The DNA binding factor Hmg20b is a repressor of erythroid differentiation

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ABSTRACT

Background
In erythroblasts, the CoREST repressor complex is recruited to target promoters by the transcription factor Gfi1b, leading to repression of genes mainly involved in erythroid differentiation. Hmg20b is a subunit of CoREST, but its role in erythropoiesis has not yet been established.

Design and Methods
To study the role of Hmg20b in erythropoiesis, we performed knockdown experiments in a differentiation-competent mouse fetal liver cell line, and in primary mouse fetal liver cells. The effects on globin gene expression were determined. We used microarrays to investigate global gene expression changes induced by Hmg20b knockdown. Functional analysis was carried out on Hrasls3, an Hmg20b target gene.

Results
We show that Hmg20b depletion induces spontaneous differentiation. To identify the target genes of Hmg20b, microarray analysis was performed on Hmg20b knockdown cells and controls. In line with its association to the CoREST complex, we found that 85% (527 out of 620) of the deregulated genes are up-regulated when Hmg20b levels are reduced. Among the few down-regulated genes was Gfi1b, a known repressor of erythroid differentiation. Among the consistently up-regulated targets were embryonic β-like globins and the phospholipase HRAS-like suppressor 3 (Hrasls3). We show that Hrasls3 expression is induced during erythroid differentiation and that knockdown of Hrasls3 inhibits terminal differentiation of proerythroblasts.

Conclusions
We conclude that Hmg20b acts as an inhibitor of erythroid differentiation, through the down-regulation of genes involved in differentiation such as Hrasls3, and activation of repressors of differentiation such as Gfi1b. In addition, Hmg20b suppresses embryonic β-like globins.

Key words: Hmg20b, CoREST, erythropoiesis, Gfi1b, Hrasls3, embryonic β-like globins.

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The online version of this article has a Supplementary Appendix.

Introduction

Erythropoiesis is a multi-step developmental process that commences with pluripotent hematopoietic stem cells in the bone marrow and culminates in the release of enucleated and hemoglobinized reticulocytes in the circulation.[1] Erythroid progenitors undergo massive expansion and pass through several differentiation steps including the morphologically recognizable proerythroblast, basophilic and orthochromatic erythroblast stages.[2] These final steps involve a series of three to five differentiation-specific cell divisions before the cells arrest in G1, enucleate and undergo terminal maturation to erythrocytes.[3]

Studies on erythropoiesis have revealed the basics of tissue-specific gene regulation in which transcription factors and chromatin modifying complexes play a major role.[4] CoREST (corepressor of repressor element-1-silencing transcription factor) is a chromatin-modifying complex that was first described as a regulator of neuronal gene expression, playing a crucial role in neuronal differentiation.[5] In these cells, the CoREST complex is recruited by REST (RE1-silencing transcription factor) that recognizes the 21-nucleotide RE-1 sequence in the promoter of more than 1,700 different target genes in human and mouse.[6] The CoREST complex is composed of multiple subunits, including RCOR1, lysine-specific demethylase I (LSD1), histone deacetylases 1/2 (HDACs 1/2) and two very similar proteins from the High Mobility Group (HMG) family, HMG20A and HMG20B.[7] In erythroblasts, the transcriptional regulator Gfi1b recruits the CoREST complex to the majority of its target gene promoters *in vivo*. Similar to neuronal cells, inhibition of CoREST disturbs differentiation of erythroid progenitors, as well as megakaryocytic and granulocytic precursors.[8]

Several studies have shown the function of various CoREST complex subunits in erythropoiesis,[9-10] but the role of Hmg20a and Hmg20b in erythropoiesis has not so far been established. It is believed that Hmg20b-containing CoREST complexes mediate chromatin remodeling and repression of specific genes involved in neuronal differentiation through modulation of chromatin structure.[11-12] In erythroblasts, the transcriptional regulator Gfi1b recognizes TAAATCAC(A/T)GCA[13-14] and recruits the CoREST complex to the majority of its target gene promoters *in vivo*. Inhibition of Gfi1b and the CoREST subunit Lsd1 disturbs differentiation of erythroid progenitors, as well as megakaryocytic and granulocytic precursors.[8, 15]

Hmg20b is an HMG domain protein with the ability of non-specific DNA binding. It is expressed in various tissues with relatively high levels in the brain,[16] and is classified in a new group of HMG proteins due to the presence of a kinesin-like coiled-coil domain with no known function.[17] The only known protein with relatively high homology to Hmg20b is BAF-57 (BRG1-associated factor 57).[16] BAF-57 is part of the SWI/SNF remodeling complex in association with the RCOR1 protein and plays a crucial role in cell proliferation and cell cycle progression.[18]

To investigate the role of Hmg20b during erythroid differentiation, we employed primary proerythoblasts from mouse fetal liver, as well as I/11 cells, a cell line derived from p53 knockout mouse fetal liver.[19] These cells can be expanded in the presence of erythropoietin (EPO), stem cell factor (SCF) and dexamethasone.[20] In the absence of SCF and dexamethasone, the majority of the cells hemoglobinize and enucleate within three days. This differentiation process requires the presence of EPO and transferrin.[21]

Here, we studied the role of Hmg20b protein in the balance of erythroid proliferation and differentiation by performing knockdown experiments. We used whole genome expression profiling to further investigate the role of Hmg20b in maintaining this balance, and we assessed the impact of Hmg20b knockdown on globin gene expression.

Design and Methods

Cell culture

I/11 erythroid progenitors and primary mouse fetal liver cells were cultured as described.[19] Primary mouse fetal liver cells were collected on embryonic day 12.5 and expanded for three days before lentiviral transduction according to an established protocol.[21] To induce differentiation, proliferating erythroblasts were washed in ice-cold PBS and reseeded at 1-1.5×10^6 cells/mL in StemPro (Invitrogen, Carlsbad, USA) supplemented with EPO (10U/ml, Janssen-Cilag BV, Tilburg, NL) and holo-transferrin (1mg/ml; SCIPAC Ltd, Sittingbourne, UK). Differentiating I/11 cells were maintained at 2-3×10^6 cells/mL. To determine the morphological changes, cells were cytospun and stained with histological dyes (Diff-Quick staining set; Medion Diagnostics International Inc., Miami, USA) and neutral benzidine.[22] To measure cell size distribution, a CASY instrument (Roche Innovatis AG, Bielefeld, Germany) was used. Hemoglobin assays were performed according to a published protocol.[23] All experiments involving mice were approved by the Erasmus MC Animal Ethics Committee.

Biotinylation and tagging, and protemics analysis

Bio-HA-HMG20B was cloned into a modified 5pRRLsin.sPPT.CMV.GFP.Wpre lentiviral vector.[24] BirA-expressing I/11 cells were transduced with Bio-HA-HMG20B and Bio-HA lentiviruses. The transduced cells were selected using puromycin (1 μg/mL) and were expanded in culture. Sample preparation and mass spectrometry analyses were carried out as previously described.[25]

Virus transduction

Short hairpin constructs were from the TRC library[26] (Sigma Aldrich, St. Louis, USA). For knockdown of Hmg20b, TRCN0000081665 (Hmg20b shRNA#1), and TRCN0000081664 (Hmg20b shRNA#2) were used. For Hrasls3 knockdown TRCN0000077660 (Hrasls3 shRNA#1), and TRCN0000077662 (Hrasls3 shRNA#2) were used. SHC002 was used as control. Lentiviruses were produced by transiently transfecting 293T cells with helper plasmids, the virus-containing supernatants were collected for three days and concentrated by ultracentrifugation.[27] Five days after transduction, the cells were collected and whole cell lysates prepared for Western blotting. RNA extraction was carried out for quantitative real-time PCR (QRT-PCR) analysis. For differentiation, cells at day 5 after transduction were washed twice with PBS and cultured in differentiation medium for four days.

Western blotting and antibodies

Whole cell extract was prepared by incubating the cells with RIPA buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 5mM EDTA) supplemented with complete protease inhibitors cocktail (Roche Diagnostics BV, Almere, NL). After 2 min centrifugation at 13 krpm, the supernatant was transferred to an equal volume of 2x

sample buffer, with a final concentration of 10^7 cells per mL. To assess Hmg20b knockdown, whole cell protein extracts of 3×10^5 cells were loaded on 10% SDS-PAGE and, after blotting on nitrocellulose membrane, incubated with Hmg20b antibody (WH0010362M1, Sigma-Aldrich) at a dilution of 1:1,000 in blocking buffer (PBS containing 1% (w/v) BSA). Secondary staining was performed using goat-anti-mouse/rabbit IR-Dye 680 or 800 antibodies in PBS with 5% (w/v) blotting grade non-fat dry milk powder (Bio-Rad Laboratories, Hercules, USA) and 0.05% (v/v) tween 20 (Roche Diagnostics).

QRT-PCR analysis and statistical analysis

RNA was extracted using TRI reagent (Sigma-Aldrich) in accordance with the protocol provided by the company. To synthesize cDNA, 2 μg of RNA was used with oligo dT, RNase OUT, SuperScript reverse transciptase (Invitrogen) in a total volume of 20 μL, and 0.3 μL of cDNA was used for further amplification by QRT-PCR. Amplification was performed with the primers listed in the *Online Supplementary Table S1* using Platinum Taq DNA polymerase (Invitrogen) and 40 cycles consisting of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. All statistical analyses including χ^2 tests, ANOVA and Bonferroni correction for globin expression analysis and Mann Whitney U test *P* value for ChIP experiments were performed using Stata 11.1 software (StataCorp, College Station, TX, USA).

Microarray analysis

RNA samples were extracted from biological triplicates using shRNA#1, control SHC002 and non-transduced I/11 cells three days after transduction. RNA samples were qualitatively controlled and microarray analysis was performed as described.[28-29] The differentially regulated genes between control SHC002 and non-transduced I/11 cells were excluded from the final comparison with Hmg20b knockdown I/11 cells. Microarray data have been deposited in the NCBI GEO database (GSE29169).

ChIP analysis

To perform chromatin immunoprecipitation (ChIP) 2×10^7 of either 1/11 or MEL cells were crosslinked with 1% formaldehyde for 10 min. The reaction was stopped with 0.125 M glycine at room temperature and the cells were washed two times with PBS. The cells were resuspended in sonication buffer (10 mM Tris pH 8, 1 mM EDTA and 0.5 mM EGTA) and sonicated with amplitude 7 for 30 cycles (10 sec on, 45 sec off, Soniprep150, MSE, UK).

After centrifugation (10 min at 13 krpm) the supernatants were incubated overnight with Hmg20b (14582-1-AP, Proteintech Group Inc, Chicago, USA) or Lsd1 (ab17721, Abcam, Cambridge, UK) antibodies and Protein A Agarose/Salmon Sperm DNA beads (Millipore, Billerica, USA). Washing and elution was performed according to the protocol provided by the manufacturer (Millipore).

Results

Hmg20b is part of the CoREST complex in I/11 cells

Hmg20b has been reported to be a subunit of two different complexes in HeLa cells, CoREST and BRCA2.[7] In mouse erythroleukemia (MEL) cell line, Hmg20b acts as part of the CoREST complex.[8] To identify Hmg20b interaction partners in growth factor-dependent erythroid progenitors we used I/11 cells, a cell line derived from p53 knockout mouse fetal liver.[19] In contrast to MEL cells, I/11 cells are dependent on EPO, SCF and dexamethasone for expansion, and can be induced to undergo terminal differentiation, including enucleation, upon withdrawl of SCF and dexamethasone.[21] Thus, these cells recapitulate normal erythropoiesis more closely than MEL cells. We used I/11 cells expressing the *E. coli* BirA biotin ligase to metabolically label a tagged version of Hmg20b (Bio-HA-HMG20B). We selected a cell population expressing Bio-HA-HMG20B at a level similar to that of the endogenous protein. This did not have any major effects on the proliferation of the cells (*Online Supplementary Figure S1A and B*). Protein extracts, streptavidin pull-down of Bio-HA-Hmg20b and associated proteins, and mass spectrometry analysis were performed as described.[25, 30-31] Streptavidin pull-down of Bio-HA-HMG20B followed by mass spectrometry analysis revealed that Hmg20b is part of the CoREST complex in proliferating and differentiating I/11 cells. Consistent with previous data,[8] we also identified the transcriptional repressor protein Gfi1b in the mass spectrometry data. Very few other interacting proteins were identified and we, therefore, conclude that CoREST is the major complex in which Hmg20b participates in I/11 cells (Figure 1A and B). We also studied the expression changes of Hmg20b and Gfi1b in differentiating I/11 cells. Western blot and QRT-PCR analyses showed a modest decrease in Hmg20b and Gfi1b expression during I-11 cell differentiation (Figure 1C-E).

Knockdown of Hmg20b induces spontaneous erythroid differentiation

To assess the role of Hmg20b in erythroid differentiation, we applied lentiviral-mediated knockdown of Hmg20b expression in I/11 cells. Western blot and QRT-PCR analysis confirmed that two independent shRNA constructs reduced Hmg20b expression to less than 20% of wild-type levels at day three after transduction (Figure 2A and B). Cell counting revealed a slower expansion of Hmg20b knockdown cells (Figure 2C). Differentiating erythroblasts go through three to five cell divisions before entering G1 arrest and terminal differentiation.[3] Hmg20b depletion resulted in G1 accumulation of I/11 cells grown in expansion medium, with concomitant decrease in proliferation rate (*Online Supplementary Figure S2*). In line with reduced proliferation, differentiating cells were clearly detected by CASY cell counting profiles at day 5 to day 7 after transduction (cells <10 μm; Figure 2D). Knockdown by shRNA #1 was more efficient than that obtained with shRNA #2 with the latter resulting in the most efficient I/11 cell differentiation. This suggests that a low level of Hmg20b protein augments completion of the differentiation and enucleation process, possibly because this allows completion of the 3-5 cell divisions required for normal differentiation.

To study the effects of Hmg20b knockdown in more depth, cytospins were prepared at different time points and stained with histological dyes and neutral benzidine.[22] The results show that by day 7 after transduction, Hmg20b knockdown cells displayed increased differentiation with more than 50% hemoglobinized and enucleated cells, while only 10% of control cells were observed at these late stages of differentiation (Figure 2D). Direct measurement of hemoglobin content showed elevated hemoglobin levels in Hmg20b-depleted cells, further supporting the requirement of Hmg20b for renewal divisions of proerythroblasts.

To confirm that the spontaneous differentiation upon

A

Proliferating I/11-BirA cells		
Hmg20b-associated proteins		
Score	Protein ID	Protein
673	IPI00323315	**Hmg20b**
640	IPI00453837	Lsd1
617	IPI00396700	Gse1
415	IPI00265217	Rcor1
276	IPI00753751	Bhc80
192	IPI00120110	Hmg20a
150	IPI00114232	Hdac1
78	IPI00116423	Gfi1b

B

Induced I/11-BirA cells		
Hmg20b-associated proteins		
Score	Protein ID	Protein
833	IPI00396700	Gse1
817	IPI00453837	Lsd1
719	IPI00323315	**Hmg20b**
470	IPI00265217	Rcor1
270	IPI00114232	Hdac1
207	IPI00120110	Hmg20a
122	IPI00116423	Gfi1b
55	IPI00124469	Bhc80

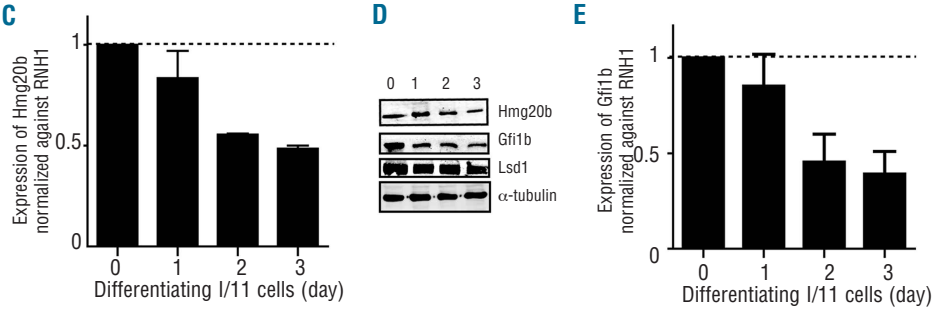

Figure 1. Bio-HA-HMG20B pull-down and mass spectrometry analysis. (A-B) Hmg20b interacting proteins in (A) proliferating and (B) differentiating I/11 cells. CoREST complex subunits are shown in gray. (C) Hmg20b mRNA expression during I/11 cell differentiation. (D) Western blot of differentiating I/11 cells showed that Lsd1 is expressed at a constant level, while a modest decrease in Gfi1b and Hmg20b expression occurs; α-tubulin serves as a loading control. (E) Gfi1b mRNA expression decreases during I/11 cell differentiation. Error bars indicate standard deviations. Ribonuclease inhibitor 1 (RNH1) was used for normalization of gene expression.

Hmg20b knockdown was not specific to the immortalized p53 *null* I/11 cell line, Hmg20b expression was knocked down in primary mouse fetal liver proerythroblasts (pMFL). The results obtained were comparable to those observed in the I/11 cells. Hmg20b depletion resulted in a marked proliferation arrest and an increased percentage of hemoglobinized cells compared to control cells (Figure 2F and G). Thus, shRNA-mediated knockdown of Hmg20b in pMFL cells induced differentiation under self-renewal conditions. Collectively, these results indicate that Hmg20b maintains proliferation and suppresses differentiation of mouse proerythroblasts.

Hmg20b regulates Gfi1b expression during erythropoiesis

Hmg20b knockdown resulted in Gfi1b downregulation in I/11 cells (Figure 3A). Since it is known that the CoREST complex regulates Gfi1b expression in erythroblasts[8] and Hmg20b is part of the CoREST complex in I/11 cells (Figure 1A and B), we performed chromatin immunoprecipitation (ChIP) analysis to clarify whether Hmg20b is part of the CoREST complex that regulates Gfi1b expression in proerythroblasts. To be able to compare previously reported results in MEL cells[8] with those obtained in I/11 cells, we first performed ChIP of the CoREST subunit Lsd1 in I/11 cells. Similar to the observations reported for MEL cells,[8] Lsd1 enrichment on the *Gfi1b* promoter was stronger in proliferating than in differentiating I/11 cells (Figure 3B). In MEL and I/11 cells, Hmg20b followed a pattern of enrichment on the *Gfi1b* promoter similar to that of Lsd1 (Figure 3C). These results suggest that Hmg20b regulates *Gfi1b* expression in erythroid progenitors as part of the CoREST complex.

The majority of genes deregulated upon Hmg20b depletion are up-regulated

To explore the changes in whole genome expression profiles of Hmg20b-depleted cells in comparison with control cells, microarray experiments were performed in biological triplicates. RNA for expression profiling was harvested from I/11 cells three days after transduction with lentiviral shRNA vectors. Following array hybridization, we first ensured that the triplicate samples were closely correlated (Figure 4A). To validate the microarray data, QRT-PCR was performed for several selected target genes including up-regulated genes such as Hrasls3, Trp53inp1, Cited2, Ccng2, and down-regulated genes such as Rcor2 and Kit. This analysis showed a generally good correlation between the microarray expression data and QRT-PCR results on the same genes (*Online Supplementary Figure S3* and *Online Supplementary Table S2*). Taking a 1.5-fold change in gene expression with *P* value less than 0.01 as a cut off, the analysis of the microarray data showed deregulation of 620 genes, the majority of which (85%; 527 out of 620) had been up-regulated (*Online Supplementary Table S3*). This strongly points to a generally repressive role of Hmg20b in erythroid gene expression. However, some of the deregulated genes (15%; 93 out of 620) were down-regulated indicating that Hmg20b may have a dual role as either an activator or a repressor in erythroid cells (Figure 4B and C).

Knockdown of Hrasls3 inhibits erythroid differentiation

We noted that the Hrasls3 tumor suppressor gene was among the up-regulated genes in the Hmg20b-depleted proerythroblasts. Microarray analysis showed an approximately 1.6-fold upregulation of Hrasls3 in Hmg20b depleted I/11 cells, which was confirmed by QRT-PCR (*Online Supplementary Figure S3* and *Online Supplementary Table S2*). Hrasls3 is involved in cell cycle arrest[32-33] and its role in adipocyte differentiation has been established.[34] It might, therefore, perform similar roles during erythroid differentiation.

To investigate this, we first established the expression pattern of Hrasls3 during erythroid differentiation. QRT-PCR showed a sharp increase (>6-fold) in Hrasls3 expression at day 2 of differentiation (Figure 5A). This is consistent with a positive contribution of Hrasls3 to erythroid

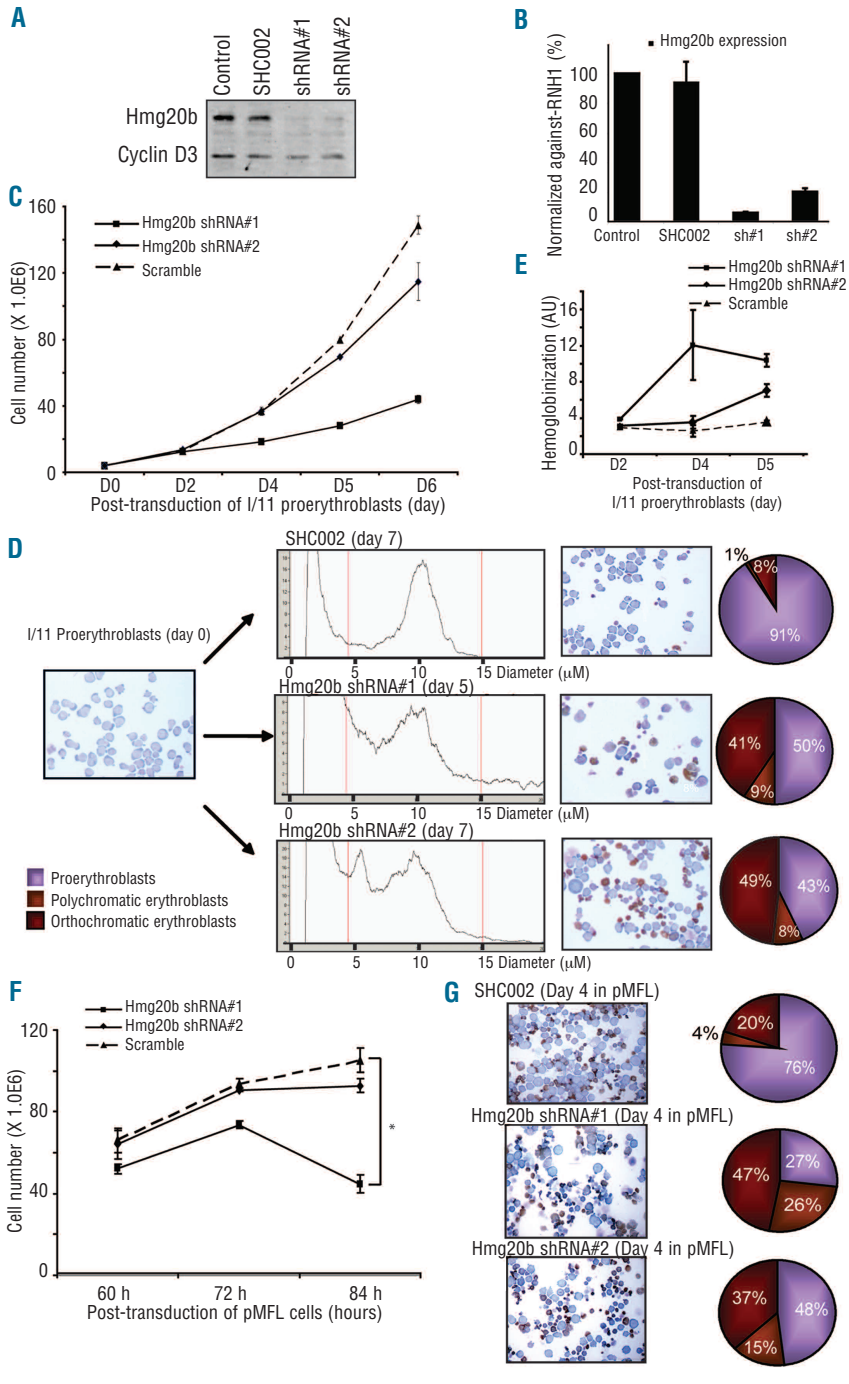

Figure 2. Hmg20b knockdown induces spontaneous differentiation of proerythroblasts. (A) Hmg20b knockdown in I/11 cells was mediated with two different shRNA constructs. Whole cell lysates were prepared and used for Western blot analysis of Hmg20b protein. Cyclin D3 served as a loading control. (B) Hmg20b knockdown was confirmed with QRT-PCR. (C) The proliferation rate of Hmg20b knockdown cells decreased compared to that of cells transduced with control shRNA virus. (D) Five to seven days after virus transduction spontaneous differentiation was observed, as shown by cell size profiles. Cytospins were used to determine the percentage of differentiating cells, tabulated in the pie charts (>300 cells counted). (E) Increased hemoglobin content of Hmg20b knockdown cells. (F, G) Hmg20b knockdown induces spontaneous differentiation in primary mouse fetal liver cells, slightly decreases primary proerythroblast proliferation (F) and increases in hemoglobinization (G). The cytospin pictures were taken with 40x magnification using an Olympus BX40F4 microscope (Olympus Optical Co. Ltd). * indicates $P<0.05$. Error bars indicate standard deviations.

differentiation. To test this putative role, we performed shRNA-mediated knockdown experiments of Hrasls3 expression. Using two different shRNA constructs, we found that Hrasls3 depletion did not interfere with cell proliferation, as Hrasls3 knockdown I/11 cells grew without any significant difference in proliferation rate compared to cells transduced with the control SHC002 shRNA virus (Figure 5B and C). Interestingly, Hrasls3 depletion severely affected differentiation of pMFL cells. The percentage of undifferentiated proerythroblasts was approximately 2-fold higher in Hrasls3 knockdown cells than that observed in controls transduced with the SHC002 shRNA construct (Figure 5D).

Next, we depleted I/11 cells for both Hmg20b and Hrasls3 to address the question of whether depletion of Hrasls3 rescues the spontaneous differentiation of I/11 cells induced by Hmg20b knockdown. Double knockdown of Hmg20b and Hrasls3 resulted in a marked decrease of differentiating proerythroblasts and the cells grew at rates similar to those observed in SHC002-transduced control cells (Figure 5E and F). As expected, expression of globin mRNAs was significantly increased in spontaneously differentiating Hmg20b-depleted cells. Notably, the fold-change in embryonic εy-globin mRNA expression was the highest (Figure 5G). This considerable increase in expression was not observed in double knockdown Hmg20b / Hrasls3 cells. In these cells, the expression of εy–globin mRNA still increased to some extent, but signif-

icantly less than in Hmg20b-only depleted cells (Figure 5G).

Collectively, we conclude that Hrasls3 contributes positively to erythroid differentiation, and that the observed spontaneous differentiation upon Hmg20b knockdown can at least partially be attributed to increased expression levels of Hrasls3.

Discussion

The current study has confirmed Hmg20b as a repressor of terminal erythroid differentiation. To address the function of this protein in erythroid differentiation we depleted Hmg20b in I/11 cells using shRNA-mediated knockdown. Interestingly, Hmg20b downregulation induced spontaneous differentiation of I/11 cells which was manifested by hemoglobinization and enucleation of the cells. Notably, previous studies showed that other subunits of the CoREST complex, including Rcor1 and Lsd1, are involved in erythro- and megakaryopoiesis.[8] Knockdown of Lsd1 but not of Rcor1 impaired erythroid differentiation in MEL cells and reduced expression of erythroid differentiation markers.[8] Redundant expression of Rcor family members may explain the apparent absence of a phenotype after Rcor1 depletion in erythroblasts.[8] From our data on spontaneous differentiation induced by Hmg20b knockdown in I/11 cells and pMFL cells, we conclude that Hmg20b plays a crucial role in maintaining the proliferative status of erythroid progenitors. Presumably, it does so in concert with the other subunits of the CoREST complex; this notion is supported by our proteomics data indicating that CoREST is the major complex in which Hmg20b participates in these cells. Differentiating erythroblasts complete a series of differentiation-specific cell divisions before undergoing G1 arrest.[3] Thus, our data

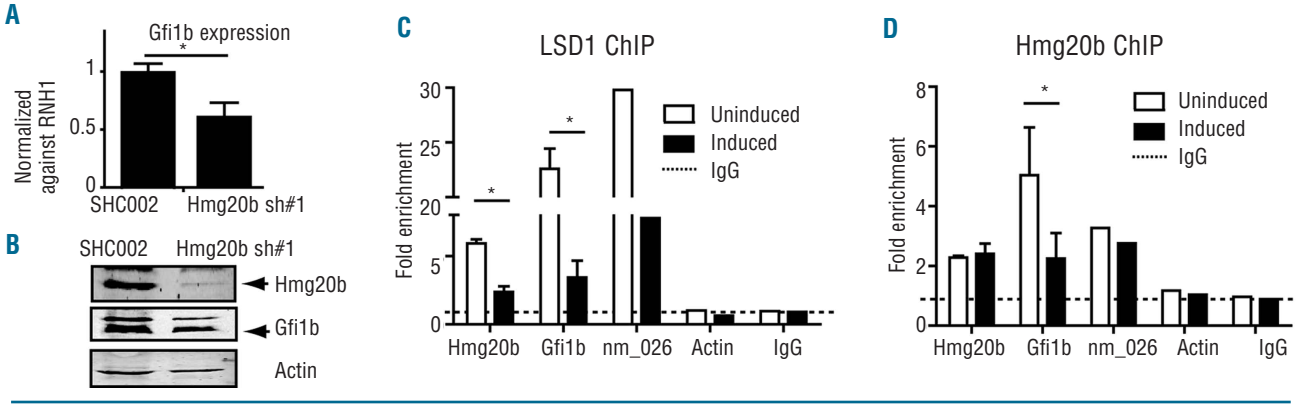

Figure 3. Hmg20b regulates Gfi1b expression during I/11 cell differentiation. (A) Gfi1b mRNA expression is down-regulated three days after Hmg20b knockdown. (B) Gfi1b expression at the protein level is decreased after Hmg20b knockdown after five days of virus transduction. (C) Lsd1 enrichment on the Gfi1b promoter in I/11 cells. (D) Hmg20b enrichment on the *Gfi1b* promoter. The *Nm_026543 (nm_026)* gene was used as a positive control.[8] White bars: proliferating cells; black bars: differentiating cells. *indicates $P<0.05$. Error bars indicate standard deviations.

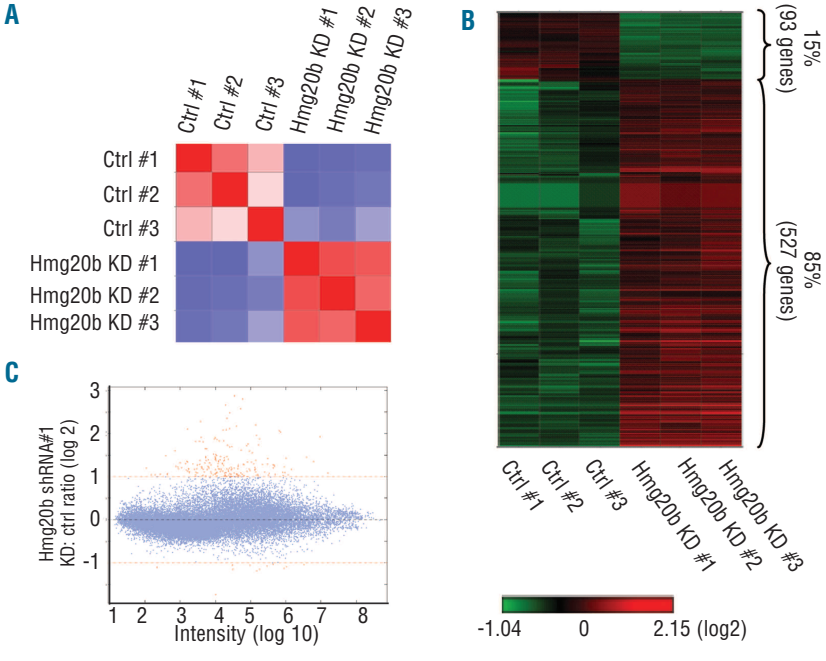

Figure 4. Expression profiling of Hmg20b knockdown proerythroblasts. (A) Correlation analysis of biological triplicates of control and Hmg20b knockdown cells. Red: positive correlation; blue: negative correlation. (B) Heatmap of differentially regulated genes with known function (GO terms) in Hmg20b knockdown cells compared with control I/11 cells. Down-regulated genes: green, upregulated genes: red. (C) MA plot of Hmg20b knockdown proerythroblasts. Blue: constantly expressed genes; red: differentially expressed genes (>2-fold).

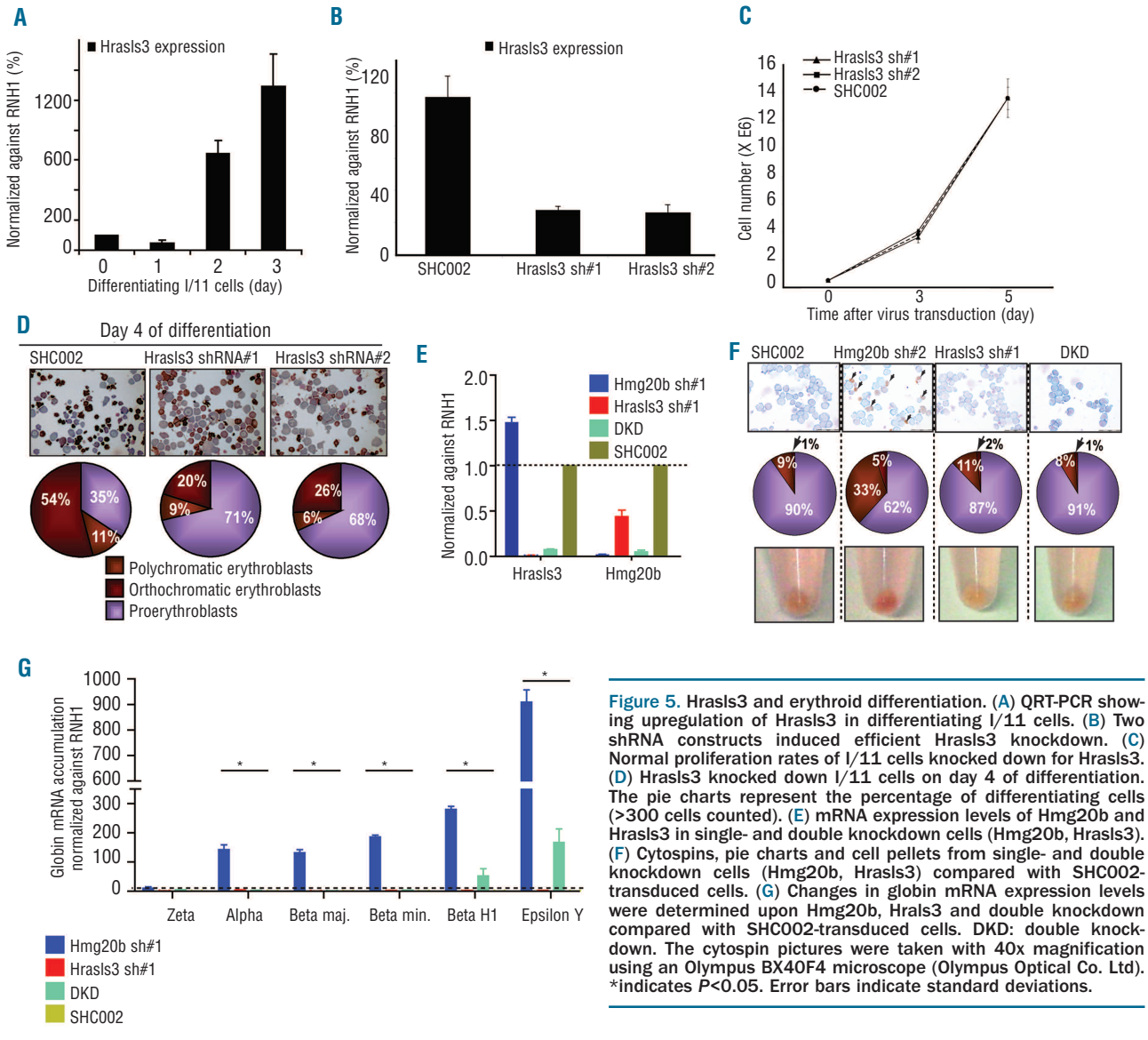

Figure 5. Hrasls3 and erythroid differentiation. (A) QRT-PCR showing upregulation of Hrasls3 in differentiating I/11 cells. (B) Two shRNA constructs induced efficient Hrasls3 knockdown. (C) Normal proliferation rates of I/11 cells knocked down for Hrasls3. (D) Hrasls3 knocked down I/11 cells on day 4 of differentiation. The pie charts represent the percentage of differentiating cells (>300 cells counted). (E) mRNA expression levels of Hmg20b and Hrasls3 in single- and double knockdown cells (Hmg20b, Hrasls3). (F) Cytospins, pie charts and cell pellets from single- and double knockdown cells (Hmg20b, Hrasls3) compared with SHC002-transduced cells. (G) Changes in globin mRNA expression levels were determined upon Hmg20b, Hrals3 and double knockdown compared with SHC002-transduced cells. DKD: double knockdown. The cytospin pictures were taken with 40x magnification using an Olympus BX40F4 microscope (Olympus Optical Co. Ltd). *indicates $P<0.05$. Error bars indicate standard deviations.

suggest that Hmg20b is involved in the switch from renewal to differentiation divisions.

We found that Hmg20b depletion results in accumulation of different globin mRNAs, including embryonic β-like globins. It has been shown that CoREST complex subunits like Hdac1, Lsd1 and Rcor1 bind to the promoters of the embryonic εy and βH1 genes, but not to the promoter of the adult β-major gene (S. Cui *et al.*, unpublished data, 2011). In addition, Lsd1 and Rcor1 do not bind to the promoters of the α-like globin genes, providing a rationale for our observation that the embryonic ζ-globin gene is not derepressed upon Hmg20b knockdown. Our data on Hmg20b suggest that it plays a role in the recruitment of the CoREST repressor complex on the embryonic β-like globin genes in adult erythroid cells. Accordingly, Hmg20b depletion removes the repressor complex giving rise to relief of suppression of the embryonic β-like globin genes. This suggests that Hmg20b maintains a repressive function on some of its targets during terminal differentiation of definitive erythroid cells. We note that the expression levels of the embryonic β-like globin genes remain low compared to those of the adult β-like globin genes, indicating that even upon knockdown of Hm20b the transcription factor milieu of adult erythroid cells is not permissive for full expression of the embryonic genes. This could be due, for example, to the presence of the Bcl11a and Sox6 repressor proteins.[35]

Collectively, the data on the role of CoREST subunits indicate that modulation of CoREST activity is essential for normal erythropoiesis.[8]

To identify more potential targets of Hmg20b in proerythroblasts, we carried out microarray analysis on Hmg20b-depleted I/11 cells. Whole genome expression profile analysis of Hmg20-depleted cells strongly supports the inhibitory role of Hmg20b in erythroid cells, since we found that 85% of differentially expressed genes are upregulated upon Hmg20b knockdown. Apparently, in the presence of Hmg20b the majority of its target genes are repressed which enables proerythroblasts to retain their undifferentiated proliferative status. In the absence of

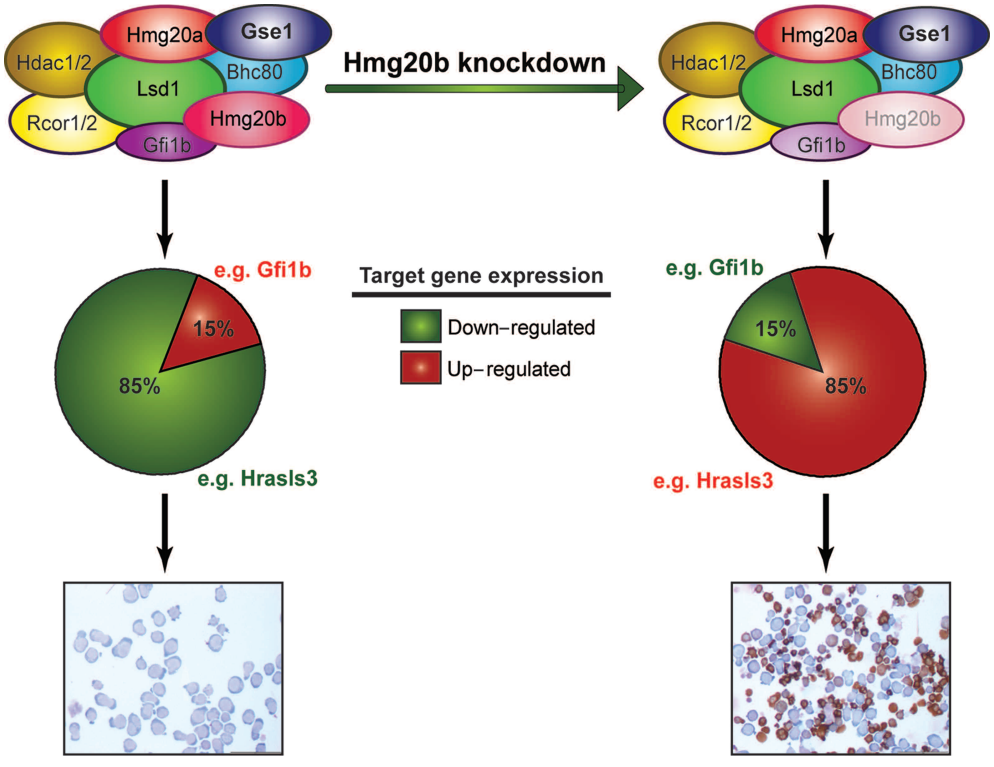

Figure 6. Proposed regulatory mechanism for Hmg20b in erythroid differentiation. Hmg20b, as part of the CoREST complex, plays a dual regulatory role in proliferating proerythroblasts. It regulates the repressor Gfi1b, which guides the CoREST complex to genes involved in differentiation, resulting in their repression. Decreased Hmg20b expression leads to reduced Gfi1b expression and release of repression of Gfi1b/CoREST target genes, including Hrasls3, allowing erythroid differentiation to ensue.

Hmg20b those targets are derepressed, leading to spontaneous terminal differentiation of the cells. Genes deregulated by Hmg20b knockdown include those involved in cell cycle arrest, globin synthesis and the erythroid differentiation process, indicating a global regulatory role of Hmg20b in erythropoiesis.

To extend our understanding of the regulatory function of Hmg20b protein, we searched for the factors that can be directly regulated by Hmg20b. Hmg20b has been purified as a subunit of the CoREST complex, mediating repression of neurogenesis through recruitment by REST protein to the RE1 sequence of the target genes.[5] In erythroid cells, Gfi1b represses gene expression through recruitment of the CoREST complex to its target genes.[8] Studies on GFP-Gfi1b-knock-in mice demonstrated that Gfi1b is highly expressed in mouse fetal liver cells, the anatomical site where hematopoiesis takes place at midgestation.[36] In the absence of Gfi1b, only a small fraction of erythroblasts is able to differentiate and the accumulation of immature erythrocytes is observed.[36-37] *In vitro* studies demonstrated that Gfi1b knockdown also impairs erythroid differentiation.[8,15] Gfi1b expression is autoregulated, and it recruits the CoREST complex to its promoter.[8,38] Proteomics analysis demonstrated association of the Hmg20b protein with the CoREST complex in erythroid cells, and we showed direct binding of Hmg20b to the Gfi1b promoter. We, therefore, conclude that Gfi1b-mediated CoREST recruitment to the Gfi1b promoter regulates Gfi1b expression[8, 38] (Figure 6).

One of the consistently up-regulated genes upon Hmg20b depletion was the Hrasls3 tumor suppressor gene. Hrasls3-overexpressing cells can not be expanded in culture, demonstrating the role of Hrasls3 in suppressing cellular proliferation.[39-40] In fibroblasts, overexpression of Hrasls3 augments adipogenesis and accordingly Hrasls3 downregulation inhibits adipocyte differentiation.[34,41-42] We find that, similar to the observations in adipogenesis, Hrasls3 expression increases during erythroid differentiation. We demonstrated that shRNA-mediated downregulation of Hrasls3 inhibits erythroid differentiation. It, therefore, appears that Hrasls3 plays a key role in distinct differentiation processes, as exemplified by adipogenesis and erythropoiesis (Figure 6). Additionally, we found that the spontaneous differentiation phenotype observed upon Hmg20b depletion is dependent on the upregulation of Hrasls3.

In conclusion, we found that Hmg20b is a repressor of erythroid differentiation. It acts in the autoregulatory loop of Gfi1b expression, helping to maintain the Gfi1b/CoREST activity that serves to repress erythroid differentiation. Upon Hmg20b knockdown the majority of differentially expressed genes are up-regulated and the terminal differentiation program ensues. Furthermore, Hmg20b knockdown leads to derepression of the embryonic β-like globin genes. We discovered that Hrasls3 is an important gene repressed by Hmg20b, and that upregulation of Hrasls3 plays an important role during terminal erythroid differentiation.

Authorship and Disclosures

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