Hypoxia-induced phenotypic modulation of human neuroblastoma cells

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Hypoxia-induced phenotypic modulation of human neuroblastoma cells

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Academic Dissertation

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Abstract Neuroblastoma is a childhood tumour derived from cells of the sympathetic nervous system, which are arrested at low differentiation stages. Low differentiation stage and high tumour stage correlate to poor outcome. In earlier studies we have found that hypoxia induces dedifferentiation of neuroblastoma cells, which implies that hypoxia evokes a more aggressive phenotype. Here we employ microarray analysis to investigate the hypoxic effects, in neuroblastoma cells, on the expression of a larger set of genes. Genes involved in survival and treatment resistance were upregulated, which support the concept of an aggressive hypoxic phenotype. The microarray results further strengthened the concept of hypoxia-induced dedifferentiation of neuroblastoma cells. Hypoxia-treated neuroblastoma cells were reoxygenated to determine the persistence of the hypoxic phenotype. Based on neuronal- and neural crest marker gene expression analysis, we conclude that the hypoxic phenotype persisted for at least 24 h upon reoxygenation. Hence, there was no selection for dedifferentiated cells; instead the hypoxic phenotype appears adaptable and dynamically reversible.

Major transcription factors that act in response to hypoxia are the hypoxia-inducible factors HIF-1α and HIF-2α. Intriguingly, HIF-2α, but not HIF-1α, was detected adjacent to blood vessels in neuroblastoma specimen, indicating a role for HIF-2α at more physiological oxygen levels. Analysis of HIF-protein levels in neuroblastoma cells exposed to hypoxia (1% O_2) or 5% O_2 (mimicking a more physiological oxygen level) revealed that HIF-1α is primarily and only transiently induced at 1% O_2. HIF-2α, on the other hand, is induced at both 1 and 5% O_2 and its protein levels increase over time. In a microarray analysis we extracted a set of genes with regulation patterns similar to that of the HIF-protein patterns seen in neuroblastoma cells grown at either 1 or 5% O_2. We propose that the differences in HIF target gene utilization are dependent on time and oxygen conditions, rather than on target gene specificity. According to that concept, we propose that HIF-1α primarily drives gene transcription at acute hypoxia, while HIF-2α is active at prolonged hypoxia and at 5% O_2, conclusions supported by HIF-1α and HIF-2α siRNA analyses and expression of the HIF-driven genes *VEGF* and *DEC1/BHLB2*.

Evaluation of a clinical neuroblastoma material showed correlation between HIF-2α immunostaining and poor patient outcome. Our results further implicate HIF-2α as a possible individual prognostic marker for neuroblastoma patients. Tumour growth of neuroblastoma cells knocked down for *HIF-2α* in nude mice was slower than neuroblastoma cells transfected with a scramble siRNA sequence. These observations support a role for HIF-2α in neuroblastoma progression. Immunostaining of neuroblastoma specimen revealed co-localization of VEGF and HIF-2α, suggesting a putative mechanism for neuroblastoma tumour growth due to HIF-2α-induced *VEGF* expression. Our results implicate an oncogenic role for HIF-2α in neuroblastoma aggressiveness, which might be exploited in the treatment of aggressive neuroblastomas.

Key words: Neuroblastoma, hypoxia, differentiation, HIF-1α, HIF-2α, reoxygenation

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Signature Linda Holmquist Mengelbier Date 23-10-2006

LIST OF PAPERS

This thesis is based on the following papers, referred to as Papers I-III.

I Human neuroblastoma cells exposed to hypoxia: induction of genes associated with growth, survival, and aggressive behavior.
Annika Jögi, Johan Vallon-Christersson, **Linda Holmquist**, Håkan Axelson, Åke Borg and Sven Påhlman. *Exp Cell Res*, 295: 469-487, 2004.

II Phenotypic persistence after reoxygenation of hypoxic neuroblastoma cells.
Linda Holmquist, Annika Jögi and Sven Påhlman. *Int. J. Cancer*, 116, 218-225, 2005.

III Recruitment of HIF-1α and HIF-2α to common target genes is differentially regulated by time and oxygen conditions in neuroblastoma – HIF-2α promotes an aggressive neuroblastoma phenotype.
Linda Holmquist Mengelbier*, Erik Fredlund*, Tobias Löfstedt, Rosa Noguera, Samuel Navarro, Helén Nilsson, Alexander Pietras, Johan Vallon-Christersson, Åke Borg, Katarina Gradin, Lorenz Poellinger and Sven Påhlman. *These authors contributed equally to this work. Accepted for publication in *Cancer Cell*.

Contents

ABBREVIATIONS

ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
DBH	dopamine β-hydroxylase
DDC	dopadecarboxylase
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ENO2	enolase 2
EPO	erythropoietin
ERK	extracellular regulated kinase
eIF-4E	eukaryotic translation initiation factor 4E
4E-BP1	eIF-4E binding protein
FGF	fibroblast growth factor
FIH	factor inhibiting HIF
GAP-43	growth associated protein-43
GCR	glucocorticoid receptor
HASH-1	Human *Achaete-scute* homologue-1
Hes	Hairy/enhancer of split
HIF	hypoxia inducible factor
HK2	hexokinase 2
HRE	hypoxia response element
ICD	intracellular domain
Id	inhibitor of DNA binding/inhibitor of differentiation
IGF	insulin growth factor
IPAS	inhibitory PAS domain protein
MAPK	mitogen-activated protein kinase
MASH-1	mammalian *achaete-scute* homologue-1
MEK	MAP/ERK kinase
MNK	MAP kinase signal-integrating kinase
mTOR	mammalian target of rapamycin
NGF	nerve growth factor
NPY	neuropeptide tyrosine Y
NSE	neuron specific enolase
ODD	oxygen dependent degradation
PDGF	platelet derived growth factor
PHD	prolyl hydroxylase
PI3K	phosphatidyl inositol 3-kinase
PNMT	phenyl ethanolamine N-methyltransferase
RCC	renal cell carcinoma
ROS	reactive oxygen species
SAP	sympatho-adrenal progenitor
SIF	small intensely fluorescent
SNS	sympathetic nervous system
TAD	transactivation domain
TGF-α	transforming growth factor α
TH	tyrosine hydroxylase
TNF-α	tumour necrosis factor α
VEGF	vascular endothelial growth factor
VHL	von Hippel Lindau

BACKGROUND

NEUROBLASTOMA

Neuroblastoma occurrence and staging

Neuroblastoma is a solid tumour afflicting children between 0 and 15 years' of age with most cases found in young children before the age of 4 (1). Median age of diagnosis is 18 months (2). Cases diagnosed at early infancy have a better prognosis than those of older children. The tumour can be located where the peripheral sympathetic nervous system branches out, the most common location being the adrenal gland. Neuroblastoma is a rare disease, but still one of the major causes of death among children afflicted by cancer, being the third most common paediatric cancer, comprising 7-10% of all childhood malignancies (2).

Neuroblastomas are clinically divided into five stages- spanning from stage 1 with localized tumours to stage 4 exhibiting extensive tumour metastasis, with stage 4s as a special diagnosis group. Children with stage 1 and 2 tumours are treated by surgery alone when harbouring an amplified MYCN gene, whereas children with the aggressive stage 4 tumours are given advanced radiation and chemotherapy. Stage 4s is an enigmatic stage comprising localized primary tumours with limited dissemination to liver, skin and /or bone marrow (3). These tumours occur during infancy and typically regress spontaneously, thereby separating from stage 4 tumours with regards to therapy (4). The overall survival rate of treated neuroblastoma patients is approximately 50%. However, for stage 4 patients the figure is only 20% and approximately 80% for patients with stage 4s neuroblastoma. Neuroblastoma is a heterogeneous disease, as it ranges from incurable aggressive tumours to tumours, which, spontaneously regress or differentiate into benign ganglioneuroma (2).

Genetic aberrations and other implications for neuroblastoma prognosis

The aetiology of neuroblastoma is unknown and has so far not been linked to any external environmental factors (5). Neuroblastoma is a tumour with immature sympathetic nervous system (SNS) features. The SNS continues to evolve for some years after birth, presenting a dynamic postnatal environment, which can be prone for genetic aberrations, although most neuroblastomas occur spontaneously. An inherited predisposition locus for neuroblastoma has been mapped to chromosome 16p (6). One common genetic abnormality of clinical importance seen in neuroblatomas is the amplification of the transcription factor and protoncogene *MYCN*, with overall prevalence of amplification of approximately 22% (2). *MYCN* amplification results in over expression and deranged growth and proliferation and is correlated to rapid tumour progression, advanced stage of disease and poor prognosis (7, 8). Deletion of 1p and 17q gain are frequent genetic abnormalities in neuroblastomas and reveal prognostic information, but so far no genes have been linked with these karyotypes (9). Still 17q is associated with more aggressive neuroblastomas, so is the chromosomal 1p deletion, which is found in around 35% of all neuroblastomas (2). Allelic loss at 1p36 gives information about disease progression but not overall survival. 1p deletions are also highly associated with *MYCN* amplification (10). No *bona fide* neuroblastoma tumour suppressor gene has been established, not even at 1p.

Cellular differentiation of neuroblastomas has been thoroughly investigated. A large number of

marker genes corresponding to normal expression in the developing sympathetic nervous system and different stages of neuroblastoma have been established (11). Low neuroblastoma tumour cell differentiation is correlated to more aggressive tumours with poor patient outcome (12).

TrkA is a tyrosine kinase- and high-affinity-receptor for nerve growth factor (NGF). Ligand-binding and induction of its signal causes cell differentiation, or with no ligand bound, apoptosis. TrkA expression in neuroblastoma is correlated with high tumour cell differentiation, low clinical stage and a favourable outcome for the patient (13, 14), and its expression is low in *MYCN* amplified tumours (13). TrkB expression, on the other hand, is correlated to poor prognosis, and is most often detected in *MYCN* amplified tumours (15, 16).

THE SYMPATHETIC NERVOUS SYSTEM

Sympathetic nervous system: its connection to neuroblastoma

The nervous system comprises the central (brain and spinal cord) and peripheral nervous system. The peripheral nervous system consists of a somatic and an autonomic part. The latter includes the sympathetic and the parasympathetic nervous system. Neuroblastoma is considered to be derived from immature cells of the sympathetic nervous system (SNS). The SNS is responsible for the "fight-and-flight" response by controlling heart rate, blood flow and respiration. It innervates organs of the abdomen and sites in the chest, pelvis and head and neck regions, locations of primary neuroblastoma tumours. To determine the origin and differentiation grade of neuroblastoma tumours knowledge about markers and factors influencing SNS development is important.

SNS structures

The SNS is comprised of sympathetic neurons that exit the spinal cord in the thoracic and lumbar regions and gather into sympathetic chain ganglia. Their axons innervate blood vessels and internal organs via the sympathetic chain ganglia, which locate along each side of the spinal column and subsequently connect with the central nervous system via branches connected to spinal nerves. The sympathetic chain and truncus ganglia, i.e. the sympathetic ganglia proper, consist of ganglion cells and, during development, small intensely fluorescent (SIF) cells. Ganglionic cells are recognized by neuronal markers, such as neurofilament (NF), neuropeptide tyrosine (NPY), GAP-43 and MYCN, and by their axons and dendrites. Chromaffin cells are the dominating sympathetic cell type of the adrenal gland, although in the human foetus scattered nests of sympathetic neuroblasts can be observed in the developing adrenal glands (17). Chromaffin cells are detected by chromaffin markers such as chromogranins A and B and synaptophysin. Phenyl ethanolamine N-methyltransferase (PNMT) is a specific marker for the chromaffin cells of the adrenal medulla, and its expression is not detected in extra-adrenal chromaffin cells. SIF cells are phenotypically intermediate between neuroblasts and chromaffin cells, containing both neuroendocrine vesicles as well as small processes. They are visualized by their strong IGF-2 expression, while SNS neuroblasts are IGF-2 negative (18). The paraganglia are formed at approximately embryonic week 6 in humans, in parallel with other sympathetic structures. They are the major catecholamine producers in the foetus and in the newborn. Catecholamines (noradrenaline and adrenaline) and acetylcholine are the main neurotransmitters of the SNS. The largest paraganglia is called the organ of Zuckerkandl. Two to three years after

birth the paraganglia regress and the adrenal glands are properly developed and take over the catecholamine production in the body. Supportive cells of the SNS are the Schwann cells. For a schematic overview of SNS cell types and their locations, see Figure 1.

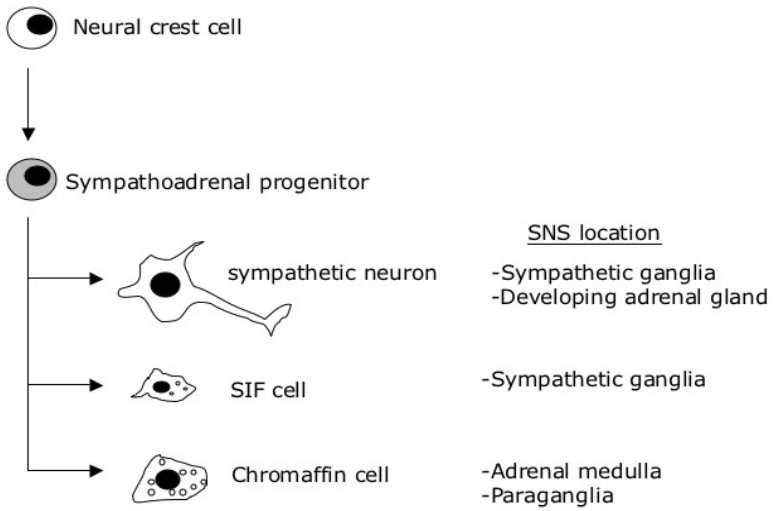

Figure 1. *The three main cell types of the SNS originate from the neural crest via a sympatho-adrenal progenitor cell. Schematic illustration of the cell types of the developing SNS and their primary locations.*

Development of the SNS

The SNS originates from neural crest cells. The developing embryo consists of three germ layers, which generate distinct types of tissues, the endoderm (gut, liver and lungs), the mesoderm (skeletomuscular system, connective tissues, kidney, heart and blood) and the ectoderm (skin and nervous system). From the ectoderm, the neural plate is formed. During neurulation, the neural plate invaginates ventrally towards the notochord. The notochord is of mesoderm origin. It secretes chemoattractant molecules that direct neural tube formation, which eventually develop into the brain and spinal cord. At the dorsal most edges of the closing neural tube, the multipotent neural crest cells form, and from there they migrate to different places in the developing embryo. Depending on where on the anterior-posterior axis they arise and on what environmental cues the neural crest cells encounter during migration and at their final destination, they can develop into diverse cell subtypes such as melanocytes of the epidermis, smooth muscle cells, sensory and autonomic cells, supportive glial cells and SNS cells (19).

Neural crest cells of the trunk region aggregate adjacent to the dorsal aorta. Cells in the wall of the dorsal aorta produce bone morphogenic proteins (BMPs), e.g. BMP-4 and –2, which induce the neural crest cells into catecholaminergic, tyrosine hydroxylase-positive sympatho-adrenal progenitor cells, also recognized by dopamine β-hydroxylase (DBH) expression (Figure 2) (20, 21). Tyrosine hydroxylase (TH) is the rate-limiting enzyme of the catecholamine synthesis, and DBH is an enzyme acting downstream in the same synthesis route, i.e. both proteins are markers of the catecholaminergic phenotype. Neurofilaments (NF) and SCG10 are other sympathetic

markers of the developing SNS.

The sympatho-adrenal progenitor cells migrate ventrally from the dorsal aorta to their end-locations, where they contribute to the sympathetic ganglia or the adrenal medulla and areas of extra-adrenal chromaffin paraganglia. During migration, sympatho-adrenal progenitor cells receive signals from the somites, the ventral neural tube and the notochord, which probably all affect the specification of the progenitor cells to eventually differentiate into either of the three previously described sympathetic neuronal/neuroendocrine lineages (Figures 1 and 2).

Cues involved in SNS cell type development

Multicellular organisms can form because of complex communication between cells within the developing embryo. Intricate gene expression programs are activated by transcription factors, which influence the fate of multipotent progenitor cells. Time and room are important decisive factors. The following arguments are extrapolations of avian and rodent studies, since, for ethical reasons, corresponding studies of human embryonic specimens are very rare.

Sympatho-adrenal progenitors are induced to proliferate and initiate neurite outgrowth, and differentiate into neurons in response to fibroblast growth factor (FGF). The neuronal phenotype is maintained by nerve growth factors (NGF) (22). A one-way transdifferentiation can appear between chromaffin cells and sympathetic neurons, as early postnatal adrenal chromaffin cells can convert into neurons, if stimulated by NGF (23, 24).

In vitro studies of cultured progenitor cells from rat embryonic sympathetic progenitors show that in culture low doses of glucocorticoids induce these cells into SIF-cells, whereas high concentrations of the hormones induce chromaffin cells (25). These and similar results have contributed to the "glucocorticoid hypothesis", encompassing the idea that sympatho-adrenal progenitors, which migrate to the developing adrenal gland, will in response to adrenal glucocorticoids lose their neuronal traits in benefit for the expression of neuroendocrine markers (reviewed in (26)). The theory has been challenged by the investigation of glucocorticoid receptor (GCR) deficient mice, as *GCR*$^{-/-}$ mice exhibited normal development of the chromaffin cell phenotype and no conversion of adrenal chromaffin cells to a neuronal phenotype was demonstrated, which would have supported the glucocorticoid hypothesis. Glucocorticoids are instead more likely to be important for the maintenance of chromaffin cells, rather than the induction of them. The only detected deviation from the adrenal chromaffin phenotype in *GCR*$^{-/-}$ mice is the lack of PNMT and secretogranin II (also called chromogranin C, related to chromogranins A and B) in chromaffin cells (27). The enzyme PNMT converts noradrenaline into adrenaline and its expression is glucocorticoid dependent. PNMT is not found in neuroblastoma specimen, thus neuroblastomas prevailing in the adrenal gland are not likely to be derived from mature adrenal chromaffin cells themselves, but rather from a transient neuroblast population of the developing adrenal medulla (17).

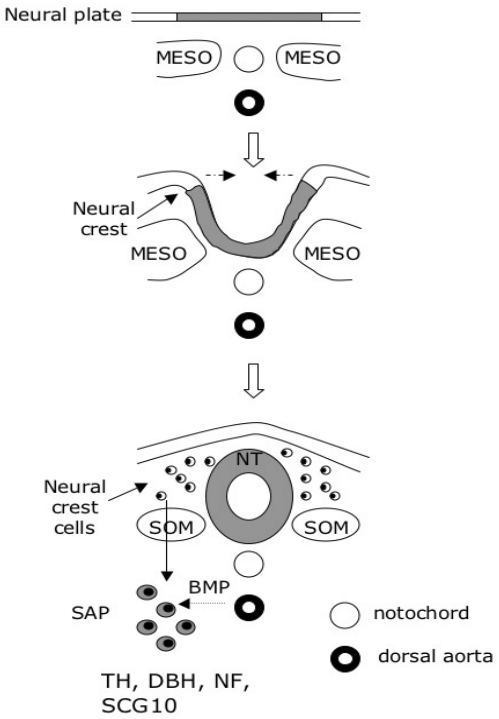

Figure 2. *Cartoon representing the formation and migration of the neural crest cells during embryonic development. The notochord induces the ectoderm of the neural plate to invaginate and form the neural tube. At the edges of the neural tube closure the neural crest cells are formed and start to migrate throughout the embryo. In response to bone morphogenic proteins (BMPs), produced by the dorsal aorta, neural crest cells develop into catecholaminergic sympatho-adrenal progenitor cells (SAPs), which express markers like tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), neurofilament (NF) and SCG10. Abbreviations: MESO=mesoderm; SOM=somites; NT= neural tube.*

Transcription factors involved in SNS development

A number of transcription factors including HASH-1 (human achaete-scute homoogue-1, MASH-1 in mice), Phox2a/b, dHAND (deciduum, heart, autonomic nervous system and neural crest derivatives) and GATA2/3 promote further development of sympatho-adrenal progenitor cells (28-30). Especially the basic helix loop helix (bHLH) transcription factors are important for the development of the SNS. They are temporally expressed and their effects can differ from one expression site in the embryo to another. They can be used as markers for a certain developmental stage of the SNS, which in turn can be correlated to neuroblastoma cells arrested at different maturation grades of the SNS development.

Class B bHLH proteins, e.g. HASH-1 or dHAND, are tissue specific factors involved in activation of neuronal and neuroendocrine genes, thereby controlling cell type specification and differentiation. They dimerize with ubiquitously expressed class A bHLH proteins, the E-proteins (E2-2, HEB, E12 and E47). The bHLH transcription factors contain a basic DNA binding domain, whereas the HLH domain is a protein-protein dimerization motif. Only when dimerization takes place between proteins, which both contain basic domains, will dimers with capacity to bind DNA form. The Id (inhibitor of differentiation) proteins are HLH proteins that lack the basic DNA binding domain and act as dominant negative regulators of bHLH proteins, such as HASH-1 and dHAND. Id proteins sequester E-proteins, hindering their potential to dimerize with lineage specifying bHLH proteins and transcativation of target genes, thus the expression of sympathetic neuronal marker genes is inhibited (31, 32).

E2-2 is highly expressed in neuroblastoma cells and might be the preferential binding partner of HASH-1 in those cells (33). *HASH-1* shows a transient expression pattern during embryogenesis, and in humans, it is found to be expressed during SNS development in sympathetic cells aggregated at the dorsal aorta, in the adrenal gland and the sympathetic ganglia at week 6.5. *HASH-1* and *dHAND* are expressed in neuroblastoma tumours as well as in cell lines (34).

Most studies on the bHLH transcription factors are done in mouse models, and below I will summarize some observations derived from these model systems. MASH-1 has been shown to be important for proper neuronal development visualised in lack of sympathetic-, parasympathetic- and enteric-ganglia in *MASH-1* deficient mice (35, 36). *Phox2a*, *TH* and *DBH* are genes involved in the development of sympatho-adrenal progenitor cells, which have been implicated to be directly, or indirectly, regulated by MASH-1 (35, 37). MASH-1 is also required for the generation of the chromaffin cell phenotype. Adrenal chromaffin cell differentiation is severely impaired in *MASH-1*$^{-/-}$ mice, exhibiting lack of chromaffin granules and featuring an immature neuroblast like phenotype (38). These different effects of MASH-1 are possibly due to spatial and temporal differences in *MASH-1* expression.

The homeobox domain transcription factor Phox2b is also essential for sympathetic neuron development and can sustain *MASH-1* transcription (39). *Phox2b* deficient mice die before birth due to severely impaired systemic noradrenaline levels, and they exhibit similar developmental defects of the sympathetic ganglia, as do *MASH-1* deficient mice. However, sympatho-adrenal progenitor cells of *Phox2b* null mice still cluster along the dorsal aorta, but the cells lack well-established sympatho-adrenal progenitor markers, such as Phox2a, TH and DBH, similar to the defects seen in *MASH-1*$^{-/-}$ mice. Thus, studies of the *Phox2b* null mice demonstrate a role for Phox2b in the activation of the catecholamine synthesis (40, 41).

An additional pathway implicated in neuronal development is the Notch cascade, which promotes an undifferentiated state, but also the glial cell fate while it hinders neuronal differentiation. (42). Thus, the Delta-Notch route is implicated in the development of the SNS and involved in neurogenesis in what is referred to as lateral inhibition. It is a mechanism employed in the embryo to generate two diverging cell types from one precursor cell. Delta is the ligand of the Notch receptor and both are expressed in neuronal cells and the developing/embryonic ganglia. Notch acts as a receptor for Delta proteins on neighbouring cells.

Activation of Nocth is followed by several cleavage steps terminating in nuclear translocation of the intracellular domain of Notch (Notch ICD) that results in the transactivation of genes of

the Enhancer of Split complex *(HES1-7*, *HEY1-2* and *HEYL1* in humans) (43). The *HES* and *HEY* genes produce bHLH transcription factors, which negatively influence the transcription of proneuronal genes such as *HASH-1*. When the equilibrium of all these components is shifted, for example a cell slightly over expresses a proneuronal gene or Delta, that cell will follow a neuronal fate, whereas the neighbouring cell will develop towards the glia cell lineage. However, the precise function of Notch depends on the context. Taken as a whole, all the different molecules and transcription factors described can be used as markers of the sympathetic cell linage and the majority of them have been detected in human neuroblastoma cells.

OXYGEN PRESSURE IN TUMOURS

Tumour hypoxia

Tumours are formed due to clonal expansions of a mutated normal cell. Inherited or spontaneous mutations cause activation of oncogenes and the inactivation of tumour suppressor genes. These changes enable tumour cells to override the balance between proliferation and apoptosis, resulting in continuous growth and formation of neoplasms. In order for tumour cells to maintain their growth, they need oxygen and nutrients as well as to discard metabolic waste products. The diffusion limit for oxygen is approximately 100 μm and oxygen-transport over further distances requires red blood cells. When available blood flow cannot fulfil the requirements for maintaining oxygen- and nutrient- homeostasis, the partial oxygen pressure of these tumour areas become low, i.e. hypoxic, or close to zero, anoxic. Metabolic hypoxia has been defined as when the oxygen partial pressure decreases below a threshold value where O_2-consumption and ATP production are slowed down and cell homeostasis is no longer maintained. Hence, hypoxia is the state where oxygen levels are below a critical and specific threshold impairing normal function of organs, tissues or cells. This hypoxic threshold differs from tissue to tissue and cannot be given with accuracy (44, 45).

In many studies, growth conditions of 10 mmHg, or 1.3% O_2, approximated to 1% O_2, is considered hypoxic. It is based on the finding that the overall survival of patients with cervix cancer is poorer if the median tumour oxygen pressure is 10 mmHg or less (46), since ATP production is severely reduced and not sufficient to maintain all aspects of cellular homeostasis. The hypoxic and anoxic regions of tumours are heterogeneously distributed, with plenty of regions approaching 5 mmHg, corresponding to approximately 0.7% O_2 (47). End-capillary blood pressure is about 5-6% O_2 and the border between this oxygen level and hypoxia is vague, emphasizing the importance of proper definitions of what oxygen levels are used in different experiments (45). Ordinary cell culturing is performed at an atmospheric oxygen pressure of 760 mmHg, or 21% O_2, and is mostly referred to as normoxia, but this oxygen pressure is in fact not at all close to physiological oxygen levels. Thus, it would be more appropriate to culture cells at lower oxygen levels, at “physioxia”, but the specific pressure is difficult to establish since it is not uniform either within or between specific tissues. In paper II and III in this thesis we have used 5% O_2 as an approximation of well oxygenated areas of neuroblastoma.

Hypoxic responses

Hypoxia elicits a myriad of biological responses. To maintain the systemic homeostasis during hypoxic conditions, the body responds by increasing the secretion of erythropoietin (EPO) from the kidney and raises blood flow and heart rate via an increase in catecholamine production, illustrated by increased transcription of *tyrosine hydroxylase*, the rate limiting enzyme of catecholamine synthesis, by sympathetic neuronal/neuroendocrine cells (48-52). Cells maintain energy supply by entering glycolysis and anaerobic metabolism. Hence, a row of glycolytic enzymes is upregulated by hypoxia, which results in lactic acid production causing acidosis. In addition, glucose transporters are upregulated at hypoxia. Hypoxia also induces the formation of new blood vessels via an increased expression of the angiogenic *Vascular Endothelial Growth Factor* (*VEGF*). At the transcriptional level, the key mediators of the hypoxic response are the hypoxia inducible factors (HIFs), HIF-1α and HIF-2α, which will be discussed further.

Tumour vascular supply

The process of tumour vascularization differs from tumour to tumour. It can start as an avascular tumour mass, which cannot grow larger than 1-2 mm until formation of new blood vessels occurs (44). These lesions can stay dormant by keeping a balance between proliferation and apoptosis. Another scenario is tumour growth initiated adjacent to pre-existing vessels, vessels with which the tumour can co-opt, and hence, be well vascularized already from the start. However, tumours supplied by co-opted vessels are bound to become hypoxic, since co-opted vessels destabilize and regress resulting in a hypoxic tumour microenvironment, which triggers the tumour cells to secure survival and re-growth of vessels (53, 54). Eventually both avascular, as well as co-opted tumours, require new blood vessel formation, angiogenesis, to support tumour cell survival.

In response to hypoxia, tumour cells release pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), platelet derived growth factor (PDGF) and epidermal growth factor (EGF), which attract endothelial cells from pre-existing vessels, which in turn proliferate and form new tumour capillary networks. The induction of tumour angiogenesis is traced to several mechanisms such as hypoxia, metabolic stress, acidic environments, hypoglycaemia, mechanical stress due to increased intratumoural pressure, tissue-infiltrating inflammatory cells, which secrete angiogenic molecules, as well as activation of oncogenes and inactivation of tumour suppressor genes (55).

Angiogenesis is finely tuned by opposing factors such as thrombospondin-1, endostatin and angiostatin. Many inhibitory molecules, e.g. the statins, are fragments derived from larger proteins, often residing in the extra cellular matrix (ECM), which cannot affect angiogenesis themselves. For instance, endostatin is a cleavage product of type XVIII collagen (56). The induction of angiogenesis is referred to as the “angiogenic switch”, which is when the balance between angiogenic and anti-angiogenic factors is shifted so that endothelial cell growth stimulation is favoured (Figure 3). An alternative route to form blood vessels is vasculogenesis, the mode used during embryogenesis where de novo vessels are formed by attracting endothelial-precursor cells, which reside in the bone marrow. This route of neovascularization seems not to be commonly used by tumour cells, although conditions have been identified, such as tumour therapy-induced recruitment of circulating endothelial-precursor cells (57, 58).

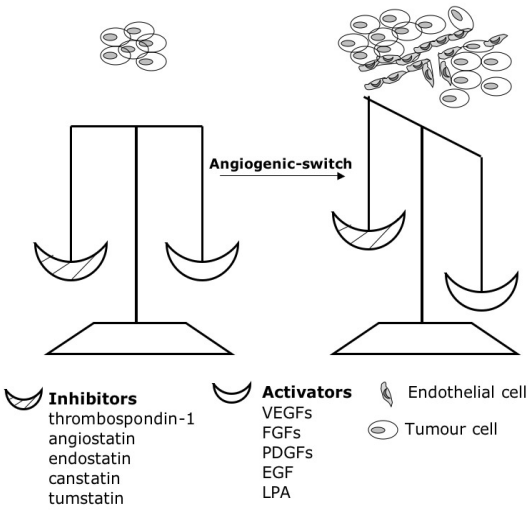

Figure 3. *The angiogenic switch in tumours is when there is a shift in the tumour from anti-angiogenic factors (inhibitors) towards angiogenic factors (activators), thus promoting angiogenesis.*

Tumour vasculature

Adult mature blood vessels consist of endothelial cells lined with supportive cells, such as pericytes and smooth muscle cells, and are in general quiescent with a low turn over of endothelial cells. However, ongoing angiogenesis is a prerequisite for female reproductive organs, as well as in wound healing. Tumour vessels, on the other hand, are immature and often malformed with abnormal branching characteristics, resulting in a chaotic structure. In addition, cancer cells are integrated into the walls of some tumour vessels (59, 60). Leaky vessels and blind ends are by themselves promoting a hypoxic milieu causing further induction of pro-aniogenic factors like VEGF, thus creating a positive paracrine loop which maintains tumour growth and continued malformation of vessels.

VEGF, angiopoietin-2 and (tumour) angiogenesis

VEGF/VEGFA is the most common member of the VEGF family, and it binds to tyrosine kinase receptors, VEGFR-1 (flt-1**)** and-2 (flk-1/KDR), whereas VEGFC and VEGFD both bind VEGFR2. VEGF receptor tyrosine kinases are primarily expressed on endothelial cells of newly formed blood vessels. Via these receptors, VEGF plays several roles in angiogenesis. It is induced by HIFs and loss of HIF-1α in endothelial cells perturbs a hypoxia-driven VEGF autocrine loop required for tumorigenesis (61). As implied by its name it is a mitogen and pro-survival factor for endothelial cells in newly formed immature vessels (62). VEGF is also depicted vascular permeability factor (VPF), which indicates its role in causing leaky blood vessels that allow

the release of plasma proteins and formation of an extravascular matrix, which is important for endothelial cell growth and motility (63). The tumour interstitial pressure is raised due to this increased vascular permeability (64). VEGF also induces chemotaxis and it has mobilizing effects on circulating endothelial precursor cells (65). In addition, it plays a prominent role in vasculogenesis during embryonic development as *VEGF* knock out mice demonstrate embryonic blood vessel malformations and embryonic lethality (66, 67).

Angiopoietins (Ang) are important for stabilization and remodelling of the vasculature. The angiopoietin receptors, Tie1 and Tie2, are both receptor tyrosine kinases, like VEGF receptors. Angiopoeitin-1 and –2 mediate their known effects by binding to Tie-2. The function of Tie-1 is uncertain. Angiopoietin-1 mediates interaction between endothelial cells and supportive cells such as pericytes and smooth muscle cells (68). *Tie-2* or *Ang-1* null mouse embryos show normal formation of the primitive vasculature, guided by VEGF, whereas stabilization and remodelling is severely disturbed and fatal (68-70). Angiopoietin-1 and Tie-2 are ubiquitously expressed in quiescent endothelial cells while Angiopoietin-2 is abundant in endothelial cells of immature- and co-opted tumour vessels (53). Angiopoietin-2 acts as natural antagonist for Angiopoietin-1/Tie-2 interaction, and transgenic over expression of *Angiopoietin-2* in mice leads to a fatal vascular phenotype similar to that seen in *Ang-1* and *Tie-2* null embryos (71). *Angiopoietin-2*, similar to *VEGF*, is induced by hypoxia and it facilitates the effects of VEGFA. Angiopoietin-2 is involved in destabilization of capillaries, which is a prerequisite for induction of vessel sprouting (53, 65).

Anti-angiogenic therapy

Tumour vessels differ from mature quiescent vessels found in healthy tissue. Upregulation of VEGF receptors and expression of ECM-binding integrin receptors are seen in tumour vessels (72). These immature vessels are sparse in endothelial supporting cells, which is another cause for increased permeability of tumour vessels. The activated endothelial cells of immature vessels are more vulnerable than those of mature vessels and are strictly dependent on VEGF for survival. This difference is being exploited in anti-angiogenic therapies. Compounds have been developed with the aim to inhibit tumour angiogenesis. Some of them are synthetic variants of endogenous angiogenic inhibitors, such as endostatin and angiostatin; others are VEGFR inhibitors (73). The net effect is inhibition of tumour growth or, even better, tumour shrinkage due to attenuated angiogenesis. Angiogenic inhibitors have also been evaluated in combination with ordinary cytostatic agents and the result is augmented tumour shrinkage. Inhibition of VEGF leads to less immature leaky vessels within the tumour, followed by decreased interstitial tumour pressure, which both promote an increased drug delivery to tumour cells. The strength of this therapy is that endothelial cells do not become resistant. On the other hand, when tumour angiogenesis is pharmacologically reduced, surviving tumour cells respond by further upregulation of VEGF, resulting in an insidious loop that selects for tumour cells which are able to persist a hypoxic environment (72, 73).

Hypoxia and malignant progression

Hypoxia in tumours correlates positively with an aggressive phenotype, which has been demonstrated in for instance cervix cancer and soft tissue sarcoma (46, 74, 75)**.** The mechanisms for increased aggressiveness are attributed to aspects, such as increased metastatic potential, high mutational frequency and promotion of angiogenesis. Hypoxia-regulated genes involved in the aggressiveness of hypoxic tumour cells are partly due to gene transcription driven by the

HIFs. HIF-1α is important for solid tumour growth in experimental models (76) and its over expression is detected in common human cancer forms, especially in metastases (77).

Metastasizing cells have to overcome several obstacles in order to invade new tissue. They need to cut interactions with adjacent cells, traverse the basal membrane, migrate through the extracellular matrix and enter blood- or lymph vessels, and eventually penetrate their new surroundings, proliferate and secure blood flow. Hypoxia affects some of the steps of metastasis and several genes involved in metastasis have been shown to be hypoxia-induced and HIF-regulated, such as lysyl oxidase (78). Other hypoxia- and HIF-1α-driven genes implicated in digestion of basal membranes and extracellular matrices are *cathepsin D*, *urokinase-type plasminogen-activator receptor* (*uPAR*) (79) and *metalloproteinase-2* (*MMP2*), and in invasion and migration are tyrosine kinase receptor *met* protooncogene and secreted factors such as *TGF-α* ((80) and reviewed in Semenza 2003 (81)).

The hypoxic tumour microenvironment induces genetic instability (82) and genes involved in DNA repair are downregulated by hypoxia, increasing the risk for hypermutability (83). Both DNA mismatch (84) and DNA double strand break repair gene expression is decreased by hypoxia (85). In addition, *MutSalpha*, which recognizes base mismatches, is repressed by HIF-1α at hypoxia (86).

Hypoxia, treatment resistance and apoptosis

Hypoxia is known to impair the effects of radiotherapy and chemotherapy on tumour growth. Radiotherapy is based on the transformation of oxygen into cell damaging reactive oxygen species (ROS). It has been debated, but still appreciated, that during hypoxia, radiation-induced tumour shrinkage is impaired due to low ROS production as a result of low oxygen availability (47, 87). During recent years, radiation has been considered to trigger apoptosis in endothelial cells, i.e. irradiation primarily targets tumour vasculature, rather than tumour cells proper (88). The hypoxia-inhibiting effects on radiation could be due to endothelial protective effects by hypoxia-induced *VEGF* expression. One report demonstrates that tumour reoxygenation and ROS-formation as consequences of radiation induce HIF-1α nuclear accumulation and activation of cytokines, like VEGF, which is capable of inhibiting endothelial cell apoptosis (89).

Hypoxic tumour cells become resistant to chemotherapeutic drugs for several reasons. Low drug penetration is caused by poor drug-accessibility of cells growing far away from functional capillary networks, and dysfunctional, leaky tumour blood vessels raise tumour interstitial pressure. Some drugs are also dependent on oxygen availability for their toxicity. Most cytotoxics target dividing cells, and low oxygen pressure slows down cell proliferation resulting in an increased number of cells in the G_0 phase, thus obstructing drug action (90). Drug resistance is augmented by hypoxia-induced expression of genes like multidrug resistant gene 1 (MDR1) (91). In addition, many cytotoxic drugs act via DNA damage-induction of p53 dependent apoptosis, but hypoxic conditions can select for p53 deficient cells (92). Hypoxia, acting via HIF-1α, induces the pro-apoptotic gene BNIP3 (93, 94). However, the effects of BNIP3 seem to be finely tuned by minor changes in oxygen levels (95). In summary, the severity of hypoxia is the key determinant of hypoxia-induced apoptosis, and there is a balance between hypoxia-induced survival and hypoxia-induced apoptosis, and only when this balance is shifted will hypoxic tumour cells die. Hypoxia balances apoptotic mechanisms by inducing anti-apoptotic genes such as apoptosis inhibitory protein, IAP2 (reviewed in (96)).

HYPOXIA-INDUCIBLE FACTORS (HIFs)

Hypoxia-inducible factors and their distribution

There are three members of the hypoxia-inducible bHLH-PAS family, HIF-1α, HIF-2α and HIF-3α. Wang and Semenza first described HIF-1α in 1993 (97), and in 1997 Fujii-Kuriyama's group described HIF-2α (98). HIF-2α is also termed HLF (HIF1α-like factor), EPAS1 (endothelial PAS1), MOP2 (member of PAS family 2) or HIF-related factor. In 1998 HIF-3α, which lacks a transactivation domain, was discovered in kidney tissue (99). The discussion to follow will focus on HIF-1α and HIF-2α.

HIF-1α is ubiquitously expressed in all tissues, whereas *HIF-2α* initially was recognized as a transcription factor selectively expressed in endothelial cells (100), although work by e.g. Talks *et al* demonstrate HIF-2α presence in several different cells, such as kidney fibroblasts, neural crest derivatives and lung type II pneumocytes as well as cancers with clear expression in tumour associated macrophages (101-104). HIF-1α over expression, detected by immunohistochemistry, is demonstrated in many human cancers (77, 101), and has been shown to be important for solid tumour formation (105). It is associated with poor patient survival in several cancers (81), but as for example epithelial ovarian tumours, HIF-1α only correlates to adverse outcome if found in combination with over expression of non-functional p53 (106). The necessity of HIF-1α in solid tumour formation is demonstrated in the reduced capacity of murine *HIF-1α*$^{-/-}$ ES cells to form teratomas as opposed to wt ES cells (76). Further more, renal cell carcinomas (RCCs) and hemangioblastomas, which lack functional von Hippel Lindau protein (VHL), results in constitutively expressed HIFs. In some RCCs both HIFs are expressed, while in others only HIF-2α is express. Kondo *et al* report that HIF is important for renal cell cancer tumour formation and that HIF-2α is the main actor (107, 108).

Hypoxia-inducible factors during development

The HIFs were initially considered to be redundant, but increasing evidence support that HIF-1α and HIF-2α indeed have distinct roles, and that they exhibit disparate roles in embryonic development. Several *HIF* knock out mice have been engineered, but due to different genetic backgrounds of the mice and different analytical methods, the conclusions have not always been confirmed. Still, they provide a fair indication of the role of these transcription factors during embryogenesis.

HIF-1α null embryos produced by Iyer *et al* resulted in developmental arrest and death by embryonic day (E) 11. The embryos demonstrated neural tube defects, cardiovascular malformations and failure in vascular integrity and the latter was probably due to deficiency in pericyte formation as a result of mesynchymal cell death (109), which was confirmed in a similar study (110). Abnormal neural development and vascular defects were disclosed at E 8 and E 8.5 in *HIF-1α*$^{-/-}$ embryos reported by Ryan *et al* (76). Compernolle *et al* also detected defective heart development in *HIF-1α* null mice. In addition, they noticed abnormal neural crest migration along with reduced levels of *Id2* (111). *Id2* is normally expressed in migrating neural crest cells (112). The lack of Id2 might explain the defective neural crest cell migration. The decrease in *Id2* is in keeping with data from our group demonstrating *Id2* as a HIF-1α target gene (113).

The *HIF-2α* knock out mice by Tian *et al* died from circulatory failure and bradycardia during mid-gestation due to disturbed catecholamine homeostasis. Examination of the animals

disclosed *HIF-2*α-expression in the sympatho-adrenal cell lineage in addition to endothelial cells. At E11.5, *HIF-2*α positivity was detected in sympathetic chain ganglia with a decline at E12.5, followed by a striking expression in developing paraganglia at E13.5 to E15.5. Lower levels were also found in the adrenal gland at that time, all implicating a role for HIF-2α in the development of the SNS (52). Jögi *et al* and Nilsson *et al* corroborate these findings by detection of HIF-2α in mouse paraganglionic cells at E14.5 and in human paraganglia at foetal week 8.5, which developmentally corresponds to mouse E16 (114, 115). It is an intriguing finding, as also neuroblastoma cells and tumours reveal presence of HIF-2α at seemingly non-hypoxic conditions ((115) and papers -II and -III in this thesis). *HIF-2*α null mice derived from different backgrounds than the first *HIF-2*α knock out mice, demonstrated defect vascular remodelling (116) and impaired foetal lung maturation, but no effect on catecholamine production (117). Duan *et al* proposed that HIF-2α expression and subsequent regulation of Tie-2 expression in endothelial cells is one important factor for proper embryonic vascularization in mice (118). Scortegana *et al* established a vital *HIF-2*$\alpha^{-/-}$ mouse phenotype, which showed multiple organ pathology, impaired homeostasis of reactive oxygen species and mitochondrial deficiency (119). Further analysis unravelled a role for HIF-2α in murine haematopoiesis (120) and the HIFs are suggested to be the key regulators of murine haematopoietic development via regulation of *erythropoietin* (*EPO*) (121).

HIF structure and complexing partners

HIFs are heterodimeric transcription factors consisting of a hypoxia-inducible α-subunit and a constitutively expressed β-subunit, HIF-1β, also called ARNT (aryl hydrocarbon receptor nuclear translocator), which resides in the nucleus (122). HIFs are instantaneously stabilized by hypoxia (123) and they bind to hypoxia response elements (HREs) in promoters or enhancers of their target genes. They are relatively large proteins of approximately 800 amino acids or 120 kDa, and the subunits belong to the bHLH-PAS (Per/Arnt/Sim) family (124, 125). They contain a nuclear localization signal (126), a basic helix-loop-helix motif (bHLH) and two PAS domains, PAS-a and PAS-b. The basic domain is important for DNA binding and the HLH domain is used for dimerization between HIF-α and ARNT (97, 127). An alternatively spliced variant of HIF-3α, IPAS (inhibitory PAS domain protein) is a dominant regulator of HIF-1α (128). IPAS sequesters HIF-1α, preventing dimerization of HIF-1α/ARNT and no DNA-binding can take place (129). The α subunit contains two transactivation domains (TADs), the N- and C-terminal TADs (130)**.** In the middle resides an oxygen dependent degradation (ODD) domain (131), which partly overlaps with N-TAD. The C-TAD is required for full HIF activity and interacts with co-activators like CBP/p300 (132) (Figure 4).

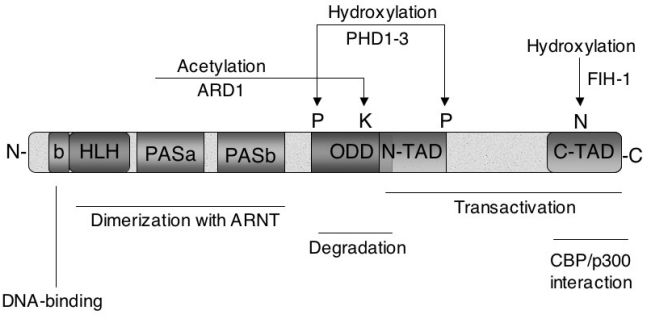

Figure 4. *Structure of the HIF-1*α *protein. Domains and their functions are indicated, as described in the text.*

Regulation of HIF transcriptional activity

HIF transcriptional activity is dependent on co-factors such as CBP/p300. The interaction between HIFα and CBP/p300 is inhibited at normoxia due to hydroxylation of an aparagine residue (Asn803 and Asn851 in HIF-1α and HIF-2α, respectively) (133). Factor Inhibiting HIF (FIH-1) is the asparaginyl hydroxylase, which hydroxylates HIFα in the C-terminal TAD and hinders HIFα from interacting with the co-activator p300 (134-136). HIF-2α is less efficiently hydroxylated by FIH-1 than is the HIF-1α CAD, resulting in increased HIF-2α basal activity (137).

CBP and p300 are ubiquitously expressed proteins, which possess histone acteyltransferase activity (138). Thus binding of p300 to HIF-1α probably facilitates transactivation by augmenting histone acetylation, resulting in stronger HIF-1 DNA binding. Steroid-receptor co-activator-1 (SRC-1) and transcription intermediary factor 2 (TIF2) are other histone acetyltransferases and co-activators of HIF-1α, and the redox regulator protein Ref-1 is also potentiating HIF-1α and HIF-2α transactivation (139).

HIF transactivation is also affected by phosphorylation, which is a well-established mechanism for regulation of transcription factor activity (140). Phosphorylation sites have been identified within the transactivating domains of both HIF-1α (140) and HIF-2α (141). These sites are suggested to be phosphorylated by the MEK-1/p42/p44 MAPK pathway (140-142). The effects of different kinases seem to differ between cells and settings, as in one report, MAPK and p38 kinase are demonstrated to positively regulate HIF-1α transcriptional activity (143), whereas another study show HIF-1α phosphorylation by p42 and p44 but not by p38 MAPK (144). In endothelial cells, ERK-dependent phosphorylation of HIF-1α increases HIF-1α transcriptional activity (145). Other groups suggest that MAPK signalling facilitates HIF activation through regulation of p300/CBP activity (146). These phosphorylations of HIFα are present at hypoxia, potentiating HIF activity but not HIFα DNA binding. HIF-1α transactivation are counterbalanced by MAPK dephosphorylation by e.g. MKP-1 (MAPK phosphatase-1) (147).

Oxygen- and stabilization dependent regulation of HIF

The degradation of HIF-1α and HIF-2α proteins is oxygen-dependent. The HIFs are stabilized at hypoxia, whereas normoxic oxygen levels promote proteosomal degradation. The ODD domain contains two prolines, p402 and p564, which at normoxia are hydroxylated by prolyl hydroxylases (PHDs) (148, 149). The tumour suppressor, protein VHL, recognizes hydroxylated HIFα subunits. The VHL protein is the recognition component of an E3 ubiquitin-protein ligase consisting of elongin-B, elongin-C, rbx1 and cullin 2 (150, 151). Except hydroxylation, the pVHL-HIFα binding is promoted by acetylation of lysine residue 532 by the ARD1 acetyltransferase. ARD1 expression is reduced at hypoxia (152). Hydroxylated and acetylated HIFα bound to pVHL, ensures HIF-ubiquitination by cullin-2, which targets the transcription factor for degradation by the 26s proteasome (149, 153). The tumour suppressor p53 negatively regulates HIF-1α ubiquitination and proteosomal degradation via recruitment of the MDM2 ubiquitin ligase (154). Nitric oxide (NO) also affects HIF-1α stability, both positively and negatively depending on cell type and NO concentration (155), and sumoylation of lysines within the ODD domain enhances HIF-1α stability (156). The list of proteins that can modulate HIFα stability does not end with these examples and highlights the complex networks involved in its function. In addition, stabilization patterns of HIF-1α vs. HIF-2α proteins differ in some cell lines ((157) and paper II and III in this thesis). HIF-regulation is summarized in Figure 5.

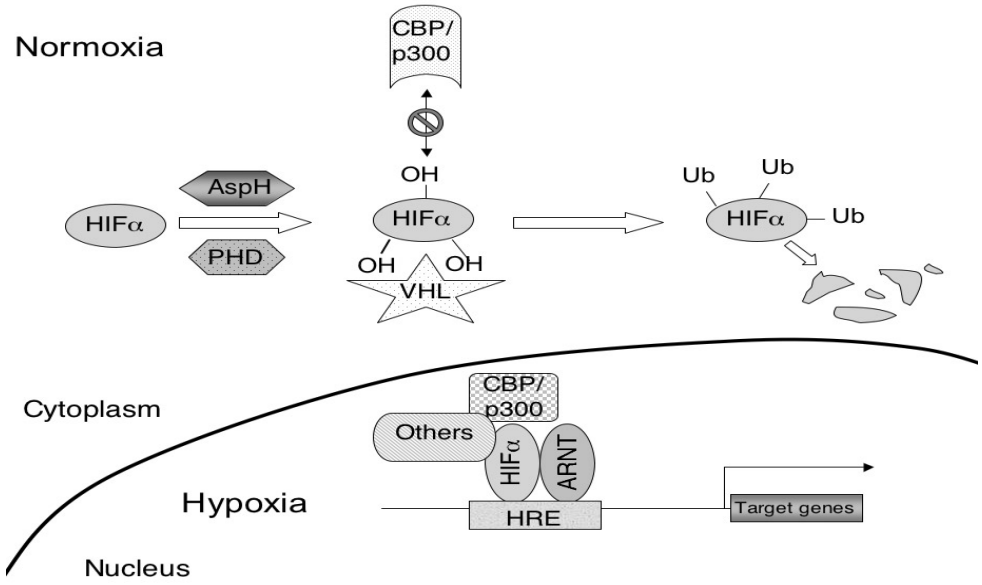

Figure 5. *Hypoxic and normoxic regulation of HIF-α function. At normoxia HIF-α is hydroxylated by prolyl hydroxylases (PHD). Hydroxylated HIF-α is recognized by pVHL, which targets HIF-α for ubiquitination (Ub) and proteosomal degradation. The asparaginyl hyrdoxylase (AspH), also called FIH (Factor Inhibiting HIF), hydroxylates a specific asparagine residue of HIF-α, rendering it difficult for HIF-α to interact with the co-activators CBP/p300. At hypoxia the oxygen-dependent regulation of HIF-α is attenuated and HIF-α can dimerize with its partner ARNT. Together, and in collaboration with for example CBP/p300, they bind hypoxia responsive elements (HREs) of their target genes causing transactivation as a response to low oxygen levels.*

Prolyl hydroxylases

The are three known prolyl hyrodylases, PHD-1, -2 and –3 (158). Their denotations can be somewhat confusing PHD1, PHD2 and PHD3, or HPH-3, HPH-2 and HPH-1, or EGLN2, EGLN1 and EGLN3, respectively. Here the abbreviation PHD will be used. They are dioxygenases and oxygen sensors, which require oxygen, 2-oxoglutarate and iron for full enzymatic hydroxylation of HIFs (159). One oxygen atom is transferred to a proline residue, whereas the other reacts with 2-oxoglutarate generating succinate. Oehme *et al* discovered a fourth proline 4-hydroxylase-related protein, PH-4 (160), which when over-expressed inhibits HIF-activity, but has no effect on the HIF-1α/VHL interaction.

Interestingly, the expression of *PHD2* and *PHD3* are upregulated by hypoxia (158, 161) and also demonstrated to be HIF-1α target genes (162-164). It was recognized that the half-life of HIF upon reoxygenation is dependent on the duration of hypoxia. Longer hypoxic exposure leads to faster degradation of HIF upon reoxygenation (162), and degradation of HIF-1α is dependent on hypoxia-induced transcription. Several other reports support this feedback mechanism, which limits HIF responses upon reoxygenation (165, 166). Today, this phenomenon can be attributed to the hypoxia-induced HIF-1α-dependent upregulation of *PHD2* and *PHD3* (163,

164), although results from other studies indicate that HIF-2α regulates PHD3 (95, 167). PHDs are also active at low oxygen levels, balancing the increased HIF stabilization at hypoxia (168). To counteract PHD activity during hypoxia, degradation of PHDs is induced at hypoxia by the E3 ubiquitin ligase Siah2 (169). The PHDs are distributed in different cellular compartments, where PHD1 is nuclear, PHD2 cytoplasmic and PHD3 is evenly distributed in the cell (170). The importance of this distribution is far from fully elucidated. PHD2 has been claimed to regulate the low steady-state levels of HIF-1α at normoxia (171). Additional studies suggest that PHD2 is the main regulator of HIF-1α and HIF-2α is most efficiently hydroxylated by PHD3 (161). A protein called OS-9 has been shown to promote oxygen-dependent degradation of HIF-1α by interacting both with the prolyl hydroxylases and the HIFα subunit (172).

Signalling-pathway-dependent regulation of HIF synthesis

HIFs are frequently present also in normoxic cells. One identified mechanism is growth-factor dependent increase in HIF translation. Hypoxia augments HIF stabilization in all cell types, whereas growth factor-dependent induction of HIF is cell type specific. Growth factors, vascular hormones, cytokines and other signalling molecules enhance HIF-1α synthesis by activating the PI3K- (phosphatidylinositol 3-kinase) or MAPK- (mitogen-activated protein kinase) pathways. The list of growth factors that stimulates HIF-1α is becoming increasingly longer and includes PDGF, EGF, IGF-2 and TNF-α. The same signalling pathways are activated by oncogenes such as HER2, RAS and v-src and by mutations in the tumour suppressor gene PTEN (81). Mammalian target of rapamycin, mTOR, a kinase acting downstream of PI3K and Akt, phosphorylates and activates factors regulating protein synthesis and promotes HIF-1α protein synthesis. HER2 belongs to the epidermal growth factor receptor family (EGFR) and, in breast cancer cells, activation of HER2 elicits signalling via the PI3K/Akt/mTOR pathway causing increased HIF-1α protein synthesis and increased VEGF mRNA levels (173). A breast cancer cell line study also shows that PI3K affects HIF-1α protein levels (but not HIF-2α) and subsequently VEGF levels, and that PI3K-effects are lacking in cell lines with *RAS* or *PTEN* mutations (174). Similar results concerning HIF-1α protein levels and PI3K and PTEN, are shown in several other reports. Some reports even show a link between RAS, PI3K and HIF levels (175-177). Fukuda *et al* show how both PI3K- and MAPK-signalling positively regulate HIF-1α protein synthesis (178). Suggested mechanisms for increased HIF-1α protein synthesis are summarized in Figure 6 (modified from Semenza 2003 and (81, 173, 175-177, 179)). It is important to stress, though, that receptor-mediated effects on HIFα are not in the same range as hypoxic stabilization of HIFα, but the growth factor-influences are still important for cancer cells to survive and divide.

HIF-1α and HIF-2α: differences and similarities

Target genes of the two HIFs include genes involved in glycolysis, angiogenesis and cellular survival (180). As HIF-1α was the first of these transcription factors to be described, most reports deal with HIF-1α mechanisms, although HIF-2α publications have increased substantially during the last few years. Presence of a transcription factor does not in itself implicate activity and the functionality of HIF-2α has been questioned. For example, HIF-2α was shown to be trapped in the cytoplasm, and to be transcriptionally inactive in mouse embryonic fibroblasts (181). HIF-2α is also non-functional in murine ES cells, possibly due to expression of a HIF-2α-specific co-repressor (182, 183). However, these results do not preclude HIF-2α function in other cells, as shown in this thesis (paper III).

The HIFs have several target genes in common. HIF-1α was once identified to be the transcription factor driving *EPO* expression in response to hypoxia (184). However, several research groups report *EPO* to be the main target of HIF-2α in specific cells, such as neuroblastoma cells, hepatoma cells and cortical astrocytes (185-187). *VEGF*, another *bona fide* HIF-1α target gene, is also regulated by HIF-2α (98). In the search for HIF-1α and HIF-2α specific target genes, Hu *et al* demonstrated that glycolytic enzymes are HIF-1α specific (183). Cell type specificity and context seem to be main determinants of HIF-subunit usage. We shall consider a few examples. In one breast cancer cell line, HIF-1α is the great contributor to HIF-target gene activation, as shown by *VEGF* induction (188). In pVHL defective renal cell carcinoma cells, HIF-2α is the main target gene regulator, whereas in those RCC cells that express both HIFs, HIF-1α is the predominant factor (188-190).

RCCs frequently lack functional pVHL, consequently HIF proteins are constantly present and as a result the tumours are highly vascularized, although in several RCC cell lines only HIF-2α protein is present. A recent report reveals that in RCC cell lines, which contain both HIF proteins, HIF-1α is the main *VEGF* regulator, whereas in those with only HIF-2α protein, HIF-2α targets *VEGF* (190). It is also shown that in RCC cell lines, which lack HIF-1α, the HIF-1α promoter is either silenced by hypermethylation, truncated HIF-1α mRNA transcripts are produced (185, 190) or a HIF-1α-dominant-negative isoform of HIF-3α, HIF-3α4, is downregulated (191). HIF-1α or HIF-2α specific targets genes unraveled from the RCC system will not necessarily be applicable to cells with a functional HIF-α degradation system (like neuroblastoma).

Promoter analysis of the genes specifically induced by HIF-2α reveals putative ETS transcription factor binding sites in proximity to HREs (192). In endothelial cells, HIF-2α is dependent on cooperation with ETS-1 in order to transactivate the VEGF-receptor 2 gene *Flk-1* (193). Similarly, the ETS-transcrition factor Elk-1 seems important for HIF-2α taget gene activation in breast cancer cells (192). The NF-κB essential modulator NEMO interacts specifically with HIF-2α to enhance normoxic HIF-2α transcriptional activity. The effect is independent of the HIF-2α TAD, and instead NEMO facilitates recruitment of the co-activators CBP/p300 to HIF-2α (194).

HIF-2α is well expressed in endothelial cells and drives the expression of hypoxia-induced genes such as VEGF, Tie-2, Flk-1, Flt-1 and GAPDH (100, 187, 195, 196). Reported HIF-2α target genes, such as PAI-1 (plasminogen activator inhibitor-1) and MT1-MMP (membrane type-1 matrix metallo-proteinase), are activated via cooperation between HIF-2α and Sp1 (197, 198), although Sp1 dependency has also been shown for some HIF-1α target genes (199, 200).

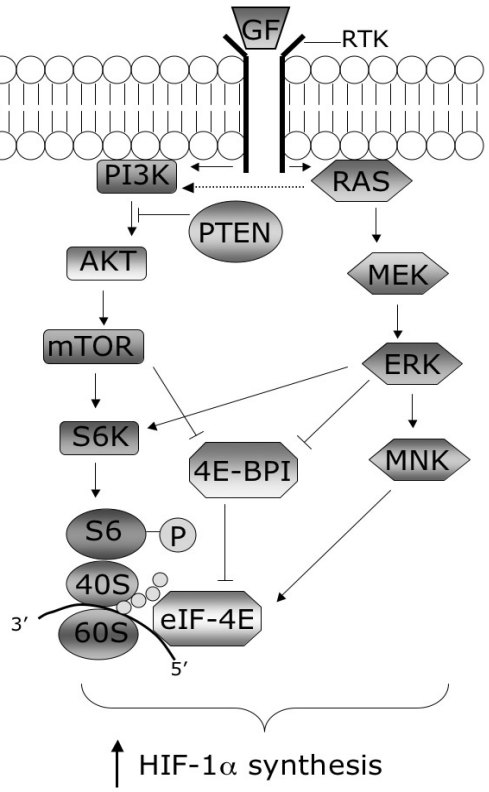

Figure 6. *Growth factor binding to a receptor tyrosine kinase causes phosphorylation of phosphatidylinositol 3-kinase (PI3K) and activation of RAS, which initiate the PI3K- and mitogen-activated protein kinase (MAPK) pathways. PI3K activates downstream serine/threonine kinase AKT/PKB followed by mTOR/FRAP activation. In the MAPK pathway, MEK activates ERK, and ERK activates MNK. Both mTOR and ERK phosphorylate the p70 S6 kinase (S6K) that phosphorylates the S6 protein of the 40S ribosomal subunit. S6 kinases are known to regulate the translation of mRNAs, like HIF-1, which contain polypyrimidine tracts within the 5'untranslated regions (UTR). Binding of 4E-BP1 to eIF-4E inhibits eIF-4E from maintaining translation of pyrimidine-rich mRNAs. Both ERK and mTOR phosphorylate 4E-BP1, and phosphorylated 4E-BP1 cannot bind eIF-4E. Also, MNK directly stimulate eIF-4E activity by phosphorylating it. The tumour suppressor and phosphatase PTEN can inhibit PI3K and PTEN loss of function enhances PI3K activity. The result of the growth factor signalling is increased transcription of pyrimidine-rich mRNAs,like HIF-1α.. (PKB=protein kinase B; eIF-4E=eukaryotic translation initiation factor 4E; 4E-BP1= eIF-4E binding protein; mTOR=mammalian target of rapamycin; ERK=extracellular signal-regulated kinase; MEK=MAP/ERK kinase; MNK=MAP kinase signal-integrating kinase)*

PRESENT INVESTIGATIONS

Aims

The specific aims, with neuroblastoma as primary model system, were to,

- Characterize the hypoxic neuroblastoma phenotype.
- Investigate the persistence of the hypoxic dedifferentiated phenotype upon reoxygenation.
- Elucidate a putative role of HIF-2α at near-physiological oxygen levels.

RESULTS AND DISCUSSION

Hypoxia and differentiation

A subset of neuroblastoma tumours forms lobular structures and, within these lobular structures, a neuronal to neuroendocrine lineage shift is seen in tumour cells adjacent to necrotic areas (201). One speculation was whether hypoxia could be the key stimulant of this transdifferentiation. Thus, we studied phenotypic changes in neuroblastoma cells in a hypoxic environment. However, neuroblastoma cells cultured at 1% O_2 reveal a downregulation of chromaffin marker genes such as *chromogranins A* and *B* and neurofilament. Furthermore, hypoxic neuroblastoma cells upregulate early neural crest marker genes such as *Notch-1*, *HES-1* and *c-kit*, whereas neuronal markers such as *NPY*, *MYCN*, *HASH-1* and *dHAND* are downregulated (114). The results did not support the initial hypothesis, but instead illustrate an interesting phenomenon of human neuroblastoma cells grown under hypoxic conditions, i.e. hypoxia induces a dedifferentiated phenotype, and the hypoxic cells seem to have adopted a more neural crest like phenotype. Xenograft tumours of neuroblastoma cells in nude mice corroborate the *in vitro* findings by exhibiting downregulation of neuronal marker genes like *dHAND* and *GAP-43* in tumour cells adjacent to necrotic areas (114, 202). Hedborg and colleagues, on the other hand, claimed that hypoxia does induce neuronal-to-chromaffin lineage shift in the specific subset of neuroblastomas (203). However, they define the chromaffin phenotype based on hypoxic induction of *IGF-2*, which is abundant in chromaffin cells, but also a gene well known to be triggered by hypoxia. In their study, the chromaffin markers chromogranin A and B did not increase at hypoxia, which is in accordance with previous findings from our group ((114) and paper I). Global gene expression studies clearly show that hypoxic cultured neuroblastoma cells dedifferentiate (paper I, paper III).

Gene expression analysis of hypoxic neuroblastoma cells (paper I)

In paper I, we further evaluate the hypoxic neuroblastoma phenotype by employing microarray analysis of SK-N-BE(2)c cells grown at either 21 or 1% O_2. Our results support the earlier discussed hypoxia-induced neural crest-like phenotype. The hallmark neuronal marker neurofilament was shown to be downregulated by hypoxia, which was further validated by immunostaining of cells from seven different neuroblastoma cell lines that were grown at hypoxia (1% O_2). Neuronal markers such as *GAP43* and *Phox2b* were also downregulated in the microarray. The hypoxia-induced neuronal and neuroendocrine changes in gene expression in

SK-N-BE(2)c cells, displayed by microarray analysis, were in accordance with those detected in previous work (114). Extra interesting was the decrease in the gene coding for the E-protein E2-2. E-proteins are mandatory dimerization partners for tissue-specific bHLH transcription factors. The hypoxia-evoked *Id2* increase (paper I, (114)), together with decreased E2-2 levels propose a mechanism for the hypoxia-induced dedifferentiation of neuroblastoma cells. At hypoxia there will be less E-proteins and more Id2 proteins that sequester E-proteins and, consequently, less E2-2 heterodimerization with tissue specific bHLH transcription factors such as HASH-1 (which also is reduced at hypoxia), presumably resulting in reduced transcription of neuronal and neuroendocrine genes. Löfstedt *et al* show that *Id2* is a HIF-1α transcriptional target, suggesting a mechanism for hypoxia-elicited *Id2* upregulation (113).

Adaptations to low oxygen (paper I)

The upregulation of the neuropeptide Chromogranin C was at a first glance contradictory to our previous conclusion about hypoxic dedifferentiation, as the related neuroendocrine markers chromogranin A and B decreased by hypoxia. However, chromogranin C/SCG2 could be grouped with some other contradictory genes, which at a closer look actually were part of a general response to changes in oxygen levels, such as neuronspecific enolase (NSE)/enolase 2 (ENO2), TH and dopadecarboxylase (DDC). SCG2 is a neuropeptide, which upon cleavage produce secretoneurin that itself is an angiogenic factor promoting proliferation and migration of smooth muscle cells, which in turn are components of blood vessels (204). NSE/ENO2 is a neuronal/neuroendocrine isoform of the glycolytic enzyme enolase that together with other genes code for glycolytic enzymes (HK2, PGK1 etc) that are upregulated as a general response to hypoxia. Both TH and DDC are enzymes involved in catecholamine synthesis, which also is part of the systemic response to hypoxia. Thus, the confusing upregulation of some neuronal genes rather illustrate the adaptive capacity of hypoxia-exposed neuroblastoma cells. Their ability to adapt were corroborated by hypoxic induction of pro-survival genes, such as *IGF-2*, *IGF-binding protein 3*, *TGF*-β, and of course *VEGF*. The VEGF coreceptor *neuropilin* was also upregulated, which was seen in five other neuroblastoma cell lines examined by Q-PCR analysis. These results might imply hypoxia-induced autocrine growth and survival loops, also seen in several other tumours ((205) and reviewed in Semenza 2003, (81)). Das *et al* describe a survival promoting autocrine loop in neuroblastoma cells, comprising VEGF and its receptor Flt-1 (VEGFR1). They show that the MAPK pathway is involved. Hypoxia-activated ERK1/2 phosphorylates HIF-1α, which in turn promotes bFGF expression, further adding to the pro-survival loop (206). Genes involved in tumour aggressiveness were also detected in our microarray as hypoxia-induced. For example, metallothioneins are associated with drug resistance (207), and several metallothioneins were hypoxia-evoked in the microarray.

Reoxygenation of hypoxic neuroblastoma cells (paper II)

We reoxygenated hypoxic neuroblastoma cells in order to determine for how long time the immature hypoxic phenotype persists. One possibility we also wished to test was a putative hypoxia-driven selection for an immature cell pool, as an explanation of an observation that hypoxic cells become immature. To mimic a more physiological oxygen level in comparison to conventionally used 21% O_2, we also cultured cells at 5% O_2, which is in the range of reported end-capillary oxygen levels (45). We considered 5% O_2 as a good estimation for a more physiological oxygen level, as neuroblastoma cells are arrested at an early differentiation stage and embryonic development takes place under low oxygen levels in utero. Ezashi *et al*

demonstrate that human embryonic stem cells (hES) show a stable pluripotent phenotype when maintained at 3-5% oxygen in contrast to the high frequency of spontaneous differentiation seen at 21% oxygen used in general cell culture work (208). Neural crest stem cells as well as central nervous system precursors show better survival, proliferation rate and potential to differentiate, if cultured at 5% oxygen (209, 210).

Based on analyses of a selection of sympathetic neuronal differentiation and neural crest markers, the hypoxic neuroblastoma phenotype at 1% O_2 could still be distinguished from that of cells grown at 5% O_2. The neuronal and neuroendocrine markers *NPY* and *chromogranin A* and *B* were consistently downregulated in 1% O_2-treated cells compared to those grown at 5% O_2. As hypoxic neuroblastoma cells appear aggressive, supported by microarray analysis (paper I), it was of interest to establish for how long time the phenotype persisted upon reoxygenation. The dedifferentiated phenotype persisted for 24 h upon reoxygenation, where after it reverted to its initial phenotype. Even when cells were exposed to long-term hypoxia of up to 12 days, the phenotype was reversible after 24 h reoxygenation. In agreement with our findings, the hypoxic neuroblastoma phenotype described by Hedborg *et al* was also reversible (203). We conclude that there was no selection for a more persistent immature neural crest-like cell type. A schematic presentation of oxygen-regulated differentiation is summarized in Figure 7.

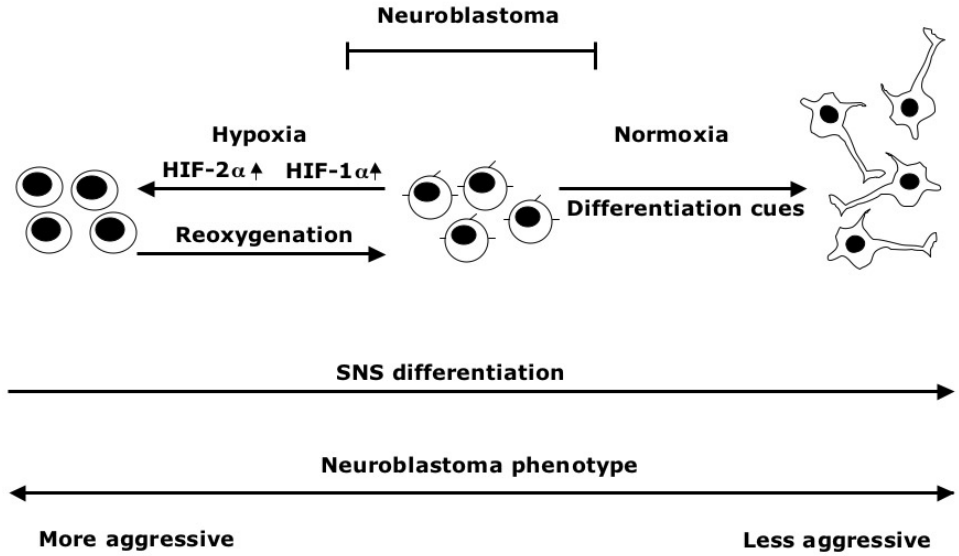

Figure 7*. Microenvironmental effects on neuroblastoma cell differentiation. The differentiation stage at which neuroblastoma cells are arrested can change in response to external factors such as hypoxia, reoxygenation and differentiation stimulating cues (pro-differentiation cues are not investigated in this thesis, they are just depicted to get the full impression). Possible early and late roles of the hypoxia-inducible factors (HIFs) involved in the differentiation mechanism are indicated. The connection between high/low differentiation stage and good/poor prognosis is indicated. The clusters of cells illustrate the dynamic phenotypical reversibility of hypoxic cells.*

Even if the immature hypoxic neuroblastoma phenotype reverses after 24 h of reoxygenation, parts of the extensive gene programs activated at hypoxia (paper I), might persist somewhat longer. Repeated exposure to hypoxia followed by reoxygenation is known to further rise the

mutation frequency seen at hypoxia (82). Considering that we focused our investigation on the differentiation status of hypoxic neuroblastoma cells, a certain selection pressure cannot be completely precluded. Weinmann *et al* show that human lung cancer cells obtain increased radiation and cytostatic resistance after 10 cycles of severe hypoxia (48 h 0.1% O_2, 120 h 21% O_2 x10), and the increased apoptosis resistance persists even after two weeks of reoxygenation (211, 212). We never measured any of these parameters, but our hypoxic cells appeared to be in good condition throughout the experiments, in addition the hypoxic condition we used was milder (1% O_2).

The fact that the hypoxic dedifferentiated neuroblastoma phenotype is reversible can be taken as an indication of highly adaptable and dynamic cells, which is an advantageous characteristic for cancer cells. Taken together, hypoxic cells might maintain their aggressive characteristics long enough to take advantage of them in the process of invasion, migration, intravasation and extravasation. Human bladder carcinoma has in an *in vitro* assay been shown to extravasate, i.e. cross an endothelial barrier, within 4 hours (213). Hypothetically, aggressive hypoxic neuroblastoma cells, which manage to migrate, intravasate and counter well oxygenated blood seem likely to within 24 h be able to adhere to and extravasate the endothelium at a secondary site.

HIF-proteins and differentiation (papers I, II and III)

Investigation of the hypoxia-inducible factors showed that prolonged hypoxia decreased initially induced HIF-1α protein levels, while HIF-2α levels kept increasing. HIF-2α, as opposed to HIF-1α, was induced at 5% O_2 and basal levels of HIF-2α was previously detected in a series of neuroblastoma cell lines (paper II and III and Nilsson 2005 (115)). In paper III, we analyzed the HIF-levels more thoroughly, which will be discussed further.

HIF-2α appears to be involved in SNS development and we have previously identified HIF-2α expression in mouse, and HIF-2α protein in human foetal paraganglia, as well as in neuroblastoma tumour specimen (52, 114, 115), which could suggest a role for HIF-2α in neuroblastoma. A possible scenario, based on HIF results from paper II and III, would be that HIF-1α initiates the acute hypoxic response where after HIF-2α maintains the hypoxic phenotype. HIF-1α would initiate the dedifferentiation phenotype by *Id2* induction and then HIF-2α would take care of persistence (113). Nilsson *et al* show that HIF-2α is reduced in neuroblastoma cells, stimulated to differentiate, which could support a role for HIF-2α in maintaining an immature phenotype in these cells (115), although HIF-2α expression might just be a differentiation-regulated gene.

The upregulation of Notch-1 seen in hypoxic neuroblastoma cells (paper I and (114)) fosters speculations concerning a role for Notch in maintaining an undifferentiated cell state. Gustafsson *et* al relate to that question in their work on the role of Notch and hypoxia during myogenic and neuronal differentiation. To maintain an undifferentiated cell state of these two cell types, hypoxia requires Notch signalling. They demonstrate a crosstalk between Notch ICD and HIF-1α and they propose that this interaction transactivates Notch target genes such as *Hes1* and *Hey-2*, thereby maintaining a stem cell like phenotype (214). Earlier, it has been shown that the tumour suppressor pVHL has a neuron-differentiating potential of human neuroblastoma cells (215). In addition, Tanaka *et al* describe how neuronal markers of neuronal progenitor cells are inhibited by anoxia and that further differentiation only takes place if pVHL is present under normoxic oxygen levels in association with the degradation of HIF-1α (216). These observations

further support a model in which hypoxia and HIF-1α-activation induce neuroblastoma cell dedifferentiation.

Taken together, these observations are intriguing and promote the concept of hypoxia and its effectors as maintainers of stem cell and neural crest-like phenotypes. Similar to our studies on neuroblastoma (paper I, II and (114)), our group has also reported that hypoxic breast cancer cells develop a less mature phenotype in comparison to well-oxygenated cells, a finding corroborated in breast cancer specimen (217). Hypoxic prostate cancer cells also show down-regulation of differentiation-specific genes (218). Contrary to these findings hypoxia, hypoxia-mimetics and HIF-1α are believed to induce leukeamic cell differentiation and not dedifferentiation (219, 220). In addition, HIF-2α has been suggested to play a part in driving adopogenesis by regulating glucose uptake and subsequent lipid synthesis (221). As with Notch-effects, the hypoxia-differentiation connection appears cell type specific as the phenomenon described in neuroblastoma is not applicable to all cancer types. However, increasing evidence suggests a role for hypoxia to maintain stemness over differentiation, illustrated by e.g. hypoxia-induced block of adipocyte-, osteogenic- and chondrogenic differentiation (222-224).

HIF-1α and HIF-2α regulation in neuroblastoma cells (papers II and III)

As discussed earlier, investigation of hypoxia-inducible factor levels in neuroblastoma cells showed differences between HIF-1α and HIF-2α at prolonged hypoxia (1% O_2) and at 5% O_2. HIF-2α protein is induced at both oxygen levels and increases with time. HIF-1α, on the other hand, is rapidly induced at 1% O_2 but culminates after 8-24 hours of hypoxia and then decreases (paper II and III). Interestingly, HIF-1α, as opposed to HIF-2α, is not stabilized at 5% O_2 (paper II and III). Time dependent regulation of HIFs has been shown earlier. One study shows how nuclear HIF-1α decreases despite ongoing hypoxia in hepatoma cells (124). Another report demonstrates the same pattern for HIF-1α at prolonged hypoxia in Hela cells, irrespective of localization, but HIF-2α proteins behaved like HIF-1α and did not remain high during prolonged hypoxic exposure (187). Further, intriguing results, concerning HIF protein levels, have been shown in PC12 cells, which respond to prolonged hypoxia in a similar pattern as neuroblastoma cells do (137). PC12 cells are neuroendocrine pheochromocytoma cells from rat and they share characteristics with adrenal chromaffin cells. Thus, both neuroblastoma and PC12 cells are of neuronal and neuroendocrine SNS origin, respectively, and show similar HIF protein levels in response to hypoxia. A study investigating lung epithelial cells (HIF-2α is implicated in lung maturation (98, 116)) reveals the same HIF pattern at prolonged hypoxia as we show in neuroblastoma cells (157). The mechanisms behind these differences in HIF-1α and HIF-2α protein levels are not fully elucidated, but HIF-2α mRNA is upregulated in lung epithelial cells exposed to 0.5% O_2, similar to our findings in neuroblastoma cells cultured at 1 and 5% O_2 for up to 72h (paper I and III). In addition, lung epithelial HIF-1α mRNA decreased over time (0-16h) when cells were exposed to hypoxia. In paper I, we see a very small decrease in HIF-1α mRNA in five out of seven neuroblastoma cell lines after 72 h exposure to 1% O_2, including SK-N-BE(2) cells. However, in paper III, HIF-1α mRNA levels appear more or less constant over time in investigated neuroblastoma cell lines. In the report on lung epithelial cells, a negative feedback loop is suggested, where hypoxia via HIFs regulates natural antisense HIF (aHIF), mRNA which destabilizes HIF-1α mRNA. We have not looked into the effect of aHIF in neuroblastoma since this mechanism is less likely in view of the minute decrease in HIF-1α mRNA levels seen at hypoxia.

PHD-1, -2 and-3 hydroxylate and prime HIFs for protesomal degradation and in neuroblastoma cells cultured at both 1 and 5% O_2, we monitored the levels of these three prolyl hydroxylases. We speculate that hypoxic induction of PHD2 and PHD3 might target HIF-1α, but not HIF-2α, for proteosomal degradation, suggesting HIF-1α-specific hydroxylation at prolonged hypoxia and at 5% O_2 (paper III). Increased *PHD2* and *PHD3* levels were seen over time at both 1 and 5% O_2, although more prominently at the lower oxygen level. Since the prolyl hydroxylases are not equilibrium enzymes we can speculate that increased levels of the PHDs could be associated with augmented PHD-enzymatic activity and, if they are HIF-1α specific under these conditions, they could partly explain the HIF-1α protein patterns. PHD2 is proposed to be the main PHD involved in maintaining low normoxic HIF-1α levels (171) and, in a follow up study, it would be interesting to knock down *PHD2* and *PHD3* in neuroblastoma cells to evaluate effects on HIF protein levels during different oxygenation conditions. However, high HIF-2α protein levels might saturate the effects of PHDs, which rises the question what then actually regulates HIF-2α protein levels.

Increased HIF-2α mRNA levels at both 1 and 5% O_2 prompt for HIF-2α promoter analyses, which might yield sites for presumptive transcription factors important to HIF-2α transcription. There are several Sp1-sites in the HIF-1α gene upstream of the transcription initiation site (179) and Sp1 sites might be abundant in the HIF-2α gene as well. Except HIFs and Sp1/Sp3, several other transcription factors are hypoxia-responsive, such as AP-1, Ets-1, NF-κB and p53 (225). Another option is increased HIF-2α translation. Both the PI3K- and MAPK pathways are implicated in increased HIF-1α protein translation (173, 174) and similar mechanisms might affect HIF-2α protein levels.

HIF-1α- and HIF-2α- target genes in neuroblastoma (paper III)

The microarray in paper III is an analysis of SK-N-BE(2)c cells exposed to 21, 5 or 1% O_2 for 0-72 h. Seven time points were monitored. Microarray and Q-PCR analyses of *TH* showed a rapid induction at 1% O_2 and a slow increase at 5% O_2, a pattern similar to the HIF-1α and HIF-2α protein patterns seen in cultured neuroblastoma cells. Using the *TH* expression pattern as a template, approximately 75 genes with similar expression patterns were extracted from the microarray. Several of the listed genes are known hypoxia-driven or HIF target genes. Using siRNA-, Q-PCR- and ChIP- analyses we could confirm that HIF-2α specifically binds to the promoters of *VEGF* and *DEC1/BHLHB2*, and that HIF-2α is the main HIF-transcription factor at prolonged hypoxia and at 5% O_2, driving the expression of these and several other genes. Among the 75 genes, we found some that have not previously been shown to be hypoxia-driven. One of them was *SERPINB9*, which is involved in metastatic melanoma (226) and can be classified as a gene involved in tumour progression. By HIF-2α knock down, *SERPINB9* was confirmed as a HIF-2α regulated gene at 5% oxygen. Whether HIF-2α has a direct or indirect impact on *SERPINB9* transcription needs to be further investigated.

The fact that HIF-2α, in neuroblastoma cells, regulates some genes at 5% O_2 and at prolonged hypoxia (1% O_2), but not at acute hypoxia, suggests involvement of co-factors uniquely or more extensively acting at 5% O_2 as opposed to at 1% O_2. As mentioned before, several other transcription factors apart from the HIFs are induced at low oxygen pressure such as Sp1, Ets-1 and NFκB (225). Several of these are known to interact with HIF-1α in target gene activation, and most likely they are able to cooperate with HIF-2α as well. NEMO, the NFκB essential modulator, interacts with HIF-2α, but not HIF-1α, resulting in enhanced normoxic HIF-2α

transcriptional activity (194). Possibly, NEMO involvement is augmented in neuroblastoma cells at 5% O_2 or at prolonged hypoxia. The discrepancies seen between HIF-1α and HIF-2α are at times indeed due to cooperating factors. The genes *membrane type-1 matrix metalloproteinase* (*MT1-MMP*) and *plasminogen activator inhibitor-1* (*PAI-1*) are suggested to be HIF-2α specific, but interestingly, the specificity is dependent on Sp1 cooperation (197, 198). Further, several HIF-2α-inducible genes have putative binding sites for ETS transcription factors in the proximity of HREs. Knock down of the ETS transcription factor *ELK-1* in breast cancer cells results in a clear reduction in HIF-2α-dependent gene activation (192). Furthermore, a study on a variety of cell lines disclosed that *EPO* is a HIF-2α target gene in hepatoma as well as in neuroblastoma cells. Interestingly, they find that while a limited region of the *EPO* promoter is sufficient to confirm HIF-1α-specific and hypoxia-dependent *EPO* transcription, a much larger part of the EPO enhancer is necessary for HIF-2α EPO induction, implying the need for, of as yet unidentified, co-transcription factors (185).

In summary, putative HIF-2α target genes extracted from our microarray analysis in paper III, should be followed up in search for HRE sites and physical HIF-2α binding sites, but also for sites with which Sp1 and ETS interact. Based on the compilation by Wenger *et al* on differences between HREs and the different HIFs, it is most likely that the core HRE is not discriminating between HIF-1α and HIF-2α. This selection is rather dependent on cell- and tissue-specific HIF-α protein expression and cooperation with other transcription factors (227). Thus, it would be of interest to further study the presumptive HIF-2α target *SERPINB9* (paper III) to elucidate if it is a direct HIF-2α target and, if this is the case, what co-factors are prerequisites for gene induction at 5% O_2.

Oncogenic potential of HIF-2α in neuroblastoma (paper III)

Our data indicate that HIF-2α most likely regulates several genes involved in malignant neuroblastoma progression. The knock down of *HIF-2α* in neuroblastoma cells grown at 5% O_2, an oxygen level mimicking that in tumour specimen, resulting in decreased *VEGF* expression, is one example supporting an active role of HIF-2α in neuroblastoma growth. When we immunostained neuroblastoma specimen for HIF-2α, we surprisingly detected HIF-2α protein in tumour cells residing close to blood vessels, and we saw co-staining of VEGF in consecutive sections. Investigation of a larger set of neuroblastoma tissue organized into a tissue microarray (TMA) revealed correlation between HIF-2α- and VEGF-staining, suggesting that HIF-2α drives *VEGF* expression also in this *in vivo* context.

In renal cell carcinoma, TGF-α is implicated in tumour progression. The ligand of epidermal growth factor (EGFR), TGF-α, is regulated by HIF-2α in some renal cell carcinomas, tumours known to be highly vascularized, and in some additional tumour types as well (183, 228). HIF-2α has been shown to promote the progression of VHL deficient renal cancer by driving *TGF-α* expression and silencing of the EGFR suppresses HIF-2α-driven tumour progression (229). Covello *et al* engineered homozygous HIF-2α knock-in/knock-in mouse embryonic stem cells, i.e. cells where the HIF-1α locus is replaced by a cDNA encoding *HIF-2α*. Teratomas, as well as embryos, derived from these cells show increased *TGF-α* and *VEGF* expression (230, 231). In addition *HIF-2α* knock-in ES cells are more prone to form teratomas than wt cells, indicating an important role for HIF-2α in tumour formation. A similar phenomenon appeared when SK-N-BE(2)C cells were transfected with either siRNA against *HIF-1α*, *HIF-2α* or a scramble sequence. Knock down of *HIF-2α* slowed down xenograft tumour growth, but not tumour

formation, in nude mice. Decreased *VEGF* expression and initial tumour vascularization are likely explanations to this result, but lack of other gene expression such as *SERPINB9* or *TGF*-α might also be involved, in accordance with the increased *TGF*-α and *VEGF* expression reported in HIF-2α knock-in teratoma cells (230, 231).

A recent report shows that inhibition of AKT diminishes tumour angiogenesis (232). Maybe HIF-2α is the missing link in such a scenario. Either HIF-2α protein levels are increased via a TGF-α-EGFR-AKT positive protein translation-loop, with the net effect of induced *VEGF* expression, and/or HIF-2α in neuroblastoma cells also causes abundant TGF-α production resulting in increased tumour growth. Hence, it would be of interest to monitor TGF-α expression in neuroblastoma cells grown at low oxygen.

In the clinical neuroblastoma material investigated, we found correlation between HIF-2α and poor prognosis of afflicted children. High HIF-2α protein levels in high stage neuroblastomas are also associated with poor outcome. Thus, HIF-2α protein levels might provide direct prognostic information beyond that of clinical staging. HIF-2α is further implicated in neuroblastoma aggressiveness by driving the expression of SERPINB9, a gene involved in tumour migration and invasion. These are compelling results, which all provide support for the involvement of HIF-2α in determining aggressive neuroblastoma behaviour. However, both HIF-1α and HIF-2α have been claimed to function as tumour suppressor proteins in glioblastoma and teratoma models (233). In agreement with our findings, their report shows that HIF-2α over expression results in increased VEGF expression and vascularization, but in disagreement with our neuroblastoma model, they report no positive correlation to xenograft tumour growth. These discrepancies might be due to tissue specific differences in response to HIF-protein levels, although there are conflicting glioma data as described in paper III. Taken together, we show that HIF-2α is important for neuroblastoma tumour progression and we propose its use as a clinical prognostic factor in determination of neuroblastoma outcome.

CONCLUSIONS

Paper I

-Hypoxia induces dedifferentiation of human neuroblastoma cells.

-Genes involved in cell survival are induced in hypoxic neuroblastoma cells, protecting them from the harsh environment of low oxygen pressure.

Paper II

-The hypoxia-induced dedifferentiated neuroblastoma phenotype persists for at least 24 h upon reoxygenation.

-HIF-1α- and HIF-2α- protein levels differ at prolonged hypoxia and at 5% O_2.

Paper III

-HIF-1α and HIF-2α are differently regulated in hypoxic and "physioxic" neuroblastoma cells. HIF-2α protein is expressed at 5% O_2 and detected adjacent to blood vessels in neuroblastoma specimen.

-HIF-2α affects gene expression at prolonged hypoxia and at 5% O_2, whereas HIF-1α primarily drives gene expression at acute hypoxia.

-HIF-2α correlates to poor outcome of neuroblastoma patients.

-High HIF-2α-protein levels correlate to worse prognosis within the subgroup of high stage neuroblastomas, and is seemingly an independent prognostic marker.

-HIF-2α positively affects xenografted neuroblastoma tumour growth.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Introduktion

Cancer uppstår när en cell i kroppen börjar dela sig okontrollerat, varmed det sker en onaturlig ansamling av celler – tumörbildning. Solida tumörer t ex bröst- och prostatacancer är till skillnad från ickesolida tumörer som blodcancer/leukemi ofta dåligt försedda med syre på grund av långa avstånd mellan tumörceller och blodkärl. För att säkra syre- och näringsförsörjning kan tumörceller sätta igång nybildandet av blodkärl in i tumören. Tumörblodkärlen är dock omogna och deformerade i sin natur, vilket resulterar i läckande och inte fullt ut funktionella blodkärl. Dessa egenskaper stärker den dåliga tillgången på syre, varmed hypoxi (syrebrist) uppstår. Hypoxi innebär ett syretryck på runt 1%, vilket är betydligt lägre än normalt syretryck i våra organ (fysiologiska syretryck på ca 5-6% syre).

I hypoxiska tumöromården aktiveras uttrycket av gener vars produkter, proteiner, hjälper cellen att anpassa sig till den onormalt låga syrenivån genom att trygga energitillgång och överlevnad. VEGF är en av de mest välundersökta generna som sätts igång vid hypoxi. VEGF driver nybildandet av blodkärl. Vid extra svår hypoxi (<1% O_2) uttrycks istället proteiner som driver programmerad celldöd, apoptos. Aktivering av genuttryck, transkription, är beroende av transkriptionsfaktorer, vilka binder till DNA. Transkriptionsfaktorerna HIF-1α och HIF-2α ansamlas i stora mängder vid hypoxi och de kan båda driva VEGF-uttryck. Vid högre syretryck bryts HIF-1α och HIF-2α ner.

Neuroblastom är en solid barntumör (barncancer) som drabbar barn i åldern 0-15 år, med flest fall i åldern 0-3 år. I Sverige får varje år runt 15 barn diagnosen neuroblastom. Trots intensiva kirurgiska ingrepp och aggressiva cellgiftsbehandlingar överlever endast ca 50% av de barn som har de mest elakartade fallen av neuroblastom. Neuroblastom uppstår på de ställen i kroppen som det sympatiska nervsystemet utmynnar, t ex i magtrakten och bäckenområdet. Mest vanligt är att den finns i binjurarna, körtlarna ovanför njurarna. Tumörcellerna kommer från just det sympatiska nervsystemet och består av nervceller som inte är färdigutvecklade utan har stannat upp i ett omoget stadie. Ju mindre mogna tumörcellerna är, desto aggressivare är de och desto sämre prognos har det drabbade barnet. I en tidigare studie har vår forskargrupp konstaterat att neuroblastomceller som utsätts för hypoxi blir än mer omogna än motsvarande celler som växer i en syrerik miljö. Detta tyder på en förhöjning av tumörcellernas aggressivitet.

Avhandlingens delarbeten

Arbete I är en fördjupad undersökning i uttrycket av ett stort antal gener i hypoxiska neuroblastomceller. Resultaten stödjer vår tidigare slutsats att hypoxi driver neuroblastomceller mot mer omogna karaktäristika. Dessutom såg vi att gener som bidrar till drogresistens uttrycks i större omfattning än vid högre syretryck. Sammantaget visar arbete I att även neuroblastomceller anpassar sig vid hypoxi genom att uttrycka gener som främjar överlevnad av tumörceller.

Framskridandet av cancer kan ske genom att den första tumören som uppstår, primärtumören, bildar öar av tumörceller, metastaser, placerade i andra vävnader i kroppen än där primärtumören uppstod. För att metastasering ska kunna ske krävs att tumörceller bryter sig loss från ursprungsvävnaden, och ut i blodomloppet. De följer blodflödet tills de stöter på en ny plast där de kan fortsätta sitt okontrollerade delande. Hypoxiska tumörceller som lyckas ta sig ut i ett blodkärl utsätts där för en omfattande ökning i syretryck. I arbete II studerade vi reoxygenering av hypoxiska tumörceller. För att se hur länge de hypoxiska neuroblastomcellerna bibehåller sina

omogna karaktäristika vid höga syrenivåer har vi utsatt tumörceller för hypoxi (1% syre) och sen åter (re-) utsatt dem för högre syre (oxygen)-nivåer. Oavsett om vi utsatte cellerna för hypoxi i några dagar eller upp till två veckor eller i cykler av hypoxi och högt syretryck (normoxi) så återvände cellerna till sitt ursprungsläge efter ett dygns reoxygenering. Alltså var de hypoxiska neuroblastomcellernas karaktäristika reversibla. De bibehöll inte sina omgogna egenskaper när de utsatts för höga syrenivåer i mer än ett dygn. Tjugofyra timmar kan verka som en kort tid men det kan tänkas vara länge nog för en hypoxisk tumörcell att metastasera.

I arbete III studerar vi mer ingående HIF-1α och HIF-2α och hur de bidrar till att neuroblastomceller anpassar sig till syrebrist. Tidigare trodde forskningsgrupper att proteinerna HIF-1α och HIF-2α hade samma funktion eftersom de är mycket lika varandra, men mer och mer forskningsresultat stödjer hypotesen att de har olika uppgifter. Våra resultat indikerar att HIF-1α utför sitt arbete vid akut syrebrist vilket kan uppstå under korta perioder i en tumör när t ex ett blodkärl tillfälligt blir tilltäppt. I motsats till HIF-1α tar HIF-2α tar över och arbetar vid långvarig syrebrist vilket tumörer utsätts för när tumörceller under en längre tid har dålig tillgång till syre. Vid närmare studier av tumörmaterial från neuroblastom fann vi dessutom HIF-2α protein i tumöromården nära blodkärl, dvs. i områden som förväntas vara välförsedda med syre. Vanligtvis hittar man bara HIF-1α och HIF-2α i dåligt syresatta (hypoxiska) tumöromården, därför var det ett oväntat resultat att se HIF-2α i väl syresatta tumöromården. Genom att i laboratoriet odla neuroblastomceller vid 5% syre, ett syretryck som motsvarar det fysiologiska syretryck som finns i kroppens friska organ, konstaterade vi att HIF-2α, men inte HIF-1α ansamlas vid bättre tillgång till syre. Genom att studera ett större patientmaterial fann vi att höga HIF-2α-nivåer är kopplat till en sämre prognos för patienterna. Dessa resultat kan komma att bli viktiga vid framtida bedömningar av neuroblastompatienters överlevnadschanser.

De tre studierna som ingår i avhandlingen utgör pusselbitar i det ständigt pågående arbetet med att kartlägga varför neuroblastom är en så aggressiv cancerform.

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