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DNA methylation-dependent suppression of *HIF1A* in an immature hematopoietic cell line HMC-1

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ABSTRACT

The HMC-1 cell line represents the phenotype of immature mast cells. The *HIF1A* gene product HIF-1α plays key roles in maintaining oxygen homeostasis in eukaryotic organisms and is involved in many processes, including immune response and hematopoiesis. In this study we investigated *HIF1A* expression in HMC-1 immature hematopoietic cells and CD34+ hematopoietic progenitors. HMC-1 cells exhibited exceptionally low levels of *HIF1A* expression compared to other cell lines as determined by real-time PCR, and multipotent CD34+ hematopoietic progenitors in bone marrow exhibited significantly lower levels of *HIF1A* mRNA compared to mature blood cells in peripheral blood. We searched for the mechanisms responsible for suppression of *HIF1A* expression in HMC-1 cells and obtained evidence for a DNA methylation-dependent process. *In vitro* methylation of the *HIF1A* promoter resulted in a decrease in its transcriptional activity and the level of DNA methylation in the *HIF1A* promoter region in analyzed cell lines was negatively correlated with *HIF1A* expression. Furthermore, the DNA demethylating agent 5′-azacytidine increased *HIF1A* expression, and MeCP2 protein was preferentially associated with the *HIF1A* promoter *in vivo*. In conclusion, we report that the *HIF1A* gene in HMC-1 immature hematopoietic cells is suppressed by a process dependent on DNA methylation, and we present evidence indicating downregulation of *HIF1A* expression in multipotent CD34+ hematopoietic progenitors.

Introduction

HIF1A encodes hypoxia-inducible factor-1α (HIF-1α), a transcription factor that mediates the adaptive response to hypoxia [1–7]. Increasing evidence also suggests a crucial role for HIF-1α in immune responses [6,8–10].

Mast cells like other blood cells originate from bone marrow multipotential progenitors [11–13]. The complex process of hematopoiesis involves changes in the pattern of gene expression regulated by epigenetic mechanisms [14]. One of the critical mechanisms of epigenetic regulation depends on the methylation of cytosines within CG-rich regions of regulatory sequences of genes. Cytosine methylation mediates transcriptional repression by interaction of the methylated cytosines with specific proteins such as MeCP2 [15], which in turn recruits histone deacetylases capable of modifying the local chromatin structure [16]. Examples of DNA methylation-dependent regulation of gene expression in differentiating hematopoietic cells include, regulation of the expression of the hematopoietic stem cell-specific SCL/TAL-1 gene in CD34+ cells [17], the γ-globin gene in erythroid cells [18], Th1/Th2-specific cytokine genes in T cells [19,20], and the L-histidine decarboxylase gene in basophils and mast cells [21].

The effects of hypoxia and HIF-1α on mast cell differentiation are largely unknown, although it has been reported that hypoxic conditions are favorable for *in vitro* culture of mast cells from CD34+, c-Kit+ peripheral blood-derived progenitors [22]. We previously reported that in human mast cells, *HIF1A* expression is transcriptionally upregulated by calcineurin- and NFAT-dependent signaling [23]. In this report, we show that the HMC-1 immature mast cell line, used as a prototypic immature hematopoietic cell line, exhibits an exceptionally low level of *HIF1A* mRNA, which could be explained by DNA methylation of a CG-rich region in the *HIF1A* promoter. Furthermore, we show evidence for low *HIF1A* expression in CD34+ hematopoietic progenitors in the bone marrow.

Materials and methods

Cell culture. HepG2 (hepatocellular carcinoma) and A549 (alveolar epithelial non-small cell lung cancer) cell lines were obtained from ATCC and maintained under standard conditions. HMC-1 [24]

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and LAD [25] mast cells were maintained as previously described [23].

Real-time RT PCR. Total RNA was isolated from cells using TRI Reagent® from Molecular Research Center and reverse-transcribed with the RevertAid™ H Minus M-MuLV Reverse Transcriptase from Fermentas primed with anchored oligo-dT18. cDNAs from BM CD34+, PB CD34+, BM MNCs, PB MNCs and BM MSCs purchased from AllCells (Emeryville, CA, USA) was obtained from a population of positive cells (purity exceeding 95%) collected from five individual donors. The level of cognate cDNA was measured by real-time PCR amplification performed on a LightCycler 480 (Roche) using SYBR Green I master mix (Roche) for detection of the PCR product. The following intron spanning primers were used for detection of cDNA sequences: 5′-GAAAGCGCAAGTCTTCAAAG-3′ and 5′-TGGGT AGGAGATGGAGATGC-3′ for *HIF1A*. We used the normalization gene selection procedure of Vandesompele et al. [26]. *HPRT1* and *HMBS* were selected as the most reliable reference genes for the cell lines included in this study. For presentation and analysis, ΔC_t values were transformed into relative copy number values (number of copies of *HIF1A* gene mRNA per the housekeeping gene index) as described previously [27].

Treatment with 5′-azacytidine. For demethylation studies, HMC-1 cells were maintained in the presence of 0.1, 0.25 and 0.5 μM 5′-azacytidine for 5 days and then collected for RNA extraction.

In vitro methylation of the HIF1A promoter sequence. Source of *HIF1A* promoter sequence was the phHIF1A(−863/+5)Luc reporter construct described in [23]. The whole promoter sequence excised from this plasmid was gel purified and subsequently methylated with the SssI DNA methyltransferase (New England Biolabs) according to manufacturer's instructions. The methylated promoter fragment was reinserted into the pGL3-basic vector. Control *HIF1A* promoter sequence was also subjected to the same manipulations with mock methylation. As an additional control, *in vitro* methylation was performed for a promoter sequence from the *ABCC6* gene known to be silenced by CpG methylation in particular cell lines [28]. The resulting constructs were transfected into A549 and HepG2 cells. Luciferase activity was measured 48 h after transfection and standardized as described [27].

In vivo methylation status of the human HIF1A promoter in different cell lines. Genomic DNA from selected cell lines was isolated using a Genomic DNA Extraction Kit from Panomics. Methylated DNA was isolated based on MeCP2 affinity chromatography using the Promoter Methylation PCR Kit (Panomics) according to the manufacturer's instructions. The methylated genomic DNA was analyzed by RT PCR with primers specific to the *HIF1A* promoter: 5′-ACAAGCCACCTGAGGAGAGG-3′ (position −211) and 5′-GAAGA GAAGGAAAGGCAAGTCC-3′ (position −84). Relative amount of PCR product was calculated by transforming C_t values according to the following formula: relative amount of PCR product = (2^{-C^t})/(input DNA concentration [μg/ml]), where input DNA concentration is concentration of DNA determined spectrophotometrically prior to MeCP2 protein affinity chromatography. The relative amount of PCR product is directly proportional to the amount of methylated DNA.

Chromatin immunoprecipitation assay (ChIP). Chromatin immunoprecipitation with normal mouse IgG (Upstate) and anti-MeCP2 (Abcam) was performed using the EZ-ChIP kit from Upstate according to the manufacturer's protocol. PCR amplification was performed using 2 μl of DNA sample with primers specific to the *HIF1A* promoter [23] for 33 cycles. Amplification of soluble chromatin prior to immunoprecipitation was used as a control for equal input of DNA.

Computational analysis and statistics. For computational analysis of the *HIF1A* promoter region, CpG Island Searcher [29] was used. Testing for statistical significance was done by one-way ANOVA followed by the Holm–Sidak test or one-way ANOVA on Ranks followed by the Dunn's test as appropriate. A p value of 0.05 or lower was considered statistically significant.

Results

Different HIF1A expression in cells with different phenotypes

Previously, we observed that the HMC-1 immature mast cell line exhibits much lower HIF-1α protein expression under normoxic and hypoxic conditions compared to other cell lines such as HepG2 [23]. We decided to investigate whether the differences observed in the level of HIF-1α protein were associated with differences in *HIF1A* expression in resting cells. To this end, we determined the level of *HIF1A* mRNA in the HMC-1 immature mast cell line, LAD-2 mast cell line, HepG2 hepatocellular carcinoma cell line and A549 lung epithelial cancer cell line and observed that the level of *HIF1A* transcript was particularly low in HMC-1 cells compared to LAD (134-fold higher), HepG2 (113-fold higher), and A549 (175-fold higher) (Fig. 1A). Because HMC-1 cells represent the phenotype of immature hematopoietic cells [30,31], we decided to investigate *HIF1A* expression in bone marrow and peripheral blood-derived hematopoietic precursor cells. Total RNA obtained

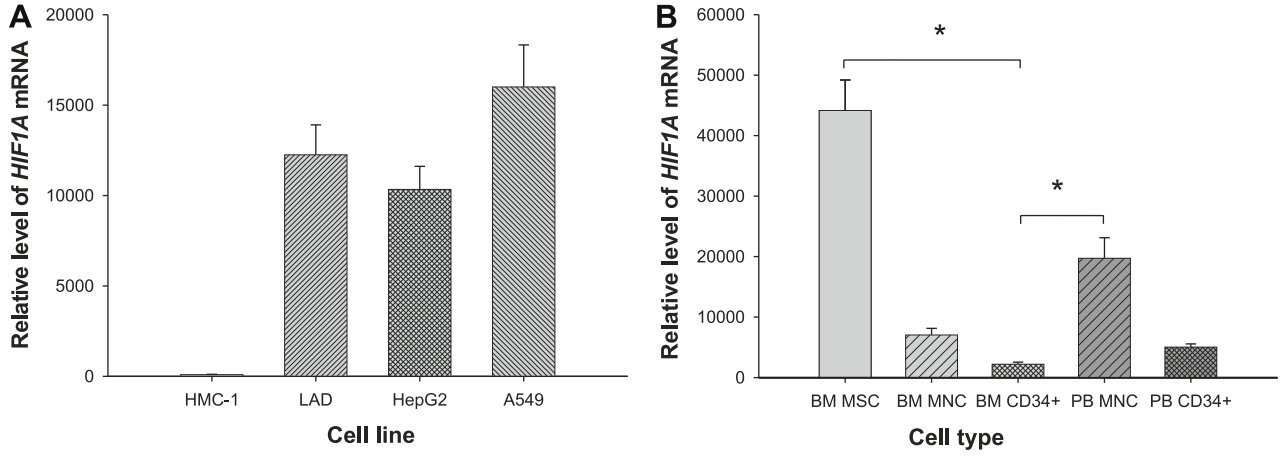

Fig. 1. Expression of *HIF1A* in different human cells. The level of *HIF1A* mRNA in (A) selected cell lines and (B) cells isolated from human blood and bone marrow obtained from individual donors was determined by real-time RT PCR as described in Materials and methods. Gene expression is presented as number of copies of *HIF1A* mRNA per the housekeeping gene index (means ± SEM, $n = 4$ for A; $n = 5$ for B). *Statistically significant difference at $p < 0.05$.

from bone marrow CD34+ cells (BM CD34+), peripheral blood CD34+ cells (PB CD34+), bone marrow mononuclear cells (BM MNCs), peripheral blood mononuclear cells (PB MNCs) and bone marrow mesenchymal stromal cells (BM MSCs) was analyzed for the level of *HIF1A* mRNA using real-time RT PCR. The resulting data (Fig. 1B) showed differences in the *HIF1A* level depending on cell type, with the lowest *HIF1A* expression in BM CD34+ (6.6% of *HIF1A* expression in BM MSCs) and the highest in BM MSCs (100%), suggesting that undifferentiated human blood progenitor cells have a lower level of *HIF1A* mRNA compared to more differentiated hematopoietic and non-hematopoietic cells.

The effect of in vitro methylation on HIF1A promoter activity

Analysis of a 4-kb 5′-upstream region of the *HIF1A* gene performed using CpG Island Searcher software revealed one large CpG island (with 64% of CpG dinucleotides) stretching from −1026 to +140 bp (counting from ATG) [32]. To show that methylation of cytosines within the identified CpG island within *HIF1A* promoter can indeed lead to tangible transcriptional outcomes (silencing), we analyzed the effect of *in vitro* methylation of the promoter sequence on its activity using reporter gene assay. As shown in Fig. 2A, methylation of the promoter led to similarly decreased (up to 50%) transcriptional activity in both cell lines tested (A549 and HepG2) when compared to control. A similar methylation-mediated decrease was observed for the control *ABCC6* gene in HepG2 cells, where high transcriptional activity of the promoter has been shown to required its unmethylated status [28], but not in A549 cells where *ABCC6* promoter activity is inherently depressed by methylation and cannot be further decreased by *in vitro* methylation (Fig. 2B). The basal transcriptional activity of the *HIF1A* promoter in HMC-1 cells in a reporter gene assay is low enough to preclude a similar assay with *in vitro* methylated DNA (data not shown).

Methylation of the human HIF1A promoter in cells of different origin

We next decided to investigate whether the *HIF1A* promoter sequences in different cell lines were differently methylated. In a series of experiments, methylated genomic DNA was isolated from HMC-1, LAD, A549 and HepG2 cells and analyzed for the presence of *HIF1A* promoter sequences using real-time PCR. As seen in Fig. 3A samples obtained from HMC-1 mast cells contained significantly more *HIF1A* promoter sequences compared to samples from LAD, HepG2 and A549 cells (1.9, 2.1 and 7.7 times more respectively), indicating a higher level of methylation of *HIF1A* promoter sequences in HMC-1 mast cells as compared other cell lines. To further verify whether *HIF1A* promoter sequence is preferentially methylated in HMC-1 mast cells, a ChIP assay with anti-MeCP2 was performed. To this end, chromatin samples obtained from HMC-1 cells and A549 cells were sonicated and immunoprecipitated with anti-MeCP2 or control antibodies, and the resultant DNA was analyzed by PCR with primers specific to the *HIF1A* promoter sequence. As shown in Fig. 3B, the specific 180 bp PCR product was detected in HMC-1 mast cells but not in A549 cells. Thus, MeCP2 is preferentially associated with the *HIF1A* promoter sequence in HMC-1 mast cells compared to A549 cells.

Effect of 5′-azacytidine on the HIF1A mRNA level in HMC-1 cells

Given that analysis of the *in vivo* methylation of the *HIF1A* promoter in different cells indicated that HMC-1 cells exhibited the highest level of DNA methylation, we decided to investigate the effects of 5′-azacytidine, known to be an inhibitor of DNA methyltransferases [33,34], on *HIF1A* expression in HMC-1 mast cells. In a series of experiments, HMC-1 mast cells were cultured with the indicated concentration of 5′-azacytidine (0–0.5 μM) for 5 days. As shown in Fig. 4, the addition of 5′-azacytidine resulted in a dose-dependent increase in the amount of *HIF1A* transcript (2.0- to 8.2-fold) compared to control cells, suggesting that DNA methylation is necessary for suppression of *HIF1A* gene expression in HMC-1 mast cells.

Discussion

Mast cells arise from bone marrow-derived hematopoietic progenitors (CD34+, c-Kit+ and CD13+) [13]. HMC-1 cells represent the phenotype of immature mast cells and exhibit an exceptionally low level of *HIF1A* expression compared to several other human cell lines of different origin and phenotypic characteristics, including LAD-2 cells, which represent the phenotype of more mature mast cells (Fig. 1A) [24,25,31,35]. These observations led us to

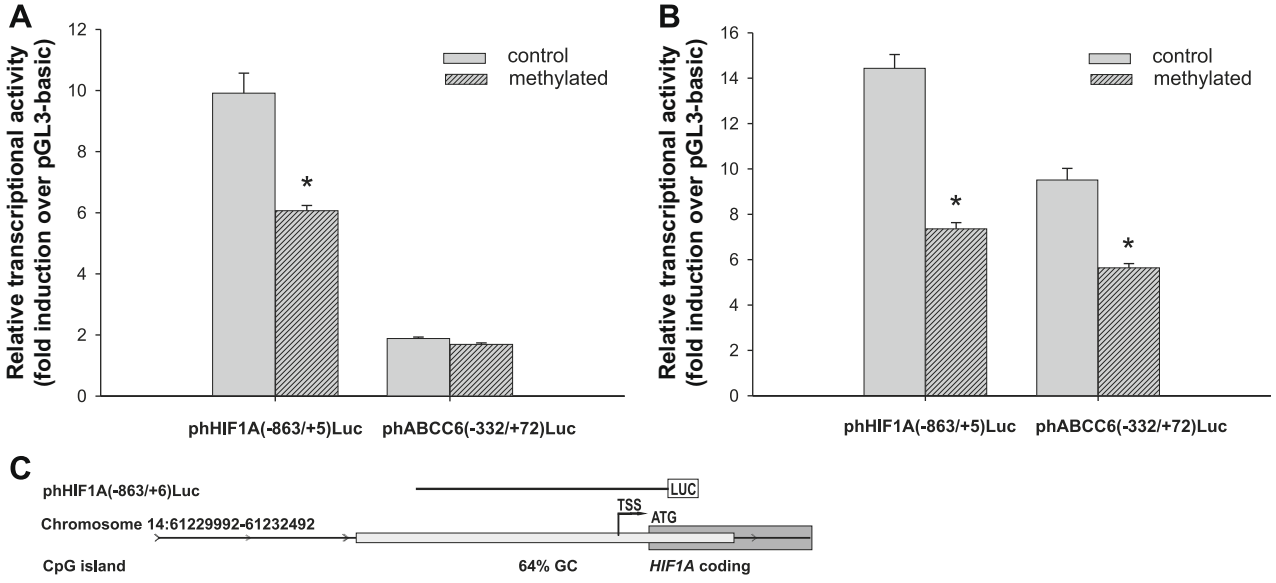

Fig. 2. Effect of CpG methylation on *HIF1A* promoter activity. A549 (A) and HepG2 (B) cells were transiently transfected with luciferase reporter gene constructs containing methylated or unmethylated *HIF1A* and *ABCC6* promoter sequences. Results are given as luciferase activity normalized to cotransfected SEAP reporter activity (mean ± SEM; $n = 6$). *Statistically significant difference at $p < 0.01$. (C) Schematic representation of the upstream region of *HIF1A* gene.

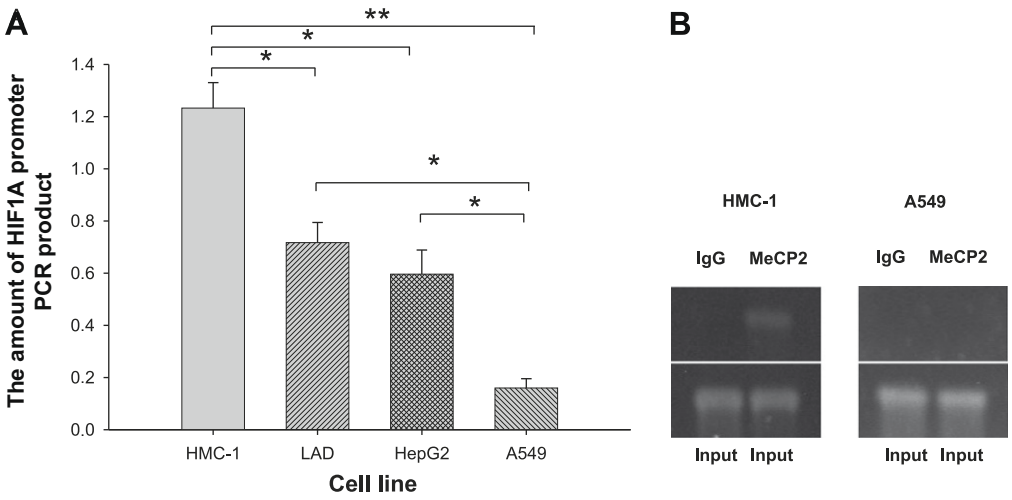

Fig. 3. *In vivo* methylation of the *HIF1A* promoter. (A) Methylated DNA fragments were isolated from genomic DNA obtained from HMC-1, LAD, HepG2 and A549 cells and analyzed with real-time PCR with primers specific to the promoter region (−211/−84 bp). The relative amount of PCR product reflects the methylation status of *HIF1A* promoter sequences (mean ± SEM; $n = 4$). *Statistically significant difference at $p < 0.05$. **Statistically significant difference at $p < 0.01$. (B) Chromatin from HMC-1 and A549 cells was analyzed using ChIP assay with anti-MeCP2 (MeCP2) or control mouse IgG (IgG). Total extracted DNA (input) prior to the immunoprecipitation and the immunoprecipitated samples (IgG and MeCP2) were amplified with PCR using primers specific to the *HIF1A* promoter. PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide.

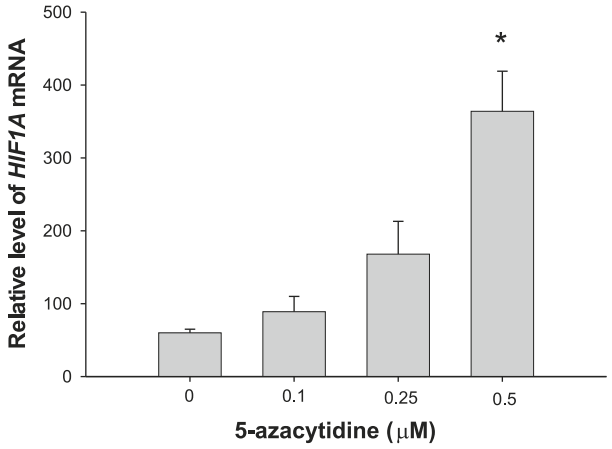

Fig. 4. The effect of 5′-azacytidine on the *HIF1A* mRNA level in HMC-1 cells. HMC-1 cells were cultured in the absence or presence of 0.1, 0.25 and 0.5 μM 5′-azacytidine for 5 days, and the level of *HIF1A* expression was measured by real-time RT PCR (mean ± SEM; $n = 4$). *Statistically significant difference at $p < 0.05$.

consider a possible relationship between the phenotype and differentiation stage of cells and the level of *HIF1A* expression. Because HMC-1 cell line originated from blood obtained from a mast cell leukemia patient [24], it is conceivable that the observed low expression of *HIF1A* is a feature of leukemic cells. While this hypothesis could not be ruled out, studies investigating gene expression in tumor cells indicate that *HIF1A* expression is frequently increased rather than downregulated compared to normal tissue [36,37]. Alternatively low *HIF1A* expression in the HMC-1 cell line could be related to its immature phenotype. This hypothesis is consistent with observations that CD34+ multipotential hematopoietic progenitors in the bone marrow exhibit a significantly lower level of *HIF1A* mRNA compared to the population of mature blood cells (PB MNCs) and stromal cells (Fig. 1B). The observed differences in *HIF1A* expression between hematopoietic progenitors and mature blood cells might be of special interest to better understand the molecular mechanisms involved in hematopoiesis because the local oxygen concentration has been shown to be an important part of the microenvironment influencing this process [38,39]. In addition, HIF-1α has recently been implicated as a nuclear transcription factor playing role in certain *in vitro* models of differentiation of hematopoietic cells [40,41].

The amount and activity of HIF-1α protein product in tissues are predominantly regulated by posttranscriptional mechanisms [3,42]. There are reports showing constitutive expression of *HIF1A* at the mRNA level in multiple tissues in humans and mice [43,44]. Therefore, a possible role of transcriptional regulation in *HIF1A* expression might be underestimated. There is increasing evidence, however, that the level of *HIF1A* mRNA varies in different tissues and different physiological and pathological situations [45]. The mechanisms of tissue and cell maturation stage-specific transcriptional regulation of gene expression depend in part on methylation of cytosines in regulatory sequences. DNA sequences that undergo methylation are known as CpG islands and are characterized by high CG content, and the presence of such sequences is a prerequisite for methylation-dependent gene silencing [46,47]. Therefore, the observation that the entire 5′-flanking region of *HIF1A* is particularly CG-rich, with 64% CG nucleotides led us to the hypothesis that the *HIF1A* gene could be silenced by DNA methylation. Consistent with this hypothesis, the reporter gene assay revealed that *in vitro* methylation of the *HIF1A* promoter resulted in decreased transcriptional activity (Fig. 2A and B) showing that *HIF1A* promoter activity could be suppressed by methylation of DNA. These observations suggest that low expression of *HIF1A* in HMC-1 cells could be mediated by methylation of the promoter sequences *in vivo*. In agreement with such hypothesis the level of DNA methylation in the *HIF1A* promoter region in analyzed cell lines *in vivo* was found to be negatively correlated with *HIF1A* expression and HMC-1 cells demonstrated the highest level of DNA methylation (Fig. 3A). Thus, the *HIF1A* promoter was preferentially methylated in cells exhibiting very low gene expression. The mechanism of methylation-mediated transcriptional repression of specific genes depends on the interaction of methylated cytosines with specific proteins such as MeCP2 [15]. We were able to demonstrate that MeCP2 is preferentially associated with the *HIF1A* promoter in HMC-1 cells *in vivo*, in contrast to control A549 cells (Fig. 3B), which further substantiates the hypothesis of methylation-dependent *HIF1A* silencing in HMC-1 mast cells. Significantly, we have also found that the DNA demethylating agent 5′-azacytidine increased *HIF1A* expression in HMC-1 mast cells but not in other cell lines expressing high amounts of *HIF1A* mRNA (Fig. 4, and data

not shown). This indicates that the very low level of *HIF1A* mRNA in HMC-1 mast cells depends on DNA methylation of the *HIF1A* promoter.

In conclusion, our data show that the *HIF1A* promoter could be regulated by DNA methylation and that suppression of *HIF1A* expression in the immature hematopoietic cell line HMC-1 is caused by DNA methylation. Furthermore, we have shown preliminary evidence indicating that downregulation of *HIF1A* expression might be associated with certain phenotypes of immature blood cells such as CD34+ multipotent hematopoietic progenitors.

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