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Promoter-associated proteins of *EPAS1* identified by enChIP-MS – A putative role of HDX as a negative regulator

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ABSTRACT

Presence of perivascular neuroblastoma cells with high expression of hypoxia inducible factor (HIF)-2α correlates with distant metastasis and aggressive disease. Regulation of HIFs are traditionally considered to occur post-translationally, but we have recently shown that HIF-2α is unconventionally regulated also at the transcriptional level in neuroblastoma cells. Regulatory factors binding directly to *EPAS1* (encoding HIF-2α) to promote transcription are yet to be defined. Here, we employ the novel CRISPR/Cas9-based engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) – mass spectrometry (MS) methodology to, in an unbiased fashion, identify proteins that associate with the *EPAS1* promoter under normoxic and hypoxic conditions. Our enChIP analysis resulted in 27 proteins binding to the *EPAS1* promoter in neuroblastoma cells. In agreement with a general hypoxia-driven downregulation of gene transcription, the majority (24 out of 27) of proteins dissociate from the promoter at hypoxia. Among them were several nucleosome-associated proteins suggesting a general opening of chromatin as one explanation to induced *EPAS1* transcription at hypoxia. Of particular interest from the list of released factors at hypoxia was the highly divergent homeobox (HDX) transcription factor, that we show inversely correlates with HIF-2α in neuroblastoma cells. We propose a putative model where HDX negatively regulates *EPAS1* expression through a release-of-inhibition mechanism.

1. Introduction

Neuroblastoma cells at low oxygen (i.e. hypoxia) de-differentiate and acquire stem cell-like features [1]. Hypoxia-inducible factors (HIF)-1 and HIF-2 are key regulators of the cellular response to hypoxia. Expression of HIF-α proteins correlate with unfavorable disease in numerous cancer forms, and in neuroblastoma expression of HIF-2α protein correlates to poor patient outcome and disseminated disease while HIF-1α expression levels lack prognostic value [2,3]. Perivascular neuroblastoma cells expressing unexpectedly high levels of HIF-2α are immature and neural crest-like [4], and similar observations have been reported in glioma [5]. Interestingly, *EPAS1* (encoding HIF-2α) is expressed during discrete periods of early developmental stages of human sympathetic nervous system (SNS) [6,7]. HIF-2α accumulation is promoted at the mRNA level [7–9] and given the expression patterns of HIF-2α during normal SNS development and the positive association to neuroblastoma tumor progression, understanding of molecular events regulating HIF-2α expression in neuroblastoma may open up for novel treatment strategies.

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The CRISPR/Cas9 system has turned out to be an extremely efficient technique to edit the genomic architecture [10]. Recently, the CRISPR/Cas9-based engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) - mass spectrometry (MS) approach was developed to combine and take advantage of the dCas9 and SILAC-MS technologies [11,12]. With enChIP-MS, quantitative changes in proteins that bind to gRNA-targeted DNA regions can be identified in an unbiased fashion.

Since accumulation of HIF-2α protein correlates with *EPAS1* mRNA levels, and the increase in mRNA is not the result of mRNA stabilization [9], we have used the enChIP-MS approach to unravel an unbiased protein signature associated with the *EPAS1* promoter after a normoxic to hypoxic shift. We confirm that individual proteins binding to *EPAS1* can be successfully isolated and identified using enChIP-MS. Among the proteins less abundantly bound to the *EPAS1* promoter at hypoxia were several groups of histones as well as nucleophosmin (NPM1), indicating a general opening of the chromatin structure. This partly explains increased *EPAS1* transcription at hypoxic conditions. As proof-of-principle for application of enChIP-MS, we show that the HDX transcription factor – with two putative binding sites in the regulatory region of *EPAS1* – is less expressed in neuroblastoma cells cultured at hypoxia. With support from mouse and human tumor gene expression data, we propose a model where HDX might be involved in a release-of-inhibition mechanism of *EPAS1* expression.

2. Materials and methods

2.1. Cell culture and transfection

Human neuroblastoma cell lines SK-N-BE(2)c and IMR-32 (ATCC) were cultured as described [7]. Cell lines were regularly screened for mycoplasma. Culture conditions and handling of neuroblastoma cell lines and tissues analyzed by global expression approach has been described [13]. Neuroblastoma patient-derived xenograft (PDX) cells were isolated and cultured as previously described [14]. HEK293T cells (ATCC) were cultured in DMEM medium supplemented with 10% FBS and antibiotics. Hypoxia was generated in a Whitley H35 Hypoxystation (Don Whitley Scientific). Transfection was performed using siRNAs (20 nM) targeting *HDX* (s44196/Exon9/10; s44198/Exon3/4; Customized/Exon1; Ambion) or non-targeting control siRNA with RNAiMAX (ThermoFisher Scientific).

2.2. Tissues

Sympathetic ganglia containing hyperplastic neuroblast foci were harvested from *Th-Mycn*$^{+/+}$ mice [15] at days 7 ($n = 4$) and 14 ($n = 4$). Advanced neuroblastic tumors ($n = 4$) were obtained after 6 weeks. As control, sympathetic ganglia were obtained from *Th-Mycn*$^{-/-}$ mice at corresponding developmental stages. Total RNA was profiled on Agilent SurePrint G3 Gene Expression Microarrays according to the manufacturer's protocols. Data were summarized and normalized with the vsn method in the R statistical programming language using the *limma* package.

2.3. Promoter evaluation

The promoter region of *EPAS1* (±3 kbp) was examined for suitable guide RNA (gRNA) target regions by BLAST search (RefSeq NM_001430). Sequence regions with required endonuclease motif were evaluated for overlap with publically available ChIP-Seq datasets; ENCODE database [16], interspersed repeats and low complexity DNA sequence; RepeatMasker, http://www.repeatmasker.org, and conservation; GERP [17]. Chromatin States were obtained from the UCSC genome browser.

2.4. enChIP preparation

To construct gRNA_hEPAS1#2 (gRNA target sequence: *aagggcacggcgccggtacgagg*), gRNA cloning vector (Addgene #41824, kind gift from George Church) was used for Gibson assembly as previously described [18]. To construct gRNA_hEPAS1#2/pSIR, the gBlock excised from gRNA_hEPAS1#2 was inserted into pSIR self-inactivating retrovirus vector (Clontech). To examine target specificity of gRNA_hEPAS1#2, 3xFLAG-dCas9/pCMV-7.1 (Addgene #47948) [11] and gRNA_hEPAS1#2 were transfected into HEK293T cells. Cells were fixed with 1% formaldehyde at 37 °C for 5 min. The chromatin fraction was extracted, fragmented by sonication, and used for enChIP as previously described [11,12,19]. Purified DNA was used for real-time PCR as previously described [11]. Primers are listed in Supplementary Table S1. The 3xFLAG-dCas9/pMXs-puro (Addgene #51240) and gRNA_hEPAS1#2/pSIR plasmids were generated and sequentially transduced into neuroblastoma SK-N-BE(2)c cells. Cells were incubated with culture medium diluted viral supernatant in presence of 5 μg/ml polybrene (Sigma) for 24 h. Puromycin (0.7 μg/ml) selection started after 72 h for 3xFLAG-dCas9/pMXs-puro. Similarly, G418 (0.8 mg/ml) selection was added 72 h post transduction of gRNA_hEPAS1#2.

2.5. enChIP-MS

enChIP-MS was performed as described previously [11,12,19]. In short, 3xFLAG-dCas9 and gRNA_hEPAS1#2 expressing SK-N-BE(2)c neuroblastoma cells were grown in MEM and dialyzed FBS (ThermoFisher Scientific) with Lysine-2HCL and L-Arginine-HCL (ThermoFischer Scientific) (Light SILAC Medium) or $^{13}C_6$ $15N_4$ L-Arginine-HCL and $^{13}C_6$ L-Lysine-HCL (ThermoFisher Scientific) (Heavy SILAC Medium). 5×10^7 cells grown in Heavy SILAC Medium were subjected to hypoxia and mixed with 5×10^7 cells grown in Light SILAC Medium at normoxia for 72 h. Cells were fixed in 1% formaldehyde and neutralized with glycine solution. Chromatin was isolated and fragmented by sonication to obtain average length of 1.5 kb fragments and used for enChIP as previously described [12]. MS was performed on enChIP samples using a nanoLC-MS/MS system at DNA-chip Development Center for Infectious Diseases (RIMD, Osaka University) as described in Ref. [12]. The MS analysis was performed in duplicate.

2.6. Quantitative real-time PCR

Total RNA was extracted using the automated extraction robot Arrow (DiaSorin) after being prepared according to the manufacturer's recommendation. cDNA synthesis and quantitative PCR analysis was performed as described in Ref. [8]. Primers are listed in Supplementary Table S1.

2.7. Western blot

Western blot analyses were performed as previously described [9]. SK-N-BE(2)c cell cytoplasm and nuclei were fractionated using NE-PER (ThermoFisher Scientific). Antibodies used: anti-FLAG (#F3165, Sigma), anti-SDHA (#Ab14715, Abcam), anti-HIF-2α (Ab199, Abcam), anti-Actin (#691001, MP Biomedical), anti-HDX (#SAB1400801, Sigma), anti-Lamin B1 (ab16048, Abcam), anti-mouse (#62–6520, Invitrogen) and anti-rabbit (#65–6120, Invitrogen).

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