Chapter 2

Hypoxic Upregulation of ARNT (HIF-1β): A Cell-Specific Attribute with Clinical Implications

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Abstract

According to the current point of view described in the literature, the transcription factor aryl hydrocarbon receptor nuclear translocator (ARNT), also designated as hypoxia-inducible factor (HIF)-1β, is constitutively expressed and not influenced by oxygen tension. However, a study published two decades ago provided early evidence regarding a hypoxia-dependent ARNT upregulation. This finding was subsequently challenged and neglected. Until now, only a limited number of publications focus on the regulation of ARNT in hypoxia. Therefore, appropriate studies and the putative mechanism mediating this cellular attribute are discussed. The advantages of an elevated ARNT expression level in tumour cells are delineated. This chapter provides an overview of hypoxia-inducible ARNT as an emerging concept in HIF biology.

Keywords: aryl hydrocarbon receptor nuclear translocator, ARNT, HIF-1β, crosstalk, cancer

1. Introduction

The name aryl hydrocarbon receptor nuclear translocator (ARNT) designates a transcription factor of the Per-ARNT-Sim family which is ubiquitously expressed. This protein is also known as hypoxia-inducible factor (HIF)-1β. The use of these two equal synonyms for the same transcription factor throughout the literature already implies its role in various signalling pathways [1]. Unfortunately, the term "hypoxia-inducible" might be misleading in this context. According to the current point of view described in the literature, ARNT expression is not affected by environmental conditions such as hypoxia. Therefore, ARNT is considered to be a constitutively expressed gene [1]. Although this notion might be true for the majority of cells/tissues investigated, numerous studies reported the capability of tumour cells to elevate

ARNT expression in response to hypoxia [1–7]. This cellular attribute was found in cells of different tumour types of both human and murine origin. These key findings clearly suggest that hypoxia-dependent ARNT upregulation might provide a certain benefit for appropriate cells [1].

ARNT and its paralogue ARNT2 [1] share a 90% identical amino acid sequence [8]. In contrast to ARNT, ARNT2 is mainly expressed in the central nervous system [8, 9]. ARNT2 expression was shown to be positively correlated with breast cancer prognosis [8]. In addition, high-ARNT2 levels in hepatocellular carcinomas are associated with a prolonged overall survival of cancer patients [8]. However, many functions of this transcription factor are still unknown [1, 8]. Moreover, the regulation of ARNT and ARNT2 varied in human hepatocellular carcinoma Hep3B cells. ARNT was elevated in hypoxia, whereas ARNT2 was not affected in this model [3].

This chapter describes the current knowledge regarding hypoxic upregulation of ARNT, which appears to be beneficial for certain types of tumour cells. Therefore, the aim of this section is to emphasise this unique cellular attribute. A potential altered ARNT expression level due to hypoxic exposure of cells should be considered and not generally excluded. In this context, the use of ARNT as loading control or as a reference gene is basically not recommended [1].

2. Regulation of ARNT

2.1. Upregulation of ARNT in response to hypoxia

First evidence for a hypoxia-dependent regulation of ARNT was provided by Wang et al. [5]. Herein, Hep3B cells were used to study the effects of HIF-1α and ARNT under hypoxic conditions. ARNT was elevated on mRNA level in this cell line due to hypoxic exposure (1% v/v O_2). In addition, treatment with hypoxia mimetics such as cobalt chloride and desferrioxamine had similar effects. Nuclear extracts prepared from Hep3B and HeLa cells were used to investigate the response of both HIF-1 subunits to oxygen deprivation. Re-oxygenation experiments were also included into the study [5]. The data revealed that both transcription factors HIF-1α and ARNT were inducible in hypoxic cells on mRNA as well as protein levels [5]. Huang et al. [10] reported that ARNT protein levels remained constant regardless of cellular oxygen tension. In this study, Hep3B, HeLa and HEK293 cells were used. Unfortunately, not all experiments were conducted with all cell lines [10], thereby making a direct comparison with the study of Wang et al. [5] complicated. Nevertheless, the latter report [10] challenged the results of the previous one [5] due to signal variations of Northern blots and discontinuities of time-course experiments [10].

However, Huang et al. [10] proposed a very graphic working model including a specific sensor for hypoxia located in the cell membrane [10]. The depicted mechanism is similar to our nowadays HIF scheme. These days the prolylhydroxylase domain enzymes (PHDs), which require O_2 as a substrate, are known to act as cellular oxygen sensors [11]. The comparison of both seminal studies conducted by Wang et al. [5] and Huang et al. [10] also requires a glance on

citation frequencies of both reports. Noteworthily, the report of Wang et al. [5], which describes the upregulation of ARNT for the first time, was cited approximately four times more often as compared to Huang et al. [10]. Despite this clear distinction of citation frequencies, the opinion that ARNT is unaffected by cellular oxygen tension became a guideline in HIF biology [1].

The capability to elevate ARNT expression in hypoxia was also found in murine L929 and Hepa1 cells. Interestingly, human Hep3B cells were also used in this study conducted by Chilov et al. [7], but ARNT was unaffected by oxygen deprivation [7]. This seemingly conflicting observation compared to a previous report [5] is likely due to different experimental conditions. Obviously, a short-term exposure of Hep3B cells to hypoxia (4 h in Ref. [7]) is not sufficient to induce ARNT protein expression in this model. However, other studies clearly confirmed the hypoxia-dependent upregulation of ARNT in Hep3B cells [3, 4].

First mechanistic clues regarding the induction of ARNT under oxygen deprivation were provided by Zhong et al. [6]. Herein, the authors tested the hypothesis whether HIF-1α and ARNT are regulated by similar signalling pathways in human prostate cancer cells. Indeed, an elevated ARNT protein level was observed in hypoxic PC-3 cells. Interestingly, this effect was attenuated by inhibition of the phosphatidylinositol-3 kinase (PI3K)/AKT-pathway by Wortmannin [6]. Another hint regarding the ARNT expression pattern in cancer cells came from Skinner et al. [12]. Herein, the authors investigated the transcriptional regulation of VEGF in ovarian cancer cell lines in response to PI3K/Akt signalling. Blocking of this pathway using the compound LY294002 specifically inhibited HIF-1α expression but had no effect on ARNT. Unfortunately, the inducibility of ARNT in hypoxia was not tested in this study [12]. Most important, the observation that PI3K/Akt inhibition decreased HIF-1α but not ARNT in one model [12] whereas both transcription factors were reduced on protein level in another model [6] might suggest a HIF-dependent regulation of ARNT in certain cell lines.

The studies discussed so far clearly show that certain cell lines are capable to induce ARNT in hypoxia and that this effect is dependent on the experimental conditions (i.e. time points). Thus, one might assume that scientists became more aware of this phenomenon over time. However, ARNT was also used as a loading control in Western blot analysis [12, 13]. This application clearly demonstrates the major opinion of a complete non-hypoxic regulation of ARNT. Of note, effects of hypoxia and hypoxia mimetics on ARNT expression should be taken into consideration when studying the HIF pathway as previously proposed [1]. Such an approach will help to identify new cell types harbouring the hypoxia-inducible ARNT attribute and might provide novel mechanistic insights. The current proposed mechanism is discussed later in the chapter.

The seminal study conducted by Choi et al. shed light on ARNT expression and turnover [14]. The authors assumed that the regulation of ARNT expression or activity might significantly affect cell metabolism. Thus, ARNT should be regarded as a drug target and appropriate compounds need to be investigated. De novo synthesis of ARNT, enhanced stability of the protein and dimerisation with HIF-1α, represents three ways how this transcription factor can be controlled. Curcumin, the major component of the spice turmeric, was tested in this study for potential inhibitory effects on HIF-1. Interestingly, curcumin facilitated the degradation

of ARNT and blocked HIF signalling [14]. Similar effects were reported by Ströfer et al. [15]. Curcumin-mediated ARNT depletion was observed in human HepG2, Hep3B and MCF-7 cells [15]. The half-life of ARNT was determined in Hep3B cells after cycloheximide treatment and calculated with approximately 5 h [14]. In contrast, curcumin exposure decreased ARNT half-life to roughly 2 h. It turned out that the curcumin-dependent degradation of ARNT was redox sensitive and could be reversed by antioxidants and the proteasome inhibitor MG-132 [14]. Remarkably, MG-132 did not affect ARNT protein level in the absence of curcumin. Therefore, the authors proposed the existence of two different mechanisms mediating ARNT turnover: a proteasome-independent mechanism under physiological conditions and a proteasome-dependent degradation in response to stress [14].

The elevation of ARNT protein expression under oxygen deprivation might not be an exclusive trait of cell lines. Exposure of primary mouse keratinocytes to acute hypoxia (1% O_2) resulted in an upregulation of ARNT after 4 and 5 h, respectively [16]. However, this effect was not statistically significant. Putative alterations on ARNT mRNA expression were also evaluated in this cell model. In contrast, time-course experiments revealed no apparent changes on mRNA level in murine keratinocytes cultured in hypoxia up to 48 h [16]. The selection of an inappropriate internal control in qPCR analysis can also lead to different expression levels in normoxia and hypoxia [17]. Therefore, Vavilala et al. determined the expression level of three housekeeping genes (ribosomal protein L32, β-actin and GAPDH) in normoxic and hypoxic cells, respectively [17]. The authors observed no significant changes on mRNA levels between both experimental settings. The aim of this study was to investigate inhibitory effects of Honokiol, a biphenolic phytochemical compound, on HIF signalling in several cell lines. The results presented in this report consist solely of gene expression data. Among them, the HIF-1α, HIF-2α and ARNT mRNA level were compared under normoxic and hypoxic conditions [17]. Remarkably, an approximately 7-fold increase in ARNT mRNA was observed in D407 human retinal pigment epithelial cells. In addition, a 2-fold upregulation was detected in HT-29 cells and a slight increase in the HEK293 cell line. MCF-7 cells showed no increase in ARNT mRNA due to hypoxic exposure. Unfortunately, the comparison of these effects among the cell lines tested in this study is limited because of different time points used (12 versus 24 h in D407 cells; 1% O_2) [17]. Nevertheless, the study provides clear evidence of a cell-specific transcriptional ARNT upregulation in hypoxia although these findings were not confirmed by Western blotting.

Further mechanistic insights into this cellular trait were provided by a research project investigating the regulation of ARNT in human melanoma cells [2]. Among a panel of five different cell lines, ARNT was rapidly elevated on protein level in 518A2 cells after treatment with the hypoxia mimetic cobalt chloride ($CoCl_2$). Interestingly, knockdown of HIF-1α in $CoCl_2$ stimulated and hypoxic 518A2 cells abolished the hypoxia-dependent upregulation of ARNT. Overexpression of a dominant-negative HIF mutant in this cell model indicated that ARNT expression is dependent on the HIF pathway itself. In agreement with these findings, overexpression of HIF-1α caused an elevation of ARNT protein in $CoCl_2$ treated 518A2 cells. Taken together, this study demonstrated a regulatory relationship between HIF-1α and its binding partner ARNT for the first time. In addition, it was concluded that this capability might prevent ARNT to become a limiting factor in hypoxia [2].

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The first comprehensive study aiming to re-evaluate the regulation of ARNT was conducted by Wolff et al. [4]. Herein, numerous cell lines were exposed to 1 and 3% O_2 for different time points. In addition, hypoxia mimetics such as $CoCl_2$ and dimethyloxalylglycine (DMOG) were used and the quantity of ARNT protein determined by Western blotting. The authors found out that ARNT expression was induced in MCF-7, HeLa and Hep3B cells. Interestingly, the ARNT level was also dependent on the hypoxic environment used. A concentration of 1% O_2 led to a faster increase in ARNT protein but also to an earlier decline to basal levels as compared to 3% hypoxia. Moreover, the appropriate mRNA levels did not correlate with the amount of protein detected. In particular, in MCF-7 and Hep3B cells, a downregulation of ARNT mRNA was observed due to hypoxia. Therefore, the authors hypothesised the existence of a reciprocal feedback regulation between ARNT protein stability and de novo synthesis. This study provides convincing evidence that the predominant point of view that ARNT is unaffected by hypoxia and hypoxia mimetics cannot be applied to all cell lines in general [4].

The first review highlighting the topic of hypoxia-inducible ARNT was published by Mandl and Depping [1]. Herein, two major questions were raised: (1) How can cells acquire this attribute? and (2) What is the benefit for these cells? [1] Both issues will be discussed below. An updated list of cell lines capable to elevate ARNT in response to hypoxia is presented in **Table 1**. Among them, the human Hep3B cell line is obviously the best studied model in this context.

Cell line	Species	Origin	References
518A2	Human	Melanoma	[2]
A375	Human	Melanoma	[2]
D407 *	Human	Retinal pigment epithelium	[17]
HEK-293*	Human	Embryonic kidney	[17]
HeLa	Human	Cervix adenocarcinoma	[4]
Hep3B	Human	Hepatoma	[3–5]
Hepa1	Mouse	Hepatoma	[7]
HT-29*	Human	Colorectal adenocarcinoma	[17]
L929	Mouse	Connective tissue	[7]
LNCaP*	Human	Prostate cancer	[20]
MCF-7	Human	Breast carcinoma	[4]
PC-3	Human	Prostate cancer	[20, 6]

*Only shown on mRNA level.

Table 1. Cell lines with hypoxia-inducible ARNT expression.

2.1.1. Purpose of hypoxia-inducible ARNT

The capability of certain tumour cells to upregulate ARNT under hypoxic conditions might provide a specific survival advantage as previously proposed [1]. Indeed, we recently discovered a relationship between ARNT and the cellular response to radiation [18]. Tumour hypoxia

is associated with radioresistance and poor patient prognosis. Therefore, we investigated the effects of an altered expression of ARNT on radioresistance and performed clonogenic survival assays. As expected, silencing of ARNT in Hep3B and MCF-7 cells by siRNA rendered these models susceptible to radiation. Interestingly, overexpression of ARNT in these cell lines promoted radioresistance. Therefore, it was hypothesised that radiation treatment might provide a selection pressure and lead to an enrichment of high-ARNT expressing cells. Taken together, these findings provide evidence to consider ARNT as a drug target in order to increase radiosensitivity in tumour cells and as a predictive marker in this context [18].

As outlined above, there is evidence that HIF-1α mediates the elevation of ARNT under hypoxic conditions in certain cell lines. This regulatory relationship is the prerequisite of a feed-forward loop (FFL) as demonstrated recently in Hep3B cells. In such a network motif, one transcription factor regulates the other and both controls the expression of a target gene cooperatively. Given the fact that HIF-1α and ARNT form the transcriptional active complex HIF-1, which regulates a plethora of target genes, the FFL definition is fulfilled. By using reporter gene assays, we were able to demonstrate that overexpression of ARNT in Hep3B cells increased the luciferase signal in hypoxia. Therefore, it was concluded that augmented HIF signalling in terms of elevated target gene expression might be beneficial for tumour cells. These findings support the concept of ARNT being a limiting factor in at least certain cell models [3].

Moreover, general considerations regarding inducible gene expression are in line with the studies discussed above. In order to respond rapidly to micro-environmental alterations required genes need to be specifically activated. Inducible genes are highly regulated and must be quickly shut down to basal expression levels once the stimulus disappeared [19].

2.1.2. Mechanism of hypoxia-dependent ARNT upregulation

The mechanism(s) underlying this unique cellular attribute is (are) unclear. There is mounting evidence indicating a pivotal role of HIF-1α [2–4]. It was demonstrated that ARNT was increased in 518A2 human melanoma cells in a HIF-1α-dependent manner under hypoxic conditions [2]. A very similar mechanism was revealed in Hep3B cells [3]. Knockdown and overexpression of HIF-1α affected the ARNT protein level accordingly. Moreover, a clear transcriptional relationship between HIF-1α and its binding partner ARNT was established in this model system. Treatment with actinomycin D, an inhibitor of RNA synthesis, diminished the induction of ARNT under oxygen deprivation. In addition, appropriate gene-silencing experiments and qRT-PCR analysis confirmed this finding [3]. Another important observation might designate HIF-1α as a mediator of this cellular attribute. The PI3K/Akt inhibitor LY294002 was shown to inhibit HIF-1α expression in ovarian cancer cell lines but had no effect on ARNT protein [12]. In contrast, several independent studies have shown that the hypoxia-dependent increase in ARNT was abolished by blocking the PI3K/Akt pathway with LY294002 or similar compounds [2, 6, 20]. This finding—the susceptibility of ARNT to PI3K/Akt inhibition in certain models—might be characteristic for cells capable to induce ARNT in hypoxia. Taken together, this suggests a linear model and might imply ARNT to be a downstream target of HIF-1α.

The cellular cause of the regulatory relationship between HIF-1α and ARNT is not known. HIF-1α can act independent of its binding partner ARNT and regulate gene expression [1].

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It was shown that HIF-1α can act as a co-activator or co-repressor on certain genes. In addition, an indirect regulatory connection between both transcription factors might exist [1]. HIF-regulated genes encode for growth factors, glucose transporters, glycolytic enzymes but also other transcription factors and miRNAs. Therefore, HIF-controlled transcription factors and miRNAs might influence ARNT expression [1, 3]. A general working concept is discussed below.

2.1.2.1. Working concept of hypoxia-inducible ARNT

Based on the studies mentioned above, a general working concept can be deduced (**Figure 1**). In addition to its oxygen regulation, the HIF pathway, that is, HIF-1α, is also controlled by growth factors via the PI3K/Akt signalling cascade leading to elevated translation [21, 22]. Upon activation HIF-1α induces the upregulation of its binding partner ARNT either on mRNA and/or protein level in appropriate cell lines. For instance, it was shown that hypoxic induction of ARNT in Hep3B cells is mediated by de novo synthesis [3]. This effect can be achieved either directly or indirectly. A direct mechanism might involve the recruitment of HIF-1α to the ARNT promoter, whereas an indirect mechanism might be mediated by other HIF-regulated transcription factors or miRNAs [1]. Indeed, a complex mutual regulatory relationship between miRNAs and PAS proteins exists. However, the physiological and pathophysiological mechanisms behind are unclear [23].

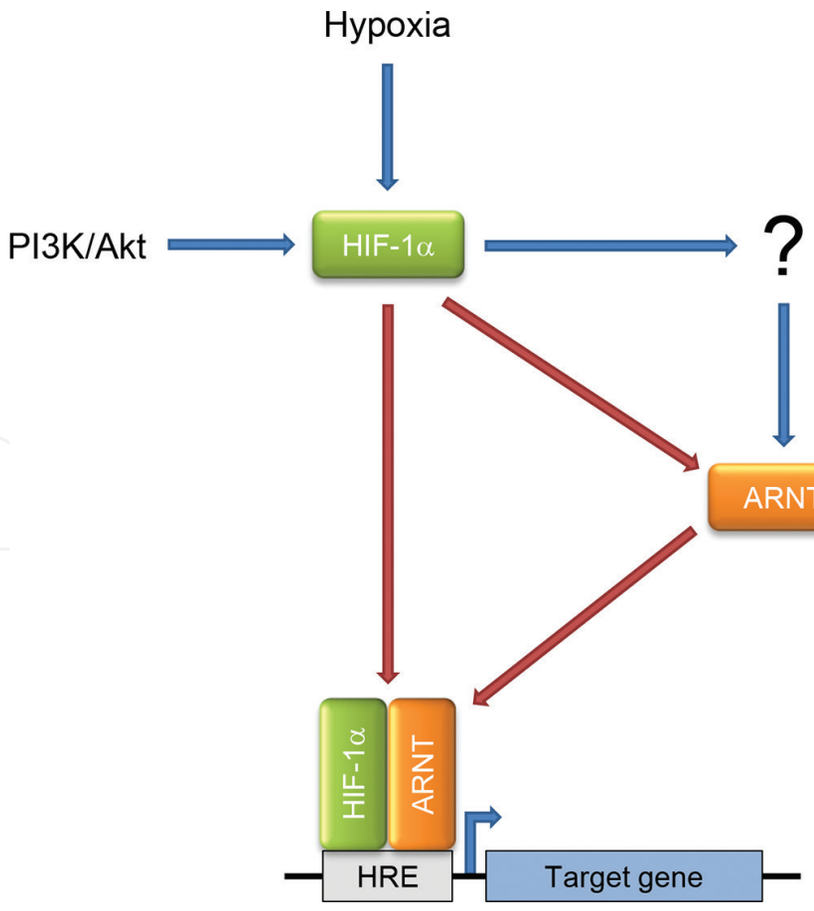

Figure 1. General working concept of hypoxia-inducible ARNT. See text for details.

Our recent experiments revealed that HIF-1α and ARNT are recruited to the ARNT gene promoter in hypoxic Hep3B cells. Deployment of CRISPR/Cas9 gene editing technology confirmed the importance of a unique genomic sequence for hypoxia-dependent ARNT upregulation. Therefore, these findings suggest a direct mechanism and render ARNT a putative HIF-1 target gene in Hep3B cells (unpublished observations; manuscript in preparation).

The regulatory relationship between HIF-1α and ARNT is part of a feed-forward loop (FFL; **Figure 1**: red arrows) as already demonstrated in Hep3B cells [3]. Subsequently, HIF-1α and its binding partner ARNT form the transcriptional active heterodimer HIF-1 and initiate the expression of various target genes. Therefore, an increased target gene expression seems to be beneficial for tumour cells [3].

2.1.3. Experimental conditions

Although the hypoxic inducibility of ARNT is described in specific cell lines by convincing data, not every study could confirm this circumstance. This obvious conflict depends mainly on the experimental conditions used. For instance, it was demonstrated that in Hep3B cells, 3% O_2 for 8 h was sufficient to elevate ARNT on protein level [3]. In contrast, a peak induction on mRNA level was observed after 5 h in the same setting [3]. Of note, these conditions need not to be appropriate in other cells. Until now, a few studies reported that ARNT mRNA and protein levels do not correlate in a number of cell lines [4, 18].

2.2. Regulation of ARNT by other factors

The regulation of ARNT or whether it responds to stimulation is poorly understood. There is evidence that ARNT expression is controlled by the NF-κB pathway in different models. It was demonstrated that ARNT mRNA was induced in HEK293 cells due to TNF-α stimulation. This effect was abrogated by pharmacological blocking or silencing of the NF-κB cascade [24]. Moreover, Per-ARNT-Sim (PAS) transcription factors belonging to different signalling circuits can compete for common binding partners such as ARNT (discussed below). Thus, misregulation of these proteins might contribute to tumour survival [9]. Noteworthily, the mutual regulation of PAS transcription factors on mRNA level was also mentioned in the literature, but appropriate citations are missing [22].

3. Crosstalk between Per-ARNT-Sim transcription factors

The HIF, AhR and BMAL1/Clock pathways respond to a decline in cellular oxygen concentration, environmental xenobiotics or govern circadian rhythms, respectively. All of these transcription factors are related. They belong to the group of Per-ARNT-Sim (PAS) transcription factors which are characterised by the presence of a PAS domain (composed of PAS-A and PAS-B subdomains) required for protein-protein interactions. Therefore, all family members are able to form homo- and heterodimers among the group [9, 25].

The transcription factor ARNT plays a pivotal role within the HIF and AhR pathways. It serves as the common binding partner for HIF-α subunits and ligands activated AhR proteins [1].

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Therefore, a competition between both signalling cascades regarding the recruitment of ARNT might be obvious. Indeed, early evidence for such an antagonism was provided by Gradin et al. [26]. By using luciferase reporter gene constructs under the control of xenobiotic-responsive elements (XRE), the effect of HIF and AhR activation was studied in HepG2 cells. As expected, stimulation of cells with an appropriate AhR ligand leads to a pronounced induction of reporter gene expression. This effect was suppressed by co-treatment with the hypoxia mimetic cobalt chloride. Co-immunoprecipitation experiments clearly indicated a competition between the HIF and AhR pathway relating to ARNT binding. In addition, it was shown that HIF-1α could efficiently compete with the AhR for dimerisation with ARNT. This study provided evidence for a HIF-1α-mediated inhibition of AhR signalling by sequestration of ARNT [26]. Vorrink et al. [27] observed similar effects again in human hepatocellular carcinoma HepG2 cells and in the human keratinocyte HaCaT cell line. One major advantage of this study was the genuine hypoxic exposure of cells instead of stimulation with hypoxia mimetics such as cobalt chloride. AhR signalling was triggered by treatment with the dioxin-like compound PCB126. Again, hypoxia inhibited CYP1A1 reporter gene activity in PCB126 stimulated HepG2 cells. Importantly, ARNT overexpression caused an elevated luminescence signal under normoxic and hypoxic conditions. Moreover, forced ARNT expression was sufficient to overcome the inhibitory effect of hypoxia on AhR signalling. The authors concluded that ARNT is sequestered by HIF-1α in hypoxia thus limiting the availability of this transcription factor for AhR heterodimerisation [27]. Noteworthily, another report published nearly two decades ago claims the complete opposite [28]. This study might provide evidence for a lack of competition between HIF and AhR signalling on ARNT recruitment. Unfortunately, the presented arguments and data are not convincing at many points [28].

Furthermore, a crosstalk between AhR and BMAL1/Clock exists. Lipophilic AhR ligands such as dioxin or dietary polyphenols bind within the AhR PAS-B domain and trigger nuclear translocation. Within the nucleus activated AhR can dimerise with BMAL1 thus disrupting the autoregulatory loop of BMAL1/Clock genes. Therefore, AhR activation leads to a suppression of circadian rhythms, whereas AhR inhibition strengthens rhythm amplitude [25]. Interestingly, there is evidence that both AhR and ARNT are expressed in an oscillatory pattern in vivo [29].

4. Subcellular dynamics of ARNT and turnover

Translocation of ARNT from the cytoplasm into the nucleus is mediated by importins as also demonstrated for other HIF family members [30, 31]. Blocking of this specific process was proposed as a novel way to suppress HIF signalling [30]. Whether ARNT shuttles, back into the cytoplasm is unknown. Under these circumstances, inhibition of the putative nuclear export might prolong HIF activity. Moreover, whether ARNT is degraded, within the nucleus is not investigated in greater depth (depicted in **Figure 2**).

In general, there is evidence for two different mechanisms leading to ARNT degradation. It was found out that ARNT was not affected by the proteasome inhibitor MG-132 under physiological conditions. In contrast, proteasomal degradation of ARNT might be triggered by reactive oxygen species [3, 14].

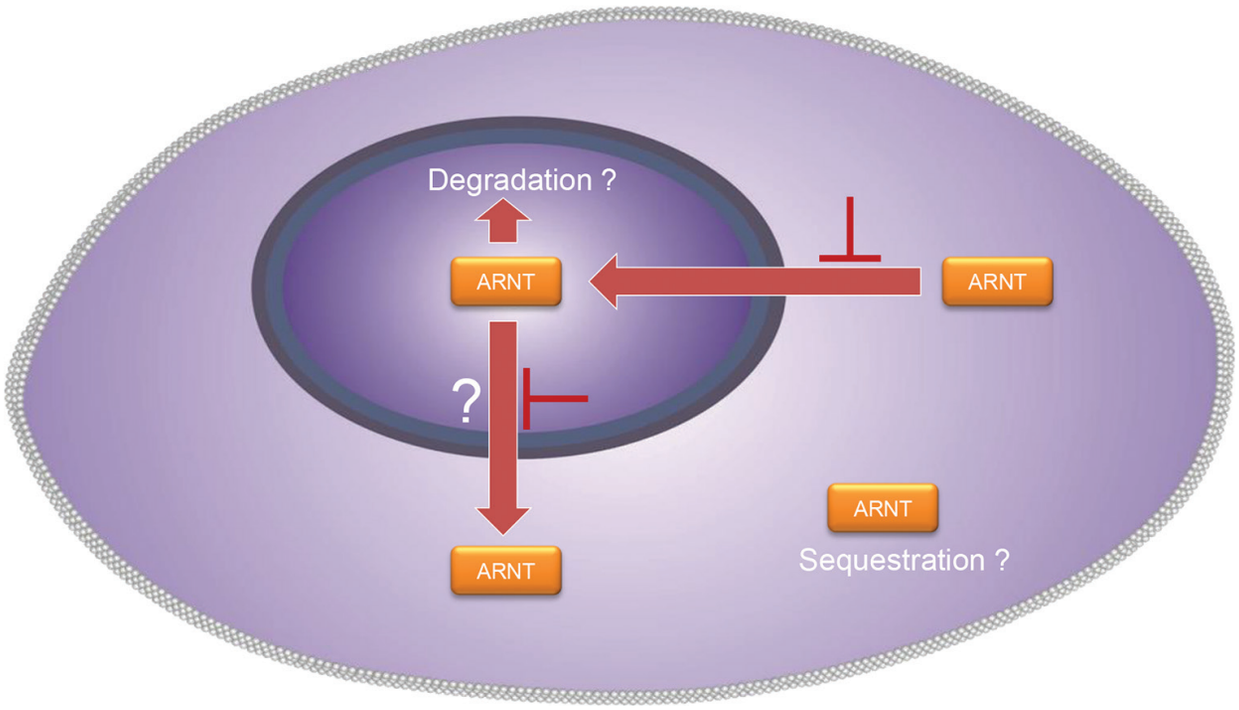

Figure 2. Subcellular logistics of ARNT. See text for details.

5. Clinical aspects

Inhibition of the HIF pathway is proposed as a treatment strategy in oncology. Several appropriate compounds have been identified and confirmed in xenograft models. These drugs are able to block different processes of the HIF signalling pathway. For instance, HIF-1α protein synthesis is diminished by rapamycin which is an inhibitor of mTOR. The antibiotic acriflavine prevents heterodimerisation of HIF-1α and ARNT subunits [32]. Moreover, a plethora of other HIF inhibitors was discovered which comprise of different chemical entities. HIF is considered as an attractive drug target, and blocking of its activity might lead to cytostatic antitumour effects. A synergistic outcome with radiotherapy is also expected [33, 34]. However, such drugs might be useful in multidrug regimes only in a subset of cancer patients. Cancers in which HIF is a strong driving force for disease progression are assumed to be susceptible for anti-HIF treatment [32].

The temporal importance of ARNT during tumour growth was investigated by Shi et al. [35]. Herein, the authors used murine hepatoma Hepa-1 cells transduced with a Tet-Off mArnt construct. Xenograft experiments conducted with these cells indicated that ARNT is particularly required during the early stage of tumour growth. The authors proposed that a profound inhibition of the HIF pathway might be achieved only by suppressing both HIF-1α and HIF-2α proteins. Therefore, it was concluded that the binding partner ARNT might represent a preferable therapeutic target rather than HIF-α subunits [35]. More convincing evidence regarding the role of ARNT in this malignancy was provided by a study using human tissue samples and cell lines [36]. ARNT expression was analysed by immunohistochemistry in hepatocellular carcinoma (HCC) and liver tissues. ARNT

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was found primarily in the nucleus but also in the cytoplasm in a minor fraction of cells. Interestingly, ARNT expression was significantly higher in normal liver samples as compared to appropriate HCC tissues. In addition, the impact of ARNT expression on overall survival (OS) of HCC patients was evaluated [36]. Surprisingly, a high intra-tumour ARNT level was associated with a prolonged OS. In agreement with this observation, stably lentiviral transduced ARNT-knockdown HCCLM6 cells showed a high proliferation rate, whereas overexpression of ARNT had the opposite effect. In addition, ARNT-suppressed cells formed smaller tumours in a murine xenograft model as compared to appropriate ARNT-overexpressing counterparts. This finding is in line with clinical data indicating a smaller tumour size in high-ARNT expressing hepatocellular carcinoma [36]. Moreover, the incidence of recurrence after surgery was significantly lower when a high intra-tumour ARNT level was detected. Taken together, this study describes an inhibitory role of ARNT in HCC progression. It was concluded that ARNT is a central regulator in HCC progression and a useful predictive marker regarding curative resection. The authors proposed that the relative balance of ARNT and its binding partners might be an important determinant in HCC [36]. In addition, another study demonstrated an important role of ARNT in this malignancy. Choi et al. [37] silenced ARNT expression in several human hepatoma cell lines by using siRNA and evaluated the effects on cell growth. It was shown that knockdown of this transcription factor inhibited proliferation and sensitised cells to apoptosis [37]. An elegant approach to target ARNT by small molecule inhibitors was conducted by Guo et al. [38]. Herein, nuclear magnetic resonance and biochemical screens were used in order to identify molecules selectively binding to the PAS domain of ARNT. The compound KG-548 was discovered to compete with the co-activator TACC3 for ARNT binding. The specific blocking of protein-protein interactions among transcription factors represents a novel technique to inhibit HIF signalling. Due to the shared use of ARNT among alpha subunits, targeting this protein was proposed to be more efficient as compared to its counterparts [38]. Evidence highlighting the importance of ARNT as a drug target was also provided by another study. Chan and colleagues [39] described that ARNT expression enhances cisplatin resistance in cancer cells. This phenotype was mediated by upregulation of MDR1, a multidrug efflux pump of the ABC superfamily, by a direct mechanism. Accordingly, knockdown of ARNT by siRNA transfection reduced cisplatin resistance in human cancer cells. Moreover, ARNT silencing increased the therapeutic efficacy of this cytotoxic drug in a murine xenograft model [39].

Targeting the HIF pathway in cancer therapy in order to achieve tumour control has been proposed by several reports [40–42]. Remarkably, the majority of HIF inhibitors described until now lack specificity. For instance, the drug topotecan blocks topoisomerase I activity but also diminished HIF signalling in preclinical models. This inhibitory effect on HIF was accomplished by preventing the accumulation of HIF-1α. In multihistology target-driven clinical trial, Kummar et al. [43] evaluated the oral use of this compound in a small number of cancer patients. Different tumour entities were diagnosed in these patients including ovarian cancer, sarcoma and melanoma among others. A complete inhibition of HIF-1α was detected in biopsies of a few patients, but inherent sampling and heterogeneous HIF-1α expression might limit this finding [43]. In contrast, despite the clear role of ARNT in tumour progression, its drug-ability and appropriate treatment effects need to be evaluated in a clinical setting.

6. Concluding remarks

The attribute of certain tumour cells to elevate the transcription factor ARNT in hypoxia was shown decades ago but since neglected in HIF biology. Only a small number of studies focus on the regulation of ARNT, especially under hypoxic conditions. Therefore, hypoxia-inducible ARNT is an emerging concept in this field. According to the major opinion, ARNT is a constitutively expressed gene. This means that ARNT expression is not effected by environmental factors such as hypoxia. Due to the fact that there are exceptions from this dogma, the statement of a constitutive ARNT expression should be revised and not used in general terms. Thus, ARNT should be regarded as a "cell-specific facultative gene" in tumour cells which indicates an expression as needed.

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