td>
<td>270 ± 5.0</td>
</tr>
</tbody>
</table>

CD34+ cells were plated in the presence of SCF, IL-3, EPO, and dexamethasone then infected with shControl or shHMGB2 at 48 (D1) and 72 (D2) hours after. Twenty-four hours later (D3) or the day of the induction of erythroid differentiation (E0), cells were plated at 100 × 10^3 cells per well and counted in the presence of trypan blue 2 days after.

D indicates day; E, day after EPO.

Discussion

Erythropoesis involves coordinated expression of regulatory and structural proteins acting in concert to direct the development of immature progenitors into differentiated erythrocytes. Gfi-1B is an important erythroid-specific Gfi-family transcriptional repressor. Inactivation of GFI1B in mice leads to failure of mature erythroid cell production, and its forced expression in immature progenitors induces erythroid differentiation in the absence of EPO. We have previously shown that Gfi-1B expression increases and remains high all along erythroid differentiation. We here identify HMGB2 as a key regulator of GFI1B transcription in erythroid cells. HMGB2 associates with the GFI1B promoter and increases GFI1B transcription by enhancing the binding of Oct-1 and, to a lesser extent, of NF-Y to the GFI1B promoter. Oct-1 in turns potentiates the transactivation of the GFI1B gene by GATA-1. HMGB2 is therefore essential to maintain GFI1B transcription in maturing erythroid cells and to achieve erythropoiesis.

Although HMGB2 binds to DNA with no sequence specificity, it controls the expression of only a limited number of genes. Accordingly, we here show that although HMGB2 enhances the transactivation of the GFI1B promoter by GATA-1 and Oct-1, it binds to but does not affect the activity of the GATA-1 promoter. This finding suggests that the architecture of the GFI1B promoter might be essential for HMGB2 function. Interestingly, the GFI1B promoter—but not the GATA-1 promoter—contains tandem sites at a distance of 60 bp, each of which includes both a GATA- and an Oct-binding sequence.

A model wherein HMGB2 promotes DNA bending at the promoter—contains 2 tandem sites at a distance of 60 bp, each of which includes both a GATA- and an Oct-binding sequence. 

CD34+ cells were plated in the presence of SCF, IL-3, EPO, and dexamethasone then infected with shControl or shHMGB2 at 48 (D1) and 72 (D2) hours after. Twenty-four hours later (D3) or the day of the induction of erythroid differentiation (D5) cells were plated at 100 × 10^3 cells per well and counted in the presence of trypan blue 2 days after.

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A model wherein HMGB2 promotes DNA bending at the GFI1B promoter and thereby facilitates the formation of a DNA loop that binds both GATA-1 and Oct-1 can thus be envisioned. Indeed, it has been shown using atomic force microscopy that the association of HMGB proteins to double-strand DNA may participate in this process.

However, the precise mechanism of loop formation has not been elucidated. Our work suggests that HMGB proteins may participate in this process.

Furthermore, we propose that HMGB2 potentiates GATA-1–dependent transactivation of the GFI1B promoter in the presence of

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In this scenario, HMGB2 bends DNA at the GFI1B promoter, facilitating the binding of Oct-1 and its association to GATA-1 for transactivation. A nonexclusive alternative hypothesis proposes that HMGB2 might form a complex with Oct-1 or Oct-1 and GATA-1 before DNA binding, thereby facilitating their association to their target sequences at the GFI1B promoter. Accordingly, it has been shown that the stimulation of the ORF50 promoter by HMGB1 relies on an Oct-1 binding site located at the promoter, strengthening the importance of the complex formation before DNA. This might be because HMGB2 requires association with Oct-1 to be efficiently recruited to its target locus. However, our results show that HMGB2 is found at the GFI1B promoter in the absence of Oct-1 and GATA-1, favoring the first model. Whether HMGB2 modifies the 3-dimensional structure of the GFI1B promoter shall now be investigated.

In conclusion, our results show that HMGB2 is an important regulator of GFI1B transcription through its cooperation with the transcription factors GATA-1 and Oct-1 and is therefore essential to erythroid and megakaryocytic development. Interestingly, inactivation of GFI1B, GATA-1, and Oct-1 leads to a similar phenotype (ie, embryonic lethality due to lack of differentiated erythroid cell production). Our findings further suggest that HMGB proteins might help remodeling the structure of DNA during both erythropoiesis and megakaryopoiesis and thereby modify the action of the specific transcription factors involved. Accordingly, it has been described that HMGA1 binds to and down-regulates GATA-1 promoter activity and would thereby play a prime role in lymphohematopoietic differentiation from embryonic stem cells. Together with ours, these results suggest that the various members of the HMGB protein family might play a key role in the regulation of hematopoiesis.
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Authorship

Contribution: B.L. and V.R.-H. performed research, analyzed and interpreted data, and wrote the paper; V.M. and I.D.-F. contributed to research strategy and interpreted data; P.M. performed mass spectrometry analysis and interpreted data; and D.D. coordinated and performed the research.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Dominique Duménil, U567, CNRS (UMR 8104), Institut Cochin, Batiment G Roussy, 27 rue du Faubourg Saint Jacques, 75014, Paris, France; e-mail: dominique.dumenil@inserm.fr.

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High-mobility group protein HMGB2 regulates human erythroid differentiation through trans-activation of GFI1B transcription

Benoît Laurent, Voahangy Randrianarison-Huetz, Vincent Maréchal, Patrick Mayeux, Isabella Dusantér-Fourt and Dominique Duménil

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