

Epigenetic regulation of *CD34* and *HIF1A* expression during the differentiation of human mast cells

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Abstract Mast cells differentiate from circulating pluripotent hematopoietic progenitors. During this differentiation, the progenitor cells are exposed to changes in oxygen availability. *HIF1A* is the major sensor of oxygen concentration in mammalian cells. We investigated the expression of *HIF1A* during the in vitro differentiation of peripheral blood-derived progenitors into human mast cells. In a series of experiments, we determined the changes in *CD34* expression, selected mast cell markers, and *HIF1A* in human mast cell cultures. While the expression of *CD34* dramatically decreased, the expression of mast cell-specific genes, including *FCER1A*, *MS4A2*, *TPSB2*, and *CMA1*, steadily increased. *HIF1A* expression similarly increased during mast cell differentiation, reaching its maximum level at five weeks of culture. The analysis of the promoter methylation status showed decreasing levels of methylation at the *HIF1A* promoter, increasing levels of methylation at the *CD34* promoter, and no significant changes in other genes. In silico analysis of the promoter regions of these genes revealed large CpG islands in close proximity to the *HIF1A* and *CD34* transcription initiation sites, but not in other investigated genes. In conclusion, in vitro mast cell differentiation was associated with decreased *CD34* expression and increased *HIF1A* expression. These changes were paralleled with changes in the methylation status of the

respective promoters, suggesting that DNA methylation-dependent epigenetic regulation mediates the gene expression changes involved in maintaining the phenotype of hematopoietic stem cells and mature mast cells. Therefore, the baseline expression of *HIF1A* is epigenetically regulated in a cell type- and differentiation stage-specific fashion.

Keywords Human mast cells · Differentiation · Hif1a · Cd34 · Epigenetic regulation

Introduction

Mast cells originate from pluripotent hematopoietic cells that are released from the bone marrow into the circulation and differentiate in the peripheral tissues (Metcalf et al. 1997). One of the factors that differentially influences cellular physiology at different sites in the human body is the partial pressure of oxygen. Relatively low oxygen concentrations, often referred to as physiological hypoxia or physioxia, are characteristic of the bone marrow and the skin (Carreau et al. 2011; MacDougall and McCabe 1967). While the partial pressure of oxygen in various tissues ranges from 5 to 15 mmHg, the partial pressure of oxygen observed in blood plasma is significantly higher, reaching 40 mmHg (Carreau et al. 2011; MacDougall and McCabe 1967). Thus, during the differentiation of bone marrow progenitors into peripheral mature mast cells, the cells are exposed to a gradation of oxygen availability. The major sensor of oxygen concentration in mammalian cells is the dimeric transcription factor HIF-1, which consists of the protein product of *HIF1A*, known as HIF-1 α , and the protein product of *ARNT*, known as HIF-1 β (Semenza 2001). The amount of HIF-1 α in the cell

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depends on the availability of oxygen, which induces HIF-1 α degradation (Semenza 2001), and cell type-specific regulation of *HIF1A* expression at the transcriptional (Gorlach 2009; Lauzier et al. 2007; Walczak-Drzewiecka et al. 2008), and epigenetic levels (Kosłowski et al. 2010; Walczak-Drzewiecka et al. 2010). The role of HIF-1 α -mediated oxygen sensing is not limited to the physiological response to hypoxic stress, but is also important for other processes, such as astrocyte differentiation (Mutoh et al. 2012), and the maintenance of a quiescent state of bone marrow hematopoietic stem cells (Takubo et al. 2010). HIF-1 α is also involved in innate immune responses to bacterial infection, where it upregulates antibacterial functions of granulocytes, macrophages, and keratinocytes (Peyssonnaud et al. 2008; Peyssonnaud et al. 2005; Zinkernagel et al. 2007).

We previously reported that CD34⁺ hematopoietic progenitors isolated from the peripheral blood and the bone marrow demonstrated significantly lower levels of *HIF1A* expression as compared to mature PBMC (Walczak-Drzewiecka et al. 2010). This, combined with the observation of epigenetic silencing of *HIF1A* in the HMC-1 human mast cell line (Walczak-Drzewiecka et al. 2010), prompted us to investigate the expression of *HIF1A* during in vitro differentiation of human mast cells from their peripheral blood-derived progenitors.

Materials and methods

Cytokines and reagents

Ficoll-Paque cell separation solution was purchased from GE Healthcare (Uppsala, Sweden). The AC133 cell isolation kit and LS⁺ cell separation columns were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). StemSpan culture medium was purchased from Stem Cell Technologies (Vancouver, Canada). Fetal calf serum (FCS) was bought from PAA (Pasching, Austria), and penicillin/streptomycin was acquired from Invitrogen/Gibco (Carlsbad, California). Human recombinant stem cell factor (rhSCF), human rhIL-6 and human rhIL-3 were purchased from R&D Systems (Abingdon, UK).

CD133⁺ cell isolation

The culture of primary human mast cells was initiated from a population of CD133⁺ cells that were obtained from buffy coat preparations as per Holm et al. (2008). Fresh

Table 1 Source of cells employed for analysis of gene expression

Donor no.	PBMC	CD133 ⁺	Week of mast cell culture				
			1st	2nd	3rd	4th	5th
1	■		■	■	■	■	■
2	■		■				
3			■			■	■
4	■		■		■	■	■
5	■		■		■	■	■
6			■			■	■
7	■		■	■	■	■	■
8			■			■	■
9	■		■	■	■	■	■
10			■			■	■
11			■			■	■
12	■		■	■	■	■	
13	■		■	■	■		
14	■		■	■	■	■	■
15	■		■	■	■		
16	■		■	■	■		
17	■		■	■	■		
18	■		■	■	■		
19	■	■	■				
20	■	■	■				
21	■		■				
22	■	■	■				
23	■	■	■				
24	■	■	■				
25	■	■	■				
26	■	■	■				
27	■	■	■				
28	■	■	■				
29	■	■	■				
30	■	■	■				
31	■		■	■	■		
32	■	■	■				
33	■		■				
34	■	■	■				
35	■		■	■	■		

buffy coats from a total of 35 anonymous blood donors (Table 1) were obtained from the Regional Center of Blood Donation and Blood Treatment in Lodz, Poland according to the center's legal guidelines. Briefly, the 50 ml buffy coats were diluted in three volumes of PBS, gently layered on a Ficoll-Paque gradient, and centrifuged at room temperature for 30 min at 450 \times g. The interface layer of mononuclear cells was harvested and washed in PBS.

The CD133+ cells were separated from the mononuclear cells using a magnetic separation column as follows: the mononuclear cells were resuspended in 300 μ l of MACS buffer and were pre-treated with an FcR blocking reagent at 100 μ l/10⁸ cells. The cells were incubated with AC133 MicroBeads for 30 min at 4 °C. After incubation, the cells were washed with MACS buffer. The pellet was then resuspended in 500 μ l of MACS buffer and passed through the separation columns. The LS+ column was washed three times with 3 ml of MACS buffer while in the magnetic field, and the CD133+ cells were retained in the column, while the lineage negative cells were eluted. The column was removed from the magnet, and the CD133+ cells were collected and counted in Trypan blue.

Cell culture and differentiation of mast cells

The mast cell cultures were performed as described by Holm et al. (2008). Briefly, the cells were maintained at a density of 5×10^5 /ml in StemSpan medium supplemented with 100 ng/ml rhSCF, 50 ng/ml rhIL-6, 1 ng/ml rhIL-3, and penicillin/streptomycin solution. IL-3 was removed from the medium after three weeks of culture, and 10 % FCS was introduced into the culture medium after six weeks. The medium was renewed once per week. For the gene expression analysis, 33 mast cell cultures were initiated from CD133+ cells that were obtained from individual donors. At the time points indicated in Table 1, aliquots of the cell suspensions were withdrawn from the culture for analysis. Seven cultures were continued until the seventh week. For the DNA methylation analysis, additional 8 cultures were initiated from CD133+ cells that were obtained from individual donors. At 3 and 5 weeks of culture, aliquots of equal numbers of cells from two of the cultures, or in one case four, were withdrawn, pooled, and analyzed.

Real-time RT-PCR

RNA was isolated using TRI Reagent[®] from Molecular Research Center. Aliquots of 1–5 μ g of total RNA were reverse-transcribed using RevertAid[™] H Minus M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania) in the presence of oligo-dT18 primers in a 20 μ l RT reaction. The level of cognate cDNA was measured by real-time PCR amplification performed on a LightCycler 480 (Roche, Basel, Switzerland) using SYBR Green I master mix (Roche). The cycling conditions were as follows: 95 °C 10 s, 55 °C 10 s, and 72 °C 20 s. The following intron spanning primers were used: 5'-GAAAGCGCAAGTCTTCAAAG-3' and 5'-TGGGTAGGAGATGGAGATGC-3' for *HIF1A*, 5'-CATCACAGAAACGACAGT

CAA-3' and 5'-ACTCCGCACAGCTGGAGG-3' for *CD34*, 5'-TGTGGCAGCTGGACTATGAGTCT-3' and 5'-ACTTCTCACGCGGAGCTTTTAT-3' for *FCER1A*, 5'-ACAGGAGCCTTCCAGTGTGCCT-3' and 5'-CAGTGGTGGGGATGAGGCCG-3' for *MS4A2*, 5'-GGTGAACAAGCAGCGGCC-3' and 5'-TTCCTGGTGCTCAGGCCCGT-3' for *FCER1G*, 5'-GGGGAGGGGAGAGCCCACTG-3' and 5'-CTTGACGTCCGGTCCCACGC-3' for *TPSAB1*, 5'-TCATGCCAGGGCGACTCG-3' and 5'-ACCCAGGTGGACACCCCAGG-3' for *TPSAB2*, and 5'-ACTTCCAACGGTCCCTCAA-3' and 5'-CCTCAAGCTTCTGCCATGTG-3' for *CMA1*, 5'-ATTCTCTGCGTTCTGCTCCTAC-3' and 5'-CGCCACGCGGACTATTA for *KIT*, 5'-AGGATGAAAACAAGCAGACATCA-3' and 5'-CAGACTGGATGGCTTGGGATT-3' for *CPA3*, 5'-GAATGACGCCCTCAATCAAAGT-3' and 5'-TCATCTGGGCAGTCACATACA for *IL1A*, 5'-ACAGATGAAGTGCTCCTTCCA-3' and 5'-GTCGGAGATTTCGTAGCTGGAT-3' for *IL1B*, 5'-AAGAGACCTTGGCACTCTTTC-3' and 5'-GGAACAGGAATCCTCAGAGTCTCA for *IL5*, 5'-TGAGGAGCTGGTCAACATCA-3' and 5'-CAGGTTGATGCTCCATACCAT-3' for *IL13*, 5'-ATGCCGACCTCAACTCCTCCACT-3' and 5'-GCCACCCAGCTGCAAGATTTC-3' for *IL16*, 5'-GACGCATGCCCTCAATCC-3' and 5'-TAGAGCGCAATGGTGCAAT-3' for *IL18*, 5'-CAAGGCTACCATGCCAAC-3' and 5'-AGGGCCAGGACCTGCTG-3' for *TGFBI*, 5'-AGGAGGAGGGCAGAATCATCA-3' and 5'-CTCGATTGGATGGCAGTAGCT-3' for *VEGFA*, 5'-GCAACCAGTTCTCTGCATCA-3' and 5'-TGGCTGCTCGTCTCAAAGTA-3' for *CCL3*, 5'-ACCACACCCTGCTGCTTTC-3' and 5'-CCGAACCCATTCTTCTCTGG-3' for *CCL5*, 5'-GGCCAGCCACTACAAGCAGCACT-3' and 5'-CAAAGGGGATGACAAGCAGAAAG-3' for *CSF2*, 5'-ACTTTGCAGCCAGGTACGTC-3' and 5'-CTCAACCGAGAGATGCGAGC-3' for *TPSG1*

HPRT1, *HMBS*, *B2MG*, *RPL13A*, *TBP*, and *ACTB* were chosen as the most reliable reference genes by a selection procedure that was developed by Vandesompele et al. (2002). The relative gene expression was calculated using $\Delta\Delta C_T$ method with the geometric mean of selected reference genes.

Genomic DNA isolation and analysis of the in vivo methylation status of the human HIF1A and CD34 promoters

The genomic DNA from human cells was isolated using the Genomic DNA Extraction Kit (Panomics, Fremont, USA). The DNA was then sheared into 500–100 bp fragments by sonication (6 sets of 10-second pulses on wet ice) using a VCX130 sonicator (Sonics Inc., Newtown, USA). The methylated DNA was isolated

based on the methylated CpG island recovery assay (MIRA) (Rauch and Pfeifer 2005) using a MethylCollector™ Ultra Kit (Active Motif, Carlsbad, USA). The recovered methylated DNA fragments were purified by phenol/chloroform extraction, followed by ethanol precipitation, and then analyzed by RT-PCR with primers specific for the *HIF1A* promoter (forward: 5'-AGGATCACCTCTTCGTCG-3' and reverse: 5'-GCGGAGAAGAGAAGGAAAGG-3'; position: -259/-80) and the *CD34* promoter (forward: 5'-ACTCGGTGCGTCTCTCTAGG-3' and reverse: 5'-CCGTGAGACTCTGCTCTGC-3'; position: -35/+172). The relative amount of PCR product was calculated by transforming the Ct values according to the following formula: relative amount of PCR product = $1,000 \times (2^{\Delta Ct})$, where $\Delta Ct = Ct_{\text{sample}} - Ct_{\text{input}}$.

Bioinformatics

The promoter sequences of all of the analyzed genes were identified and downloaded from the UCSC genome browser (Kent et al. 2002). For the analysis of potential methylation sites in the selected gene promoters, the CpG Island Searcher was applied (Takai and Jones 2003). The putative HIF1 α binding motifs were identified using MatInspector Software (Cartharius et al. 2005).

Statistics

The statistical significance of the data was evaluated with the Student parametric test and the Mann–Whitney and Kruskal–Wallis nonparametric tests using the SPSS 10 software. The statistical analysis of the *in vivo* methylation data was performed using a one-way ANOVA followed by the Dunn's post hoc test. A *p* value of 0.05 or lower was considered statistically significant.

Results

In this study, we employed published experimental protocols for the culture of human mast cells. To verify the cell culture protocol, CD133+ cells were purified from the buffy coat preparations of five independent donors (>85 % purity), and $1\text{--}2 \times 10^6$ cells were cultured for 6 weeks. The cells were then analyzed for the presence of metachromatically stained granules and for surface expression of c-Kit and Fc ϵ RI. Greater than 92 % of the cells stained positive for toluidine blue, c-Kit, and Fc ϵ RI (data not shown). Next, the cells were harvested at the indicated time points and were analyzed by real-time PCR for the expression of several genes that are preferentially expressed either in hematopoietic

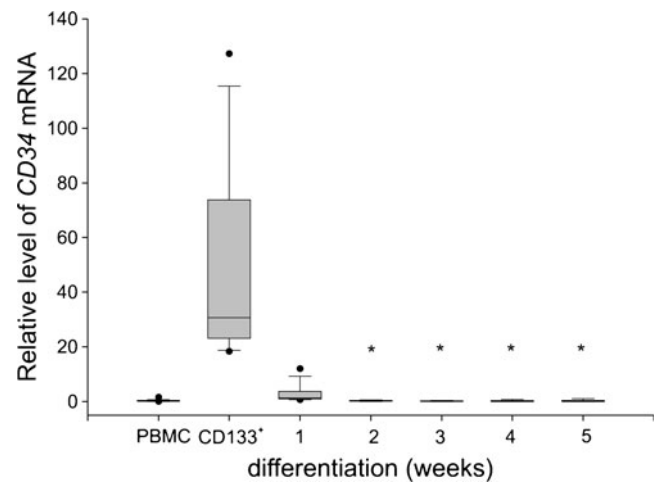


Fig. 1 *CD34* expression in differentiating human mast cells. Aliquots of PBMC obtained from anonymous blood donors were lysed for RNA isolation (PBMC), and the remaining cells were used for the purification of CD133+ cells by magnetic separation. Aliquots of the purified CD133+ cells were lysed for RNA isolation (CD133+), and the remaining cells were cultured under mast cell differentiating conditions for up to five weeks. At the indicated time points, the cells were lysed for RNA purification. The RNA was used to determine *CD34* expression by real-time RT-PCR. The data obtained from a total of 35 donors and 33 mast cell cultures, each initiated from a single donor, are presented as median, lower and upper quartile, and minimum and maximum for each experimental condition from at least 11 cultures. * indicates significant differences at $p < 0.05$ as compared to the CD133+ cells

progenitors or in mature mast cells. As seen in Fig. 1, *CD34* (the marker of hematopoietic progenitors) was hardly detectable in PBMC but showed a high level of expression in the purified CD133+ cells 48 h post-magnetic bead separation. This expression level decreased dramatically after the first week of culture and reached a level comparable to that observed in the PBMC by the second week of culture. We next analyzed the expression of a series of genes that are known to be specifically expressed in mature human mast cells: *FCERIA*, which codes for the alpha polypeptide of the Fc fragment of the high affinity IgE receptor; *MS4A2*, which codes for the beta chain of the Fc fragment of the high affinity IgE receptor; *TPSB2*, which codes for the mast cell granule-specific tryptase; and *CMA1*, which codes for the mast cell granule-specific chymase, and *KIT* which codes for c-Kit receptor. Expression of *KIT* has steadily increased from the low level observed in CD133+ cells (median 0.02) to the significantly higher expression at the third (median 11.56) and the fourth (median 19.66) week of culture (data not shown). As seen in Fig. 2a, the expression of *CMA1* in the PBMC and CD133+ cells was below the level of detection. However, it increased significantly following two weeks of culture and showed the highest level of

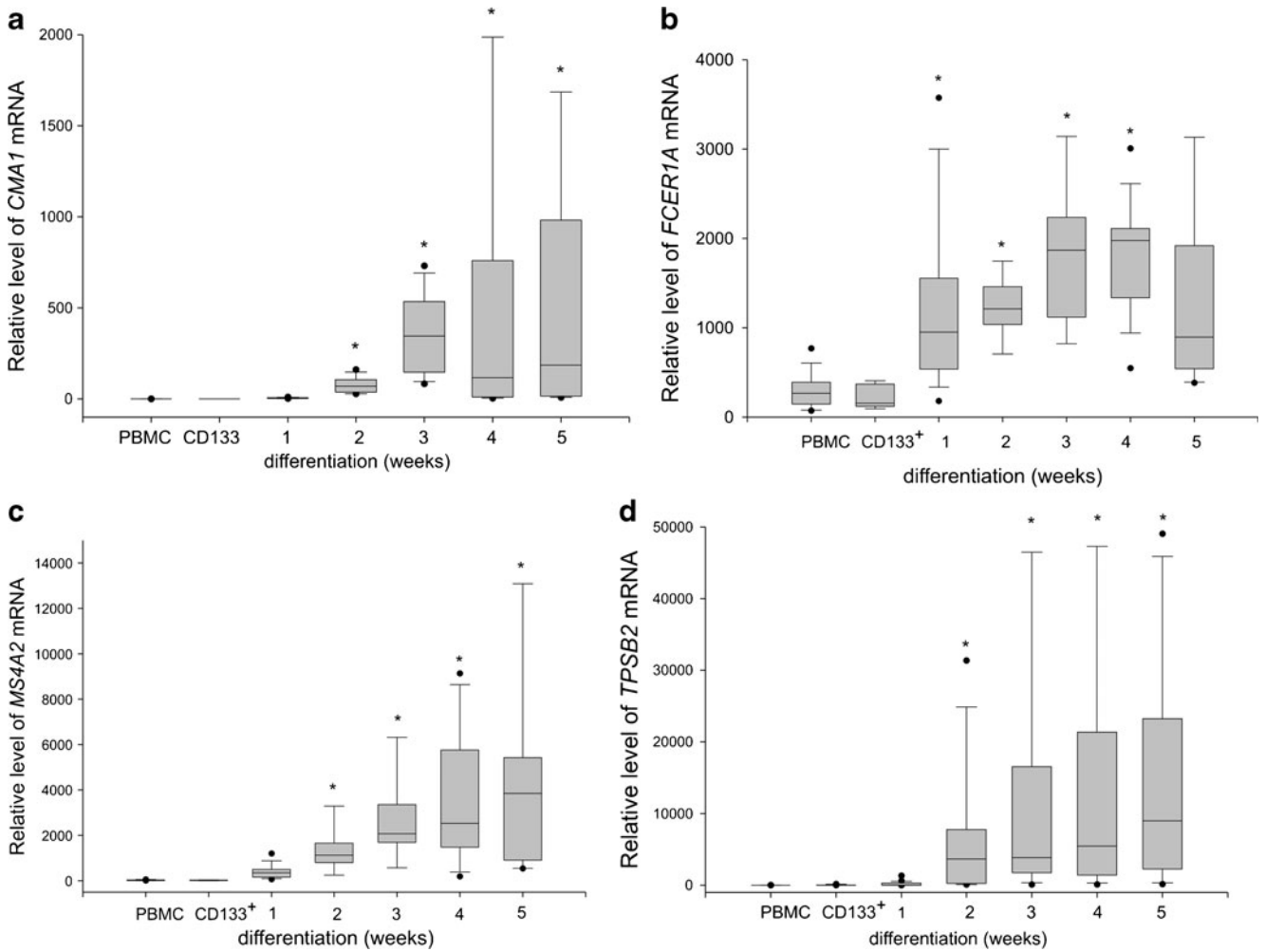


Fig. 2 Mast cell-specific gene expression in differentiating human mast cells. CD133+ cells obtained from PBMC of individual blood donors by magnetic separation were cultured under mast cell differentiating conditions for up to five weeks. Aliquots of PBMC (PBMC), purified CD133+ (CD133+), and cell cultured for indicated time were lysed for RNA purification and the RNA was used to determine *CMA1*

(a), *FCER1A* (b), *MS4A2* (c), and *TPSB2* (d) expression by real-time RT-PCR. The data obtained from a total of 35 donors and 33 mast cell cultures, are presented as median, lower and upper quartile, and minimum and maximum for each experimental condition from at least 11 cultures. * indicates significant differences at $p < 0.05$ as compared to the CD133+ cells

expression in the third week of the differentiating culture. The expression of *FCER1A* in the PBMC was above the level of detection, likely due to the presence of a small fraction of FcεRI positive cells, but was below the limit of detection in the purified CD133+ cells. *FCER1A* expression significantly increased within the first week of the differentiating culture and further increased, reaching the maximum expression level, in the fourth week of culture (Fig. 2b). Similar kinetics were observed for the expression of *MS4A2*, which was also undetectable in CD133+ cells: the expression significantly increased in the first week of culture and reached the maximum expression in the third week of culture (Fig. 2c). The expression of *TPSB2* was initially undetectable in the CD133+ cells. However, it increased significantly by the second week of culture and steadily

increased until the fifth week, when it reached the maximum expression level (Fig. 2d) Continuation of culture until the seventh week previously reported as optimal time for development mast cell-specific phenotype (Holm et al. 2008) did not result in significant differences in *FCER1A* and *TPSB2* expression as compared to the fifth week (data not shown). Thus, we observed increased expression of mast cell-specific genes upon culturing CD133+ cells under mast cell differentiating conditions. We next investigated the changes in *HIF1A* expression during culture. As seen in Fig. 3, the purified CD133+ cells revealed lower expression of *HIF1A* as compared to the PBMC. During in vitro differentiation, the expression of *HIF1A* significantly increased following three weeks of culture as compared to the CD133+ cells, and remained

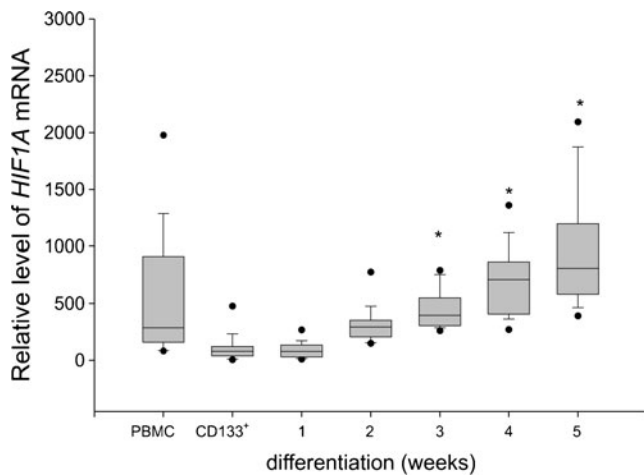


Fig. 3 *HIF1A* and *CD34* expression in differentiating human mast cells. CD133⁺ cells were obtained from PBMC of individual blood donors by magnetic separation and were cultured under mast cell differentiating conditions for up to five weeks. Aliquots of PBMC (PBMC), purified CD133⁺ (CD133⁺), and cell cultured for indicated time were lysed and the lysates were used for RNA purification. The RNA obtained from the different cell populations was used to determine *HIF1A* expression by real-time RT-PCR. The data obtained from a total of 35 donors and 33 mast cell cultures, are presented as median, lower and upper quartile, and minimum and maximum for each experimental condition from at least 11 cultures. * indicates significant differences at $p < 0.05$ as compared to the CD133⁺ cells

at this level until the seventh week of culture (data not shown). Thus, the expression of *HIF1A* paralleled the expression of several genetic markers of mast cell differentiation. We then analyzed the status of DNA methylation at the promoters of all the analyzed genes. We first performed a bioinformatics search for putative CpG islands in the upstream region of the analyzed genes. As seen in Table 2, only the upstream regions of *CD34* and *HIF1A* unambiguously demonstrated the presence of CpG islands. There were no potential CpG islands detected in the upstream regions of the *TPSB2*, *CMA1*, *MS4A2*, and *FCERIA* genes. We experimentally determined the level of DNA methylation and found that the methylation status of the promoter regions of *FCERIA*,

MS4A2, *TPSB2*, and *CMA1* did not change during the differentiating culture (data not shown). In contrast, the methylation of the *HIF1A* and *CD34* promoters changed during cell differentiation. Upon three weeks of culture under differentiation conditions, the level of DNA methylation at the *CD34* promoter significantly increased (Fig. 4a), and the level of DNA methylation at the *HIF1A* promoter significantly decreased (Fig. 4b). Thus, we observed that during mast cell differentiation, both the expression and the methylation levels of the *CD34* and *HIF1A* promoter sequences were inversely related. It has been previously shown that HIF1 α is engaged in the regulation of mast cell cytokine expression (Sumbayev et al. 2012). To further assess the potential role of *HIF1A* in mast cell function, we analyzed genes of known function that are preferentially expressed in mast cells (Metcalf 2008) for potential HIF-1 binding to hypoxia responsive elements (HRE). As seen in Table 3, this analysis identified several cytokine, chemokine and protease genes that are potentially regulated by HIF-1A. We next used the established human mast cell line LAD-2 to investigate the effects of hypoxia on the expression of genes that are potentially regulated by HIF-1 α . We observed significant increases in the expression of several genes important for mast cell function, such as: c-Kit, IL-1, and RANTES (Table 4).

Discussion

We report here that *HIF1A* expression significantly increased during the differentiation of peripheral blood-derived CD133⁺ cells into mast cells. While the level of *HIF1A* expression in undifferentiated CD133⁺ cells was significantly lower than in the PBMC, the level observed in mast cells was comparable to the PBMC, which consist mainly of differentiated hematopoietic cells (Fig. 3). This is similar to our previously reported observations that CD34⁺ cells isolated from the bone marrow and the peripheral

Table 2 Summary of bioinformatics-based analysis of promoter regions of investigated genes and experimental analysis of their methylation status during mast cell differentiation

Gene	CpG island	%GC	ObsCpG/ExpCpG	Length [bp]	Observed change in methylation upon differentiation
<i>FCERIA</i>	No	–	–	–	None
<i>TPSAB1</i>	No	–	–	–	None
<i>TPSB2</i>	No	–	–	–	None
<i>CMA1</i>	No	–	–	–	None
<i>CD34</i>	–197/+200	66.8	0.782	397	Increase
<i>HIF1A</i>	–775/+200	68.2	0.809	978	Decrease
<i>MS4A2</i>	No	–	–	–	None

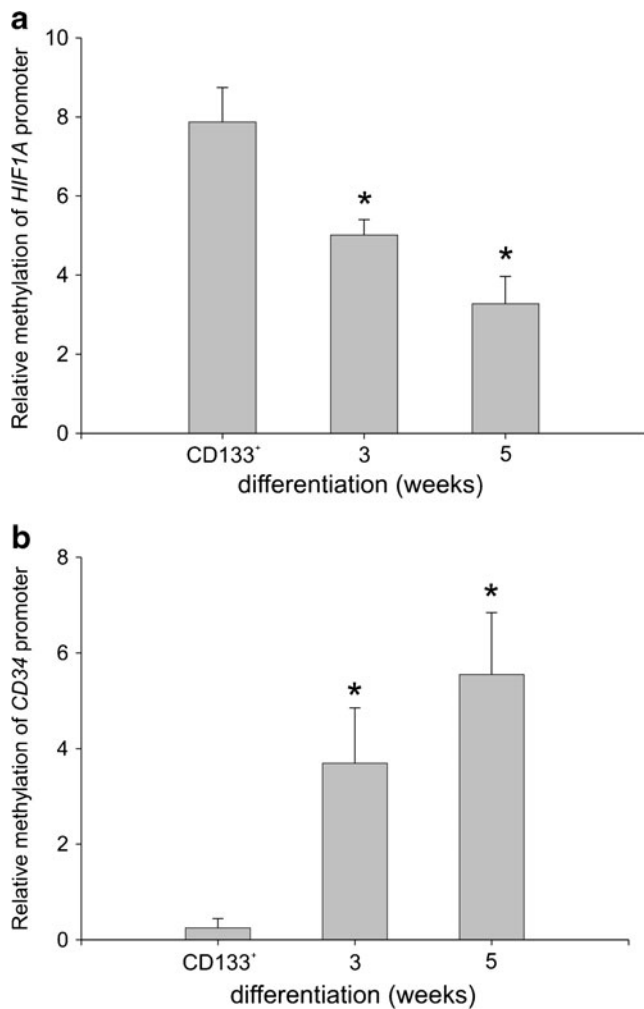


Fig. 4 The methylation status of the human *HIF1A* and *CD34* promoters in differentiating human mast cells. Aliquots containing equal numbers of CD133⁺ cells purified from the PBMC of two to four anonymous blood donors were pooled and lysed for the extraction of genomic DNA (CD133⁺). The remaining CD133⁺ cells were seeded into separate flasks and cultured under mast cell differentiating conditions. After 3 and 5 weeks of culture, aliquots containing equal numbers of cells originating from two to four blood donors were pooled and lysed for the extraction of genomic DNA. The DNA was analyzed by a methylated CpG island recovery assay followed by real-time PCR to determine the number of recovered *HIF1A* (a) promoter and *CD34* (b) promoter sequences. The data represent the mean \pm SEM of three DNA pools representing a total of eight donors

blood revealed lower levels of *HIF1A* expression than mature hematopoietic and non-hematopoietic cells (Walczak-Drzewiecka et al. 2010). Upon culture under differentiating conditions, the level of *HIF1A* expression increased in parallel to genes encoding characteristic mast cell proteins, such as the surface receptor for Fc IgE and granule associated proteases (Fig. 2). This suggested that the observed changes in *HIF1A* expression are related to the process of mast cell differentiation and that increased *HIF1A* expression relative to progenitor cells is a marker of mature mast

cells. The level of *HIF1A* expression in cultures predominantly containing differentiated mast cells was similar to that observed in PBMC. This is consistent with the hypothesis that increased *HIF1A* expression is not necessarily a phenomenon specific to mast cell differentiation, but might represent a general phenomenon of the differentiation of hematopoietic progenitors into mature blood cells.

One possible explanation for the increased baseline *HIF1A* expression in resting cells during differentiation is an epigenetic regulatory process. This hypothesis is consistent with the presence of large CpG islands in the *HIF1A* promoter region (Table 2). It also agrees with the observed decrease in methylation at the *HIF1A* promoters of differentiated mast cells at three and five weeks of culture (Fig. 3), which paralleled a significant increase in *HIF1A* expression. Interestingly, the *CD34* promoter, which also contains CpG islands (Table 2), simultaneously demonstrated increased methylation that paralleled decreased *CD34* expression. The opposing methylation statuses of these two genes suggests that they represent components of a complex epigenetic mechanism, which mediates cell maturation by silencing the genes involved in maintaining the phenotype of hematopoietic stem cells and upregulating the genes necessary for mature mast cell functions.

There is experimental evidence that *HIF1A* is essential for the cell cycle quiescence of hematopoietic and other stem cells, possibly due to its role in Notch-dependent signaling (Bar et al. 2010; Mukherjee et al. 2011; Takubo et al. 2010). This implies that the amount of HIF-1 α mRNA in hematopoietic stem cells is distinct from mature hematopoietic cells and is tightly regulated, at least in part, by methylation of the *HIF1A* promoter region. The change in the level of *HIF1A* promoter methylation upon mast cell differentiation, resulting in increased levels of HIF-1 α mRNA, might be important for the regulation of multiple *HIF1A* regulated genes that are necessary to maintain a mature mast cell phenotype. This hypothesis is consistent with the previously reported observations that the *HIF1A* product, HIF-1 α , plays a role in LPS-mediated TNF release in human mast cells (Sumbayev et al. 2012) and our observations that genes important for mast cell function, such as *Kit* and *IL1A*, contain promoter HRE motifs (Table 3) and are influenced by changes in oxygen concentrations (Table 4).

Previously reported epigenetic regulation of *HIF1A* expression has involved the methylation of DNA in the region overlapping the *HIF1A* proximal promoter (Koslowski et al. 2010; Walczak-Drzewiecka et al. 2010). Considering the sequence of the upstream region that showed decreased methylation levels, one possible mechanism for the upregulation of *HIF1A* is the demethylation of cytosines that neighbor the HRE binding sites. These cytosines have previously been shown to regulate the accessibility of the

Table 3 HIF1 α binding motifs identified in the promoters of genes relevant for mast cell function. -2000/+100 sequences were scanned for Putative HIF1 α binding sites for each gene. Locations of putative binding sites are relative to the ATG initiation codon

Class	Gene symbol	Number of HRE	Matrix similarity	Sequence	Strand
Preformed mediators	<i>CMA1</i>	1	0.894	-124gaagaaaaCGTGaccat-108	(+)
	<i>CPA3</i>	2	0.974	-1763catggaCGTGtttt-1749	(+)
			0.698	-603ttcacCACGttggccag-587	(-)
			0		
	<i>TPSAB1</i>	3	0.973	-776gggtctcaCGTGttggccag-760	(+)
			0.946	-731cccaccccCGTGgatte-715	(+)
			0.976	-368cgggggcaCGTGttaca-352	(+)
	<i>TPSB2</i>	2	0.946	-732gccaccccCGTGgatte-716	(+)
			0.976	-368gggggCGTGttaca-716	(+)
			<i>TPSG1</i>	5	0.982
	1.000	-1598cacccaCGTGgctg-1585			(+)
	0.980	-839ctccccaCGTGctg-823			(+)
	0.976	-816gcggcCACGtctgtg-800			(-)
	0.989	-515ctgccggaCGTGgggac-499			(+)
Cytokines	<i>INFG</i>	0			
	<i>IL1A</i>	1	0.931	-786gtagcCACGTagccacg-770	(-)
	<i>IL1B</i>	1	0.948	-857tcttctaaCGTGggaaa-841	(+)
	<i>IL4</i>	0			
	<i>IL5</i>	2	0.945	-1502accaaaCGTGgtaca-1488	(+)
			0.955	+40gctgcctaCGTGtatgc+56	(+)
	<i>IL6</i>	0			
	<i>IL8</i>	0			
	<i>IL13</i>	2	0.975	-1998cggCAAGTgcgcgc-1985	(-)
			0.945	-809gccagacaCGTGcacac-793	(+)
			0.973	-1725gagCACGTagacc-1712	(-)
	<i>IL16</i>	1	0.892	-1600gggcaaCGTGgtgaa-1586	(+)
	<i>IL18</i>	1	0.989	-1855gccccCGTGgcggc-1841	(+)
			0.871	-1645ctccaACGTcaccac-1631	(+)
<i>TGFB1</i>	2	0.975	+19atccgggaCGTGgagct+35	(+)	
Chemokines	<i>CCL2</i>	0			
	<i>CCL3</i>	1	0.955	+65ctgcatCACGtgagtct+81	(+)
	<i>CCL5</i>	1	0.973	-41agacagcaCGTGgacct-25	(+)
Growth factors	<i>CSF2</i>	1	0.986	-1141tgggcaCGTGtgggc-1127	(+)
	<i>VEGFA</i>	2	0.987	-1552tgaggaCGTGtgtgt-1538	(+)
0.892			-1353tccgaACGTaacctc-1339	(+)	
Receptors	<i>FCER1A</i>	0			
	<i>FCER1G</i>	0			
	<i>KIT</i>	4	0.916	-814ggggaaaaCGTGtatga-798	(+)
			0.979	-496cgcccgaCGTGctcga-480	(+)
			0.916	-424tgccttaaCGTGtgcgt-408	(+)
		0.897	-45agctggaaCGTGgacca-29	(+)	
	<i>MS4A2</i>	0			

HRE binding motif, which has been shown to positively autoregulate *HIF1A* expression in colon cancer cells exposed to hypoxia (Koslowski et al. 2010). However, the observed increase in *HIF1A* expression in differentiating human mast

cells occurred under high concentrations of oxygen, and the same HRE motif was also reported to mediate negative regulation of *HIF1A* in other cell types (Xu et al. 2012). Furthermore, there are other binding motifs at close proximity

Table 4 Changes in gene expression in LAD-2 mast cells under hypoxia

Gene symbol	Relative gene expression	
	Normoxia	Hypoxia
<i>KIT</i>	112±24	401±118*
<i>CPA3</i>	4 914±239	102 130 ±
<i>IL1A</i>	0	50±16*
<i>IL1B</i>	0	1±1
<i>IL5</i>	0	4±2*
<i>IL13</i>	0	4±4
<i>IL16</i>	29±3	24±10
<i>IL18</i>	96±8	139±48
<i>TGFB1</i>	58±6	1 281±1054
<i>VEGFA</i>	25±4	444±209*
<i>CCL3</i>	106±5	91±5
<i>CCL5</i>	7±2	21±5*
<i>CSF2</i>	87±23	65±15
<i>CMA1</i>	254±83	346±66
<i>TPSB2</i>	62±18	100±42
<i>TPSG1</i>	1±0	2±0

LAD-2 mast cells were cultured for 5 days under normoxia (21 % O₂) or hypoxia (1 % O₂) and the gene expression was determined by real-time RT-PCR using $\Delta\Delta C_T$ method with the geometric mean of selected reference genes. Data are presented as the mean±SEM from four independent experiments ($n=4$)

$p<0.05$ statistically significant difference

to the HRE motif, including SP1, that are necessary for *HIF1A* promoter activity (Iyer et al. 1998; Minet et al. 1999).

In summary, we present evidence that during in vitro mast cell differentiation, *HIF1A* expression is upregulated by an epigenetic mechanism involving DNA methylation. This suggests that the baseline expression of *HIF1A* is epigenetically regulated in a cell type- and differentiation stage-specific fashion. Such precise regulation of *HIF1A* expression may be important for the cellular response to varying oxygen availability in particular organs and tissues.

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