Hypoxic Adaptation and Arsenic Trioxide Treatment in Small Cell Lung Carcinoma

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Hypoxic Adaptation and Arsenic Trioxide Treatment in Small Cell Lung Carcinoma

Matilda Thorén

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Abstract

Small cell lung carcinoma (SCLC) is a very aggressive solid tumor and is often widely metastasized by the time of diagnosis. Despite good response to the initial chemotherapy, SCLC cells often develop multidrug resistance to conventionally used chemotherapeutic drugs, which cause almost all SCLC tumors to relapse. The 5-year survival rate for patients with limited disease is around 20% while it is only a few percent for patients with a disseminating disease.

Arsenic trioxide (As_2O_3) is one of the oldest medicines used for treatment of different diseases and it is today used as first-line treatment for patients with relapsed or refractory acute promyelocytic leukemia. Here, we demonstrate that As_2O_3 is cytotoxic to SCLC cells and xenotransplanted SCLC tumors at clinically relevant concentrations and the effect is also sustained at hypoxic conditions.

Areas of low oxygen tensions, hypoxia, are a common characteristic in solid tumors and are associated with aggressive tumor behavior, treatment resistance and poor outcome in several tumor forms. In response to hypoxia, tumor cells induce a transcriptional shift which is mainly regulated by the transcription factors hypoxia-inducible factor (HIF)-1 and HIF-2. HIF proteins consist of two subunits, an oxygen-regulated α-subunit and a constitutively expressed β-subunit. Previous reports have shown that the transcription factors are differentially regulated over time; HIF-1 primarily mediates the acute hypoxic response, whereas HIF-2 dominates during more chronic phases of hypoxia. We found that SCLC tumor specimens and cells lack expression of HIF-2α protein while HIF-1α is expressed at both acute and prolonged hypoxia. In addition, SCLC cells have a high adaptive capacity to hypoxia including a high proliferation rate and low cell death, even though we demonstrated a modest induction of well-known hypoxia-driven genes. We further show that knockdown of *HIF1A* using siRNA or shRNA, is not significantly affecting the cell viability of cultured SCLC cells at moderate and severe hypoxia or tumor take and tumor growth in SCLC xenografts.

We found that SCLC cells are dependent on glutamine metabolism for cell viability and proliferation, in a HIF-independent fashion. The SCLC cells used here are *MYC* and *MYCL* amplified and *MYC* overexpression is known to stimulate glutaminolysis and lipogenesis. In *HIF1A* repressed cells that overexpress *MYC,* genes involved in these pathways are further up-regulated at hypoxic conditions. Taken together, our data indicate that the adaptive capacity to hypoxia is partially HIF-independent in *MYC* amplified SCLC cells.

Key words	small cell lung carcinoma, arsenic trioxide, hypoxia, hypoxia-inducible factors, survival, proliferation, glycolysis, glutamine metabolism, MYC

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Table of contents

List of papers

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

I Arsenic trioxide is highly cytotoxic to small cell lung carcinoma cells

Helen M. Pettersson, Alexander Pietras, **Matilda Munksgaard Persson**, Jenny Karlsson, Leif Johansson, Maria C. Shoshan and Sven Påhlman

Mol Cancer Ther 2009, 8(1):160-170

II HIF-2α expression is suppressed in SCLC cells, which survive in moderate and severe hypoxia when HIF-1α is repressed

Matilda Munksgaard Persson, Martin E. Johansson, Nastaran Monsef, Maria Planck, Siv Beckman, Michael J. Seckl, Lars Rönnstrand, Sven Påhlman and Helen M. Pettersson

Am J Pathol 2012, 180(2):494-504

III Increased glutaminolysis and lipogenesis in HIF-repressed SCLC cells support cell viability and proliferation at hypoxia

Matilda Munksgaard Thorén, Marica Vaapil, Sofie Mohlin, Helen M. Pettersson, Martin E. Johansson and Sven Påhlman

Manuscript

Paper not included in the thesis

MYC inhibition induces metabolic changes leading to accumulation of lipid droplets in tumor cells

Hanna Zirath, Anna Frenzel, Ganna Oliynyk, Lova Segerström, Ulrika K. Westermark, Karin Larsson, **Matilda Munksgaard Persson**, Kjell Hultenby, Janne Lehtiö, Christer Einvik, Sven Påhlman, Per Kogner, Per-Johan Jakobsson and Marie Arsenian Henriksson

Proc Natl Acad Sci U S A 2013, 110(25):10258-10263

Abbreviations

AC	Atypical carcinoid
ACACA	Acetyl-CoA carboxylase alpha
ACLY	ATP citrate lyase
ALDOA	Aldolase A
APL	Acute promyelocytic leukemia
ARNT	Aryl hydrocarbon receptor nuclear translocator
As_2O_3	Arsenic trioxide
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
ATRA	All-trans retinoic acid
bHLH	basic helix-loop-helix
BiP/GRP78	Heat shock 70kDa protein 5
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3
ccRCC	Clear cell renal cell carcinoma
CITED2	CBP/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2
EGFR	Epidermal growth factor receptor
ENO1	Enolase 1
EPO	Erythropoietin
ER	Endoplasmic reticulum
FASN	Fatty acid synthase
FIH-1	Factor inhibiting HIF-1
GLS	Glutaminase
GLUT1	Solute carrier family 2, member 1
GLUT3	Solute carrier family 2, member 3
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HK2	Hexokinase 2
HRE	Hypoxia-response element
IDH	Isocitrate dehydrogenase
IRE1	Endoplasmic reticulum to nucleus signaling 1
JNK	c-Jun N-terminal kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LCNEC	Large cell neuroendocrine carcinoma
LDHA	Lactate dehydrogenase A
LOX	Lysyl oxidase
MAPK	Mitogen-activated protein kinase
MAX	Myc associated Factor X
MCT	Monocarboxylate transporter
MGA	Max dimerization partner
MIZ-1	Myc-interacting zink finger protein 1

MNT	MNT, Max dimerization protein
MXD1	Max dimerization protein 1
MXI1	MAX interactor-1, dimerization protein
MYC	v-myc myelocytomatosis viral oncogene homology (avian)
MYCL	v-myc myelocytomatosis viral oncogene lung carcinoma derived homolog (avian)
MYCN	v-myc myelocytomatosis viral oncogene neuroblastoma derived homolog (avian)
NADPH	Nicotinamide adenine dinucleotide phosphate
NEB	Neuroepithelial bodies
NF-κB	Nuclear factor kappa B
NOXA	Latin for damage
NSCLC	Non-small cell lung carcinoma
OCT4	POU class 5 homeobox 1
ODD	Oxygen-dependent degradation domain
OXPHOS	Oxidative phosphorylation
PARP	Poly ADP-ribose polymerase
PAS	PER/ARNT/SIM
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PERK	Eukaryotic translation initiation factor 2-alpha kinase 3
PHD	Prolyl hydroxylase domain protein
PI3K	Phosphatidyloinositide 3-kinase
PKM2	Pyruvate kinase, muscle
PML	Promyelocytic leukemia protein
PNEC	Pulmonary neuroendocrine cell
PUMA	p53 up-regulated modulator of apoptosis
RARα	Retinoic acid receptor-α
RB1	Retinoblastoma 1
ROS	Reactive oxygen species
SCLC	Small cell lung carcinoma
SLC1A5	Solute carrier family 1, member 5
TAD	Transactivation domain
TC	Typical carcinoid
TCA	Tricarboxylic acid
TP53	Tumor protein p53
TRB3	Tribbles homolog 3
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
VHL	von Hippel Lindau
XBP1	X-box binding protein 1

Introduction

Today cancer is one of the major causes of deaths worldwide and the incidence is increasing yearly. More than 100 different types of cancer exist as well as various subtypes of tumor forms are found within specific organs (Hanahan and Weinberg, 2000). A common feature of all tumor forms is the malignant transformation of normal human cells and Hanahan and Weinberg have described ten hallmarks that drive this multistep process (Hanahan and Weinberg, 2000) (Hanahan and Weinberg, 2011). The certain traits that lead to the malignant growth stimulates several cellular processes, for instance uncontrolled growth, evasion of apoptosis, metastatic growth, sustained angiogenesis, shift in energy metabolism and evading immune destruction. How tumor cells acquire their traits and the underlying mechanisms can differ between various tumor forms.

As a consequence of tumor cells rapid and uncontrolled growth, the oxygen tension is often reduced in solid tumors. Insufficient oxygen supply, hypoxia, is associated with more aggressive phenotype, poor outcome and treatment resistance in several tumor forms. Cells are responding to the hypoxic environment by altering the transcriptional program to maintain the intracellular homeostasis, which is mainly regulated by the transcription factors HIF-1 and HIF-2. In this thesis the overall aim was to characterize the adaptive response of human SCLC cells to hypoxic conditions and to investigate the importance of HIF-1 and HIF-2 activity for cell viability and growth.

Small cell lung carcinoma

Lung cancer

Lung cancer is the leading cause of cancer deaths worldwide and more than 1.3 million people die yearly of the disease. The incidence is highest in more developed countries of Northern America, Europe and Eastern Asia (GLOBOCAN 2008: Cancer Incidence and Mortality Worldwide, *http://globocan.iarc.fr*, last accessed August 9, 2013). A strong risk factor for developing lung cancer is cigarette smoking (Brownson et al., 1992). Based on clinical and histological criteria, lung cancer is classified into non-small cell lung carcinoma (NSCLC) and small cell lung carcinoma (SCLC). The majority of the tumors are classified as NSCLC, which is further subdivided into three major histological subtypes; adenocarcinoma, squamous cell and large cell carcinomas. NSCLC is a heterogeneous group of tumors, though a common feature is the tumor cells relative insensitivity to chemotherapy and therefore surgery is primarily used. Adenocarcinoma is the most common type of lung cancer and this subgroup may also be found in non-smokers. Approximately 15-18% of the lung tumors are diagnosed as small cell lung carcinoma (Hoffman et al., 2000) (Travis, 2011).

Small cell lung carcinoma

Overview

SCLC is the most aggressive lung tumor form and characterizes by the tumor cells high proliferation rate. The majority of the primary SCLC tumors are located centrally in the lung. By the time of diagnosis SCLC is often widely metastasized and the most common sites for distant metastasis are liver, bone and brain. Distant metastasis is found in approximately 60% of the patients (Nakazawa et al., 2012) (Jackman and Johnson, 2005). SCLC is classified in a 2-stage system as limited or extensive stage according to the Veterans Administration Lung Study Group (VALSG). Limited stage is defined by restricted disease into one hemithorax and to regional lymph nodes that can be included in a single radiation field. Extensive disease is characterized by wide tumor spread throughout the body. The International Association for the Study of Lung Cancer

(IASLC) has discussed to recommend the tumor, node, metastasis (TNM) staging system for SCLC patients. This staging system is used for NSCLC and will hopefully, if adopted give better prognostic information and treatment for SCLC patients (Kalemkerian and Gadgeel, 2013) (Zarogoulidis et al., 2013). The 5-year survival rate for patients with limited disease is around 20%, but around two-thirds of the patients present a disseminating disease and the 5-year survival rate for these patients is only a few percent (Sørensen et al., 2010). The treatment has remained largely the same for the last 25 years and consequently, the prognosis for SCLC patients has not been improved during this period.

Development of SCLC is strongly associated with tobacco smoking and the majority of the patients are current or former heavy smokers. The risk increases with duration and the intensity of smoking. Since smoking habits has decreased during the past 50 years and changed to more similar smoking habits between men and women in developed countries, the incidence today has declined and is more equal between the genders (Govindan et al., 2006) (Brownson et al., 1992). In China, the increase of cigarette use occurred much later than in Northern America and Europe, and consequently the smoking-related lung cancer deaths will first be seen during the next decades (Liu et al., 1998).

Common genetic alterations

Genetic alterations are not well studied in SCLC patient material since the patients are mostly treated with chemotherapy and not surgery, leading to shortages in tumor specimens for scientific studies. The most common chromosomal aberrations in SCLC involve tumor suppressor genes, which are genetically affected in two events, loss of DNA regions and/or mutations. Several chromosomal aberrations have been described in SCLC, such as deletion or mutation in the *TP53* gene, which appears in around 90% of the tumors. *TP53* is the most common mutated gene in human cancers. In 70-90% of the SCLC tumors, the retinoblastoma (*RB1)* gene is also lost or mutated. Both TP53 and RB1 proteins are important for cell cycle regulation and the aberrant expression leads to uncontrolled cell growth (Toyooka et al., 2003) (Girard et al., 2000) (Wistuba et al., 2001) (Meuwissen et al., 2003). Interestingly, mRNA levels of the cell cycle inhibitor *p130* have been shown to be down-regulated in SCLC cells by microRNA from the miR-17-92 cluster, which often is overexpressed in SCLC tumors. Loss of p130 in *RB1*/*TP53*-mutant mice demonstrates increased tumor progression with similar features as human SCLC (Schaffer et al., 2010).

Other common chromosomal aberrations in SCLC are loss of chromosome 3p, which is found in more than 90% of the tumors. The fragile histidine triad gene (*FHIT*) located at 3p14.2 is deleted in the majority of the SCLC tumors. Several other tumor suppressor genes are located at this chromosome, for example RAS effector homolog (*RASSF1*) and retinoic acid receptor-β (*RAR*β). However, all the tumor suppressor genes affected on

chromosome 3p are not completely characterized. Alterations in the *TP53* gene and chromosomal deletion of 3p are often a consequence of, or associated with the patient's tobacco smoking (Naylor et al., 1987) (Wistuba et al., 2000) (Rong et al., 2004) (Lin et al., 2000).

Also of great interest is the frequently occurring overexpression of the *MYC* family oncogenes (*MYC, MYCN, MYCL*). One of the *MYC* family genes is amplified in approximately 20-30% of the tumors (see *The Myc pathway*) (Takahashi et al., 1989) (Wistuba et al., 2001).

Bronchopulmonary neuroendocrine tumor

SCLC is defined by small cells with very high mitotic rate, scant cytoplasm, vague cell borders, frequently absence of nucleoli and large areas of necrosis in the tumors. SCLC is subdivided into pure and combined SCLC. Combined tumors consist of a mixture of cells with SCLC and NSCLC histology. The majority of the tumors are classified as pure SCLC, and only a few percentages of the tumors are classified as combined (Travis, 2012) (Babakoohi et al., 2013).

SCLC is also characterized as a neuroendocrine tumor, expressing neuroendocrine markers such as neural cell adhesion molecule, synaptophysin and chromogranin A. According to the World Health Organization classification system in 2004, the bronchopulmonary neuroendocrine tumors include four different subgroups; typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma (LCNEC) and SCLC. This group of tumors is very heterogeneous, including the well differentiated carcinoids with good prognosis to the poorly differentiated LCNEC and SCLC (Travis, 2012) (Swarts et al., 2012).

SCLC cell-of-origin

The cellular origin of SCLC has not yet been identified and it is not clarified whether SCLC and NSCLC originate from a common cell of origin. The combined histology seen in some SCLC supports the model that a common pulmonary progenitor cell can give rise to the different lung cancer forms (Yesner, 2001). However, since SCLC expresses neuroendocrine markers it has been suggested that the rare pulmonary neuroendocrine cells (PNECs, see below) are the progenitor cells. A distinct origin of SCLC is supported by several mouse models, showing that the SCLC mouse tumors derive mainly from PNECs (Park et al., 2011) (Sutherland et al., 2011) (Song et al., 2012). This suggestion is also supported by experimental data showing that both airway and alveolar epithelial linages can arise from a multipotent lung epithelial cell from a normal mouse lung, whereas no differentiation into neuroendocrine cells is seen (McQualter et al., 2010). Instead the cell heterogeneity seen in some SCLC tumors may be driven by mutations in

the PNECs that will result into both neuroendocrine and non-neuroendocrine cells (Calbo et al., 2011 307).

A small number of PNECs are located individually or in clusters, called neuroepithelial bodies (NEB), in the epithelium from the larynx down to the terminal airways in the lung (Linnoila, 2006). The role of PNECs is poorly understood, but today several studies are ongoing to establish their functions. Interestingly, mouse studies have demonstrated that after a lung injury the PNECs start to proliferate and can regenerate Clara cells in the bronchioles (Song et al., 2012) (Reynolds et al., 2000). However, major studies are investigating the function of PNECs and NEB as airway chemoreceptors and their involvement in oxygen sensing in the control of breathing (Buttigieg et al., 2012) (O'Kelly et al., 1998) (Youngson et al., 1993) (Wang et al., 1996).

Treatment

Treatment of SCLC includes chemotherapy and in patients with limited disease it is combined with adjuvant radiation therapy. SCLC patients are rarely treated by surgery because of the broad spread of the disease at diagnosis. The first-line of chemotherapy treatment is a platinum-based agent, such as cisplatin or carboplatin, in combination with another cytotoxic drug, such as etoposide (Spigel, 2012) (Sørensen et al., 2010). Despite good response to the initial chemotherapy nearly all of the tumors will eventually relapse since a selection of multidrug-resistant cells occurs (Demedts et al., 2010). Today topotecan is the only approved agent for treatment of patients with relapsed SCLC (Spigel, 2012). Clinical trials involving patients must be approved before new drugs can be used in the clinic. Several new therapies for SCLC have been tested but none have made their way into daily practice (Rossi et al., 2008) (Demedts et al., 2010) (Hurwitz et al., 2009).

Arsenic trioxide

Overview

Arsenic is found in the nature as unstable sulfides, oxides and arsenates of potassium, sodium or calcium. There are three inorganic forms of arsenic, the red arsenic/arsenic disulfide (As_2S_2), the yellow arsenic/arsenic trisulfide (As_2S_3) and white arsenic/arsenic trioxide (As_2O_3) (Konkola, 1992). A major health problem is exposure to arsenic through inhalation and ingestion and in several countries the ground water is contaminated with arsenic. Exposure to arsenic during longer time periods can eventually induce cell transformation and chronic exposure is associated with increased risk of developing skin, lung, bladder and liver cancer (Germolec et al., 1998) (Morales et al., 2000).

Despite these carcinogenic effects, arsenic is one of the oldest medicines used for treatment of different diseases. For more than 2400 years ago, arsenic was used for treatment of ulcers by Hippocrates. In the 1700s, As_2O_3 dissolved in potassium bicarbonate was discovered by Thomas Fowler (Fowler´s solution) and was used to treat various diseases, such as asthma, eczema, psoriasis, chorea, anemia and leukemia. As a consequence of the known toxic effects of arsenic and that more modern therapies were introduced in the clinic, the arsenic containing treatments were not used in the Western world during several decades in the 20th century (Waxman and Anderson, 2001).

As_2O_3 in treatment of APL

In China, arsenic medicine has a long tradition for treatment of different diseases. In 1992 Sun *et al.* showed that As_2O_3 was efficient for treatment of patients with acute promyelocytic leukemia (APL) (Zhang et al., 2001). Despite the successful introduction of all-trans retinoic acid (ATRA) in combination with chemotherapy, 20-30% of the APL patients relapse (Cohen et al., 2001). Later on, several reports showed that As_2O_3 has the capacity to induce complete remission in patients with relapsed or refractory APL (Zhang et al., 2001) (Shen et al., 1997) (Soignet et al., 1998) (Soignet et al., 2001) (Niu et al., 1999). Today, the As_2O_3 agent Trisenox is used as first-line treatment for these patients which has improved the clinical outcome (Cohen et al., 2001). As_2O_3 treatment is often well tolerated and not frequently associated with severe side effects (Soignet et al., 2001).

Interestingly, As_2O_3 is also effective in patients with newly diagnosed APL and a synergistic effect has also been shown between As_2O_3 and ATRA. In clinical trials, the combination between these two agents showed high efficiency and better complete remission rate in newly diagnosed APL patients than using the agents alone (Mathews et al., 2006) (Shen et al., 2004) (Hu et al., 2009).

As_2O_3 treatment in other malignancies

The satisfying clinical results with As_2O_3 treatment in APL patients have led to increased studies in other hematologic malignancies, such as myeloma, acute myeloid leukemia and lymphoma as well as in in solid tumors, such as bladder cancer, prostate cancer, ovarian cancer, neuroblastoma and lung cancer. Several preclinical studies have showed that As_2O_3 inhibits cell growth and promotes cell death in cultured cancer cells (Emadi and Gore, 2010) (Yang et al., 1999) (Uslu et al., 2000) (Ora et al., 2000) (Akao et al., 1999) (Qu et al., 2009) and *Paper I*. Today, several clinical studies are ongoing and at Skåne University Hospital a clinical trial with As_2O_3 treatment for SCLC patients is planned.

Mechanisms of As_2O_3

The mechanisms of As_2O_3 chemotherapeutic actions are not exactly known, though several cellular pathways are affected (Figure 1). In APL cells As_2O_3 have dual effects, by inducing differentiation at lower concentrations whereas at higher concentrations apoptosis occur (Chen et al., 1997). APL is characterized by a translocation between chromosomes 15 and 17, which results in a fusion protein between the promyelocytic leukemia protein (PML) and retinoic acid receptor-α (RARα). This PML-RARα fusion protein blocks the differentiation of the myeloid cells (Wang and Chen, 2008). As_2O_3 induce differentiation and promotes cell death by mediating sumoylation and degradation of the PML-RARα protein (Shao et al., 1998) (Sternsdorf et al., 1999).

As_2O_3 also promote cell death in cells lacking the PML-RARα fusion protein. In APL cells and other tumor forms, As_2O_3 has been shown to increase cellular levels of reactive oxygen species (ROS) (Gupta et al., 2003) (Chen et al., 1998). The mechanisms behind the increased production of ROS are not completely understood. The ROS production is suggested to occur in the mitochondria, as a consequence of As_2O_3 effect on the mitochondria. However, in APL cells enhanced activation of NADPH oxidase has shown to contribute to the increased ROS production (Chou et al., 2004). The induced ROS levels are among others activating the c-Jun N-terminal kinase (JNK) that mediates apoptosis in the cell (Davison et al., 2004).

ROS is also activating the pro-apoptotic Bax protein and an early event of As_2O_3 treatment is the conformational change of Bax and translocation from the cytosol to the mitochondria (Zheng et al., 2005). As_2O_3 also down-regulates the anti-apoptotic protein Bcl-2. *In vitro* studies have demonstrated that Bax negative cells and Bcl-2 overexpressing cells are not as sensitive to As_2O_3 treatment as wild-type cells (Zheng et al., 2005) (Gupta et al., 2003). The changes in the pro- and anti-apoptotic proteins affect the mitochondria by opening the permeability transition pore complexes and consequently affecting the mitochondrial potential. As_2O_3 may also act directly on the mitochondria and affect the transmembrane potential. The voltage-dependent anion channels in the permeability transition pores increases by As_2O_3 treatment and are important for the cytochrome c release. Cytochrome c and apoptosis-inducing protein are then released and poly ADP-ribose polymerase (PARP) becomes cleaved. An initiation of the apoptotic program occurs and caspases 3 and 8 becomes activated. The intrinsic pathway mediates the As_2O_3-induced apoptosis and the cell death is independent of the extrinsic pathway (Larochette et al., 1999) (Gupta et al., 2003) (Zheng et al., 2005) (Scholz et al., 2005a).

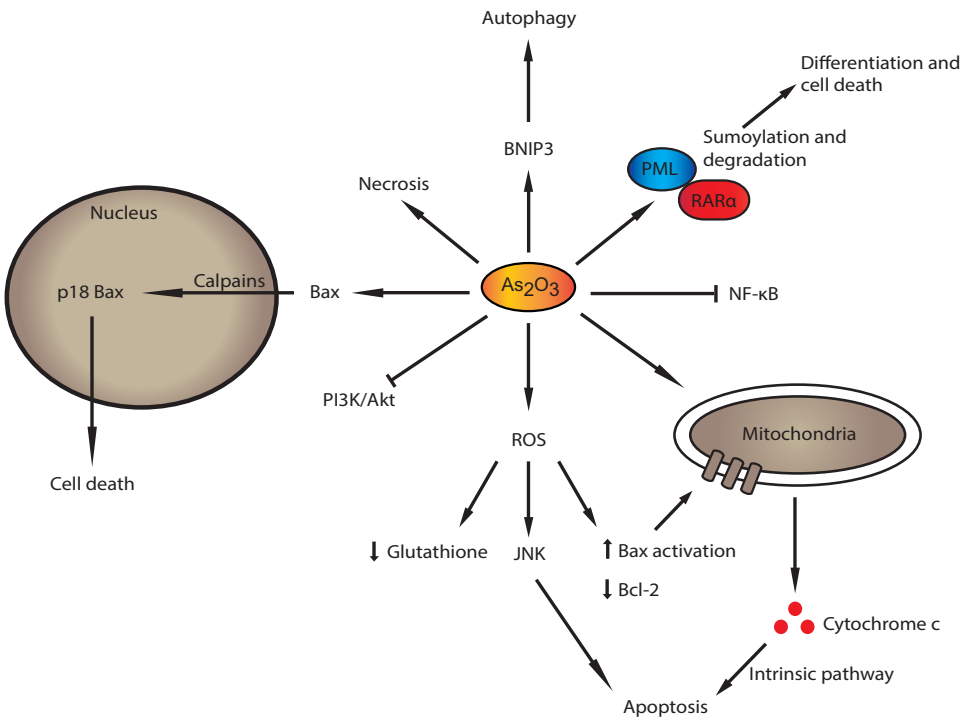

Figure 1. Potential mechanisms and effects of As_2O_3 treatment.
Examples of cellular pathways involved in the chemotherapeutic actions induced by As_2O_3 treatment.

The response to As_2O_3 seems to differ from one tumor form to another and the involvement of caspases in As_2O_3-induced cell death has been discussed. In neuroblastoma cells, As_2O_3 induces a proteolytic cleavage of Bax to a p18 form. This cleavage occurs by calpains in the nuclei, and the intrinsic apoptotic pathway seems not to be involved in the cell death mechanisms (Karlsson et al., 2004) (Karlsson et al., 2007). In several tumor forms, inhibition of caspases is not preventing the As_2O_3-induced cell death. Altogether, both caspase-dependent and -independent pathways have been reported to be involved in the cell death mechanisms (Karlsson et al., 2004) (Scholz et al., 2005b) (McCafferty-Grad et al., 2003).

Glutathione is an antioxidant and is important for the cellular redox status. The intracellular levels of glutathione have been shown to be important for the tumor cells sensitivity to As_2O_3 treatment. Cells with lower levels of glutathione are associated with better sensitivity to As_2O_3. During As_2O_3 treatment the glutathione levels diminish due to e.g. elevated intracellular levels of ROS (Gupta et al., 2003) (Yang et al., 1999) (Brambila et al., 2002) (Dai et al., 1999).

Several other signaling pathways have been reported to be involved in As_2O_3-induced cell death and growth arrest. Inhibition of the PI3K/AKT and NF-κB pathways have been demonstrated to be involved in the apoptotic function of As_2O_3 (Li et al., 2009) (Mathieu and Besancon, 2006). Autophagic cell death by an up-regulation of BNIP3 has also been reported as well as necrotic cell death has also been predominant in some tumor forms (Scholz et al., 2005b) (Kanzawa et al., 2005).

Tumor hypoxia

Overview

The oxygen tension in the body differs depending on the tissue, from high arterial oxygen tension to the end-capillary oxygen pressure at around 45-50 mmHg (5-6% O_2). Hypoxia occurs when the oxygen supply is insufficient to the demand, leading to impaired functions of organs, tissues and cells. Reduced available oxygen occurs due to various reasons, such as low oxygen tension because of pulmonary diseases or high altitude, reduced tissue perfusion, impaired ability of blood to carry oxygen or inability of cells to use oxygen. When the oxygen tension declines, the available ATP reduces and energy consuming processes in the cells are affected. There is no precise oxygen tension defining hypoxia, however metabolic hypoxia has been estimated to occur at approximately 8-10 mmHg (~1% O_2) (Höckel and Vaupel, 2001).

Hypoxia is frequently present in tumors due to uncontrolled tumor growth and malformed vasculature. When solid tumors increase in size, the oxygen tension is often reduced because of impaired blood flow, insufficient development of new blood vessels as well as functional and structural abnormalities of the vessels (Carmeliet and Jain, 2000). Since the diffusion limit of oxygen is around 100-150 µm (approximately 5-10 cell layers), the severity of hypoxia within tumors varies from cellular areas with low to no (anoxia) access of oxygen (Höckel and Vaupel, 2001).

Tumor cells have the ability to survive and proliferate in hypoxic environments since genetic and adaptive changes occur. The adaptive response contributes to a more aggressive phenotype and increased resistant to radiation and chemotherapy (Harris, 2002). Hypoxia is associated with therapeutic problems and several mechanisms are involved in decreased sensitivity to chemotherapy treatment, such as longer distance for the therapeutic agents, low penetration, induction of multidrug-resistance genes and selection of resistant cells. Hypoxic tumors are also less sensitive to radiation therapy, since oxygen is involved in the production of ROS, which causes the DNA damage (Shannon et al., 2003) (Moeller et al., 2007).

Hypoxia-inducible factors

Structure of HIF

Tumor cells as well as non-malignant cells are adapting to a hypoxic environment by changing their transcriptional program. Expression of genes involved in various cellular processes, such as angiogenesis, anaerobic glycolysis, cell survival and metastasis are induced at hypoxia (Löfstedt et al., 2007). The hypoxic response is primarily mediated by the hypoxia-inducible factor (HIF)-1 and HIF-2. HIF proteins are heterodimeric transcription factors consisting of two subunits, an α-subunit and a β-subunit. The stability of the α-subunit is oxygen-dependent regulated and today there are three known isoforms, HIF-1α (Semenza and Wang, 1992) (Wang et al., 1995) (Wang and Semenza, 1995), HIF-2α (Ema et al., 1997) (Tian et al., 1997) (Wiesener et al., 1998) and HIF-3α (Makino et al., 2001). The α-subunits are encoded by three different gene loci and HIF-1α and HIF-2α proteins share 48% amino acid sequence homology, whereas HIF-3α is not as closely related (Tian et al., 1997). HIF-1β or aryl hydrocarbon receptor nuclear translocator (ARNT) is non-oxygen-dependent and is constitutively expressed (Wang et al., 1995).

Both the α-subunit and β-subunit are basic helix-loop-helix proteins (bHLH) of the PAS domain family. The basic region recognizes and is responsible for the DNA-binding and the HLH and the PAS domains (PAS-A and PAS-B) mediate protein-protein interactions including ARNT dimerization (Figure 2). The bHLH domain and the PAS domains are the most conserved sequences in HIF-1α and HIF-2α (Tian et al., 1997). HIF-1α and HIF-2α also contain an oxygen-dependent degradation domain (ODD) involved in regulation of the HIF-α protein stability. The proteins also contain two transactivation domains (TADs), N-terminal and C-terminal, and the N-terminal TAD overlaps with the ODD. The function of TAD is to bind co-activators (Maxwell et al., 1999) (Semenza, 2000).

The HIF-1α and HIF-2α isoforms have mostly been studied, while HIF-3α is less well characterized and the function is not fully understood. However, alternative splicing variants of the HIF-3α subunit have been shown, and all are lacking the C-terminal TAD. The splice variants inhibit the HIF response since non-functional heterodimers with HIF-1α and HIF-2α are formed (Makino et al., 2001) (Makino et al., 2002) (Maynard et al., 2007).

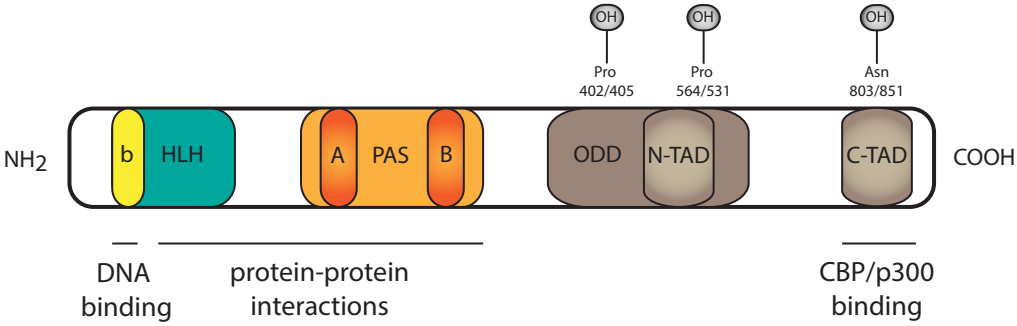

Figure 2. Domain structure of HIF-1α and HIF-2α.
HIF-α contains a DNA-binding basic region at the N-terminal, HLH and PAS domains that mediate protein-protein interactions including the interaction with ARNT, two transactivation domains (TADs) that bind co-activators and an oxygen-dependent degradation domain (ODD). At normoxia, the HIF-α subunit is hydroxylated at two proline residues (Pro-402 and Pro-564 in HIF-1α and Pro-405 and Pro-531 in HIF-2α) in the ODD and at an asparagine residue in the C-terminal TAD (Asn-803 in HIF-1α and Asn-851 in HIF-2α).

Regulation of HIF

A key factor in the hypoxic adaptation response is the stabilization and activation of the HIF transcription factors. The *HIF1A* and *HIF2A* mRNA are constitutively translated and an oxygen-dependent regulation occurs at the post-translational level. In the presence of oxygen, the prolyl hydroxylase domain proteins (PHDs) 1-3 hydroxylate two proline residues in the ODD of the HIF-α subunit (Pro-402 and Pro-564 in HIF-1α and Pro-405 and Pro-531 in HIF-2α). The PHD enzymes require oxygen, 2-oxoglutarate and ferrous for their function and ascorbate for enhanced activity (Bruick and McKnight, 2001) (Epstein et al., 2001). The abundance of PHD1-3 proteins differs between tissues, though PHD2 seems to be the predominately prolyl hydroxylase regulating HIF-1α (Appelhoff et al., 2004) (Berra et al., 2003). The tumor suppressor protein von Hippel Lindau (VHL) has a strong affinity to the hydroxylated prolines in the ODD and binds and mediates degradation of HIF-α subunits at oxygenated conditions (Figure 3). VHL is part of the large E3 ubiquitin ligase complex and interacts and target the HIF-α subunits for proteasomal degradation. (Ivan et al., 2001) (Ohh et al., 2000) (Jaakkola et al., 2001) (Maxwell et al., 1999) (Huang et al., 1998).

In addition to the regulation of the protein stability, the transcriptional activity of HIF-α is also regulated in an oxygen-dependent manner. An asparagine residue in the C-terminal TAD (Asn-803 in HIF-1α and Asn-851 in HIF-2α) is hydroxylated by the enzyme factor inhibiting HIF-1 (FIH-1). FIH-1 is just like the PHD enzymes dependent on oxygen, 2-oxoglutarate and iron. Hydroxylation of the asparagine residue prevents binding of co-activators p300 and CBP to the TAD and the transcriptional activity of HIF-α is inactivated (Figure 3) (Lando et al., 2002a) (Lando et al., 2002b). In HIF-2α there is a

substitution of a conserved amino acid within the C-terminal TAD immediate to the hydroxylated asparagine residue. Due to this amino acid replacement, the hydroxylation of the HIF-2α subunit is less efficient compared to HIF-1α (Bracken et al., 2006) (Koivunen et al., 2004). In addition, the enzymatic activity of PHD is inhibited at lower oxygen tensions, whereas more severe hypoxia is required for full inactivation of FIH-1 (Dayan et al., 2006).

At low oxygen tension, the PHD and FIH-1 are inhibited and no hydroxylation of the HIF-α subunits occurs. Consequently, the HIF-α subunits accumulate and dimerize with HIF-1β. A functional transcriptional complex is formed and binds together with co-activators to hypoxia-response elements (HRE) of target genes and induces the transcription (Semenza, 2003) (Weidemann and Johnson, 2008).

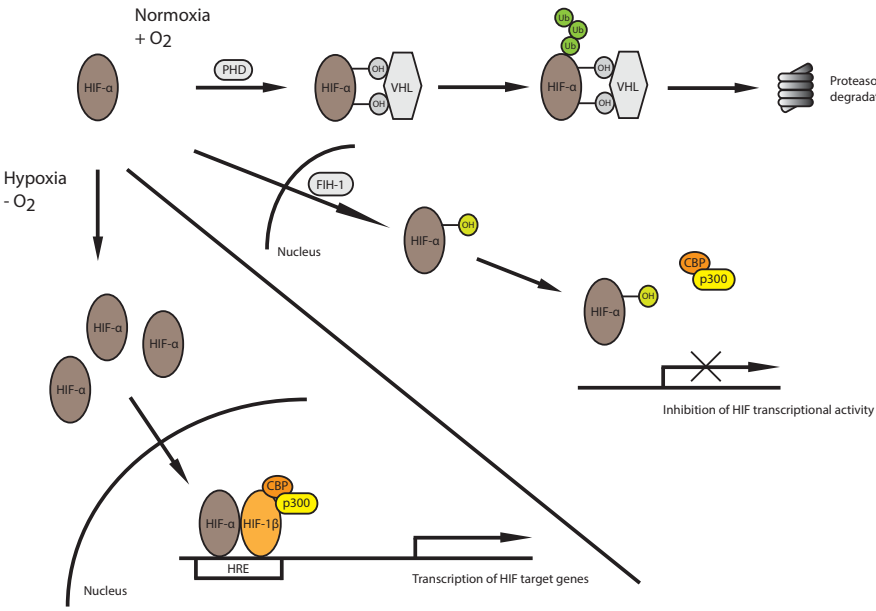

Figure 3. Oxygen-dependent regulation of HIF activity by prolyl and asparaginyl hydroxylation.
In the presence of oxygen, PHD proteins hydroxylate two proline residues in the HIF-α subunit leading to interaction with the VHL protein and ubiquitination by the E3 ubiquitin ligase. The ubiquitination mediates degradation by the ubiquitin-proteasome system. The transcriptional activity of HIF is regulated through hydroxylation of an asparagine residue in the HIF-α subunit by FIH-1. This hydroxylation prevents interaction between HIF-α and co-activators. At hypoxia, the activity of the oxygen-dependent enzymes PHD and FIH-1 are inactive leading to HIF-α and HIF-1β heterodimerize and forms a transcriptional complex that binds together with co-activators to HRE.

HIF transcription and translation is also regulated in an oxygen-independent manner. Genetic alterations such as loss of VHL, contributes to activation of signaling pathways and increased expression of HIF-α (Semenza, 2003) (Shen and Kaelin, 2013). Activation of growth factor signaling has also been shown to induce HIF-α expression, and signaling pathways reported to be involved in this increased expression are e.g. VEGF in colon cancer (Calvani et al., 2008), HER2 in breast cancer (Laughner et al., 2001) and EGFR in prostate cancer (Zhong et al., 2000). Growth factor receptor signaling is primarily activating the PI3K/AKT and MAPK pathways. The growth factor-induced HIF-α expression is more cell-type specific expression compared to the hypoxia-induced stabilization mechanisms (Semenza, 2003).

HIF-1 versus HIF-2

Despite similarities in structure and regulation of HIF-1α and HIF-2α, the subunits seem to have distinct expression and functions in development and tumor formation. In several knockout mouse models, no redundancy has been demonstrated between the proteins. Homozygous deletion of HIF-1α causes embryonic lethality at embryonic day E11 and the mice display failure in closing the neural tube, less number of somites, abnormalities in the cardiac development and defects in the vascular network (Ryan et al., 1998) (Iyer et al., 1998). In contrast, several knockout mice for HIF-2α have been reported and the embryonic phenotypes are divergent between these. The broad variation is probably explained by differences in the genetic background of the mice used in these studies. Overall, HIF-2α seems to be important for development of the vascular network in several organs (Scortegagna et al., 2003) (Compernolle et al., 2002) (Tian et al., 1998) (Peng et al., 2000).

Differences between HIF-1α and HIF-2α are also demonstrated by the distinct expression pattern of the subunits. During development, *HIF1A* mRNA is widely expressed, whereas the *HIF2A* expression is more restricted. At embryonic day E9.5 *HIF2A* mRNA expression is limited to endothelial cells of the developing vasculature and the precursor cells of the sympathetic nervous system. At later time points, the *HIF2A* expression is also present in the non-endothelial derived tissue such as lung, kidney and olfactory epithelium (Jain et al., 1998). In adult tissue, *HIF1A* mRNA is ubiquitously expressed (Wiener et al., 1996) (Wenger et al., 1996), while *HIF2A* mRNA is more limited to cell populations of organs such as lung, heart, liver, brain and kidney (Wiesener et al., 2003) (Tian et al., 1997) (Ema et al., 1997).

HIF-1α and HIF-2α bind together with HIF-1β and co-activators to the HRE in the enhancer or promoter region of target genes and activate the transcription. The two transcription factors share many target genes involved in a variety of processes, such as angiogenesis, pH regulation, apoptosis, genomic instability and survival (Löfstedt et al., 2004). However, HIF-1 and HIF-2 also have specific target gene preferences. The transcription of these distinct genes seems to be due to time, oxygen and cell context-

dependent expression of HIF-1α and HIF-2α. HIF-1α is stabilized at acute hypoxia in culture and the expression is degraded over time, while HIF-2α is gradually accumulated and is important at more prolonged response to hypoxia (Holmquist-Mengelbier et al., 2006) (Uchida et al., 2004) (Helczynska et al., 2008). This explains how HIF-1α and HIF-2α can regulate the same gene e.g. *VEGF* during different time points (Holmquist-Mengelbier et al., 2006). A large number of studies have shown a predominate role of HIF-1α in the regulation of the transcription response. An early effect of tumor hypoxia is induction of genes involved in anaerobic glycolysis, and consequently HIF-1α regulates the transcription of these genes (see *Tumor metabolism*) (Hu et al., 2003). HIF-2α on the other hand seems to primarily mediate transcription of genes induced at chronic hypoxia and at physiological oxygen tensions (Holmquist-Mengelbier et al., 2006). The stem cell marker Oct-4 has been shown to be specifically expressed by HIF-2α (Covello et al., 2006). However, potential HIF-2α target genes are scarcer, though *EPO, LOX,* and *CITED2* are genes regulated mainly by HIF-2α (Warnecke et al., 2008) (Raval et al., 2005) (Wang et al., 2005).

Hypoxia and HIFs in tumor tissue

The oxygen tensions in solid tumors can be measured by Eppendorf electrodes and several reports have shown that the oxygen concentration in tumor tissue is much lower than the levels in the adjacent non-malignant tissue (Brown and Wilson, 2004). Expression and activation of HIF-1α and HIF-2α is observed in the majority of solid tumors (Talks et al., 2000). Tumor hypoxia is associated with more aggressive phenotype, treatment resistance and poor outcome in several tumor forms. In addition to a hypoxic environment, HIF can be activated due to genetic alterations such as loss of VHL. Clear cell renal cell carcinoma (ccRCC) is characterized by lack of functional VHL protein. This occurs frequently due to mutation or hypermethylation of the VHL gene, and it is an early event in the tumor progression. This results in increased accumulation of HIF-α proteins in an oxygen-independent manner (Gnarra et al., 1994) (Herman et al., 1994). The majority of the tumors show a preference towards HIF-2α expression, although the VHL protein recognizes and degrades both HIF-1α and HIF-2α. HIF-2α overexpression has been described to promote tumor progression in ccRCC, while HIF-1α instead reduces tumor growth (Shen and Kaelin, 2013) (Raval et al., 2005) (Maxwell et al., 1999) (Kondo et al., 2003) (Krieg et al., 2000) (Lidgren et al., 2005).

There are contradicting results between the association of HIF-1α expression and prognosis in breast cancer. In several independent studies, overexpression of HIF-1α is correlated to poor overall survival (Dales et al., 2005) (Trastour et al., 2007) (Yamamoto et al., 2008), but certain studies have only found an association in specific breast cancer subgroups (Generali et al., 2006) (Giatromanolaki et al., 2004) (Kronblad et al., 2006). In a more recent study, HIF-1α expression was not linked to outcome in breast cancer (Helczynska et al., 2008). HIF-2α protein expression is less studied, but high HIF-2α

expression has been shown to correlate with distal metastasis. HIF-2α expression is an independent prognostic marker associated with breast cancer-specific death (Helczynska et al., 2008).

The childhood tumor neuroblastoma induces a hypoxic phenotype with HIF-1α and HIF-2α accumulation and the tumor cells dedifferentiate as well at lower oxygen tensions (Jögi et al., 2002). The two transcription factors are differentially expressed over time and at different oxygen tensions (Holmquist-Mengelbier et al., 2006). HIF-1α protein is transiently expressed at lower oxygen tensions and degraded over time, while HIF-2α responds to more prolonged hypoxia and is expressed at both physiological oxygen tensions and at hypoxia. In tumor tissue, HIF-2α protein has been detected at high levels in perivascular areas of the tumors (Pietras et al., 2008). Furthermore, different results are shown when *HIF1A* or *HIF2A* are knocked down by siRNA in SK-N-BE(2)c neuroblastoma cells and subcutaneously injected into mice. The *HIF2A* knockdown resulted into impaired tumor growth and delayed tumor formation, while *HIF1A* knockdown was not affecting the tumor growth (Holmquist-Mengelbier et al., 2006). In addition, HIF-2α overexpression is associated with worse overall survival, while a negative correlation between high HIF-1α expression and poor outcome is shown (Holmquist-Mengelbier et al., 2006) (Noguera et al., 2009).

HIF expression in normal lung tissue and lung cancer

During the development of the fetal lung, the physiological low oxygen tension in the environment is important for the lung organogenesis and vascular development (Groenman et al., 2007) (van Tuyl et al., 2005). During the maturation of the lung, *HIF2A* mRNA increases and is strongly expressed at later stages and remains high in adult lung tissue. On the other hand, *HIF1A* mRNA is constitutively expressed and the levels are low compared to *HIF2A* (Rajatapiti et al., 2008) (Ema et al., 1997) (Compernolle et al., 2002). HIF-1α and HIF-2α are important for the maturation of the lung, which are shown in knockout mice. Mice with a lung specific deletion of HIF-1α develop respiratory distress syndrome because of impaired differentiation and lung morphology of alveolar epithelium and decreased expression of surfactant proteins (Saini et al., 2008). Compernolle *et al* show that loss of HIF-2α diminishes VEGF expression and surfactant production (Compernolle et al., 2002). Overall, HIF-1α and HIF-2α seem to have important roles in the later stages of lung maturation, since early events during development are not affected.

Lung alveolar epithelial cells are normally well oxygenated, nevertheless the cells are very tolerant to low oxygen tensions and have the ability to survive at hypoxic conditions (Clerici and Planes, 2008). In lung tissue, the expression of HIF-1α protein is induced in the majority of pulmonary cell types, whereas HIF-2α is stabilized primarily in type II pneumocytes (Yu et al., 1998) (Wiesener et al., 2003).

Tumor hypoxia is a common feature of NSCLC and the expression of HIF-1α and HIF-2α proteins are regulated similarly to neuroblastoma and breast cancer (Le et al., 2006) (Uchida et al., 2004) (Holmquist-Mengelbier et al., 2006) (Helczynska et al., 2008) and *Paper II*. There is conflicting data whether HIF-1α is correlated with poor overall survival in NSCLC (Lee et al., 2003) (Kim et al., 2005) (Enatsu et al., 2006) (Hung et al., 2009) (Yohena et al., 2009) (Giatromanolaki et al., 2001) (Swinson et al., 2004) (Wu et al., 2011) (Ilie et al., 2010). The contradicting results in these several independent studies may be explained by different conditions, such as the size of patient materials, antibodies used, differing frequency of the NSCLC subtypes and differences in scoring nuclear and cytoplasmic staining. However, expression of HIF-2α is less frequent but has been shown to be correlated with shortened overall survival (Giatromanolaki et al., 2001) (Wu et al., 2011). Kim *et al* have showed that lung specific expression of HIF-2α in a $Kras^{G12D}$-driven NSCLC model caused reduced survival, due to increased tumor growth and vascularity (Kim et al., 2009). Using this $Kras^{G12D}$-driven NSCLC model, specific deletions of HIF-1α or HIF-2α in lung tumors were performed. Surprisingly, Mazumdar *et al* showed that HIF-2α deletion increased the numbers of tumors and tumor growth by elevated proliferation and reduced apoptosis. Xenograft growth was also promoted when *HIF2A* was knocked down by shRNA in the lung adenocarcinoma cell line A549 with a KRAS mutation. An explanation to this contradicting data was that different sets of target genes were activated and mediating these effects. Though, HIF-1α deletion in the $Kras^{G12D}$-driven NSCLC model did not affect tumor growth (Mazumdar et al., 2010).

In SCLC the expression of HIFs has not been studied in large materials. However, in two studies HIF-1α expression was linked to poor overall survival, while associations between HIF-2α and clinically parameters are not well characterized in SCLC (see also *Present investigation*) (Ioannou et al., 2009) (Luan et al., 2013).

HIF-independent adaptation responses to hypoxia

HIF transcription factors are generally found to contribute to cell survival and tumor growth at hypoxic conditions. However, several other signaling pathways have been identified that stimulate tumor cells tolerance to survive and propagate at lower oxygen tensions. A number of these signaling pathways are HIF-independent, including unfolded protein response (UPR), autophagy, cyclic AMP-responsive element-binding protein signaling and NF-κB, but not all of them will be discussed here.

Unfolded protein response

Tumors frequently contain regions with severe hypoxia (≤0.1% O_2) and as a result of the stressful condition, tumor cells induce UPR. This response is a conserved adaptive cellular pathway, regulated independent of the HIF transcription factors. UPR is activated at unfavorable environments such as metabolic stress, hypoxia and oxidative stress and by inflammatory cytokines (Wang and Kaufman, 2012) (Feldman et al., 2005).

Synthesis and folding of proteins occur in a specific coordinated pathway, to ensure that only properly folded proteins exit the endoplasmic reticulum (ER). When cells are exposed to a stressful condition, accumulation of unfolded proteins occurs in the ER. This leads to stimulation of intracellular signaling pathways from the ER and the UPR is activated. The UPR involves three distinct ER membrane-associated proteins, the IRE1, PERK and ATF6. These proteins work as stress sensors and are activated when unfolded proteins bind and sequester the ER chaperone binding proteins (BiP/GRP78) located at the sensors (Bertolotti et al., 2000) (Shen et al., 2002) (Liu and Kaufman, 2003). Each of these sensors activates a downstream signaling pathway. Upon activation, IRE1 splices the *XBP1* mRNA which encodes a stable and active transcription factor, whereas PERK promotes, among others translation of ATF4. The third arm of the UPR is progressed through cleaving and activation of ATF6 in the Golgi apparatus, which then translocate to nucleus. The activated transcription factors XBP1, ATF4 and ATF6 induce transcription of common and distinct target genes in the nucleus. The UPR promotes cell survival and growth by e.g. decreasing the protein synthesis, increasing proteasomal degradation of misfolded proteins and increasing the expression of ER chaperons (Ron and Walter, 2007) (Wouters et al., 2005). The three arms of the UPR show different kinetics and sensitivity to the various forms of stress (DuRose et al., 2006). UPR is primarily a survival response but prolonged or severe ER stress stimulates the UPR to promote apoptosis (Szegezdi et al., 2006). The importance of UPR in tumor growth is supported by increased cell death and impaired proliferation at hypoxia upon inhibition of IRE1, XBP1 and PERK signaling (Romero-Ramirez et al., 2004) (Cojocari et al., 2013).

Autophagy

Hypoxia is also activating autophagy, which is a tightly regulated process involving lysosomal degradation of proteins, organelles and cytoplasmic contents. Basal levels of autophagy are essential for normal cell growth, development and energy homeostasis. At environmental stress, such as hypoxia and nutrient starvation, autophagy contributes to the adaptation of tumor cells as well as tumor progression. Though, in response to certain stress factors, autophagy can lead to cell death (Levine and Kroemer, 2008) (Bellot et al., 2009). Autophagy begins with formation of double-membrane structures, autophagosomes, which sequester cytoplasm and organelles. The membrane of the autophagosome fuses with a lysosome, forming an autolysosome, and the intracellular content is degraded (Kondo et al., 2005) (Rosenfeldt and Ryan, 2009). Autophagy has been shown to be activated at hypoxia in both a HIF-dependent and an independent manner (Pursiheimo et al., 2009) (Bellot et al., 2009) (Zhang et al., 2008). The presence of autophagy at hypoxic conditions is important since this pathway is a survival response in stress situations that otherwise could end with cell death.

Tumor metabolism

Overview

A common feature of tumor cells is their rapid and uncontrolled proliferation. To supply the energy demands and synthesis of macromolecules, tumor cells have the ability to adjust their metabolism. The two major metabolic pathways to produce ATP are glycolysis and oxidative phosphorylation (OXPHOS). OXPHOS is the primary metabolic pathway in normal differentiated cells and contributes to efficient ATP production. In the presence of oxygen, pyruvate is synthesized via glycolysis and further converted into acetyl-CoA through the activity of pyruvate dehydrogenase (PDH) complex of enzymes (Figure 4). The conversion of pyruvate to acetyl-CoA is irreversible and consequently an important regulatory point. Acetyl-CoA then enters the TCA cycle and combines with oxaloacetate to generate citrate. A sequence of reactions occurs and electrons generated through the cycle form an electrochemical gradient within the inner mitochondrial membrane. Oxygen is the last acceptor in the electron transport chain and the proton gradient drives ATP production. Around 85-90% of the available oxygen in the cells is used by the mitochondria to produce ATP through the respiratory chain (Solaini et al., 2010) (Ward and Thompson, 2012).

For more than 70 years ago, Otto Warburg observed that proliferating tumor cells displayed high rate of glucose metabolism regardless of the oxygen tension in the tumor. This has been demonstrated in various forms of human tumors and is today referred to as the Warburg effect or aerobic glycolysis. These tumors produce their energy primarily through glycolysis and convert pyruvate into lactate instead of using respiratory metabolism (Figure 4). Warburg assumed that this appears in tumor cells due to defects in the mitochondria. However, the function of the mitochondria is not often impaired in tumor cells and today several mechanisms are suggested to cause the phenotype such as oncogenic activation, tumor suppressor loss and as an adaption response to the tumor microenvironment. It is not understood why tumor cells are using a less effective ATP production, but a high rate of glucose metabolism is associated with rapidly dividing cells (Ferreira, 2010) (Vander Heiden et al., 2009) (Gatenby and Gillies, 2004) (Bartrons and Caro, 2007). The increased glucose uptake in tumor cells is useful in the clinic for detection of tumors by positron emission tomography, using the glucose analog ^{18}F-fluorodeoxyglucose. This analog is taken up by glucose transporters, but is accumulated in

the cell since it cannot be fully metabolized and reflects the glucose uptake in the body (Kelloff et al., 2005).

Anaerobic glycolysis

Under hypoxic conditions, tumor cells induce a change from oxidative to anaerobic metabolism, which is associated with cell survival and tumor progression. The rate of glycolysis is primarily regulated by the availability of substrates and products. To compensate for the energy demands, hypoxic tumor cells have a higher rate of glucose uptake and glycolysis (Solaini et al., 2010) (Brahimi-Horn et al., 2007). By making this energy compensation, the tumor cells can meet the metabolic demand despite the low oxygen tension. During anaerobic metabolism, glucose is metabolized into two pyruvate molecules, which are further converted into lactate by lactate dehydrogenase A (LDHA) (Figure 4). The produced lactate is transported out of the cell by monocarboxylate transporters (MCTs) due to changes in cytosolic pH. Anaerobic glycolysis is less energy efficient and only 2 ATP molecules per glucose molecule are produced, compared to 36 ATP molecules generated at OXPHOS (Solaini et al., 2010) (Brahimi-Horn et al., 2007). Proliferating tumor cells not only require energy for survival and growth, the metabolism is also needed for synthesis of macromolecules such as nucleotides, fatty acids, and proteins (Hsu and Sabatini, 2008).

The metabolic shift to anaerobic glycolysis is primarily regulated by HIF-1, which binds to HRE of target genes and induces the expression of important genes. HIF-1 activation leads to a repression of the respiration in the cell, while glucose uptake, glycolysis and lactate production is stimulated (Semenza et al., 1994) (Semenza, 2007) (Semenza, 2010). The expression of glucose transporters and glycolytic enzymes such as GLUT1/SLC2A1, GLUT3/SLC2A3, HK2, ALDOA, ENO1 and PKM2 are induced by HIF-1 and the production of pyruvate is promoted (Semenza et al., 1994) (Semenza et al., 1996) (Iyer et al., 1998) (Luo et al., 2011). The entry of pyruvate into the TCA cycle is blocked since HIF-1 stimulates the expression of pyruvate dehydrogenase kinase (PDK). PDK phosphorylates and inactivates PDH, which leads to the production of lactate, and the conversion of pyruvate to acetyl-CoA is blocked. There are four PDKs (PDK1-4) and reports have shown that PDK1 and PDK3 are regulated by HIF-1. This regulation results in repressed mitochondrial respiration followed by reduced oxygen consumption and decreased production of ROS. The reduced OXPHOS at hypoxia is not due to insufficient oxygen levels in the tumor cells, but rather that the cells need to reduce the oxygen consumption and maintain the redox status (Korotchkina and Patel, 2001) (Papandreou et al., 2006) (Kim et al., 2006) (Lu et al., 2008).

The produced pyruvate at hypoxia is converted to lactate via LDHA and at the same time is NAD+ regenerated for continued glycolysis. The expression of LDHA is induced by HIF-1 and high concentrations of the enzyme is linked to poor prognosis (Semenza et al.,

1996) (Koukourakis et al., 2003) (Hermes et al., 2010). In addition, accumulation of lactate in tumors is associated with metastasis and poor survival (Brizel et al., 2001) (Walenta et al., 2000). Inhibition of LDHA in tumor cells dependent on glycolysis has been shown to increase oxygen consumption, ROS production and ATP turnover, due to increased mitochondrial respiration both at normoxic and hypoxic conditions. This inhibition results in impaired cell proliferation and increased cell death as well as tumor progression is inhibited *in vivo* (Fantin et al., 2006) (Le et al., 2010) (Xie et al., 2009).

To maintain the intracellular pH and the high rate of glycolysis, the produced lactate is transported out of the cell by proton-linked MCTs into the extracellular space. The MCTs are a family of transport proteins and the MCT4 (SLC16A3) has been shown to be up-regulated by HIF-1 at hypoxia (Halestrap, 2013) (Ullah et al., 2006). The acidification of the extracellular environment promotes tumor invasion through destruction of extracellular matrix (Robey et al., 2009) (Rofstad et al., 2006) (Rozhin et al., 1994). Interestingly, a relationship between lactate producing and lactate consuming cells has been reported. The lactate produced by hypoxic cells is then taken up through MCT1 and used as substrate for mitochondrial respiration in cells located in the adjacent normoxic conditions (Sonveaux et al., 2008).

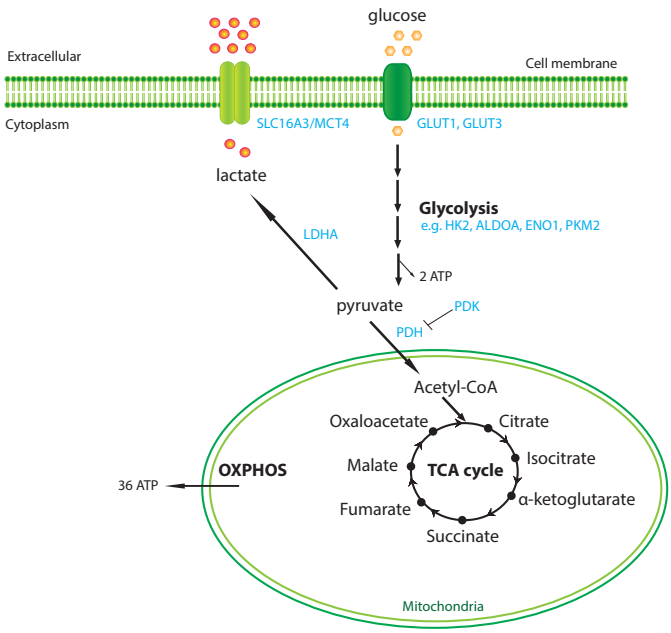

Figure 4. HIF-1 activity stimulates a metabolic shift to anaerobic glycolysis at hypoxic conditions.
At hypoxia, HIF-1 promotes glucose uptake, glycolysis and lactate production by inducing transcription of genes, such as *GLUT1, GLUT3, HK2, ALDOA, ENO1, PKM2, LDHA* and *SLC16A3*. HIF-1 repress mitochondrial respiration by stimulating the expression of PDK, which inhibits PDH, leading to the conversion of pyruvate to acetyl-CoA is blocked.

Glutaminolysis and *de novo* lipogenesis

In addition to glucose, glutamine is metabolized in tumor cells to support cellular growth and proliferation. Glutamine is the most abundant amino acid in the body and contributes to synthesis of macromolecules such as lipids and nucleotides. Glutamine is also involved in the synthesis of glutathione, regulating the redox status. Glutamine is primarily taken up by the transporter SLC1A5 and in the first step of glutaminolysis, glutamine is metabolized to glutamate and ammonia in the mitochondria by the enzyme glutaminase (GLS) (Figure 5). Glutamate is then converted to α-ketoglutarate and at the same time the amino group are removed from glutamine and released as ammonia or used in the synthesis of e.g. amino acids and proteins. The α-ketoglutarate can then enter the TCA cycle or undergo reductive carboxylation to produce citrate (Vander Heiden et al., 2009) (Deberardinis et al., 2008) (Wise and Thompson, 2010) (DeBerardinis et al., 2007). The reductive metabolism is mediated by the enzymes isocitrate dehydrogenases (IDHs) 1 and 2. IDH1 is localized in the cytoplasm and IDH2 in the mitochondria, whereas IDH3 has an irreversible function in the pathway. Oncogenic mutations of IDH1 and IDH2 have been reported in for instance glioma and acute myeloid leukemia (Ward et al., 2010) (Mardis et al., 2009) (Parsons et al., 2008) (Yan et al., 2009) (Dang et al., 2009).

Citrate stimulates proliferation by supporting *de novo* lipogenesis and citrate can be synthesized both from glucose and glutamine. Fatty acids are mainly produced from citrate derived from condensation of oxaloacetate and acetyl-CoA. At hypoxic conditions, PDK blocks the production of lipids from glucose-produced citrate, and tumor cells primarily use the reductive carboxylation of glutamine to produce citrate for fatty acid synthesis (Metallo et al., 2012) (Wise et al., 2011) (Filipp et al., 2012). The citrate levels are important for the regulation of reductive carboxylation by IDH (Gameiro et al., 2013). In HIF-1α suppressed ccRCC cells, the reductive metabolism is impaired whereas citrate synthesis through glucose metabolism is increased (Wise et al., 2011). Interestingly, tumor cells cultured in glutamine deficient medium at hypoxia have reduced viability. Suppression of glutamine metabolism, by inhibition of IDH1, IDH2 or GLS expression has demonstrated to reduce cellular proliferation and tumor growth (Wise et al., 2011) (Lobo et al., 2000) (Ward et al., 2010). In addition, enhanced activity of Myc has been reported to drive the glutaminolysis at normoxic and hypoxic conditions (see *The Myc pathway*).

During *de novo* lipogenesis the cytosolic citrate is cleaved by ATP citrate lyase (ACLY) into oxaloacetate and acetyl-CoA, which is a lipogenic precursor (Figure 5). The following steps in the fatty acid pathway are catalyzed by acetyl-CoA carboxylase alpha (ACACA) and fatty acid synthase (FASN), respectively. The synthesized fatty acids contribute to formation of lipid droplets and to membrane synthesis and saturation, which are involved in e.g. cell growth, proliferation, survival under oxidative stress and regulation of the $NADH/NAD^+$ ratio (Santos and Schulze, 2012).

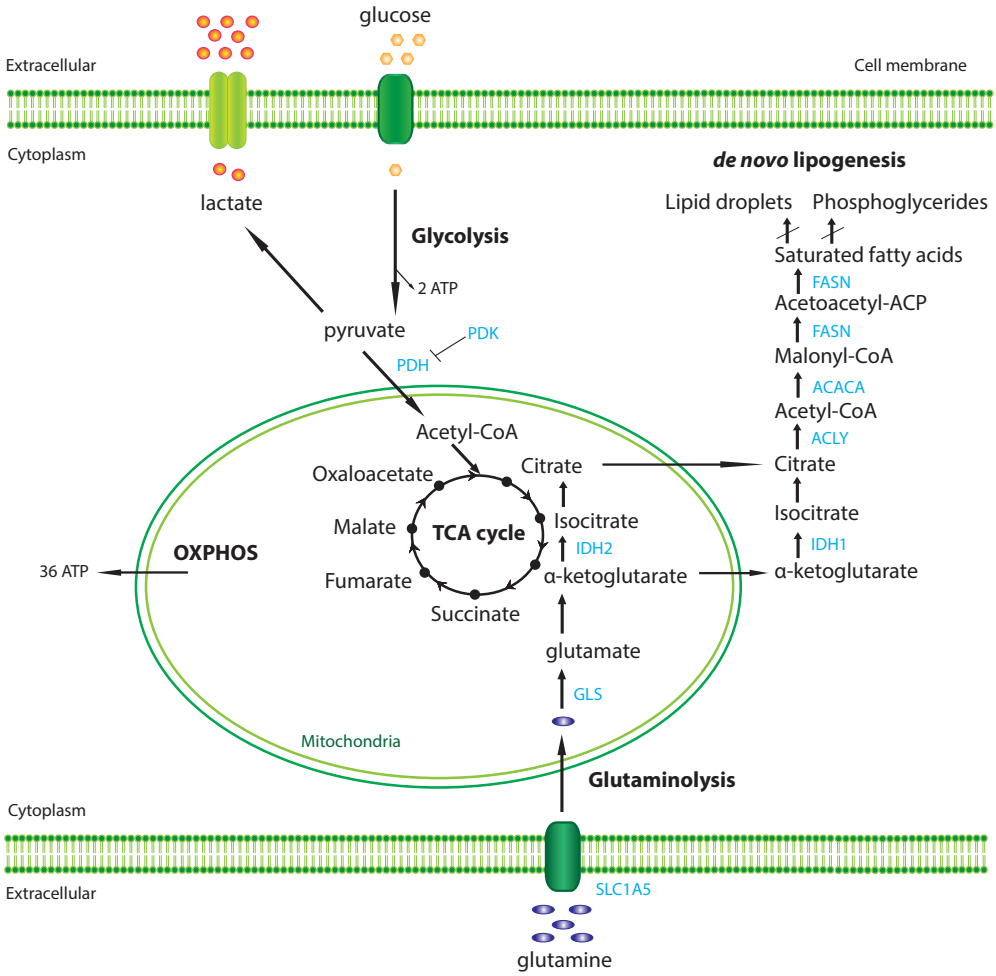

Figure 5. Glucose and glutamine metabolism promotes d*e novo* lipogenesis.
Glutamine is taken up by transporters and converts in the mitochondria to α-ketoglutarate, which then enter the TCA cycle or undergo reductive carboxylation to produce citrate. The reductive metabolism is either mediated in the mitochondria by IDH2, or in the cytoplasm by IDH1. The produced citrate from both glucose and glutamine metabolism support the fatty acid synthesis, which stimulates cellular growth and proliferation. At hypoxia, HIF-1 blocks the production of glucose-produced citrate by inducing the expression of PDK and tumor cells primarily use glutamine metabolism for synthesis of fatty acids. Myc stimulates transcription of genes involved in glutamine metabolism at both normoxic and hypoxic conditions.

Several steps in the lipid synthesis are required for and may even promote tumorigenesis. Inhibition of ACLY prevents proliferation and viability of tumor cells and suppresses tumor growth *in vivo* (Hatzivassiliou et al., 2005). The rate-limiting step in the synthesis of fatty acids is regulated by ACACA, which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA. Repression of *ACACA* using siRNA reduces the proliferation of prostate cancer cells and the numbers of apoptotic cells are up-regulated (Brusselmans et al.,

2005). Most investigations have focused on FASN, which synthesizes long-chain fatty acids in two steps by using acetyl-CoA as a primer and malonyl-CoA as a two-carbon donor. FASN is expressed at low levels in normal cells, whereas overexpression of the enzyme is seen in various human tumors such as colorectal cancer, prostate cancer and breast cancer (Rashid et al., 1997) (Pizer et al., 1996) (Swinnen et al., 2002). The enhanced expression is induced by several oncogenes or tumor suppressor genes and reports have shown that HIF-1 is involved in the induction of FASN at hypoxia (Furuta et al., 2008). It has been demonstrated that inhibition of FASN leads to diminished synthesis of fatty acids and inhibition of DNA replication, followed by reduced proliferation and induced apoptosis (Pizer et al., 1998) (Kuhajda et al., 2000) (Brusselmans et al., 2005).

Altogether, metabolic reprogramming is a common feature of tumor cells and glucose metabolism and reductive carboxylation of glutamine are promoted at hypoxic conditions. Today there are many attempts to disrupt the metabolism in tumor cells and many targets are investigated for potential novel treatment protocols.

The Myc pathway

Overview

The *MYC* family of genes includes *MYC*, *MYCN* and *MYCL*. Initially, *MYC* was identified as the cellular homology of the transforming gene from an avian retrovirus. The two structurally related genes *MYCN* and *MYCL* were discovered to be amplified in neuroblastoma cell lines and tumors (Kohl et al., 1983) (Schwab et al., 1984) and in SCLC, respectively (Nau et al., 1985). These genes are proto-oncogenes and enhanced expression is associated with a variety of human solid tumors. The Myc activation in tumor cells is due to amplification or chromosomal translocation of the *MYC* gene or overexpression of *MYC* due to constitutive activation of signaling pathways regulating the transcription. These alterations increase Myc protein through increased mRNA transcription and stability (Grandori et al., 2000).

Myc proteins are transcription factors containing a basic helix-loop-helix leucine zipper domain at the C-terminal. The basic region primarily mediates DNA binding and the helix-loop-helix leucine zipper domain is responsible for protein-protein interactions. The N-terminal contains a TAD and Myc-boxes that recruits various co-activators. Myc proteins heterodimerize with Max, a basic helix-loop-helix leucine zipper protein that is constitutively expressed. The Myc-Max heterodimer forms an active transcriptional complex, which recognizes and binds with high affinity to specific DNA E-box sequence (CACGTG) and activates gene transcription. Myc proteins have also the ability to repress transcription and several mechanisms are involved in this inhibition. Myc represses transcription by binding and inhibit the transcriptional activator Miz-1 and by transcriptionally activating or repressing expression of microRNAs. MAX also interacts with other basic helix-loop-helix leucine zipper protein including Mnt, Mga and the Mxd protein family. These heterodimers bind to the E-box sequences as well and repress the transcription by recruiting histone deacetylases, which blocks the function of the Myc-Max heterodimer (Grandori et al., 2000) (Dang, 2012) (Hurlin and Huang, 2006) (Chang et al., 2008).

Enhanced Myc protein expression contributes to tumorigenesis and is associated with poor prognosis (Gurel et al., 2008) (Murphy et al., 2008) (Yustein et al., 2010). Myc stimulates transcription of a broad spectrum of genes involved in several cellular processes e.g. proliferation, cell cycle progression, growth, energy metabolism, apoptosis and DNA repair (Fernandez et al., 2003). Different attempts have been used to inhibit Myc. For

instance, knockdown of *MYC* with shRNA in several human tumor cell lines results in growth arrest and in some of the cell lines apoptosis is induced (Wang et al., 2008). The inhibitor 10058-F4 is disrupting the N-Myc-Max complex in neuroblastoma cells, which results in growth arrest and apoptosis as well as accumulation of lipid droplets due to reduced oxidation of fatty acids (Zirath et al., 2013). Inhibition of c-Myc *in vivo* using a mouse model expressing a c-Myc mutant or using the c-Myc inhibitor AVI-4126 has been shown to repress lung tumors and reduce metastasis (Soucek et al., 2008) (Sekhon et al., 2008).

L-Myc in SCLC

The *MYC* family genes are amplified in 20-30% of SCLC tumors, and whole genome profiling have demonstrated that the *MYC* family members are overexpressed in about 62% of the SCLC cell lines and 82% of the primary SCLC tumors that were tested (Takahashi et al., 1989) (Wistuba et al., 2001) (Voortman et al., 2010). *MYCL* was discovered to be amplified in SCLC and regions of the nucleotide sequence were identified to be highly homologous to sequences conserved in the *MYC* and *MYCN* genes (Nau et al., 1985). In contrast to the more generalized expression of *MYC*, *MYCL* has more restricted expression and is confined to the fetal brain, kidney and lung and adult lung tissue (Zimmerman et al., 1986). The function of L-Myc protein is not well characterized. However, Dosaka-Akita *et al* have shown that the proliferation is inhibit when the translation of L-Myc protein is blocked in human SCLC cells overexpressing the protein (Dosaka-Akita et al., 1995).

Myc and tumor metabolism

Myc has an important role in regulating metabolism in tumor cells. c-Myc induces expression of genes involved in glycolysis, glutaminolysis and mitochondrial biogenesis for synthesis of ATP and macromolecules to stimulate proliferation and drives the fatty acid synthesis for cell growth and propagation (Dang, 2013) (Miller et al., 2012) (Li et al., 2005). N-Myc has also been found to stimulate aerobic glycolysis (Qing et al., 2010).

Both HIF and Myc are involved in tumor metabolism and interactions have been showed, but this crosstalk seems to be very complex. At lower oxygen tensions, HIF-1 and c-Myc have shown to promote glucose metabolism both independently and cooperatively. In contrast to HIF-1, c-Myc stimulates mitochondrial biosynthetic functions and anabolic pathways at normoxic and hypoxic conditions (Kim et al., 2007) (Wise et al., 2008) (Le et al., 2012). Reports have demonstrated that HIF-1α and HIF-2α have opposite effects on the mitochondrial activity and cell cycle progression induced by

c-Myc. HIF-1α inhibits c-Myc induced activity through disrupting the Myc-Max complex and the binding to co-factors. HIF-1 can also induce the expression of MXI1, which cause transcriptional repression of several genes (Zhang et al., 2007) (Gordan et al., 2007b) (Koshiji et al., 2004). In contrast, HIF-2α forms a complex with Max, which causes increased Myc-Max interactions and mediates expression of c-Myc target genes in ccRCC cells (Gordan et al., 2007a).

c-Myc is involved in enhancing the glycolytic pathway by activation of several enzymes involved in glucose metabolism, such as ENO1, HK2, LDHA and PDK1, both at normoxic and hypoxic conditions. Most of the glycolytic enzymes contain conserved consensus Myc-binding sites or E-boxes among their regulatory DNA sequences (Kim et al., 2007) (Kim et al., 2004) (Shim et al., 1997). Wise *et al* demonstrated that c-Myc is also involved in the regulation of glutamine metabolism to support a high proliferation rate (Wise et al., 2008). c-Myc regulates glutamine catabolism both directly and indirectly, directly by stimulating transcription of genes involved in glutamine metabolism such as SLC1A5 and indirectly by transcriptionally repressing miR-23a and miR-23b, which leads to increased expression of GLS (Wise et al., 2008) (Gao et al., 2009).

Enhanced c-Myc and N-Myc expression have reported to make tumor cells addicted to glutamine and suppression of c-Myc has shown to reduce the glutamine levels and ammonia production (Wise et al., 2008) (Qing et al., 2012). Under aerobic conditions, glutamine deprivation or knockdown of *GLS* in cells overexpressing c-Myc, reduces cellular proliferation, decrease ATP levels and oxygen consumption, increases ROS levels and diminishes the glutathione levels. These changes are associated with increased cell death and tumor cells overexpressing c-Myc are more sensitive to glutamine starvation (Gao et al., 2009) (Yuneva et al., 2007). Le *et al* showed in a Burkitt lymphoma model with inducible *MYC*, that glycolysis increases and glutamine metabolism in the TCA cycle is continued at hypoxia. At glucose deprivation under hypoxic conditions, c-Myc overexpressing cells are not proliferating, but glutamine is continually metabolized to citrate in the TCA cycle and glutathione is produced to stimulate cell survival. Inhibition of GLS by the inhibitor BPTES at normoxic and hypoxic conditions results in cell death and impaired tumor progression, suggesting that glutamine metabolism is promoting cellular survival in c-Myc overexpressing cells (Le et al., 2012). Cell death upon glutamine deprivation in N-Myc overexpressing neuroblastoma cells depends on increased transcription and translation of the transcription factor ATF4, which is involved in the UPR. ATF4 stimulates transcription of PUMA, NOXA and TRB3 which induces apoptosis (Qing et al., 2012). Since all tumors do not have increased glycolysis, glutamine metabolism could be a good target for both detection of tumors in the clinic as well as a therapeutically target.

Present investigation

Aims

The overall aim of this thesis was to characterize the adaptive response of human SCLC cells to hypoxia, and to evaluate the importance of activation of the transcription factors HIF-1 and HIF-2 for cell viability and proliferation. A modest aim was to unravel the cytotoxic effects of As_2O_3 treatment on human SCLC cells *in vitro* and *in vivo*.

Paper I: Arsenic trioxide is highly cytotoxic to small cell lung carcinoma cells

Aims

As_2O_3 is very efficient for treatment of patients with relapsed APL and preclinical and clinical studies with As_2O_3 have further demonstrated cytotoxic effects in a variety of human tumor forms. The general aim of this paper was to investigate SCLC cell-sensitivity to As_2O_3 treatment and the responsiveness of SCLC cells to As_2O_3 *in vivo*. Specific aims were to study the mechanisms behind As_2O_3-induced cell death and to investigate the effect of hypoxia during treatment.

Summary

Previously it has been demonstrated that clinical relevant concentrations of As_2O_3 induce cell death in neuroblastoma cells *in vitro* and inhibit tumor growth *in vivo.* The cytotoxic effect was shown both in drug-sensitive and in chemotherapy-resistant cell lines, as well as the effect was sustained at lower oxygen tensions (Karlsson et al., 2005) (Ora et al., 2000). We investigated SCLC cell-sensitivity to As_2O_3 treatment, since SCLC and neuroblastoma have several phenotypic and genotypic similarities. These tumor forms both have neuronal/neuroendocrine traits, genetic alterations including amplification of *MYCN* in neuroblastoma and *MYC*, *MYCN* and *MYCL* in SCLC, and show a good initial response to chemotherapy treatment.

We found that several SCLC cell lines were sensitive to As_2O_3 treatment at clinically relevant concentrations and that pronounced cell death was induced. We further showed that the mechanism behind the As_2O_3-induced cell death was primarily necrosis. Apoptosis was involved but to a less extent, demonstrated by weak activation of apoptotic proteins, as well as limited rescue effect of As_2O_3 treated cells by a pan-caspase inhibitor. The cytotoxic effect was also sustained when we cultured the SCLC cells at hypoxic conditions (1% O_2). Furthermore, we injected SCLC cells subcutaneously into nude mice and when 5 mm tumors were established the animals were treated daily with PBS or clinically relevant concentrations of As_2O_3. The mean tumor growth was significantly repressed by As_2O_3 treatment. A small fraction of the tumors did not respond to the treatment and cells of these tumors demonstrated a different morphology and organization compared to the responding tumors. Interestingly, the responding tumors showed large areas of necrosis, less dividing cells, impaired vascularization and increased staining of the neuroendocrine markers chromogranin A and synaptophysin compared with control and non-responding tumors.

Discussion

The mechanisms of As_2O_3 are not fully understood and seem to be very complex (see *Arsenic trioxide*). Various cell death-induced pathways have been reported to be activated in different tumor forms, indicating that the mechanisms of As_2O_3 to some extent are cell-dependent. We found that the cell death response was diverging slightly between the SCLC cell lines we investigated, and the effect was also in some degree time- and concentration-dependent. Both necrosis and apoptosis were induced in the SCLC cells, though the cell death mechanism was not fully elucidated. Only a moderate activation of caspase 3 was shown and caspase-induced cell death did not seem to be of major importance since the pan-caspase inhibitor did not prevent As_2O_3-induced cell death. Furthermore, caspase-independent cell death by cleavage and activation of the Bax p18 form was moderately induced. Our data indicates that As_2O_3-induced cell death in SCLC was primarily caused by a caspase-independent necrotic cell death. Interestingly, As_2O_3 appears to activate another cell death-induced pathway compared to conventional chemotherapy. Our study demonstrated a homogenous sensitivity to As_2O_3 treatment in the several SCLC cell lines we tested, compared to the response to the conventional chemotherapy agents etoposide and carboplatin. The NSCLC and breast cancer cell lines we investigated were less sensitive to As_2O_3 treatment, indicating that SCLC have a genotype and phenotype that make the tumor cells more sensitive for As_2O_3 treatment.

Solid tumors frequently develop regions of hypoxia due to rapid and uncontrolled proliferation and malformed vasculature (Carmeliet and Jain, 2000). The oxygen tensions in solid tumors is lower than in adjacent non-malignant tissue, and tumor hypoxia is frequently associated with more aggressive phenotype and treatment resistance (see *Tumor hypoxia*) (Harris, 2002). To be a potential strategy in the treatment of solid tumors it is necessary that As_2O_3 treatment is effective both at normoxia and hypoxia. We demonstrated that As_2O_3 was equally efficient under hypoxic conditions as under normoxic conditions. Even though the SCLC tumor cells were adapting to the lower oxygen tensions for 72 hours before treatment the tumor cells were still sensitive, which is clinically important.

Our *in vivo* data demonstrated that SCLC tumor growth was significantly impaired by using clinically relevant concentrations of As_2O_3 (5 mg/kg). As_2O_3 treatment is not frequently associated with severe side effects (Soignet et al., 2001) and no general intoxication was demonstrated in our nude mice-xenograft model. However, a small fraction of the U-1690 xenograft tumors were not sensitive to the treatment. We found a more organized growth pattern in these tumors and the cells demonstrated a different morphology such as larger cells, varied nuclei size, disintegrated chromatin and prominent nucleoli compared to the responding tumors. This variant phenotype was also found in a small number of the untreated control group which indicates that this phenotype was not developed due to an As_2O_3-induced selection. These results rather suggest that the U-1690 cell line contains at least two distinct populations of cells. In addition, a combined SCLC cell line composed of a mixture of cells with small and large

cell morphology was not as sensitive to As_2O_3 treatment as the pure SCLC cell lines. This further indicates that pure SCLC cells have a genotype and phenotype that make the tumor cells sensitive for As_2O_3.

The As_2O_3 responding tumors demonstrated fewer blood vessels, compared to the control and non-responding tumors. This antiangiogenic effect of As_2O_3 treatment was consequently leading to hypoxia since an accumulation of HIF-1α protein was detected in these tumors. In addition, SCLC is characterized as a neuroendocrine tumor and the neuroendocrine markers chromogranin A and synaptophysin were up-regulated in the drug-responding tumors. In cultured SCLC cells, As_2O_3 treatment for 6 days was slightly reducing the expression of the neuroendocrine markers, whereas hypoxic growth conditions induced the expression independently of As_2O_3. This suggests that the *in vivo* effect of As_2O_3 may be due to the diminished angiogenesis and activation of HIF-1, which increases the expression of the neuroendocrine differentiation markers.

SCLC is primarily treated with chemotherapy and despite good response to the initial treatment the majority of the tumors will eventually relapse (Demedts et al., 2010). Our results in *paper I* indicate that As_2O_3 may be a good complement to conventional treatments for patients with relapsed SCLC. Interestingly, a clinical trial to test As_2O_3 treatment in relapsed SCLC patients has been planned to be initiated at Skåne University Hospital.

Paper II: HIF-2α expression is suppressed in SCLC cells, which survive in moderate and severe hypoxia when HIF-1α is repressed

Aims

In *paper II*, the aims were to characterize the expression of HIF-1α and HIF-2α proteins in SCLC tumor specimens and in a panel of SCLC cell lines, as well as to study the adaptive capacity to hypoxia. Furthermore, we wanted to investigate the importance of activation of HIFs in SCLC cells for survival and propagation and to investigate alternative adaptation response to severe hypoxia (0.1% O_2) in HIF-repressed SCLC cells.

Summary

The expression of HIFs is not well characterized in SCLC cells, and therefore we immunohistochemically stained 35 SCLC and 9 NSCLC human tumor specimens organized in a tissue microarray to characterize the expression of HIF-1α and HIF-2α. We demonstrated that SCLC and NSCLC expressed HIF-1α to a similar degree, ~65% of the SCLC and ~67% of the NSCLC tumor specimens contained tumor cells positive for HIF-1α. However, HIF-2α was only detected in 1 out of the 35 (~3%) SCLC tumor specimens while ~56% of the NSCLC specimens showed positive staining for HIF-2α. Furthermore, we investigated a panel of SCLC cell lines and cultured them at normoxic and hypoxic conditions during different time periods. In line with our tumor data, we found that SCLC cells expressed extremely low levels of *HIF2A* mRNA and no HIF-2α protein could be detected. In contrast, *HIF1A* mRNA was detected during the entire culture period at both 21% and 1% oxygen. HIF-1α protein was stabilized at 4 hours exposure to hypoxia and was sustained and accumulated up to 96 hours, whereas no HIF-2α was detected. Despite the lack of HIF-2α protein expression, SCLC cells displayed a good capacity to adapt to low oxygen tensions and showed cell propagation at both modest and severe hypoxia. The proliferation was to some extent reduced at lower oxygen tensions, which was due to a slower rate of cell divisions, a larger fraction of undivided cells and delayed progression of the cell cycle. We investigated the expression of various genes involved in hypoxic adaptation using quantitative real-time PCR, and generally only a modest induction was demonstrated in the SCLC cells. As expected, genes involved in anaerobic glycolysis and predominately transcriptionally activated by HIF-1 were moderately up-regulated. To elucidate the importance of HIF-1 for cell survival and propagation in SCLC cells, we knocked down *HIF1A* using siRNA. To our surprise and interest, we found that repression of *HIF1A* at both 1% and 0.1% oxygen did not significantly affect the number of dead cells and the viable cells continued to divide. The

total number of viable cells was to some extent reduced in the cell line U-1906, which showed the most efficient knockdown.

The adaptive response to hypoxia is primarily regulated by HIF-1 and HIF-2, though several other signaling pathways have been reported to promote cellular survival and proliferation at lower oxygen tensions. We could not detect increased activation of autophagy or NF-κB at 1% oxygen, indicating that these pathways are not of major importance for the hypoxic adaptation of SCLC cells. However, at severe hypoxia an induced expression of spliced *XBP1* mRNA involved in UPR was detected and this induction was sustained in *HIF1A* knockdown cells.

Discussion

Previously, it has been demonstrated in lung adenocarcinoma, neuroblastoma and breast cancer cells that HIF-1α and HIF-2α are differentially expressed over time. HIF-1α protein is stabilized during the acute phase of hypoxia and the expression is decreased over time, whereas HIF-2α is gradually accumulated and is important at more chronic phases of hypoxia (Holmquist-Mengelbier et al., 2006) (Uchida et al., 2004) (Helczynska et al., 2008). We found that SCLC tumor specimens and cell lines virtually lacked HIF-2α protein at hypoxic conditions, while HIF-1α expression remained high over time, which may be as a compensation for the absence of HIF-2α expression. The observed immunohistochemical staining of HIF-1α and HIF-2α in the small number of evaluated NSCLC tumor specimens and the expression patterns in the NSCLC cell lines were in line with previous investigations (Giatromanolaki et al., 2001) (Uchida et al., 2004). Interestingly, the expression kinetics of HIF-1α and HIF-2α in SCLC cells differ compared to NSCLC, neuroblastoma and breast cancer cells, indicating that SCLC cells adapt to hypoxia by different mechanisms.

The expression of HIF-1α and HIF-2α are not well characterized in SCLC cells. Since SCLC patients are rarely treated with surgery, this leads to shortages in SCLC tumor specimens for scientific studies. However, in two small independent studies that have been performed, HIF-1α expression is associated with poor overall survival (Ioannou et al., 2009) (Luan et al., 2013). Our results suggest that HIF-1α is not involved in sustaining cell viability and proliferation at hypoxia, though HIF-1α will possibly be important for other aggressive properties. In lung adenocarcinoma cells, HIF-1α has been reported to enhance the tumor cells invasive ability (Shyu et al., 2007). Recently, Luan *et al* showed that 24.4% of the tested SCLC tumor specimens were positive for HIF-2α staining and the protein expression was correlated with shortened overall survival (Luan et al., 2013). Our contradicting results may be explained by different antibodies as well as differences in scoring nuclear and cytoplasmic staining. The specificity of HIF-2α immunohistochemical staining is difficult and it is of great importance to evaluate the antibody thoroughly before use. We verified our antibodies in normoxic and hypoxic cultured neuroblastoma cells, known to have a robust hypoxic response, as well as HIF-

2α-positive infiltrating immune cells were used as internal positive controls. The positive immune cells were not included in our scoring and may explain the observed differences.

Although SCLC cells have a high ability to adapt to moderate and severer hypoxia, we only demonstrated a modest induction of hypoxia-driven genes (*Paper II* and unpublished microarray data). This may suggest that SCLC cells are less sensitive to fluctuations of the oxygen tensions, which may be explained by the origin of SCLC (see *Small cell lung carcinoma*). The cellular origin of SCLC is not identified, though it has been suggested that SCLC are mainly derived from the PNECs (Park et al., 2011) (Sutherland et al., 2011) (Song et al., 2012) (McQualter et al., 2010). PNECs have been reported to function as airway chemoreceptors, and these cells therefore have a high tolerance to low oxygen tensions and ability to survive at hypoxic conditions (Buttigieg et al., 2012) (O'Kelly et al., 1998) (Youngson et al., 1993) (Wang et al., 1996). In addition, HIF-1α protein is induced in the majority of pulmonary cell types at hypoxic conditions, while HIF-2α is expressed primarily in type II pneumocytes (Yu et al., 1998) (Wiesener et al., 2003). Interestingly, all the pure SCLC cell lines that we have investigated lacked the expression of HIF-2α. This suggests that SCLC is not derived from the same cell type as type II pneumocytes, or indicates that deregulation of HIF-2α is important for SCLC tumorigenesis. HIF-2α protein is needed during the maturation of the lung and the mRNA levels of *HIF2A* remains high in the adult lung tissue, which may also suggest that lack of HIF-2α is involved in SCLC tumorigenesis.

Despite the abundant and exclusive expression of HIF-1α in SCLC cells, repression of *HIF1A* at both 1% and 0.1% oxygen did not significantly affect the number of dead cells and the viable cells were still proliferating. Our data indicates that the adaptive capacity to hypoxia is HIF-independent in SCLC cells, and that the adaptation to hypoxia is mediated via other signaling pathways known to be involved in the hypoxic response. The adaptation process can be very complex to visualize since several of the pathways can be activated and cooperate. We observed that mRNA levels of the transcription factor *XBP1* involved in UPR was spliced at lower oxygen tensions and the activation was sustained in *HIF1A* knocked down cells. The UPR is known to stimulate viability and propagation at stressful conditions in a HIF-independent manner (Ron and Walter, 2007) (Wouters et al., 2005) (Romero-Ramirez et al., 2004) and seems to be important for SCLC cells with or without repressed *HIF1A* at severe hypoxia.

Paper III: Increased glutaminolysis and lipogenesis in HIF-repressed SCLC cells support cell viability and proliferation at hypoxia

Aims

The aim of this paper was to investigate the importance of HIF-1α for tumor formation and growth *in vivo*. In addition, we wanted to elucidate the potentially metabolic mechanisms underlying the high survival capacity of SCLC cells with repressed *HIF1A* to hypoxic conditions (1% O_2).

Summary

In *paper II*, we demonstrated that SCLC cells lacked the expression of HIF-2α, whereas HIF-1α was expressed at both acute and prolonged hypoxia. We also concluded that the response of SCLC cells to severe hypoxia (0.1% O_2) was HIF-independent, since repressed *HIF1A* expression using siRNA did not affect the number of dead cells, and the cells continued to proliferate. To further elucidate how SCLC cells with repressed *HIF1A* expression survive and propagate at hypoxia *in vitro* and *in vivo*, cells with a stable knockdown of *HIF1A* using shRNA were generated. As reported in *paper II* when siRNA was used, shRNA against *HIF1A* showed no significant effect on cellular survival or propagation at hypoxic conditions. To investigate if repressed *HIF1A* expression could affect *in vivo* growth, we injected control and shHIF1A cells subcutaneously into nude mice. Interestingly, no significant differences in xenograft tumor take or tumor growth, including size and weight, were obtained between control or *HIF1A* knocked down cells after either 14 or 27 days. However, HIF-1α staining was obtained in a few regions in the shHIF1A tumors, though these areas were less abundant and generally in these tumors blood vessels were less frequent. In conclusion, HIFs were not required for SCLC cell survival and proliferation and xenograft tumor growth was not significantly impaired by repressed *HIF1A* expression.

Since HIF-1 is promoting anaerobic glycolysis at hypoxic conditions, we have examined how *HIF1A* knockdown in SCLC cells affected metabolic gene transcription and growth. As expected, *HIF1A* knockdown resulted in diminished expression of genes involved in glucose metabolism, as well as reduced glucose utilization and less transportation of lactate out of the cell. Interestingly, the ATP levels were not affected by *HIF1A* knockdown, despite reduced glucose metabolism. Furthermore, culturing the cells in glucose-free medium at normoxic and hypoxic conditions reduced the proliferation equally in shC and shHIF1A cells, but did not increase the number of dead cells. In contrast, in these SCLC cells glutamine deprivation drastically impaired the proliferation

and increased the number of dead cells, in a HIF-independent manner. Previously, reports have demonstrated that c-Myc is stimulating glutamine metabolism and fatty acid synthesis to support cell growth and proliferation (Wise et al., 2008) (Morrish et al., 2010). SCLC cells generally have one of the three *MYC* family genes amplified and the cell lines used here have a *MYC* (U-1906) and *MYCL* (U-1690) amplified gene. In *HIF1A* knockdown cells, genes involved in glutaminolysis were slightly induced at 1% oxygen and genes in *de novo* lipogenesis were significantly up-regulated. Knockdown of *MYC* using siRNA decreased the proliferation substantially and knockdown of *GLS* resulted in both reduced propagation and increased cell death.

Discussion

In this paper we created cells with a stable knockdown of *HIF1A* with shRNA, and consistent with our siRNA data in *paper II*, we demonstrated an efficient repression of *HIF1A* mRNA, protein and activity in the SCLC cell lines U-1906 and U-1690. In the tumors generated by shHIF1A cells no significant difference in tumor take and tumor growth compared to controls were obtained, suggesting that HIF has a limited role in cellular viability and proliferation of SCLC cells. Several reports have investigated the role of HIF-1α in survival and propagation of lung cancer cells *in vitro* and in tumor growth *in vivo*. For instance, repression of *HIF1A* using siRNA in the NSCLC cell line A549 resulted in growth arrest at hypoxia (Hanze et al., 2003). SCLC cells orthotopically injected into the lungs of mice treated with the HIF-1α inhibitor PX-478 demonstrated impaired tumor growth and progression. A disadvantage with several inhibitors is the specificity, and in this study no significant reduction of HIF-1α expression was displayed *in vivo* (Jacoby et al., 2010). In contrast, in the $Kras^{G12D}$-driven NSCLC model, HIF-1α deletion did not affect tumor growth (Mazumdar et al., 2010). However, HIF-1α seems to be involved in angiogenesis in our nude mice-xenograft model, since shHIF1A tumors displayed fewer blood vessels than the controls. In line with this data, it has previously been reported that HIF-1α stimulates angiogenesis in SCLC (Wan et al., 2011).

In the *HIF1A* knocked down cells the glucose metabolism was consequently reduced since HIF-1 activation stimulates glucose uptake, glycolysis and lactate production and represses mitochondrial respiration (Semenza, 2010). However, the glucose metabolism was still working in the shHIF1A cells, and the expression of genes involved in anaerobic glycolysis was not repressed to the same levels as in the normoxic control cells. This may be explained by other transcription factors, such as c-Myc, are stimulating this pathway. To our surprise and interest, the ATP levels were not diminished in shHIF1A cells despite reduced glucose metabolism. This suggests that these cells are using OXPHOS in addition to glycolysis for ATP production. One explanation could be that the entry of pyruvate into the TCA cycle is not blocked by PDK in the *HIF1A* knocked down cells. The cells can then use mitochondrial respiration to maintain the ATP levels and produce macromolecules, such as citrate for fatty acid synthesis. The increased production of ATP

through OXPHOS in shHIF1A cells would lead to increased oxygen consumption and consequently increased ROS levels (Papandreou et al., 2006). We were not detecting more cell death in the *HIF1A* knocked down cells, and one explanation could be increased production of glutathione. Glutathione is involved in regulating the redox status and stimulating cell survival, and glutamine is a source for glutathione synthesis.

To elucidate the importance of glucose metabolism for tumor cell viability and proliferation, we cultured control and *HIF1A* knocked down cells in glucose-free medium. To our surprise, there were no differences in growth rates and cell survival between *HIF1A* expressing and shHIF1A cells. However, the reduced proliferation we demonstrated was reflected by the diminished ATP levels in the cells. Interestingly, the SCLC cells were not dependent on glucose for cell viability. Recently, it has been demonstrated that glucose deprivation was not affecting the viability of *MYC*-inducible B-cell cells at hypoxia. Glutamine was continually metabolized in these cells and ATP, citrate and glutathione were synthesized and promoting cell survival (Le et al., 2012).

Glutamine is involved in several metabolic pathways to support cellular growth and proliferation. Myc is involved in the regulation of glutamine metabolism, and enhanced Myc expression has been reported to make tumor cells dependent on glutamine (Wise et al., 2008) (Qing et al., 2012). Consistent with data obtained in other tumor forms, *MYC* and *MYCL* amplified SCLC cells were dependent on glutamine for proliferation and survival at both normoxic and hypoxic conditions, independently of HIF. In addition, expression of genes involved in glutaminolysis and *de novo* lipogenesis were further increased in shHIF1A cells. Our results indicate that Myc overexpression overrides the need for HIF-1 for cell survival and propagation in response to hypoxia by inducing glutamine metabolism and *de novo* lipogenesis. This may explain why we cannot detect differences in cell survival and growth between control and *HIF1A* knocked down cells *in vitro* and *in vivo*.

Future perspectives

To study the metabolic pathways is very complex since they are often cooperating and the rate of the pathways is primarily regulated by the availability of substrates and products. Interestingly, since one of the *MYC* genes is frequently amplified in SCLC (Takahashi et al., 1989), the glutamine metabolism could be of great interest to study and maybe a good target in these cells. However, in this paper there are many unresolved issues. We aim to continue with the experiments where we inhibit the glutamine metabolism with siRNA or specific inhibitors against *MYC* or *GLS* and to investigate cell viability, ATP, glutathione and ROS levels in these cells compared to the control cells. Our results indicate that OXPHOS is up-regulated in the *HIF1A* knocked down compared to the control cells, and we would investigate this further using the Seahorse method. We are also interested to compare the expression levels of GLS and other enzymes involved in glutamine metabolism and *de novo* lipogenesis between *MYC* and non-*MYC* amplified

cell lines, and in the control and shHIF1A SCLC xenografts. In addition, we are interested in studying the hypoxic response more thoroughly in non-*MYC* amplified SCLC cells. It is tempting to speculate that these cells are more sensitive for cell death at hypoxia after *HIF1A* knockdown, or when culturing them in glucose-free medium. To further investigate the role of enhanced Myc expression in the adaptation response of SCLC cells would increase our understanding of the high survival capacity of SCLC cells to hypoxia.

Conclusions

In this thesis we have identified that SCLC lack the expression of the HIF-2α protein, whereas HIF-1α is abundantly expressed. We have also gained knowledge of the hypoxic adaptation response, and we found that the response is partially HIF-independent in *MYC* amplified cells. In addition, we have demonstrated that SCLC cells are sensitive for As_2O_3 treatment and the effect is sustained at hypoxic conditions.

We conclude that:

- As_2O_3 is cytotoxic to SCLC cells at clinically relevant concentrations both *in vitro* and *in vivo* and the effect is sustained at hypoxia (*Paper I*)
- HIF-2α protein is virtually absent in SCLC tumor specimens and cell lines, while HIF-1α protein expression is sustained and accumulated over time in hypoxic cells (*Paper II*)
- SCLC cells have a high capacity to adapt to moderate and severe hypoxia, despite a modest induction of hypoxia-inducible genes (*Paper II* and unpublished microarray data)
- The adaptive response to hypoxia is partially HIF-independent and the UPR is important for SCLC cell survival at severe hypoxia (*Paper II*)
- Limited effect on cell survival and growth *in vitro* and *in vivo*, although HIF-1α expression is substantially repressed (*Paper II and III*)
- *MYC* and *MYCL* amplified SCLC cells are dependent on glutamine for cell viability and propagation at normoxic and hypoxic conditions (*Paper III*)

Populärvetenskaplig sammanfattning

Cancer är ett samlingsnamn för en grupp av sjukdomar som karakteriseras av att cellerna delar sig okontrollerat, till skillnad mot kroppens friska celler. Det leder till att en tumör kan bildas och cellerna kan därifrån sprida sig till andra delar av kroppen och ge upphov till dottertumörer, även kallade metastaser. I Sverige uppskattar man att cirka var tredje person kommer någon gång under sin livstid att drabbas av cancer. Lungcancer är den tumörform som flest människor dör utav och i världen mister cirka 1,3 miljoner människor livet årligen av sjukdomen. Lungcancer klassificeras i två subgrupper beroende på tumörcellernas specifika kännetecken och utifrån det bestäms valet av behandlingsmetod. Majoriteten av lungcancerpatienter drabbas av icke-småcellig lungcancer, medan 15-18% av tumörerna klassificeras som småcellig lungcancer (SCLC). En starkt bidragande orsak till lungcancer är tobaksrökning och SCLC är en av de tumörformer som är starkast kopplad till rökning. SCLC är en mycket aggressiv tumörform på grund av att tumörcellerna delar sig väldigt fort och sprider sig snabbt till andra organ i kroppen, till följd av det är prognosen väldigt dålig för dessa patienter. Dagens behandling för SCLC-patienter innefattas av cellgifter, vilket patienterna till att börja med svarar väldigt bra på. Tyvärr får en stor grupp av patienterna återfall inom en tvåårsperiod på grund av att tumörcellerna utvecklar okänslighet mot flera cellgifter. Det är ett av de största hindren mot effektiv behandling och idag finns det inget bra läkemedel som kan användas mot återfallen.

I tusentals år har arsenikföreningar använts för behandling av flertalet sjukdomar, men när modernare behandlingsformer som cellgifter och strålning kom in i bilden slutade man använda arsenik. På 1970-talet började man undersöka arseniktrioxids celldödseffekt på cancerformen akut promyelocytisk leukemi (APL). Det visade sig senare vara en mycket effektiv behandling med lindriga biverkningar och idag används arseniktrioxid som behandling av APL-patienter som har fått återfall. Arseniktrioxids effekt på flertalet andra tumörformer har sedan dess studerats och forskning i vår grupp har tidigare visat att tumörceller etablerade från barncancerformen neuroblastom är känsliga för arseniktrioxid, trots att de inte är känsliga för cellgifter. I *artikel I* har vi studerat arseniktrioxids celldödseffekt på SCLC-celler i jämförelse med cellgifter som används i kliniken idag. Vi har funnit att SCLC-celler dör när vi utsätter dem för kliniskt relevanta koncentrationer av arseniktrioxid och att tumörtillväxten hämmas hos möss. Vi visar även att effekten bibehålls vid låga syretryck, vilket är känt att minska tumörcellers känslighet mot cellgifter.

Kroppens alla celler behöver näring och syre för att kunna växa och dela sig. Syrefattiga (hypoxiska) område uppstår ofta i tumörer till följd av tumörcellernas snabba delningsförmåga och försämrad blodtillförsel. Det beror på att nya blodkärl inte hinner bildas i samma takt som tumören växer samt att de nybildade kärlen oftast inte är lika funktionella. Cellerna anpassar sig till omgivningen genom att stabilisera och aktivera proteinerna Hypoxia-Inducible Factor (HIF)-1α och HIF-2α. Det bidrar till förändrad aktivitet i cellen och processer som kräver mindre energi samt nybildande av kärl främjas för att kunna trygga cellens överlevnad och tillväxt. Tidigare studier har visat att HIF-1α och HIF-2α reglerar anpassningen till syrebrist vid olika tidpunkter, då HIF-1α aktiveras vid korta tidpunkter medan HIF-2α vid mer långvarig hypoxi. Tumörcellers anpassning till syrebrist är starkt kopplat till ökad tumöraggressivitet och sämre prognos.

I denna avhandling har jag framförallt studerat förekomsten av HIF-1α och HIF-2α i SCLC samt tumörcellernas anpassningsförmåga till den låga syretillgången. Vi har funnit att SCLC saknar HIF-2α, medan HIF-1α förekommer vid både kortare och längre tidpunkter av syrebrist (*Artikel II*). Tumörcellerna anpassar sig mycket väl till miljön trots att regleringen endast drivs av HIF-1α. Genom att behandla tumörcellerna med en substans så att förekomsten av proteinet HIF-1α hämmas, har vi kunnat studera betydelsen av HIF-aktivitet för överlevnad och celldelning vid syrebrist (*Artikel II och III*). Till vår förvåning fann vi att SCLC-celler utan HIF-1α överlever vid syrebrist och delar sig i samma hastighet som de celler som har proteinet HIF-1α. Vi kunde även visa att tumörtillväxten i möss inte påverkas, vilket tyder på att SCLC-cellers anpassning till syrebrist inte är beroende av HIF-aktivitet.

Vid syrebrist främjar HIF-1α en nedbrytningsprocess av sockerarten glukos för att tumörcellerna ska kunna producera energi. Intressant är att de SCLC-celler som inte har HIF-1α fortfarande kan behålla samma energinivå som de tumörceller som har rikligt med HIF-1α (*Artikel III*). Våra resultat pekar på att SCLC-celler även använder sig utav glutamin för att kunna bilda energi och byggstenar, oberoende av syretillgången. Det stimulerar SCLC-cellernas överlevnad och dess snabba celltillväxt. Våra resultat tyder på att nedbrytningsprocessen av glutamin stimuleras när tillgången till proteinet HIF-1α hämmas. Denna process är inte beroende av HIF-1α utan denna reglering styrs av proteinet Myc och på grund av genetiska förändringar finns ofta Myc-proteinet i stor mängd i SCLC celler. Våra resultat antyder att SCLC-celler som har höga nivåer av proteinet Myc anpassar sig till en syrefattig miljö genom att stimulera glutamin-nedbrytning, vilket är oberoende av HIF-aktivitet.

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