Potential role of hypoxia in early stages of Hodgkin lymphoma pathogenesis

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ABSTRACT

A unique feature of the germinal center B cell-derived Hodgkin and Reed/Sternberg cells of classical Hodgkin lymphoma is their lost B cell phenotype and the aberrant expression of factors of other hematopoietic cell types, including ID2 and NOTCH1. As cellular dedifferentiation and upregulation of ID2 and NOTCH1 are typical consequences of a hypoxic response, we wondered whether hypoxia may impose an HRS cell-like phenotype in B cells. Culturing normal B cells or cell lines of germinal center-type diffuse large B-cell lymphoma under hypoxic conditions caused partial downregulation of several B cell markers, ID2 upregulation, and increased NOTCH1 activity. The hypoxic cells acquired further features of Hodgkin and Reed/Sternberg cells, including increased JUN expression, and enhanced NFκB activity. The Hodgkin and Reed/Sternberg cell-expressed epigenetic regulators KDM4C and PCGF2, as well as the phosphatase DUSP1 were partially induced in hypoxic B cells. Inhibition of DUSP1 was toxic for classical Hodgkin lymphoma cell lines. Thus, hypoxia induces key Hodgkin and Reed/Sternberg cell characteristics in mature B cells. We speculate that hypoxic conditions in the germinal center may impose phenotypic changes in germinal center B cells, promoting their survival and initiating their differentiation towards a Hodgkin and Reed/Sternberg cell-like phenotype. These may then be stabilized by transforming events in the Hodgkin and Reed/Sternberg precursor cells.

Introduction

In classical Hodgkin lymphoma (cHL), the rare Hodgkin and Reed/Sternberg (HRS) tumor cells are derived from mature B cells, presumably pre-apoptotic germinal center (GC) B cells that had acquired unfavorable immunoglobulin V gene mutations.[1,2] However, HRS cells phenotypically no longer resemble B cells, as they lack expression of the majority of B-lymphocyte markers, including the B-cell receptor (BCR) and important B-cell transcription factors.[3-5] This loss of the B cell gene expression program represents a phenomenon hereafter termed as dedifferentiation. Additionally, HRS cells express multiple genes that are not normally expressed by B cells. The aberrantly expressed genes include NOTCH1, a T cell transcription factor that regulates the differentiation of lymphoid precursors into T but not B cells, and ID2, which is expressed by natural killer cells and suppresses B cell genes.[6-8] Hence, HRS cells have undergone a dramatic reprogramming in comparison to the B cells they stem from.[6,7,9] The loss of the B cell gene expression program may be a strategy of the HRS (precursor) cells to escape the selectional forces on GC B cells to express a high-affinity BCR or otherwise undergo apoptosis.[5] Indeed, partial reexpression of the B cell program in cHL cell lines impaired their survival.[10]

Expression and activity of ID2, NOTCH1, AP-1, NFκB and JAK/STAT signalling, which are all hallmarks of HRS cells,[2,11,12] are induced upon hypoxia in various cell types.[13-16] Moreover, a general dedifferentiation is typically seen in hypoxic cancer cells, and ID2 and NOTCH1 contribute to this phenomenon.[14,17,18] Thus, there are key features of the HRS cell phenotype that resemble hypoxic cells. The main regulator of the hypoxic response is the transcription factor hypoxia-inducible factor (HIF)-1. HIF-1 is composed of an oxygen-regulated α-subunit and a constitutively expressed β-subunit. Under well-oxygenated conditions, HIF-1α or its homologue HIF-2α are constitutively produced but post-translationally hydroxylated by prolyl hydroxylases. Hydroxyproline HIF-α is polyubiquitinated and degraded. Decreased hydroxylase activity under hypoxic conditions allows the accumulation of HIF-1/2α, and after dimerization with HIF-1β, HIF-1 acts as a master regulator of the hypoxic response.[19]

HIF-1α is expressed in some GC B cells, and moderately expressed in many B cell non–Hodgkin lymphomas (B-NHL).[20] The pronounced angiogenic activity observed in cHL lymph nodes[21,22] is presumably due to hypoxic conditions. However, it seems unlikely that HRS cells are constantly under hypoxic conditions and need this to stabilize their reprogrammed phenotype. Indeed, HL cell lines retain the typical HRS cell gene expression program under normoxia.[23] It is, however, an intriguing possibility that HIF-1α signaling during early stages of HL development may lead to a transient downregulation of B cell differentiation factors, and to the upregulation and activation of factors like ID2 and NOTCH1, thereby initiating, and/or contributing to, reprogramming of HRS cells. In later stages of lymphoma development, the reprogramming may be stabilized, so that constant hypoxia is not needed to sustain the HRS cell phenotype. Indeed, in HRS cells downregulation of many B cell genes is stabilized and enforced by epigenetic mechanisms.[24,25]

Here, we analyzed whether hypoxia leads to acquisition of

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an HRS cell-like phenotype in human B cells, including downregulation of B cell genes and upregulation of non-B-cell genes. Furthermore, we analyzed hypoxia-induced gene expression that may support the malignant transformation of GC B cells to HRS cells.

Methods

Cell culturing under hypoxic conditions included oxygen displacement by nitrogen to maintain hypoxic conditions of 1% O_2 concentration. Normoxia refers to identical cell incubation conditions with 5% CO_2 and 37°C, but atmospheric O_2 pressure. For normal cell culture, qRT-PCR, Western blot, cell viability after DUSP1 inhibition and flow cytometric analysis standard methodology was used. Peripheral blood (PB) B cells were isolated using a CD19-MACS strategy (Miltenyi Biotec, Bergisch Gladbach, Germany) to a purity of >98%. Usage of peripheral blood B cells from healthy donors was approved by the internal review board of the University Hospital in Essen, Germany. A detailed description of the methods used is supplied in the *Online Supplementary Appendix*.

Results

Hypoxic conditions induce HIF-1 in GCB-DLBCL cell lines and PB B cells

To study the consequences of hypoxia on the phenotype of human B cells, we cultured PB B cells and the two GCB-DLBCL cell lines SUDHL4 and SUDHL6 in a pre-established standard condition of 1% O_2 for 24 h. The hypoxic reaction is evident by the accumulation of HIF-1α, whereas cells cultured under normoxic conditions do not show detectable levels of HIF-1α protein (Figure 1A). PB B cells and SUDHL6 cells showed no HIF-2α accumulation under hxpoxia (data not shown). Induction of HIF-1α activity was validated for each experiment by detecting upregulation of the classical HIF-1α target VEGF (Figure 1B).

Neither of the DLBCL cell lines displayed changes in terms of fitness or apoptosis under hypoxic conditions for up to 24 h (Figure 1C). 48 h of hypoxia led to a marginal increase of apoptotic DLBCL cells. PB B cells did not show increased apoptosis upon culturing under hypoxic conditions for up to 48 h. Hence, hypoxia for 24 h does not induce apoptosis in the B cells and B cell lines investigated, showing that phenotypic changes in these cells are not simply a consequence of death induction, but represent a specific hypoxic response.

Hypoxia induces a partial downregulation of B cell markers

For the analysis of a potential downregulation of typical B cell genes in PB B cells and GCB-DLBCL cell lines upon hypoxia, we selected *CD19, CD79b, PAX5, EBF1, BOB1*, and *LCK*, as these are also downregulated in HRS cells in comparison to normal GC B cells.[5,23,26-28] The results of the qRT-PCR analysis for these markers after incubation of the cells for 24 h under normoxia or hypoxia were heterogeneous. CD79b transcription showed a moderate downregulation under hypoxic conditions in SUDHL4 cells, and LCK was 3.2-fold downregulated in PB B cells. SUDHL6 downregulated CD19 at early time points of 6 h and 14 h, and PAX5 at 6 h (Figure 2A,B). However, most genes did not show a significant and consistent regulation under hypoxic conditions for the PB B cells and DLBCL lines. EBF1 and BOB1 even showed a 2-fold and 4-fold upregulation in normal B cells, respectively, and a 2-fold upregulation in SUDHL6 cells (Figure 2B).

With regards to CD19 and CD79b, we also studied their regulation at the protein level by flow cytometry. Hypoxic SUDHL4 and SUDHL6 cells displayed a significantly reduced expression of CD19 and CD79b after 24 h (Figure 2C,D). When tested only for SUDHL6, that effect was even more significant upon prolonged hypoxia for 48 h (Figure 2D). PB B cells did not downregulate CD19 or CD79b.

Taken together, exposure to hypoxia did not result in a consistent downregulation of all B cell genes tested. Nevertheless, a clear downregulation of CD19 and CD79b in the GCB-DLBCL cell lines and of LCK in the PB B cells was observed.

Key factors of HRS cell dedifferentiation and survival are HIF-1 inducible

Hypoxia-induced expression of ID2 and NOTCH1 in B cells was tested because of their aberrant expression in

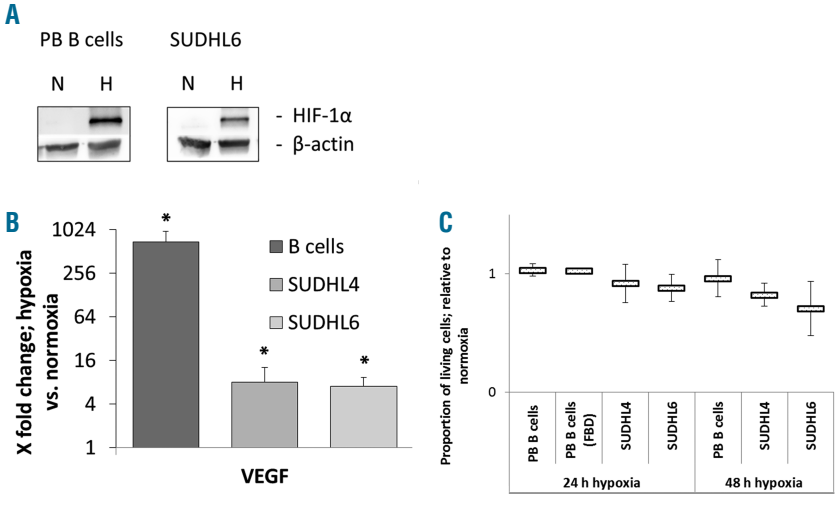

Figure 1. Hypoxic induction of HIF-1α. (A) Western blot analyses of PB B cells and the SUDHL6 GCB-DLBCL cell line that display stable HIF-1α protein upon culturing with 1% O_2 for 24 hours. β-actin was used as loading control. Normoxia: N; Hypoxia: H. (B) Normal B cells and DLBCL cell lines were analysed by qRT-PCR after hypoxic (1% O_2) or normoxic culture conditions after 24 h. At least three independent experiments were performed; VEGF upregulation was significant (*P<0.05) for all cells analysed. (C) FACS assay to quantify the proportion of living cells as defined by an annexin V- and propidium iodide-negative phenotype. Duration of culturing under hypoxia is indicated in hours (h). "PB B cells" indicates PB B cells isolated from buffy coats, whereas "PB B cells (FBD)" indicates PB B cells isolated from full blood donations. At least three independent experiments of each condition were performed.

HRS cells and their role in maintaining "the lost B cell identity" of HRS cells.[7-9] We also studied the NOTCH1 target genes *HES1, HEY1,* and *DTX1*. *DTX1* and *HEY1* are NOTCH1 target genes known to be highly expressed in HRS cells.[9,29] ID2 mRNA was significantly upregulated in B cells and both DLBCL cell lines upon 24 h of hypoxia (Figure 3A). In SUDHL6, ID2 was already expressed at 3-fold increased level after 6 h (Figure 3D). NOTCH1 mRNA levels were not significantly modulated upon hypoxia (Figure 3A). However, the NOTCH1 target genes *DTX1, HES1* and *HEY1* were expressed 4.8 – 8.1 times higher in hypoxic B cells, likely reflecting an active NOTCH1 pathway under hypoxic conditions. The DLBCL lines displayed the same tendency, but the effect was moderate.

In HRS cells, ID2 expression is partly caused by the AP-1 transcription factor JUN.[7] The aberrantly expressed JUN stimulates proliferation and contributes to the differentiation status of HRS cells.[11] JUN expression is inducible by low oxygen conditions.[30] We detected higher protein levels of JUN in hypoxic PB B cells, and hypoxia further increased JUN protein levels in the HRS cell line L1236 (Figure 3B). B-NHL derived cell lines BL41 and SUDHL6 remained JUN-negative under hypoxic conditions.

The NFκB pathway is constitutively active in HRS cells, and NFκB function is essential for the survival of HRS cells.[2,12] NFκB activity is inducible by hypoxia in various cell types.[13,16] Under hypoxic conditions, PB B cells showed a significant upregulation of the three NFκB targets IL6 (24.7-fold), IRF4 (7.9-fold) and BCL2 (7.8-fold) (Figure 3C). In DLBCL lines this effect was either weaker or not visible, but SUDHL4 showed a 6-fold induction of IRF4.

As HRS cells are very large granular cells, we analyzed whether hypoxic conditions induced an increase in cell size and/or granularity by measuring forward and side scatter, respectively, in flow cytometric analysis. No increase in forward scatter was observed, but hypoxic SUDHL4 cells showed a significantly higher side scatter (*Online Supplementary Figure S1*).

Taken together, hypoxic B cells and GCB-DLBCL lines upregulated ID2, and normal B cells upregulated JUN and showed increased activity of NOTCH1 and NFκB, all of which are key factors of HRS cell pathophysiology.

Hypoxia-induced deregulation of growth factors

We tested MYC expression due to its role as an oncogene and driver of proliferation in many lymphomas,[31] and its known expression in HRS cells.[23,32] MYC was upregulated by over 6-fold in hypoxic primary B cells (Figure 4A). No upregulation was observed for the NHL cell lines, perhaps because DLBCL cells already express considerable levels of MYC.[33]

The dual specificity phosphatase 1 (DUSP1) is involved

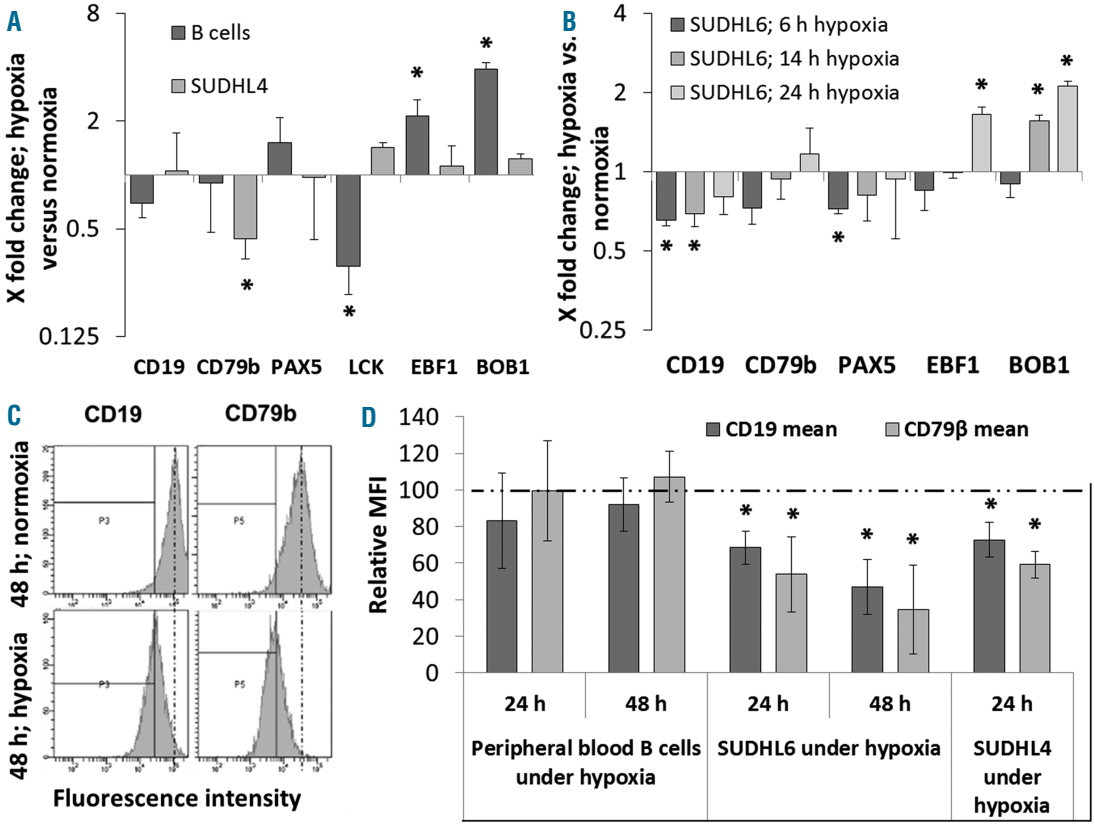

Figure 2. The hypoxia dependent partial downregulation of B cell markers. (A) B cell typical genes were analysed by qRT-PCR after hypoxic or normoxic culture conditions after 24 h for PB B and SUDHL4 cells or (B) for SUDHL6 cells at several time points. (C) Flow cytometric examples of the DLBCL cell line SUDHL6 for CD19 and CD79b expression, quantified by the mean fluorescence intensity (MFI). (D) Only annexin V- and propidium iodide-negative cells were analysed to determine the MFI of CD19 and CD79b staining at 24 h and 48 h. At least three independent experiments were performed for each experiment; *P<0.05.

in regulating extracellular signal-regulated kinase (ERK) and AP-1.[34,35] Additionally, DUSP1 has been functionally linked to HIF-1α,[36,37] and is induced by hypoxia in solid tumors.[38] As DUSP1 transcript levels are upregulated in primary HRS cells in comparison to normal GC B cells,[23] we tested whether DUSP1 is also hypoxia-inducible in B cells. Indeed, in hypoxic B cells DUSP1 was expressed as 8.6-fold higher than in control cells (Figure 4A). Even though the expression levels of DUSP1 of the HL cell lines were not much higher, or even slightly lower than those of GC B cells,[23] (*data not shown*), we tested the role of DUSP1 in HL cell lines. Incubating various cell lines with an inhibitor mainly targeting DUSP1, and with lower efficiency DUSP6, hereafter referred to as DUSP1/6i, revealed a strong attenuation of cell activity specifically for the HL cell lines (Figure 4B). Burkitt lymphoma cell lines were affected by DUSP1 inhibition only at high concentrations of the inhibitor, and two other cell lines were resistant even at high concentrations (Figure 4B)

Taken together, transcription of the growth and proliferation factor MYC is induced in normal B cells under hypoxia, and HL cell lines show a strong survival dependency on DUSP1, which is inducible in B cells under hypoxia.

Modulation of epigenetic factors in B lineage cells under hypoxia

As epigenetic factors may stabilize changes in gene expression induced by hypoxia, we investigated the epigenetic regulator KDM4C, which is inducible under hypoxia,[39] and PCGF2, which regulates the hypoxic repsonse.[40] Both factors are known to be expressed by HRS cells.[41,42] KDM4C was significantly upregulated in PB B cells and the two GCB-DLBCL lines under hypoxic conditions at certain time points of analysis (Figure 4C,D). For SUDHL4, KDM4C was weakly but significantly induced after 48 h (*data not shown*). The highest induction (3.7-fold) was observed for PB B cells after 24 h. PCGF2 (polycomb group ring finger 2), is a polycomb repressive complex 1 (PRC1) component expressed in primary HRS cells[42] and HL cell lines.[43] PRC1 together with PRC2 repress genes involved in differentiation and thereby support an undifferentiated phenotype.[44,45] PCGF2 was moderately upregulated in hypoxic DLBCL cells (Figure 4C,D). In SUDHL6 cells, PCGF2 induction became more pronounced upon longer exposure to hypoxia. In PB B cells, however, the moderate induction did not reach statistical significance. Overall, hypoxia leads to moderate upregulation of epigenetic regulators in mature B cells.

Discussion

cHL is unique among B cell lymphomas due to the extent to which the lymphoma cells have downregulated most B cell-typical genes, and upregulated numerous genes of other hematopoietic cell types, including NOTCH1 and ID2.[7-9,23,46] Intriguingly, dedifferentiation and upregulation of ID2 and NOTCH1 are hallmarks of the hypoxic response.[14,15,17,47] This prompted us to speculate

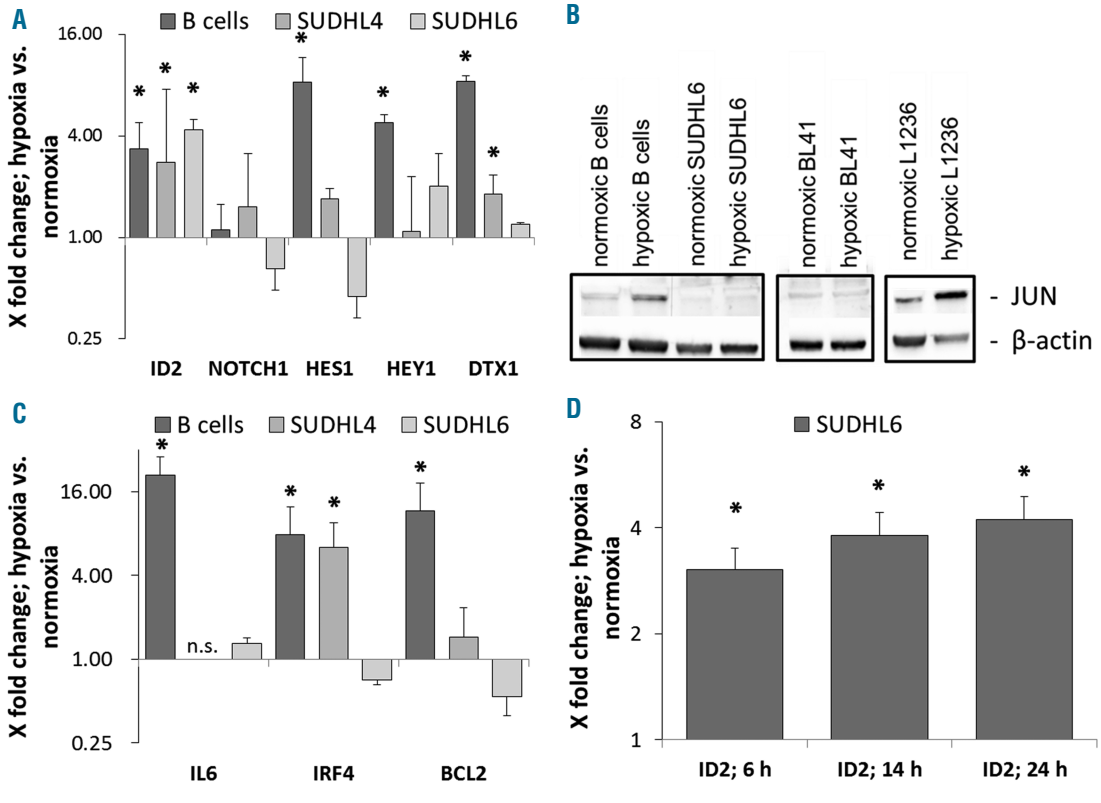

Figure 3. Hallmarks of HRS cell phenotype and pathophysiology are hypoxia-inducible. (A). qRT-PCR analysis of hypoxia-dependent expression of ID2, NOTCH1 and the NOTCH1 target genes *HES1, HEY1*, and *DTX1*. (B). Representative Western blot of JUN expression in PB B cells, SUDHL6, BL41 and L1236 cells. β-actin served as loading control. (C). qRT-PCR analysis of the NFκB target genes *IL6, IRF4*, and *BCL2*. (D). Time kinetics of ID2 expression in SUDHL6 cells, measured by qRT-PCR. At least three independent experiments were performed; *P<0.05. n.s.: no signal.

that a hypoxic response might play a role in the initial phase of HL pathogenesis, by initiating the dedifferentiation program and promoting the upregulation of non-B-cell markers. Ideally, we would have performed the experiments with GC B cells, the presumed origin of HRS cells.[1] However, these cells could not be used here, as GC B cells undergo spontaneous apoptosis in culture within one day.[48] We therefore used normal mature B cells, which survive in culture for several days. In addition we used two GCB-DLBCL cell lines, which have a gene expression pattern resembling GC B cells.[49] A caveat when using these lines is that they represent genetically altered GC B cells. However, by studying these two types of B cells, we expected to gain insight into the behavior of B cells under hypoxic conditions.

Upon hypoxia, we observed a downregulation of the B cell markers CD19 and CD79b in the DLBCL cell lines, of LCK in normal B cells, and of PAX5 in SUDHL6 cells. However, PAX5 was not significantly downregulated in normal B cells and SUDHL4 cells, and EBF1 and BOB1 were actually upregulated. It may well be that mature resting B cells are relatively resistant to downregulating the B cell program, and GCB-DLBCL are indeed considered to be "frozen" at the differentiation stage of GC B cells because of genetic lesions.[50] Normal GC B cells, on the other hand, are highly plastic cells, because they can undergo dramatic fate switches between remaining proliferative GC B cells, or differentiate into resting memory B cells or plasma cells. Thus, we speculate, but cannot directly test, that hypoxia may have a much stronger dedifferentiation effect on normal GC B cells than on PB B cells or GCB-DLBCL cells. Hence, the moderate downregulation of B cell genes we see can be considered as a proof of principle for perhaps a much greater effect of hypoxia on normal GC B cells.

In HRS cells, ID2 is one of several aberrantly expressed factors that were suggested to be central for the lost B cell phenotype.[7,8] We found a consistent upregulation of ID2 upon hypoxia of the PB B cells and DLBCL cell lines. Apart from ID2, NOTCH1 is another prominent negative regulator of the B cell program in HRS cells,[9,29,46] that is known to be induced by hypoxia in neuroblastoma and cancer stem cells.[15,18,51] Although we did not observe upregulation of NOTCH1 mRNA, key NOTCH1 target genes were consistently upregulated under hypoxic conditions. Hence, with the induction of ID2 and NOTCH1 activity, two key features of HRS cells were induced in mature B cells under hypoxic conditions.

Hypoxia caused further alterations, representing steps towards a HRS cell phenotype. The upregulation of the NFκB target genes BCL2, IL6 and IRF4 indicates NFκB activation. Constitutive NFκB activation is essential for HRS

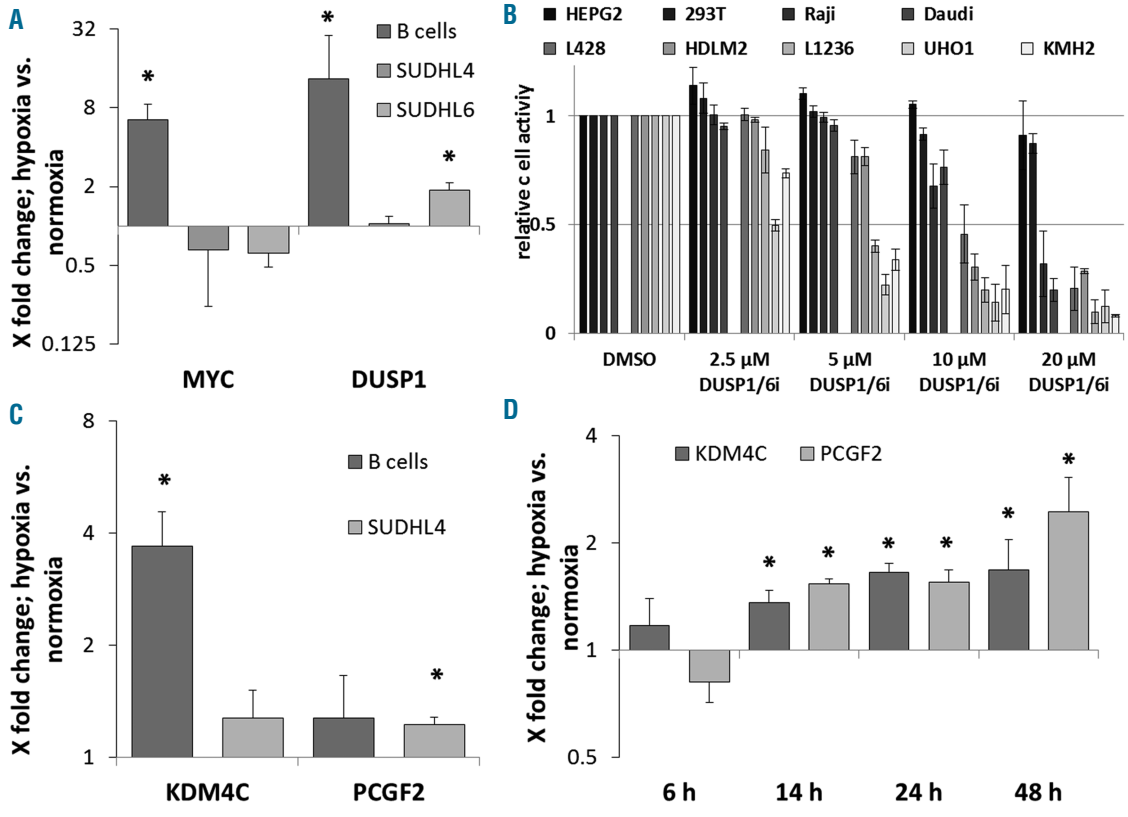

Figure 4. Factors associated with growth and epigenetic regulations are increased by hypoxia. (A). Hypoxic primary B cells, SUDHL4 and SUDHL6 cells were tested for expression changes of MYC and DUSP1 after 24 h of incubation. (B). Cell activity assay with increasing concentrations of the DUSP1/6 inhibitor reveal an increased sensitivity of five HL cell lines in comparison to the Burkitt lymphoma lines Raji and Daudi and the adherent cell lines HEPG2 and 293T to DUSP1 inhibition. (C). Hypoxic primary B cells and SUDHL4 cells were tested for expression changes of the epigenetic regulators KDM4C and PCGF2, after 24 h. (D). Time kinetic for KDM4C and PCGF2 expression changes under hypoxic cell culture conditions using SUDHL6 cells. For each experiment aliquots of cells was cultured under normal atmospheric or under hypoxic (1% O_2) conditions for 24 h. At least three independent experiments were performed; *P<0.05.

cell survival.[11,52] Also crucial for HRS cell survival and proliferation is the AP-1 protein JUN.[11] We observed increased protein levels of JUN in hypoxic PB B cells. The upregulation of MYC in normal B cells under hypoxia is an additional alteration in the B cells that brings them closer to the phenotype of HRS cells, because HRS cells show MYC activity.[23,41] As normal GC B cells express MYC only transiently in some centrocytes,[53] hypoxia in the GC may enforce and/or prolong MYC expression in these cells.

DUSP1 is a further factor upregulated in primary HRS cells[23] that was induced by hypoxia. DUSP1 expression is functionally relevant for HRS cells, because all five HL cell lines tested showed a strong impairment of metabolic activity upon DUSP1 inhibition. This may seem counterintuitive initially, because DUSP1 inhibits MEK/ERK signaling, which is important for HRS cell survival.[54] However, DUSP1 may balance ERK signaling strength, and may also have a prosurvival role in HRS cells by regulating other signaling pathways. Inhibition of DUSP1 may be of interest for targeted therapy of cHL.

As epigenetic alterations are involved in the lost B cell identity of HRS cells,[24,25] we questioned whether the short term hypoxic conditions could ultimately promote alterations in the expression of epigenetic regulators, which in the situation of HL pathogenesis may lead to stabilization of hypoxia-induced alterations. We focused on the epigenetic regulators PCGF2 and KDM4C that are expressed by HRS cells.[41,42] Hypoxia resulted in induction of PCGF2 in the two GCB-DLBCL cell lines, and of KDM4C in PB B cells. In a fraction of cHL, enforced expression of KDM4C is likely due to the 9p24 amplification in HRS cells,[41] suggesting a way in which an initial hypoxia-induced upregulation of this factor could be fixed in HRS cells. Thus, although it remains unclear what consequences of a hypoxia-induced upregulation of epigenetic regulators would have in GC B cells *in vivo*, it is conceivable that some HIF-1α-induced effects are stabilized by epigenetic alterations.

In conclusion, exposing PB B cells and GCB-DLBCL cell lines to hypoxic conditions initiates numerous changes in these cells that bring their phenotype closer to that of HRS cells. Most of the changes were moderate. However, PB B cells are less plastic than normal GC B cells, the cellular origin of HRS cells, and it is also likely that the transformed GCB-DLBCL cell lines are less responsive to microenvironmental stimuli than normal GC B cells. Therefore, we find the multitude of changes – downregulation of some B cell markers, upregulation of ID2 and NOTCH1 activity, increased JUN protein, increased NFκB activity, upregulation of MYC, DUSP1, KDM4C and PCGF2 – although often only seen in the normal B cells or the DLBCL cell lines, as being really remarkable overall. The detection of the HIF-1α protein in normal GC B cells in lymph nodes[20] supports the idea that GC B cells may be transiently under hypoxic conditions. We therefore propose a scenario in which normal GC B cells under hypoxia may transiently acquire features of HRS cells that are then stabilized by epigenetic and/or genetic alterations. This scenario is clearly speculative. Nevertheless, as none of the known genetic lesions in HRS cells explains the dramatic reprogramming of HRS cells, we offer herein a hypothesis for one way in which this process could be initiated in GC B cells.

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Authorship and Disclosures

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