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Differential regulation of HIF-1α and HIF-2α in neuroblastoma: Estrogen-related receptor alpha (ERRα) regulates HIF2A transcription and correlates to poor outcome

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A B S T R A C T

Hypoxia-inducible factors (HIFs) are differentially regulated in tumor cells. While the current paradigm supports post-translational regulation of the HIF-α subunits, we recently showed that hypoxic HIF-2α is also transcriptionally regulated via insulin-like growth factor (IGF)-II in the childhood tumor neuroblastoma. Here, we demonstrate that transcriptional regulation of HIF-2α seems to be restricted to neural cell-derived tumors, while HIF-1α is canonically regulated at the post-translational level uniformly across different tumor forms. Enhanced expression of HIF2A mRNA at hypoxia is due to de novo transcription rather than increased mRNA stability, and chemical stabilization of the HIF-α proteins at oxygen-rich conditions unexpectedly leads to increased HIF2A transcription. The enhanced HIF2A levels do not seem to be dependent on active HIF-1. Using a transcriptome array approach, we identified members of the Peroxisome proliferator-activated receptor gamma coactivator (PGC)/Estrogen-related receptor (ERR) complex families as potential regulators of HIF2A. Knockdown or inhibition of one of the members, ERRα, leads to decreased expression of HIF2A, and high expression of the ERRα gene ESRRA correlates with poor overall and progression-free survival in a clinical neuroblastoma material consisting of 88 tumors. Thus, targeting of ERRα and pathways regulating transcriptional HIF-2α are promising therapeutic avenues in neuroblastoma.

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1. Introduction

Solid tumors frequently develop regions of hypoxia—low oxygen tensions—as highly proliferating tumor cells tend to outgrow the formation of new blood vessels within the tumor. Transformed and normal cells mainly adapt to hypoxic surroundings via stabilization of the oxygen sensitive α-subunits of the hypoxia inducible transcription factors (HIFs). Expression of HIF-1α and HIF-2α, as well as overall tumor hypoxia, correlates with metastasis, drug resistance and poor outcome in a number of different tumor forms, including neuroblastoma [1–7]. Although HIF-1α and HIF-2α have high sequence homology and share a large proportion of target genes, accumulating evidence shows that the regulation, and action, of these two proteins significantly differ in tumor cells [2,3,5,6,8–10].

The childhood tumor neuroblastoma is believed to originate from dividing precursor cells of the sympathoadrenal cell lineage during formation of the sympathetic nervous system (SNS). We have previously shown that HIF-1α and HIF-2α proteins are differentially expressed in neuroblastoma cells, with acute and transient stabilization of HIF-1α, and prolonged expression of HIF-2α during hypoxic conditions [2]. Presence of tumor cells with intense HIF-2α staining correlates with aggressive and metastatic disease, whereas HIF-1α expression rather correlates to favorable disease in neuroblastoma [2,6]. In addition, HIF-2α is expressed in peri-vascular niches in both neuroblastoma and glioblastoma [5,11], whereas HIF-1α expression mainly is restricted to hypoxic regions [11]. We have recently shown that HIF-2α is regulated at the transcriptional level by insulin-like growth factor (IGF)-II in neuroblastoma, and that these two proteins are co-expressed in embryonic neuroblasts of the developing human SNS [10].

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Here, we show that hypoxia-induced expression of HIF2A mRNA mimics protein expression patterns in a time-dependent manner with increased levels over time and a rate shift around 48 h after the hypoxic onset. While HIF1A mRNA levels decrease under prolonged hypoxia (at 1% O2) uniformly in a number of cultured tumor cells (neuroblastoma, breast cancer, prostate cancer, renal cell carcinoma (RCC), and non-small cell lung carcinoma (NSCLC)), increased HIF2A expression is only observed in neuroblastoma cells. The observed changes in HIF mRNA at hypoxia are not due to alterations in overall mRNA stability. In addition, chemical stabilization of the HIF proteins at normoxia (21% O2) leads to increased HIF2A, and decreased HIF1A mRNA levels, in conformity with results observed when cells are cultured at hypoxia. Using a quantitative real time (qRT)-PCR based array, we identified members of the Peroxisome proliferator-activated receptor gamma coactivator (PGC)/Estrogen-related receptor (ERR) complex families as potential regulators of HIF2A. Knockdown or inhibition of specifically one member, ERR2, downregulates HIF2A mRNA levels in normoxic and hypoxic neuroblastoma cells. Supporting a role for ERs in tumors with an HIF-2α-driven aggressive phenotype, high expression of the gene encoding ERRs, ESRRA, correlates with poor overall and progression-free survival in a clinical neuroblastoma data set consisting of 88 tumors.

2. Material and methods
2.1. Cells and reagents

Human neuroblastoma cell lines SK-N-BE(2)c and KCN-69n (kind gifts from Drs. June Biedler, Memorial Sloan Kettering Cancer Institute and Robert Ross, Fordham University), breast cancer cell line MCF7 (ATCC), prostate cancer cell line DU 145 (ATCC), renal cancer cell adenocarcinoma cell line 786-0 (ATCC), and non-small cell lung cancer cell line A549 (ATCC) were routinely grown as cancer cell adenocarcinoma cell line 786-0 (ATCC), and non-small cell lung cancer cell line A549 (ATCC) were routinely grown as monolayers in Minimal Essential (SK–N-BE(2)c, KCN-69n, MCF7), RPMI-1640 (DU 145), F-12 Kaighn’s modification (A549), or Dulbecco’s Modified Eagle’s (786-0) medium supplemented with fetal bovine serum and antibiotics. For culturing of MCF7 cells, growth medium was supplemented with sodium pyruvate, essential amino acids and insulin. Hypoxia was generated in an InvivO2 hypoxia workstation (Ruskin Technology) or a Whitley H35 Hypoxystation (Don Whitley Scientific). Cells were cultured with Actinomycin D (5 μg/ml, Sigma–Aldrich), 2,2’-dipyridyl (DIP) (200 μM, Sigma–Aldrich), or XCT790 (1–5 μM, Sigma–Aldrich).

2.2. Transfection

Transfections were carried out in serum- and penicillin-free OPTI-MEM medium (Gibco) using Lipofectamine 2000 (Invitrogen) as transfection reagent. For HIF knockdown studies, cells were transfected with siRNA duplexes (50 nM, Ambion) targeting HIF-1α or HIF-2α respectively, and as a control, the inverted or scrambled HIF-1α sequence was used. Sequences as previously described [12]. For ESRRA knockdown studies, siRNA against ESRRA (s4830; Ambion) or a non-targeting control siRNA (sic #2, Ambion) was used at final concentrations of 5 nM.

2.3. Western blotting

Cells were lysed in RIPA supplemented with Complete Protease Inhibitor (Roche Diagnostics). Proteins were separated by SDS/PAGE and transferred to Hybond-C-Extra Nitrocellulose membranes (Amersham). Antibodies are listed in Supplementary Table S1.

2.4. Quantitative real-time PCR

Total RNA was extracted manually using the QiA shredder and RNasy Mini Kits (Qiagen) according to the manufacturer’s recommendation or automatically using Arrow (DiaSorin) with Arrow RNA (Tissue Kit -DNA Free) Kit (DiaSorin). cDNA synthesis and quantitative PCR analysis was performed as described [10]. Expression levels of reference genes (YWHAZ, SDHA and UBC for SK-N-BE(2)c, KCN-69n, 786-0 and MCF7 cells; GAPDH for DU 145 cells; and UBC, TBP and HPRT1 for A549 cells) were used to normalize gene-of-interest expression [13]. Primer sequences are listed in Supplementary Table S2.

2.5. Expressed transcription factor knockdown transcriptome PCR array

The expressed transcription factor knockdown transcriptome PCR array (Sabiosciences) was used to simultaneously investigate a total of 270 known transcription factors. SYBR Green quantitative-PCR analysis was performed in a 7300 Real-Time PCR system (Applied Biosystems), to quantify expression of HIF2A and HIF1A using GAPDH as a reference gene. Fold changes in expression as a result of each siRNA treatment relative to negative siRNA control were calculated and normalized to GAPDH in accordance to manufacturer’s instructions.

2.6. Statistical analyses

All values are reported as mean ± SEM from at least three independent experiments, unless otherwise stated. Two-sided student’s unpaired t test was used for statistical analyses and three levels of significance were used, *p < 0.05, **p < 0.01, ***p < 0.001. No asterisk (*) or n.s. indicates no significance. A publically available data set containing 88 neuroblastomas [14] was acquired from R2: microarray analysis and visualization platform (http://r2.amc.nl). Kaplan–Meier and logrank survival analyses were performed using R statistical language and the survival package [15]. Bonferroni correction for multiple testing was employed to account for survival group optimization.

3. Results

3.1. Hypoxia-induced transcriptional regulation of HIF2A is restricted to neural-derived tumors

HIF proteins are differentially regulated in neuroblastoma with HIF-1α present mainly at acute phases of hypoxia (4–24 h) after which protein levels rapidly decline. HIF-2α, on the other hand, is predominantly expressed at prolonged phases of hypoxia (48–72 h) [2]. Here, we show that mRNA expression patterns of HIF2A mimic HIF-2α protein expression in neuroblastoma cells with continuous increase over time (Fig. 1A and [2]). HIF1A mRNA levels on the contrary decrease over time, despite stabilization of the protein at acute hypoxia (Fig. 1B). To verify transcriptional activity of HIF-2α even after prolonged exposure to hypoxia, we analyzed the expression of well-established [2] and strongly HIF-2α-driven target genes in neuroblastoma. Indeed, VEGFA and DEC1 mRNA expression closely follows HIF2A expression, with highest expression at later time points (Fig. 1C–D).
We sought to establish if the observed increase in HIF2A mRNA expression in hypoxic neuroblastoma cells was a general tumor cell phenomenon. In glioblastoma, another neural cell-derived tumor with an apparent dependency on HIF-2α [5,16,17], it has been suggested that HIF2A transcription is induced at hypoxia, in particular in glioblastoma stem cell-enriched primary cultures [5,18]. On the other hand, long-term hypoxic culturing of breast cancer, prostate cancer, renal cell carcinoma, and non-small cell lung carcinoma cell lines did not result in any significant effects on HIF2A mRNA (Fig. 1E). Transcription of HIF1A, however, is unchanged or even decreased uniformly in all tumor forms investigated (Fig. 1B,F and [5]).

3.2. Differential changes in HIF mRNA levels are not due to mRNA stability

To address if the increase in HIF2A mRNA levels at hypoxia was due to altered mRNA stability or de novo transcription, we treated neuroblastoma cells with actinomycin D, an inhibitor of RNA synthesis [19,20] for 2, 4, and 6 h after an initial priming of cells for 72 h at normoxia or hypoxia. There was no marked difference in either HIF2A or HIF1A mRNA levels between cells cultured at normoxia or hypoxia when RNA synthesis was blocked (Fig. 2). Thus, the overall actinomycin D-induced decrease in both HIF2A and HIF1A mRNA rather supports active transcription of the HIF genes. If the...
elevation of HIF2A mRNA in hypoxic neuroblastoma cells would be explained by increased mRNA stability, the difference in mRNA levels between actinomycin D treated normoxic and hypoxic cells would be substantial.

3.3. HIF protein stabilization leads to induced HIF2A mRNA expression

Cells exposed to hypoxia initiate a switch in their transcriptional program, mainly mediated via HIF-1 and HIF-2. In order to determine the effects on HIF mRNA expression solely by stabilization of the HIFs and not by activating the complete hypoxic machinery, we treated two neuroblastoma cell lines with 2,2’-dipyridyl (DIP), an iron chelator, at normoxia. This leads to inhibition of the iron-dependent hydroxylation and subsequent degradation of the HIF alpha subunits and instead, HIF-1α and HIF-2α are stabilized (Fig. 3A–B and reviewed in Ref. [21]). Surprisingly, also HIF2A mRNA expression is induced by DIP treatment (Fig. 3C), whereas HIF1A mRNA levels decrease (Fig. 3D), mimicking the hypoxic response (cf. Fig. 1A–B).

Considering that HIF2A and HIF1A mRNA levels consistently display opposing expression patterns at hypoxia (Figs. 1A–B and 3 C–D), we knocked down these two transcription factors individually and looked at their mRNA expression. Indeed, knockdown of HIF1A lead to increased transcription of HIF2A, whereas knockdown of HIF2A increased the transcription of HIF1A (Fig. 3E).

3.4. Transcription of HIF2A is regulated by ERRα

To further investigate the possible interregulation between HIF2A and HIF1A, we used a commercially available quantitative real time (qRT)-PCR based array, where 270 different transcription factors (including HIF-1α and HIF-2α) have been knocked down using RNAi techniques (Expressed transcription factor knockdown transcriptome PCR array, Sabiosciences). When analyzing the effects on HIF mRNA after transcription factor knockdown, we could conclude that HIF2A transcription seemed to be more complex than that of HIF1A (ten vs. two hits, fold change cut-off at ± 4) (Table 1). However, neither HIF1A nor HIF2A were identified as hits for potential regulation of each other (Table 1).

Instead, we looked at the list of potential regulators of HIF2A transcription and identified coactivator PGC-1α/β. Coactivators constitute a complex array of factors on which transcriptional regulation by gene- and cell-specific DNA-binding transcription factors depend upon. Coactivators of the PGC-1 family, PGC-1α and PGC-1β in particular, respond to multiple signals and enhance the transcriptional activity of key transcription factors involved in mitochondrial biogenesis, fatty acid utilization, electron transport chain assembly and angiogenesis [22]. Coactivation of ERRα by PGC-1α/β has been shown to play a role in the angiogenic and proliferative processes in tumors. More specifically, all members of the ERR family have been reported to physically interact with the HIFs in breast cancer, and the specific interaction between ERRα/PGC-1α is known to drive HIF2A transcription in skeletal muscle [22–24]. Since HIF-2α and HIF-1α are well-established inducers of VEGF-A and other pro-angiogenic factors, the PGC and ERR family members as potential regulators of HIF2A transcription were of particular interest. We could not establish a link between HIF2A expression and PGC-1α, PGC-1β or ERRα based on knockdown studies in neuroblastoma cells. However, RNAi-mediated knockdown of ERRα resulted in downregulated HIF2A mRNA levels both at normoxia (Fig. 4A) and hypoxia (Fig. 4B). To further validate the possible effects of ERRα on HIF2A transcription, we treated neuroblastoma cells with the ERRα inverse agonist XCT790. Expression of HIF2A mRNA decreased in an inhibitor concentration-dependent fashion (Fig. 4C). Of note, ESRRA mRNA levels were not affected by hypoxia (Fig. 4C).

3.5. Expression of ESRRA correlates to disease stage and poor prognosis in neuroblastoma

Having identified ERRα as a regulator of HIF2A transcription, we sought to pinpoint if ERRα expression levels carried any prognostic value in neuroblastoma. Using a publically available data set consisting of 88 tumors [14], we could show that high expression of ESRRA significantly correlates to poor overall and progression-free survival in neuroblastoma (bonf. p-values equal 0.014 and 0.00044, respectively) (Fig. 4D–E).

4. Discussion

We have recently established that HIF-2α is regulated at the transcriptional level in neuroblastoma cells, likely in addition to the canonical post-translational regulation that occurs in normal and tumor cells [10]. Here we establish that transcriptional regulation of HIF2A is not a general tumor cell phenomenon, but rather appears to be restricted to neurally derived tumors such as neuroblastoma and glioblastoma. In addition, these tumors share the characteristics of restricted areas with a pseudo-hypoxic phenotype with HIF-2α positive tumor cells located in peri-vascular niches in tumor specimens. High HIF-2α levels in neuroblastoma and glioblastoma correlate with aggressive, disseminating disease and as knockdown leads to impaired tumor growth in vivo in both these tumor forms [25,11], HIF2A qualify as an oncogene. Interestingly, gain-of-function mutations have been reported in two neuroblastoma-related tumor forms, paraganglioma and pheochromocytoma [25–30], further supporting an oncogenic role of HIF2A in neural cell-derived tumors. These results highlight the importance of
understanding and exploring the transcriptional regulation of HIF-2α in order to unravel novel therapeutic strategies for HIF-2α-driven aggressive tumor phenotypes.

There was no difference in the overall HIF mRNA stability between normoxic and hypoxic neuroblastoma cells, indicating that the observed increase in HIF2A mRNA is explained by de novo transcription. This result is supported by previous findings by Lin et al., showing that HIF mRNA expression is insensitive to changes in mRNA stability [31]. In addition, these results support the notion that induced expression of the HIF-2α protein at hypoxia to a large part can be explained by enhanced HIF2A transcription and not solely by protein stabilization.

Despite an acute stabilization of the HIF-1α protein, HIF1A mRNA expression decreases in hypoxic neuroblastoma cells. Since the effects on HIF2A mRNA is not explained by decreased mRNA stability, it is tempting to speculate that the degradation of HIF2A is increased at hypoxia.

Fig. 3. HIF1A and HIF2A show opposing mRNA expression patterns upon stabilization of HIF protein and knockdown of HIF mRNA. (A–B) SK-N-BE(2)c and KCN-69n cells were treated with 2′,2′-dipyridyl (DIP) for 4 h at 21% O2 and protein expression was determined by western blot. Actin was used as loading control. (C–D) Cells were treated as in (A–B) and HIF2A (C) and HIF1A (D) mRNA was quantified. (E) Knockdown of HIF2A and HIF1A using siRNAs in SK-N-BE(2)c cells, followed by 72 h at 1% O2. As an internal control (siC) a scrambled HIF-1α sequence was used. Quantification of mRNA levels was performed using qRT-PCR. Data are presented as mean ± SEM from three independent experiments. Statistical significance was calculated using student's t test, **p < 0.01, ***p < 0.001. No asterisk (*) indicates no significance.

Table 1
Genes altering the expression of HIF1A and HIF2A upon knock-down.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>Possible effect</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF1A</td>
<td>Hypoxia inducible factor 1, alpha subunit</td>
<td>Internal control for HIF1A</td>
<td>9.0</td>
</tr>
<tr>
<td>RUNX1</td>
<td>Runt-related transcription factor 1</td>
<td>Negative regulator candidate of HIF1A</td>
<td>4.6</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
<td>Positive regulator candidate of HIF1A</td>
<td>6.3</td>
</tr>
<tr>
<td>EPAS1</td>
<td>Endothelial PAS domain protein 1</td>
<td>Internal control for HIF2A</td>
<td>4.4</td>
</tr>
<tr>
<td>PRMT5</td>
<td>Protein arginine methyltransferase 5</td>
<td>Negative regulator candidate of HIF2A</td>
<td>5.2</td>
</tr>
<tr>
<td>PPARGC1B</td>
<td>Peroxisome proliferator-activated receptor gamma, coactivator 1 beta</td>
<td>Negative regulator candidate of HIF2A</td>
<td>7.6</td>
</tr>
<tr>
<td>SMARC4</td>
<td>SWI/SNF related, matrix associated, actin dependent regulator of chromatin</td>
<td>Negative regulator candidate of HIF2A</td>
<td>7.1</td>
</tr>
<tr>
<td>SMARCB1</td>
<td>SWI/SNF related, matrix associated, actin dependent regulator of chromatin</td>
<td>Negative regulator candidate of HIF2A</td>
<td>6.0</td>
</tr>
<tr>
<td>TCEAL1</td>
<td>Transcription elongation factor A</td>
<td>Negative regulator candidate of HIF2A</td>
<td>5.2</td>
</tr>
<tr>
<td>ZKSCAN1</td>
<td>Zink finger with KRAP and SCAN domains 1</td>
<td>Negative regulator candidate of HIF2A</td>
<td>4.9</td>
</tr>
<tr>
<td>YY1</td>
<td>YY1 transcription factor</td>
<td>Negative regulator candidate of HIF2A</td>
<td>4.8</td>
</tr>
<tr>
<td>SMAD1</td>
<td>SMAD family member 1</td>
<td>Negative regulator candidate of HIF2A</td>
<td>4.7</td>
</tr>
<tr>
<td>HDAC1</td>
<td>Histone deacetylase 1</td>
<td>Positive regulator candidate of HIF2A</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Screening of HIF1A and HIF2A gene expression modulators using transcription factor knockdown transcriptome PCR arrays. Quantification of HIF1A and HIF2A gene expression levels using qRT-PCR are expressed as fold changes based on Ct calculations using GAPDH as a reference gene. Non-target siRNA treated sample wells served as negative internal control. Data presented represent one experiment.
Fig. 4. Transcription of HIF2A depends on ERRα activity. (A–B) Knockdown of ESRRA in SK-N-BE(2)c cells using siRNA, followed by 72 h of normoxia (21% O₂, A) or hypoxia (1% O₂, B).

(C) Treatment with ERRα inverse agonist XCT-790 for 72 h at hypoxia with indicated concentrations of XCT-790. Normoxic cells were used as an internal control for HIF2A expression. Relative mRNA expression was measured by qRT-PCR and data are presented as mean ± SEM from three independent experiments. Statistical significance was calculated using student’s t test, *p < 0.05, **p < 0.01, ***p < 0.001. No asterisk (*) or n.s. indicates no significance. (D–E) High ESRRA mRNA expression in a clinical neuroblastoma material consisting of 88 tumors correlate with poor overall (D) and progression free (E) survival. Bonferroni corrected logrank p-values are provided.
synthesized HIF1A mRNA at hypoxia is enhanced. In addition, decreased HIF1A mRNA expression patterns are repeated in a number of different tumor forms that all stabilize the HIF-1α protein at hypoxia, confirming an exclusive post-translational regulatory mechanism of HIF-1α in tumor cells.

Unexpectedly, stabilization of the HIF-α proteins at normoxic conditions (by inhibiting the iron-dependent hydroxylation of the α subunits), leads to increased HIF2A, and decreased HIF1A, mRNA levels, mimicking the response observed when neuroblastoma cells are cultured at hypoxia. This might suggest an autocrine-/paracrine regulatory loop of the HIF transcription factors. Indeed, knockdown of HIF-1α enhanced expression of HIF2A and vice versa, indicating either direct or indirect interplay between these two proteins to at least some extent. However, when analyzing the effect on HIF mRNA levels after knockdown of 270 different transcription factors (including HIF-1α and HIF-2α), neither HIF1A nor HIF2A were identified as hits for possible regulation of each other. Considering the somewhat modest effect seen after HIF knockdown using siRNAs (Fig. 3E), and the crude cut-off (>4-fold change in expression levels) used for the transcriptome array, the effects that HIF-1α and HIF-2α might have on each other would probably be too small for detection by this methodological approach.

Of the potential regulators that were identified, PGC-1β was of particular interest due to the known role of the PGC-1/ERR family members in angiogenesis [32], and regulation of HIF expression in skeletal muscle [24]. The consequent downregulation of HIF2A mRNA expression after knockdown or inhibition of ERRx, and the strong correlation between high expression of ESRRα and poor overall and progression-free survival in neuroblastoma strongly suggest that ERRx plays a role in aggressive neuroblastoma behavior. Interestingly, ESRRα was recently identified as one of the most strongly expression-correlated regulators in an integrative analysis of 111 reference human epigenomes [33]. When each regulator was linked to the cell/tissue type that they most highly correlated with, ESRRα was found to have a regulatory relationship exclusively with the fetal adrenal gland [33], the organ in which more than half of all neuroblastomas arise. Hence, ERRx appears highly relevant in neuroblastoma and serves as a potential therapeutic target for aggressive tumor growth in patients.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.083.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.083.

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