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Hypoxia and Differentiation in Human Neuroblastoma Cells

Helén Nilsson

Academic dissertation
By due permission of the Faculty of Medicine, Lund University, Sweden, to be defended at the main lecture hall, Pathology building, University Hospital MAS, Malmö, on Friday 10th of June, 2005, at 9.15 for the degree of Doctor of Philosophy, Faculty of Medicine

Faculty opponent
Professor Joachim Fandrey, Department of Physiology, University of Duisburg-Essen, Germany
Hypoxia and Differentiation in Human neuroblastoma cells

The childhood tumour neuroblastoma is derived from immature cells of the sympathetic nervous system, which have become arrested at different maturation stages. Neuroblastoma is a malignancy with a high degree of heterogeneity, and there is a correlation between a poor differentiation status and a more aggressive phenotype. The MYCN gene is amplified in approximately 25% of neuroblastoma tumours and is correlated to an aggressive tumour phenotype. A role of MYCN in keeping these tumours at an immature stage has been suggested. However, we show that over-expression of MYCN in non-amplified neuroblastoma cells do not restrain their capacity to differentiate. Furthermore, in a panel of 28 neuroblastoma tumours and 27 cell lines, we do not see a correlation between the degree of MYCN expression and the expression of neuronal or neuroendocrine marker genes.

Previously hypoxia and/or nutrient deprivation has been suggested to induce a neuroendocrine lineage shift in neuroblastoma tumours. However, when growing neuroblastoma cell lines at hypoxia and/or low glucose conditions, we observe a down-regulation of both neuronal and chromaffin marker genes. Instead, genes normally expressed in early neural crest cells are induced. We therefore propose that hypoxia and/or glucose deficiency induce a dedifferentiation of neuroblastoma cells, thereby rendering them a more aggressive phenotype. In addition, when growing neuroblastoma cells at hypoxia and/or without glucose, we find that hypoxia protects from glucose-deprivation induced cell death. This further adds to the malignant potential of hypoxic neuroblastoma cells.

We have further investigated the hypoxia inducible transcription factors HIF-1α and HIF-2α in neuroblastoma cells and find that they have separate patterns of activation over time and in response to different oxygen levels.
LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Neuroblastoma cells with overexpressed MYCN retain their capacity to undergo neuronal differentiation

II Hypoxia alters gene expression in human neuroblastoma cells toward an immature and neural crest-like phenotype

III HIF-2α expression in human fetal paraganglia and neuroblastoma: relation to sympathetic differentiation, glucose deficiency, and hypoxia

IV Hypoxia rescues neuroblastoma cells from glucose deficiency-induced cell death
Helén Nilsson*, Jenny Karlsson*, Marie Ovenberger, Erik Fredlund and Sven Påhlman
*These authors contributed equally to this work. Manuscript

V Gene regulation in hypoxic neuroblastoma cells - different roles of HIF-1α and HIF-2α
Erik Fredlund*, Linda Holmquist*, Helén Nilsson, Tobias Löfstedt, Johan Vallon-Christersson, Bengt Persson, Åke Borg, and Sven Påhlman
*These authors contributed equally to this work. Manuscript

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## Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>C-TAD</td>
<td>C-terminal trans-activating domain</td>
</tr>
<tr>
<td>dHAND</td>
<td>deciduum, heart, autonomic nervous system, and neural crest derivatives</td>
</tr>
<tr>
<td>EPO</td>
<td>erythropoietin</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FIH-1</td>
<td>factor inhibiting HIF-1</td>
</tr>
<tr>
<td>GAP43</td>
<td>growth associated protein 43</td>
</tr>
<tr>
<td>HASH-1</td>
<td>human achaete-scute homologue-1</td>
</tr>
<tr>
<td>HES-1</td>
<td>hairy/enhancer of split homologue-1</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>HRE</td>
<td>hypoxia responsive element</td>
</tr>
<tr>
<td>ID2</td>
<td>inhibitor of differentiation/DNA binding 2</td>
</tr>
<tr>
<td>IPAS</td>
<td>inhibitory PAS domain protein</td>
</tr>
<tr>
<td>MKP-1</td>
<td>mitogen-activated protein kinase phosphatase-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide tyrosine</td>
</tr>
<tr>
<td>N-TAD</td>
<td>N-terminal trans-activating domain</td>
</tr>
<tr>
<td>ODDDD</td>
<td>oxygen-dependent degradation domain</td>
</tr>
<tr>
<td>PAS</td>
<td>per/arnt/sim</td>
</tr>
<tr>
<td>PHD</td>
<td>prolyl hydroxylase</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SNS</td>
<td>sympathetic nervous system</td>
</tr>
<tr>
<td>SIF</td>
<td>small intensely fluorescent</td>
</tr>
<tr>
<td>TH</td>
<td>tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRK</td>
<td>tropomyosine receptor kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Lindau</td>
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The Sympathetic Nervous System

BACKGROUND

The Sympathetic Nervous System

SNS organisation and function

The vertebrate nervous system is divided into the central nervous system that consists of the brain and the spinal cord, and the peripheral nervous system containing ganglia that connect muscles, organs and other tissues to the central nervous system. The peripheral nervous system is further sub-divided into the somatic and autonomic nervous systems, where the somatic system controls skeletal muscles and the autonomic system innervates the inner organs. The sympathetic nervous system (SNS) is the division of the autonomic nervous system that controls functions such as heart rate, blood flow and respiration that are involved in the so-called “fight-and-flight” stress response.

Except for the supportive schwann cells, the SNS is made up of three related cell types, sympathetic neurons, small intensely fluorescent (SIF) cells, and chromaffin cells. The sympathetic neurons are located along the spinal cord and in the truncus, in sympathetic chain ganglia from where their axons protrude into the target organs. In the adult SNS, chromaffin cells are mainly located in the adrenal medulla and chromaffin cells that are located outside the adrenal gland form the paraganglia. SIF cells are found in small numbers interspersed among the sympathetic neurons in the ganglia. SIF and chromaffin cells release their transmitter substance into the bloodstream, and they are therefore called neuroendocrine cells. The main transmitter substances of the SNS are acetylcholine and the catecholamine noradrenaline.

The structure of the embryonic and early postnatal SNS differs to some extent from the adult. In the early SNS, extra-adrenal chromaffin cells form large paraganglionic structures that are thought to be the main source of catecholamines before the adrenal gland is properly formed. The largest of the paraganglia is called the “organ of Zuckerkandl” and is located next to the abdominal aorta. The amount of SIF cells is higher in embryonic SNS, and in the adrenal gland bundles of immature neuroblasts are found, however, their function is not clear. In the adult SNS, only traces of these structures are found and they are thought to regress either by apoptosis or by differentiation into mature neurons or chromaffin cells [1, 2].

Development of the SNS and factors involved in sympathetic differentiation

The SNS is derived from the neural crest that starts to form during the third week of human development. Pluripotent migratory progenitor cells are induced by different growth factors, hormones and steroids to become restricted to specific cell fates and differentiate terminally. The neural crest gives rise to a number of diverse tissues such as melanocytes of the skin, smooth muscle cells, supportive glial cells and sensory as well as autonomic neurons (reviewed in [3] and [4]). The neurons and neuroendocrine cells of the SNS belong to the sympatho-adrenal lineage. Depending on what growth factors and hormones these cells
encounter, they can develop into either neurons, chromaffin or SIF cells. In response to fibroblast growth factor (FGF) and nerve growth factor (NGF) sympatho-adrenal precursor cells differentiate into neurons, while glucocorticoids produced by the adrenal cortex are thought to induce differentiation into catecholamine producing chromaffin cells [5, 6].

The cells of the sympatho-adrenal lineage are unusual in that even terminally differentiated cells retain a certain degree of pluripotency. In vitro, chromaffin cells can be converted into neurons when treated with NGF [7].

At various differentiation stages these immature cells express distinct sets of genes, and the identification of these genes can be used in the characterisation of the mechanisms involved in development and as tools in the determination of origin and maturation grade of neuroblastoma cells and established cell lines. A schematic picture of the expression patterns over time in developing human/mouse neural crest and sympathetic precursor cells of a few such genes is shown in Figure 2.

bHLH transcription factors
Transcription factors involved in cell fate decisions and maturation processes during the development of SNS include several members of the basic helix-loop-helix (bHLH) family. dHAND (deciduum, heart, autonomic nervous system, and neural crest derivatives) and human achaete-scute homologue-1 (HASH-1), act as inducers of neuronal differentiation. HASH-1 is expressed in early human sympathetic cells up to fetal week 10 [8], and is thought to be required for a proper neuronal development, as mice deficient for MASH-1 (mammalian achaete-scute homologue-1) principally lack sympathetic ganglia as well as parasympathetic and enteric ganglia [9].

Other transcription factors such as HES-1 (hairy/enhancer of split homologue-1), and ID2 (inhibitor of differentiation/DNA binding) have a counteracting effect. They inhibit
The Sympathetic Nervous System

the transcriptional activity of pro-neuronal bHLHs and act as repressors of differentiation [10, 11].

MYCN

MYCN is a bHLH leucine zipper transcription factor that belongs to the MYC family of proto-oncogenes. The transcriptional activity of MYCN is regulated via the MAD-MAX-MYC network. MYC forms transcriptionally active heterodimers with MAX and together they bind a consensus sequence known as an E-box in target gene promoters. While MYC up-regulates genes involved in cell growth and proliferation, and is well known to have an inhibiting effect on differentiation in various cell systems, the role of MYCN is less clear. During development, MYCN has a more restricted expression pattern than MYC, indicating a specific function of this gene, although no exclusive MYCN target gene has been identified as yet. MYCN expression has been shown in migrating neural crest cells and a role of MYCN has been implicated in both neural crest migration and differentiation [12].

Neurotrophins and their receptors

Neurotrophin signalling is crucial for neuroblast survival and differentiation during development of the SNS [13, 14]. The neurotrophins involved in SNS maturation are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 and -4 (NT-3 and NT-4). They signal through the tyrosine kinase receptors TRKA, TRKB and TRKC, which belong to the tropomyosin receptor kinase family. Some overlap exists in the binding to these receptors, but NGF binds with highest affinity to TRKA, BDNF and NT-4 to TRKB and NT-3 to TRKC. Depending on the differentiation status of the neuroblast/neuroendocrine precursor, and on the presence of different neurotrophins, sig-

![Figure 2. Schematic summary of the spatial expression patterns in the developing SNS of a number of genes involved in sympathetic differentiation, based on human and mouse studies.](image-url)
nalling through TRK receptors results in sustained survival, proliferation, or differentiation [13, 15]. Proliferating, TRK-negative, sympathetic precursor cells are initially dependent on growth factor signalling to become neurotrophin dependent. bFGF induces TRKC expression, and the precursor cells become NT-3 responsive. NT-3 and ciliar neurotrophic factor induce TRKA expression, which makes these cells NGF responsive and halts proliferation. The terminally differentiated non-dividing neuronal cells are TRKA positive and require NGF for their survival [16].

Neuroblastoma

The childhood tumour neuroblastoma affects approximately 1 in 7000 live births [17], corresponding to 10-15 children in Sweden per year, which makes it the most common solid extra-cranial tumour among children. The median age at diagnosis is 18 months, and less than 2% of the cases are diagnosed after the age of ten. Neuroblastoma is a malignancy of the SNS and tumours can occur at any site of its location. Primary tumours are mainly located to the adrenal medulla or sympathetic paraspinal ganglia, but metastases are found in bone, liver and lung as well as in lymph nodes [17]. Symptoms are rather vague but include fatigue, abdominal pain, loss of appetite and diarrhea [18]. As a consequence of their sympathetic origin, most neuroblastoma tumours produce catecholamines, and the ratio of vanilmandelic and homovanillic acid levels in blood or urine can be used in the diagnosis of neuroblastoma [19]. Initiation of neuroblastoma tumorigenesis is thought to occur in immature neuroblastic cells, which become arrested at various stages of differentiation [20]. More differentiated tumours are correlated to a better prognosis [21].

Neuroblastoma - a heterogeneous malignancy

One intriguing characteristic of neuroblastoma is its high degree of heterogeneity. Based on criteria first proposed by Evans et al in 1971, the International Neuroblastoma Staging System (INSS) was agreed on in 1993, which divides neuroblastoma tumours according to localisation and pattern of metastasis into four main groups. Stage 1 and stage 2 tumours are localised and have a good prognosis while tumours of stage 3 and in particular stage 4 are extremely aggressive with extensive metastases and a poor prognosis. An additional stage termed 4S, denotes tumours that despite metastases in mainly liver and skin, have a very good prognosis and are even known to regress spontaneously. These tumours are only found in children under the age of one [22, 23]. Together with INSS stage, age at diagnosis is the strongest prognostic factor for neuroblastoma, where most children diagnosed before the age of one has a good prognosis and almost 80% of older children have high stage tumours with a poor prognosis [24]. Other
Neuroblastoma

strong prognostic factors include MYCN amplification, 1p deletion and TRKA expression (see below).

Spontaneous regression occurs in neuroblastoma to a higher degree than in other human tumours [17]. The regression seems to occur either by spontaneous apoptosis, as during neuronal development, or through differentiation into benign ganglioneuromas [25, 26]. Increased knowledge regarding the mechanisms behind gives hope for the development of a therapy able to induce this process also in aggressive high stage tumours. However, tumour regression occurs in low stage tumours only, and low stage tumours do not develop into high stage. Instead, two distinct subtypes of neuroblastoma tumours seem to exist [27, 28]. This is further supported by the fact that several screening programs for neuroblastoma among young children have increased the number of detected low stage tumours dramatically, but not been able to reduce the number of deaths in neuroblastoma [24, 29]. The existence of different subtypes with disparate prognosis and treatment requirements further stress the importance of increased knowledge of the characteristics of these tumours in order to avoid over-treatment of low-stage tumours and to improve the outcome for high-stage patients.

Neurotrophin receptors in Neuroblastoma

Expression of the neurotrophin receptors has a role in neuroblastoma tumours [13, 14]. High expression of TRKA is correlated to a good prognosis. During development TRKA is expressed in NGF-dependent neuroblasts, and the function of the receptor is reflected in the tumour cells, where in the presence of NGF; TRKA will induce differentiation, while in the absence of NGF; these cells will undergo apoptosis [30].

TRKB is not normally expressed in sympathetic neuroblasts [31], but found in a small population of high stage neuroblastoma tumours, in contrast to TRKA. These tumour cells can also produce BDNF, which via autocrine signalling is thought to promote the survival of TRKB expressing tumour cells [32, 33]. Although TRKB expression is found mainly in tumours with amplified MYCN, there do not seem to be a causative link between MYCN amplification and TRKB expression, as introduction of MYCN in non-amplified neuroblastoma cells is not sufficient to induce TRKB expression [34]. Interestingly, TRKB expression is up-regulated at retinoic acid induced differentiation, however, it is questionable what implications this has in neuroblastoma tumours considering that TRKB do not have a role in neuroblast differentiation in vivo [13, 32, 35].

Like TRKA, TRKC expression is mainly found in low stage tumours [36].

MYCN amplification and other genetic aberrations

Hereditary neuroblastoma is very rare, less than 1% of patients have affected relatives and there is no single genetic defect identified that is common to all neuroblastomas [37]. However, several genetic alterations are found in sporadic neuroblastomas and can be correlated to prognosis.

The MYCN gene is amplified in approximately 25% of all neuroblastomas, and amplifica-
Neuroblastoma

Neuroblastoma is highly correlated with an aggressive phenotype [38, 39]. Weiss et al. showed in 1997 that targeted over-expression of MYCN to the neural crest via the TH promoter induces tumours with neuroblastoma characteristics in mice [40]. In MYCN amplified tumours, both RNA and protein levels are high and correlated to a bad prognosis. However, it is debated whether MYCN protein levels have a prognostic value in non-amplified neuroblastoma tumours, and the exact mechanism by which MYCN contributes to tumourigenesis is not clear [41, 42].

A rather large part of chromosome 2p, where MYCN is located, is often co-amplified together with MYCN, and it has been speculated whether MYCN is the main target of amplification. However, although other genes with tumourigenic effects that are co-amplified with MYCN probably confer a growth advantage when over-expressed, these genes are never found to be amplified independent of MYCN, and are therefore probably not the driving force for amplification [43].

Other common genetic aberrations in neuroblastoma include 17q gain and deletions of the short arm of chromosome 1. 1p deletions are common in high stage tumours and are thought to involve the loss of an as yet un-identified tumour suppressor gene. There is an association between 1p deletion and MYCN amplification, however whether there is a mechanistic linkage between these events is not known [44, 45].

17q gain, which is the most common genetic alteration in neuroblastoma tumours, is also correlated to a poor prognosis [46]. The lack of direct targets implied in these genetic alterations open up for the possibility that the chromosomal aberrations found in high stage tumours are consequences of the increased genetic instability associated with an aggressive tumour phenotype, rather than being initiating events.

Neuroblastoma cell lines

A large number of cell lines have been established from human neuroblastoma tumours over the years. Most of these cell lines carry MYCN amplification, which appear to give a survival advantage for in vitro growth conditions. The immature phenotype of these cell lines and the tumours they are derived from is reflected in their expression of early sympathetic marker genes such as dHAND and HASH. dHAND is expressed in sympathetic neurons and chromaffin cells until birth [8] after which it can no longer be detected in normal SNS tissue.

Some of the neuroblastoma cell lines can be induced to differentiate into a neuronal phenotype when treated with the phorbolester TPA, or different combinations of growth- and neurotrophic factors. The neuronal differentiation is characterised by a marked morphological alteration with outgrowth of neurite-like processes carrying varicosities, as well as by an up-regulation of neuronal marker genes such as GAP-43 (growth associated protein 43) and NPY (neuropeptide tyrosine) [47, 48].

Interestingly, at induced differentiation of neuroblastoma cells, HES-1 is transiently up-regulated while HASH-1 and MYCN are down-regulated. This expression pattern probably reflects the specific time frames that these factors are expressed at during the development of the SNS. Due to the comprehensive characterisation of the expression of marker gene
Neuroblastoma

expression at various time periods in the developing SNS, it is possible to determine the differ-
entiation status of neuroblastoma tumours as well as of neuroblastoma cell lines follow-
ing exposure to various growth conditions. These cell lines are essential tools in the search
for increased knowledge regarding neuroblastoma tumour behaviour.

In vivo trans-differentiation in neuroblastoma lobules

As mentioned above, neuroblastoma is a very heterogeneous disorder, with diverse genetic,
molecular and clinical behaviour. In a subset of low stage neuroblastomas, the occurrence
of lobular structures with a central necrotic zone has been observed [1, 49]. Within these lo-
bular structures, an interesting trans-differentiating phenomenon seems to occur. Tumour
cells adjacent to the fibrovascular stroma, supposedly with a good supply of oxygen, nutri-
ents and growth factors, display an immature neuronal phenotype based on histology and
marker gene expression. In contrast, as the distance from the vasculature increases, cells
appear more mature, cease to proliferate and the expression of chromaffin marker genes is
up-regulated. In the area closest to the necrotic zone, cells undergo apoptosis. This behav-
iour is mainly found in low stage tumours of extra-adrenal origin, which together with
expression of neuroendocrine marker genes could suggest that these tumour cells are com-
mitted to a paraganglionic or SIF phenotype [2, 20]. Perhaps this phenomenon mimics
the process by which embryonic SNS structures regress during normal development, and
perhaps this is the same process seen in regressing tumours, only delayed.

What the driving force behind this process is can only be speculated, but given the con-
nection between increased maturation and distance to the vasculature might suggest a role
of hypoxia, starvation and growth factor deficiency. Interestingly, Tian et al show specific
expression of one of the main regulators of the hypoxic response, the hypoxia inducible
transcription factor HIF-2α, restricted to embryonic mouse sympathetic ganglia and para-
ganglia, which opens up for the possibility of an involvement of this transcription factor
in the development of these structures [50]. This will be discussed in more detail under
Present Investigations.

Hypoxia, the Hypoxia Inducible Transcription Factors, and their
Role in Tumour Progression

Hypoxic tumours

Tumour formation

Tumours arise when the normal control of cell death and/or proliferation becomes deregu-
lated. In a normal cell, these functions are under tight regulation, but the accumulation
of inherited or obtained mutations can lead to activation of oncogenes or loss of tumour
suppressor gene functions (reviewed in [51]). The tumour cells gain a survival advantage
compared to their neighbours and often start to proliferate in an uncontrolled manner.
Mutations that allow the cell to escape cell death through apoptosis are for example very
Hypoxia and HIFs

common in cancers. The increased mutation rate in tumour cells also gives them an advantage upon changes in the microenvironment.

Tumour growth is restricted by the need for a continuous supply of nutrients, oxygen and growth factors from the vasculature, and in order to grow beyond a certain size, tumour cells must be able to induce the formation of new blood vessels, a process known as angiogenesis. Malignant tumours are also characterised by their ability to metastasise to new locations. An extensive cross-talk between tumour cells and the surrounding stroma is essential in these processes [52].

Tumour cell adaptation

Solid tumour vasculature tends to be malformed and inefficient, therefore areas of poor oxygen and nutrient supply occur. Measurements of oxygen pressure in normal compared to tumour tissue have revealed that most solid tumours possess areas of acute or chronic hypoxia, and the average partial oxygen pressure in tumours has been estimated to approximately 1.5% compared to 5% in normal tissue [53]. To counteract the poor oxygen and nutrient supply, tumour cells up-regulate the expression of genes such as erythropoietin (EPO) and tyrosine hydroxylase (TH), that have systemic effects on red blood cell production, heart rate and blood flow, as well as of angiogenic factors such as vascular endothelial growth factor (VEGF) that promote the formation of new blood vessels (reviewed in [54]).

An adaptation of the cell metabolism also occurs to endure the harsh environment. Tumour cells are known to have an increased glycolytic activity, which contributes to the acidic environment of tumour cells. This is known as the Warburg effect. A majority of genes involved in glucose uptake and the glycolytic pathway are induced at hypoxia (reviewed in [55]). Adaptation to hypoxia occurs mainly through the action of the hypoxia-inducible transcription factors HIF-1α and HIF-2α.

**Hypoxic tumours are more aggressive and resistant to therapy**

Apart from the survival advantage induced by glycolysis and growth factor signalling, hypoxic tumours tend to be more resistant to conventional treatment such as radio-and chemotherapy [56, 57]. Radiotherapy relies on the formation of radical oxygen species (ROS), and even though the role of ROS during hypoxia is debated, hypoxic tumours are less sensitive to ionising radiation.

The effect of chemotherapy can also be impaired due to poor vascularisation, which negatively affects drug delivery. A low proliferation rate makes tumour cells insensitive to conventional drugs such as topoisoomerase inhibitors that targets dividing cells. Hypoxia is positively correlated to increased probability of invasion, metastasis, and death, due to the induction of pro-metastatic factors, an increased mutation rate and the selection of a more aggressive phenotype [56]. Hypoxic tumour cells have also been shown to down-regulate genes involved in DNA repair mechanisms and induce genes involved in multidrug resistance [58-60]. We, and others have also suggested that hypoxia induces a more immature tumour cell phenotype. This would be yet another mechanism for selection of a more aggressive hypoxic phenotype ([61, 62] and Paper II in this thesis). In order to circumvent
Hypoxia and HIFs

The hypoxia inducible transcription factors and their regulation

The HIF transcription factors

Key regulators of the hypoxic response are the hypoxia inducible transcription factors, HIFs. These factors are involved in the regulation of diverse functions such as cell survival, apoptosis, cell motility and adhesion, erythropoiesis, drug resistance, glucose metabolism and pH regulation (reviewed in [54]). The diversity of these functions reflects the importance for cell survival to adapt to or to counteract the effects of oxygen deprivation, and HIF seem to be highly conserved evolutionarily [63].

The HIF transcription factor is a heterodimer with one beta subunit (HIF-1β, or aryl hydrocarbon receptor nuclear translocator, ARNT), and one alpha subunit (HIF-1α, HIF-2α or HIF-3α). The first report describing this transcription factor came from Wang and Semenza in 1993 [64]. In 1995 the 120 kDa HIF-1α subunit was purified and characterised by the same group [65], and it became apparent that it was the alpha subunit, which is induced at low oxygen conditions, that confers the hypoxic regulation to the transcription complex, in contrast to the beta subunit, which is constitutively present in the cell nucleus. Before that, little was known about the mechanisms of oxygen-dependent gene regulation.

In 1997, a second hypoxia-induced transcription factor, HIF-2α, also known as HLF (HIF1αt-like factor), EPAS1 (endothelial PAS1), MOP2 (member of PAS family 2) or

Figure 3. Schematic illustration of the HIF-1α and HIF-1β/ARNT subunits indicating the main sites of oxygen-dependent regulation, as described in the text.

and even take advantage of the negative effects of hypoxic tumours, drugs that target hypoxic cells specifically and pro-drugs that become activated at hypoxia are under development.
HIF-related factor, was described by Ema et al [66] and a third alpha subunit, HIF-3α, was discovered in 1998 [67]. During the years to follow an increasing knowledge regarding the regulation of these transcription factors and their role in hypoxic gene response and adaptation has developed.

Both alpha and beta subunits are constitutively expressed in most cell types, but while the beta subunit mRNA and protein levels appear unaffected by oxygen pressure, the alpha subunit protein is rapidly degraded and nearly undetectable in most cell systems at a normal oxygen pressure [68]. At hypoxia, the alpha subunit is stabilised and translocates to the nucleus where it forms a transcriptional complex together with the beta subunit and co-activators such as CBP/p300, and bind to hypoxia-responsive-elements (HREs) within the promoters of different target genes [69, 70].

The alpha and beta subunits belong to the PER-ARNT-SIM (PAS) family of bHLH transcription factors [71]. As depicted in Figure 3, both alpha and beta subunits contain an N-terminal DNA-binding bHLH domain, a PAS domain that confers dimerisation and target gene specificity, and a C-terminal trans-activating domain (C-TAD), where co-factors such as CBP/p300 bind [72]. In addition, the alpha subunit possesses an additive N-terminal trans-activating domain (N-TAD) and a central oxygen-dependent degradation (ODD) domain that confers the main oxygen-dependent regulation to this protein [73].

Oxygen dependent regulation of HIF

The ODD domain within HIF-1α was first identified by Huang et al, who showed that removal of the entire domain was necessary for normoxic stabilisation of the alpha subunit, and that fusion of this domain to other proteins rendered them oxygen-sensitive [73]. Degradation of the alpha subunit was also shown to occur via the ubiquitin-proteasome pathway [73-75]. The mechanism of this oxygen-dependent degradation is now more established.

At normoxia, prolyl residues within the ODD domain are recognised and hydroxylated by specific prolyl hydroxylases (PHDs) [76, 77]. This hydroxylation makes the alpha subunit recognisable for binding to the tumour suppressor protein von Hippel-Lindau (VHL). VHL is part of a large complex of enzymes that upon binding mediates poly-ubiquitination of the HIF-alpha subunit and thereby targets it for degradation by the proteosome.

The PHDs are dependent on molecular oxygen to function, and therefore the alpha subunit is only hydroxylated when oxygen is abundant. At low oxygen levels, the ODD domain remains un-hydroxylated, VHL does not bind, and the HIF-alpha protein becomes stabilised and is translocated to the nucleus via a nuclear localisation signal in its C-terminus, as illustrated in Figure 4 [70, 78].

The interaction of PHDs and VHL with HIF-alpha is also regulated by an acetyl-transferase, ARD1. Acetylation of K532 within the ODD domain of HIF-alpha by ARD1 seems to be necessary for the interaction with VHL, and ARD1 expression is down-regulated at hypoxia. The exact involvement of PHDs in this mechanism is not clear [79].
Hypoxia and HIFs

Within the C-terminal TAD, another site for oxygen-dependent regulation of the alpha subunit is located. Here lies an asparaginyl residue that becomes hydroxylated at normoxia by an asparaginyl hydroxylase known as factor inhibiting HIF-1, or FIH-1 [80-83]. This enzyme has the same substrate requirements as the PHDs, and when active it interferes with the binding of cofactors CBP/p300 to the HIF transcriptional complex, and thereby inhibits its trans-activating capacity.

Mahon et al have also showed that FIH-1 interacts with VHL, which recruits histone deacetylases to the complex and thereby further represses the trans-activating ability of HIF [81]. These data are supported by Lee et al who have solved the crystal structure of FIH-1 and who suggest a model for the formation of a multicomponent complex of HIF-1α, FIH-1, VHL and several histone deacetylases [84].

The stabilisation and transcriptional activity of HIF is also regulated by phosphorylation (reviewed in [85]). The mechanisms involved are highly cell type specific and involve kinases of either the phosphatidylinositol-3 kinase (PI3K)/Akt pathway or members of the mitogen-activated protein kinase (MAPK)/Erk family. Phosphorylation-sites within the trans-activating domains of HIF-1α [86] and HIF-2α [87] have been identified and activation of these sites is suggested to facilitate the interaction with CBP/p300. Whether or not the phosphorylation of Akt or Erk is induced by hypoxia also seem to be cell type specific, and in some cases, it is not clear whether HIF-induction or kinase-activation is the initial event. Phosphorylation can be reverted by the action of phosphatases. Interestingly, the dual specificity MAPK phosphatase MKP-1 is up-regulated at hypoxia and renders the HIF-alpha protein yet another level of regulation by a negative feedback mechanism [88].

Figure 4. Oxygen-dependent regulation of the HIF transcription factor. At normal oxygen levels, the HIF-alpha subunit is recognised by VHL and targeted for destruction via the proteasome. When oxygen supply is restricted, HIF-alpha translocates to the nucleus where it together with the beta subunit (ARNT) forms a transcriptional complex binding to hypoxia-responsive-elements in target genes.
An inhibitory PAS domain protein, IPAS, which lacks a trans-activating domain [89], provides another mechanism for regulation of the HIF transcription factor. Upon dimerisation with the HIF-alpha subunit, IPAS acts as a dominant negative regulator by inhibiting binding to target gene HREs. IPAS was shown by Makino et al to be an alternative splice variant of the HIF-3α mRNA that is induced by hypoxia, providing a negative feedback mechanism for regulation of HIF [90].

The exact function of HIF-3α needs to be further characterised, but like HIF-1α and HIF-2α, HIF-3α is stabilised at hypoxia, dimerises with ARNT and together they bind to target gene HREs. HIF-3α contains a sequence corresponding to the ODD domain of HIF-1α, but has no hypoxia-responsive TAD element, which may indicate that the HIF-3α-ARNT complex has a decreased transcriptional potency relative to the other HIF heterodimers [67]. It has also been suggested that HIF-3α acts as a negative regulator of the hypoxic response by sequestering ARNT [91]. In the following discussion, the focus will be on HIF-1α and HIF-2α.

All together, the main mode of regulation of the HIF transcription complex occurs via inhibition of degradation of the alpha subunit rather than via stimulation of its expression and protein synthesis. This makes the activation of HIF a very rapid process, HIF-alpha becomes stabilised and active within minutes of hypoxic exposure [92], which again points to the importance of a fast adaptation to hypoxia in order to remain energy homeostasis.

Prolyl hydroxylases

The prolyl hydroxylases mentioned above play an important role in the regulation of HIF activity. They belong to a family of 2-oxoglutarate-dependent dioxygenases and were first identified in C. elegans as EGL9 [76]. There are three established human orthologs to EGL-9, PHD1, PHD2 and PHD3, also known as EGLN2, EGLN1 and EGLN3 or HPH3, HPH2 and HPH1, respectively [76, 77, 93].

Apart from dioxygen, these prolyl hydroxylases require 2-oxoglutarate and Fe(II) as co-substrates. When oxygen is abundant, the PHDs recognise and hydroxylate proline residues P402 and P564 in the consensus sequence LXXLAP within the ODD domain of HIF-1α [76, 94]. As mentioned above, hydroxylation at these sites enables the binding of VHL to the HIF-alpha subunit, which is thereby targeted for degradation.

A forth proline 4-hydroxylase-related protein, PH-4, was identified by Oehme et al in 2002 [95]. Over-expression of PH-4 inhibits HIF-activity, but PH-4 can not induce interaction between HIF-alpha and VHL like the other PHDs, and whether PH-4 has a direct role in HIF-regulation is still unclear.

The expression of PHD2 and PHD3 but not of PHD1 is up-regulated at hypoxia ([76, 97] and Paper V), and interestingly, Berra et al [100] show that the rate of degradation of HIF-1α upon re-oxygenation is dependent on the duration of the hypoxic treatment, where longer exposure to hypoxia resulted in a shorter half-life of HIF-1α, presumably due to the accumulation of PHDs in hypoxic cells. They conclude that degradation of HIF-1α is dependent on hypoxia-induced transcription and this again gives an example of the strict regulation of HIF activity. However, a hypoxia-induced negative regulation mechanism of PHD activity is reported by Nakayama et al, who demonstrate that degradation of PHDs
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is induced at hypoxia by the E3 ubiquitin ligase Siah2 [101].
Recent work by Semenza’s group identifies a novel function of the protein OS-9 as an enhancer of the binding of PHD2 or PHD3 to HIF-1α where these proteins form a ternary complex increasing the degradation of HIF-1α [102].
The regulation and function of the different PHDs seem to be very cell type specific (reviewed in [96]). According to Epstein et al, PHD3 does not hydroxylate HIF-1α as efficiently as PHD1 and PHD2, and several groups suggest that while PHD2 is the main regulator of HIF-1α, PHD3 gives the most efficient hydroxylation of HIF-2α [97, 98]. The intracellular localisation of the PHDs also differs. PHD1 is mainly localised to the nucleus, PHD2 to the cytoplasm, and PHD3 seems to be equally distributed throughout the cell [99]. What implications this have on their functions and different roles in HIF-regulation needs to be further investigated.

Non-hypoxic regulation of HIF
HIF can be activated at normoxia in vitro by inhibiting the function of the PHDs using transition metals such as cobalt or iron chelators such as 2,2'-dipyridyl and thereby prevent the degradation of the alpha subunit. Increasing evidence also indicate that HIF can be activated at normoxia in vivo. Several different stimuli have been suggested to elicit a HIF-response at normoxia, including growth factors and cytokines, oncogenes, mechanical stress and small signalling molecules such as nitric oxide (reviewed in [103] and [104]).

Growth factor-induced HIF-activation seems to act by increasing the protein synthesis and transcriptional activity of HIF, rather than by inhibiting its degradation, and growth factor-induced activation of HIF is detected in cells with functional VHL [105] [106]

The exact mechanism behind this phenomenon remains unclear, but the PI3K/Akt and MAPK/Erk pathways seem to be involved, acting both as inducers of HIF-translation and by increasing the trans-activating capacity of HIF by phosphorylation, as described above.

In a report from 2002, Chan et al show that v-Src and Ras oncogenes induce HIF-activation by inhibiting the hydroxylation of HIF-alpha, while constitutively active Akt induces an increase in HIF-alpha protein translation without any effect on its hydroxylation or stability [107].

Given the instantaneous degradation of the HIF-alpha subunit at normoxia, and the multiple steps of oxygen-dependent regulation of HIF, it might be argued that the normoxic activation of HIF is only very weak compared to its hypoxic activity, but even so, the normoxic induction of HIF that do occur might have large effects on the phenotype of for example tumour cells with deregulated growth factor signalling.

Different biological functions of HIF-1α and HIF-2α

HIF knock-out phenotypes
Although HIF-1α and HIF-2α are highly homologous and in large are regulated by the same mechanisms, they do not seem to be able to fully replace each other. During development, HIF-1α is expressed at a number of sites such as heart, gut, kidneys and thymus from mouse embryonic day 9.5 (E9.5), until approximately E15.5, when the expression starts to
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HIF-2α is more restricted and mainly found in endothelial cells of the developing vasculature [108]. Interestingly, HIF-2α is also found in the adrenal gland at E15.5, and Tian et al, who demonstrated HIF-2α expression in the developing sympathetic ganglia (E10.5-E12.5), and paraganglia (E14.5-16.5), show that HIF-2α-null mice die around E12.5-E15.5 due to impaired catecholamine production and bradycardia [50].

We have confirmed the presence of HIF-2α in mouse paraganglionic cells at E14.5, and in human paraganglia at fetal week 8.5 (Paper II and III in this thesis). In these cells, HIF-2α is co-expressed with tyrosine hydroxylase (TH), which is the rate-limiting enzyme in catecholamine production and a well-known hypoxia-induced gene [109]. Other groups have described lethal vasculature defects in HIF-2α-null mice [110], and Scortegagna et al describe a phenotype with multiple organ pathology, mitochondrial abnormalities, an impaired response to oxidative stress and mal-functional haematopoiesis [111, 112]. HIF-1α knock-out mice display defective cardio-vasculature and abnormal neural folding [113-115].

Varying levels of mRNA of HIF-1α and HIF-2α have been detected in most if not all normal adult tissue, in both human and mouse [116, 117]. The presence of HIF-1α and HIF-2α proteins in normoxic tissue is more debated. Talks et al detect no HIF-1α protein in normoxic human tissue and report that normoxic HIF-2α protein is restricted to bone marrow macrophages, while Stroka et al show the presence of HIF-1α protein in normoxic mouse brain, kidney, liver and heart, and Heidbreder with co-workers detect both HIF-1α and HIF-2α in normoxic brain and lung from rat [117-119]. The discrepancy of these reports might be explained by the use of different model systems and antibodies with different detection limits, however a low level of HIF-alpha expression in normoxic tissue might be important to maintain a basal energy homeostasis.

HIF-1α vs HIF-2α target genes

HIF-1α was identified by its binding to the promoter of EPO at hypoxia. A large number of target genes have now been identified, involved in angiogenesis, glycolysis, migration, cell survival and apoptosis. The different HIFs were initially thought to have overlapping functions, but HIF-1α, which has been most thoroughly studied, was assumed to be the main regulator of the hypoxic response. However, increasing evidence now imply that even though there exist a certain degree of redundancy, HIF-1α and HIF-2α have distinct biological functions.

Hu et al report that HIF-1α and not HIF-2α is the main regulator of glycolytic enzymes [120], while several studies suggest that over-expressed HIF-2α, but not HIF-1α, is able to overcome the tumour-suppressing function of VHL in renal carcinoma cells and promote tumour growth in xenografted mice [121-123]. Using siRNA directed against either HIF-1α, HIF-2α, or both, in cell lines of different origin, Sowter et al support the predominant role of HIF-2α in the hypoxic response in renal carcinoma cells, while HIF-1α seems to be more important in breast and endothelial cells [124]. Their observation that HIF-1α and HIF-2α do not replace each other even when one of the subunits is eliminated supports the theory that HIF-1α and HIF-2α do indeed have distinct functions.

We and others have shown differences in the kinetics of HIF-1α and HIF-2α degrada-
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tion during prolonged exposure to hypoxia, which suggests that HIF-1α is responsible for the acute hypoxic response and HIF-2α for the sustained phenotype (Paper III and V in this thesis and [125, 126]). Uchida et al suggest that HIF-1α mRNA stability is decreased during prolonged hypoxia through the action of an anti-sense HIF transcript, aHIF, which is up-regulated at hypoxia and that destabilises HIF-1α but not HIF-2α mRNA due to differences in their 3’-untranslated regions.

Inhibition of PI3K has been shown to reduce the expression of HIF-1α and its target gene VEGF, without affecting the expression of HIF-2α [127], and according to Lando et al, HIF-1α and HIF-2α DNA-binding is differently affected by redox conditions, where HIF-2α, but not HIF-1α, requires Ref-1 (redox factor with reducing activity) for full DNA-binding activity [128].

The separate regulation of HIF-1α and HIF-2α might be conferred through specific targeting by the PHDs, and thus be regulated by their specific expression and localisation in different cell types at various conditions, as mentioned above. By further study the specific targets of HIF-1α and HIF-2α respectively, it might be possible to determine whether their apparently different biological functions are due to individual gene targets or a result of cell-type specific expression levels and regulation of these two transcription factors.

HIFs in tumour progression

As mentioned previously, hypoxic tumours tend to be more aggressive and resistant to treatment. Part of this phenotype can be contributed to the action of HIFs, and over-expression of these factors has been shown in a number of different tumours [54, 129]. Several target genes of the HIFs have been suggested that would contribute to tumour progression, and in many aspects HIF and oncogene pathways overlap [130]. For example, the tumour suppressor gene PTEN negatively regulates HIF-1α activity by inhibiting signalling via the PI3K pathway, and loss of PTEN induces HIF-1α mediated expression of genes involved in angiogenesis in glioblastoma cells [131]. Petrella et al report a HIF-2α mediated induction in VHL-negative renal cell carcinoma cells of the metalloproteinase MT1-MMP, which is involved in tumour invasion and metastasis [132], and the multidrug resistance gene MDR1 is shown to be a HIF-1α target by Comerford et al [60].

Over-activation of HIF in tumours can be due to a hypoxic tumour microenvironment or to genetic alterations that stimulate HIF stabilisation and activation, such as oncogenic activation, increased growth factor signalling or tumour suppressor inactivation as in VHL disease.

As mentioned above, HIF has been shown to have tumour-promoting effects in renal cancer. The hereditary cancer syndrome known as von Hippel-Lindau disease is caused by mutations in the tumour suppressor gene VHL, and is characterised by the development of a wide range of tumours, including retinal and central nervous system haemangioblastomas, pheochromocytomas and clear-cell renal carcinomas (reviewed in [133]). Due to the inactivated VHL, HIF is constitutively present and these tumours are highly vascularised.

Because of the tumour promoting effects of HIF, including induction of angiogenesis, invasion and cell survival, HIF has been suggested as a therapeutical target, and inhibition of HIF-1α using siRNA has been shown to improve the effects of radiation therapy in an in vivo mouse study [134].
Present Investigations

Aims

The general aim of this work was to investigate the influence of hypoxia and MYCN over-expression on neuroblastoma cell differentiation. Specifically, we wanted to determine

-the effect of MYCN over-expression on neuroblastoma cell differentiation capacity

-how neuroblastoma cell differentiation status is affected by hypoxia and glucose depletion

-the roles of HIF-1α and HIF-2α transcription factors in normoxic and hypoxic human neuroblastoma cells

-the protective effect of hypoxia on glucose starved neuroblastoma cells
Results and Discussion

The role of MYCN expression in neuroblastoma cell differentiation (Paper I)

MYCN amplification is one of the strongest prognostic indicators of aggressive neuroblastomas, and in MYCN amplified tumours, there is a correlation between high MYCN expression and a poor prognosis. However, the precise mechanism by which MYCN contributes to the aggressive phenotype is still not known. One characteristic of high stage neuroblastomas is their immature phenotype and a role in inhibition of differentiation has been suggested for MYCN. Could the deregulated MYCN be responsible for keeping the tumour cells in an immature stage? MYC, the founding member of the MYC gene family, is well known to have an inhibiting effect on differentiation in various cell systems. In MYCN amplified neuroblastomas, MYC is down-regulated and it is believed that MYCN takes over the role of MYC [135]. In addition, an inhibition of differentiation has been reported when MYCN is over-expressed in human neuroblastoma cell lines [136, 137].

In contrast to those results, we show in Paper I that stably transfected MYCN over-expressing neuroblastoma cells retain their capacity to undergo neuronal differentiation upon stimulation with a variety of differentiation protocols. We show a marked morphological differentiation as well as an up-regulation of neuronal marker genes. We further see no correlation, positive or negative, between MYCN mRNA levels and mRNA levels of sympathetic neuronal marker genes such as TRKA and GAP43 in a panel of 28 neuroblastoma tumours and 27 cell lines. The same results were obtained both in MYCN amplified and in non-amplified tumours and cell lines.

Our results are supported by the finding by Nakagawara et al [14] that neurotrophin stimulation induce terminal differentiation also in some MYCN amplified neuroblastomas. Mouse and avian studies have implicated the requirement of MYCN for a proper neuronal development [12, 138], and we show MYCN expression in human fetal sympathetic ganglia at a developmental stage when these cells have ceased to migrate and have become committed to a neuronal phenotype.

These results indicate that over-expression of MYCN in high stage neuroblastoma tumours might not be the factor keeping these cells at an immature stage, but rather a symptom of their immature and genetic instable features. In order to fully understand the role of MYCN in neuroblastoma cell differentiation and tumour progression, further studies need to be performed.

In a recent paper, Slack et al report MDM2, which negatively regulates p53 and thereby apoptosis, as a direct MYCN target [139]. They suggest that the MYCN induced activation of MDM2 results in loss of apoptotic cell death via p53. Wiedemeyer et al further report that ataxin-2, which promotes apoptosis in neuroblastoma cells, is down-regulated in MYCN amplified tumours and cell lines [140].

Perhaps the immature cells that give rise to neuroblastoma tumours are neuroblasts that during normal development would regress by apoptosis or differentiation. Blocking apoptosis results in remaining neuroblasts, which give rise to neuroblastoma tumours. Although
MYCN do have a role in the development of SNS, the role of MYCN in neuroblastoma tumourigenesis is perhaps more coupled to inhibition of apoptosis of already immature cells rather than having a strong impact on the differentiation process itself.

The effect of hypoxia and glucose depletion on neuroblastoma cell differentiation (Paper II and III)

Early findings from our group show that a subset of neuroblastoma tumours possesses the capacity to trans-differentiate from an immature, neuronal, into a mature chromaffin phenotype [2, 20]. The fact that this phenomenon seems to occur as cells become distant from the fibrovascular stroma and blood supply, indicates that the maturation process could be induced by the shortage in nutrients, growth factors and oxygen, that is likely to occur in these cells. Interestingly, culture at reduced oxygen levels (5% O₂) has been shown to enhance survival and catecholaminergic differentiation capacity of neuronal progenitor cells of central as well as peripheral origin [141, 142]. The finding by Tian et al that HIF-2α knockout mice die from catecholamine shortage further implies a requirement of a hypoxia/HIF response for proper SNS development [50]. Together these results prompted us to investigate the effect of hypoxia on the differentiation status of human neuroblastoma cells.

In line with the observed trans-differentiation in lobular tumours, we observe a down-regulation of neuronal marker genes when neuroblastoma cell lines are grown at 1% O₂ (Paper II). However, in contrast to the expected induction of neuroendocrine marker genes, we also observe a down-regulation of chromaffin genes such as chromogranin A/B. Instead, marker genes normally expressed in early neural crest cells are induced. The discrepancy of these results could be explained by the fact that the in vivo trans-differentiation is only observed in low stage tumours, whereas most cell lines are established from high stage aggressive neuroblastomas. We also speculated that hypoxia alone was not enough to induce chromaffin differentiation. In order to better mimic the tumour microenvironment, we extended our study by including growth at a combination of hypoxia and different glucose levels (Paper III). However, the same expression pattern is found in cells grown at hypoxia alone or in combination with low glucose. In conclusion, hypoxic/glucose deprived human neuroblastoma cells do not develop a chromaffin phenotype, on the contrary, these cells loose their neuronal characteristics and become more immature.

These results are also supported by a recent study demonstrating that human embryonic stem cells maintain their pluripotency when cultured at hypoxic conditions [143]. Whether hypoxia and/or glucose deprivation induce a pluripotent state in neuroblastoma cells is intriguing and needs to be further investigated.

The hypoxic induction of ID2 seen in Paper II, gives a possible mechanism behind the observed dedifferentiation, as ID2 is known to inhibit the actions of pro-neuronal bHLH members such as HASH and dHAND involved in sympathetic differentiation and lineage specification [8, 11]. ID2 has been shown by Löfstedt et al to be a direct HIF-1α target [144].
Present Investigations

Increased malignant behaviour
As mentioned earlier, hypoxic tumours tend to be more aggressive with increased angiogenic and metastasising capacity [145]. In the case of neuroblastoma there is also a correlation between poor prognosis and low differentiation stage [21]. The induced de-differentiation observed in hypoxic neuroblastoma cells would thereby contribute to the aggressive phenotype of these tumours. Similar results have been reported in ductal breast carcinoma in situ and in prostate cancer cells, which suggest that this could be a general phenomenon in hypoxic tumours [61, 62]. Further support for the hypoxic induction of an aggressive immature phenotype in neuroblastoma tumours comes from Tacconelli et al, who report the occurrence of an alternative splice variant of TRKA in hypoxic neuroblastoma cells. This splice variant, denoted TRKAIII, appears unable to bind NGF and correlates to an immature phenotype and increased angiogenesis [146].

In a recently published study, Holmquist et al show that the immature phenotype of neuroblastoma cells induced at hypoxia remains for at least 24 h after reoxygenation. This time frame indicate that following hypoxic challenge, tumour cells remain in an immature aggressive state long enough to affect their migratory/metastasing capacity [126]. In order to confirm this theory, invasion studies of hypoxic neuroblastoma cells in vitro and in vivo would be a useful tool.

HIF-2α and differentiation
In accordance with the above mentioned finding by Tian et al that HIF-2α is expressed in developing mouse sympathetic ganglia and required for proper catecholamine production, [50], we show in Paper III the presence of HIF-2α protein in TH-positive human fetal paraganglia, as well as in neuroblastoma tumours. We further show a down-regulation of HIF-2α protein at induced differentiation of normoxic neuroblastoma cell lines. A role of hypoxia and/or HIFs in neuronal development is further implicated in studies regarding the VHL tumour suppressor protein and neuroblastoma cell differentiation capacity. VHL over-expression in SH-SY5Y neuroblastoma cells, presumably resulting in inhibition of HIF-stabilisation, induce neuronal differentiation of these cells [147, 148].

These results raise questions regarding the role of hypoxia and/or HIF-2α during sympathetic development. Given the presence of HIF-2α in normoxic neuroblastoma cell lines and tumours (Paper III and V), the induction of this protein in immature sympathetic structures is not necessary hypoxia-induced. It also remains to determine whether the presence of HIF-2α in neuroblastoma tumours has any influence on their differentiation state or merely is a consequence of an immature phenotype. A difference in the biological functions of HIF-1α and HIF-2α in neuroblastoma cells is supported by these results, as discussed below.

Protective effect of hypoxia (Paper IV)
When growing human neuroblastoma cells in hypoxic and glucose-depleted conditions we observed an interesting phenomenon. While neuroblastoma cells grown without glucose at normoxia display a massive cell death, cells grown without glucose at 1% O₂ appear to
be unaffected. Hypoxia therefore seems to promote cell-survival in starved neuroblastoma cells. In Paper IV, we try to unravel the mechanisms behind this phenomenon. Hypoxia is known to induce the expression of growth factors such as VEGF and IGF-II, which act as mitogenic factors and promote cell survival in several systems [149, 150]. We show that the cell death induced at normoxic glucose-deprivation of neuroblastoma cells is inhibited to some extent by the addition of VEGF or IGF-II. We speculate that hypoxia induces the production and secretion of these factors, giving rise to an auto- or paracrine loop that promotes the survival of hypoxic cells. The observed increase in survival of normoxic glucose-depleted cells treated with medium from hypoxic neuroblastoma cells supports this theory.

The involvement of the MAPK pathway in this process is implied by the finding that inhibition of Erk-phosphorylation reverts the protective effect of hypoxia-conditioned medium on normoxic glucose-deprived neuroblastoma cultures. Whether the survival-promoting signalling is conferred exclusively by MAPK signalling or if the PI3K pathway is activated as well needs to be further investigated. Akt has been shown to play a central role in promoting cell survival and opposing apoptosis in other cell systems.

Risbud et al report that hypoxia protects nucleus pulposus cells from serum starvation-induced cell death. They show an involvement of both PI3K and MAPK pathways, and suggest that activation of these pathways results in phosphorylation and inactivation of the pro-apoptotic protein GSK-3β (glycogen synthase kinase-3 beta) [151]. Interestingly, Risbud et al also report that desferrioxamine treatment has a similar protective effect as hypoxia, which implies a role of HIF activation in the process. Any involvement of HIF-1α or HIF-2α in the protection of neuroblastoma cells from glucose-deprivation induced cell death is as yet unknown, however given the reciprocal positive effects of growth factor signalling and HIF-1α activation, it would not be surprising [152]. In support of this

Figure 5. Pathways possibly involved in the protection of glucose-starved neuroblastoma cells at hypoxia.
Present Investigations

theory, VHL over-expressing SH-SY5Y neuroblastoma cells show an increased sensitivity to retinoic acid induced apoptosis, while HIF-1α over-expression rescues pancreatic cells from nutrient-deprivation induced cell death [148, 153].

Interestingly, the bHLH gene \( \text{DEC1} \) is also upregulated in neuroblastoma cells at hypoxia. \( \text{DEC1} \) has been shown to contain HREs and is upregulated in glioma cells at hypoxia, and given its function as a caspase inhibitor in colon carcinoma cells, hypoxia-induced \( \text{DEC1} \) might contribute to the survival-promoting effect of hypoxia in neuroblastoma cells [154-156]. A schematic illustration of the suggested hypoxia-induced survival-promoting pathways is shown in Figure 5.

In a tumour perspective the protective role of hypoxia would be a mechanism that further increase the aggressive behaviour of hypoxic tumours.

Different roles of HIF-1α and HIF-2α (Paper V)

In Paper V, we have further investigated the different roles of HIF-1α and HIF-2α in hypoxic and normoxic neuroblastoma cells. Interestingly, as indicated in Paper III and by Holmquist et al [126], HIF-1α and HIF-2α induction patterns differ both over time and with different oxygen pressure in neuroblastoma cell lines.

Growing neuroblastoma cells at 1% \( O_2 \) results in a rapid stabilisation and activation of HIF-1α, while HIF-2α shows a somewhat slower induction rate. After 72 h growth at 1% \( O_2 \), HIF-1α protein levels are decreased, while HIF-2α levels continue to increase. At 5% \( O_2 \), which is considered to be a more physiological oxygen level, HIF-2α but not HIF-1α is detected and presumably active based on its presence in the nucleus. These results imply different roles of HIF-1α and HIF-2α in neuroblastoma cells. We suggest that while HIF-1α is involved in the acute response to hypoxia, the role of HIF-2α in neuroblastoma cells is more complex and seem to involve both the regulation of a chronic hypoxic phenotype as well as to be involved in differentiation.

As discussed above, HIF-2α is detected at physiological oxygen levels in neuroblastoma tumours and cell lines (Paper III and V). This could be as a consequence of their immature phenotype, as HIF-2α but not HIF-1α is detected in developing paraganglia (Paper III and [50]). Nevertheless, the presence of HIF-2α in neuroblastoma cells at physiological oxygen levels implies that HIF-2α escapes the oxygen-dependent degradation machinery acting on HIF-1α. How this is accomplished needs to be further investigated. Perhaps HIF-1α and HIF-2α are differently targeted by the PHDs in these cells, or perhaps a higher expression level of HIF-2α mRNA is sufficient to saturate the degradation system.

As a tool in finding specific HIF-1α and HIF-2α target genes, array analyses of neuroblastoma cells grown at 1, 5 and 21% \( O_2 \) were performed. In combination with siRNA directed against HIF-1α or HIF-2α respectively, these array data might contribute to the understanding of the role of HIF-1α and HIF-2α in hypoxic neuroblastoma cells, and their respective contributions to the hypoxic phenotype. Such results might also give clues about the role of HIF-2α during the development of the SNS and what the consequences of the presence of HIF-2α protein in human neuroblastoma tumours are.
Summary

Paper I

- Over-expressed MYCN is not sufficient to inhibit induced differentiation of neuroblastoma cell lines.

- We find no connection between MYCN levels and the expression of neuronal or neuroendocrine marker genes in neuroblastoma cell lines and tumours.

Paper II and III

- HIF-2α is present in mice and human embryonal sympathetic ganglia at corresponding developmental periods.

- Hypoxia alone or in combination with glucose depletion induces dedifferentiation of human neuroblastoma cells.

Paper IV

- Hypoxia protects neuroblastoma cells from glucose deficiency induced cell death.

- Some sort of para/autocrine signalling induced at hypoxia seem to be involved in this process.

Paper III and V

- HIF-2α is detected at physiological oxygen levels in human neuroblastoma tumours and cell lines.

- HIF-1α and HIF-2α are differently regulated in hypoxic and normoxic neuroblastoma cells and we suggest that they also have different biological functions.
Tumörer uppstår när celler i kroppen på ett eller annat sätt förlorar sin normala reglering och börjar dela på sig okontrollerat. Det kan bero på genetiska förändringar som är tvets eller på ansamling av mutationer som orsakats av yttre faktorer. Tumörcellerna delar sig och växer snabbare än de omgivande cellerna och i solida tumörer hinner inte nybildningen av blodkärl ske i samma takt. Celler som befinner sig i områden inom tumören som ligger långt ifrån fungerande blodkärl får en sämre försörjning av syre och näringsämnen. Även om syre är livsnödvändigt för alla celler i kroppen så klarar särskilt tumörceller sig förvånansvärt bra vid låga syrehalter. Som svar på syrebrist aktiverar cellerna ett genprogram som anpassar dem till den nya miljön. Denna anpassning sker till stor del med hjälp av transkriptionsfaktorn HIF. Transkriptionsfaktorer är protein som kan aktivera eller stänga av gener som i sin tur ger upphov till andra protein som styr olika funktioner i cellen. HIF aktiveras vid låga syre-koncentrationer, och aktiverar då gener som är involverade i bland annat nybildning av kärl och resistens mot programmerad celldöd.


**MYCN** är en annan transkriptionsfaktor, vars gen ofta finns amplifierad i många kopior i aggressiva neuroblastom-tumörer och man tror att en stark signalering via **MYCN** bidrar till dessa cellers aggressiva beteende. Exakt hur det går till vet man inte, men man har till exempel föreslagit att **MYCN** skulle styra gener som är inblandade i cellers mognadprocess och en överdriven **MYCN** signalering skulle hindra neuroblastomcellerna från att mognas. I arbete I har vi studerat hur neuroblastomceller med normal mängd **MYCN** påverkas om man inför extra **MYCN** i cellerna. Vi visar att trots att dessa celler har höga nivåer av aktivt **MYCN** i sig, kan de differentiera/mognna när man behandlar dem med olika tillväxtfaktorer. Detta visar alltså att ett överdrivet **MYCN** uttryck inte hindrar neuroblastomceller från att mognas.

Vi har även studerat vilken effekt syrebrist har på neuroblastomcellernas mognad. I arbete II och III visar vi att neuroblastomceller som odlas vid 1% syre (till skillnad från 21% som i vanlig luft), och/eller utan tillgång till glukos, anpassar sig genom att bli mer omoga, vilket i de flesta tumörtyper även betyder att de blir mer aggressiva. Vi visar även i arbete IV att neuroblastomceller som odlas vid lågt syretryck dessutom klarar sig bättre utan glukos än celler som odlas vid 21% syre. Det skulle kunna vara ytterligare en förklaring till varför celler i solida tumörer är mer aggressiva och svårbehandlade än celler i tumörer med god syre- och näringstillförsel.
Hur anpassningen till ett lägt syretryck går till studerar vi i arbete V, genom att försöka ta reda på vilka olika gener som styrs av de två transkriptionsfaktorerna HIF-1α och HIF-2α, som aktiveras vid låga syrekoncentrationer styr. Dessa transkriptionsfaktorer är mycket lika varandra, och när de först upptäcktes trodde man att de hade samma funktion, men nu verkar det mer och mer som att de har specifika roller i olika celltyper och vid olika biologiska förhållanden.

I neuroblastomceller verkar HIF-1α styra de gener som behövs vid akut syrebrist, medan HIF-2α kontrollerar gener som är involverade i cellens svar på långvarig syrebrist. Vad detta har för konsekvenser för cellerna och hur den olika regleringen av dessa faktorer går till försöker vi förklara i arbete V.
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