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Hypoxia and Stem Cells in Normal and Tumor Development

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DEPARTMENT OF LABORATORY MEDICINE | LUND UNIVERSITY

Hypoxia and Stem Cells in Normal and Tumor Development

Hypoxia and Stem Cells in Normal and Tumor Development

Camilla Persson

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended at Building 302 Lecture hall, Medicon Village, Lund.

Thursday 21st of November 2019, at 09:00 am.

Faculty opponent
Senior Professor Bengt Westermark
Immunology, Genetics and Pathology (IGP)
Uppsala University
Uppsala, Sweden

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Abstract

Neuroblastoma is a childhood malignancy of the sympathetic nervous system and accounts for 15% of all cancer-related deaths in children. To understand the biology behind neuroblastoma, clarify timing of cellular events and expression profiles of proteins preceding neuroblastoma initiation, we need more relevant preclinical models.

In the first part of this thesis, we established two preclinical *in vitro* models of neuroblastoma: patient-derived xenograft (PDX) cells derived from an orthotopic PDX model of high-risk neuroblastoma and chick embryo derived trunk crestospheres. We demonstrate that PDX cells can be cultured as spheres in stem cell promoting medium with retained patient tumor characteristics and maintained tumorigenic and metastatic capacity. However, addition of serum to the culture media resulted in loss of their immature phenotype and induced neuronal differentiation, while adherent culture on laminin maintained cells in an undifferentiated state. Further, we also isolated and optimized culture conditions for chick embryo derived trunk crestospheres, comprised of both neural crest stem and progenitor cells. We demonstrate that these crestospheres are multipotent, display self-renewal capacity over several weeks *in vitro* and can be manipulated via lentiviral transduction.

In the second part of this thesis, we first demonstrate that immature mesenchymal-type neuroblastoma cells are resistant to retinoic acid (RA), a differentiating agent used as a component for treatment of high-risk neuroblastoma. We further demonstrate that mesenchymal-type neuroblastoma cells had endogenous synthesis of RA, is dependent on RA for their proliferation and migration and clustered closely with normal peripheral glia stem cells called Schwann cell precursors (SCPs). Together these data indicate that the endogenous dependency on RA in mesenchymal-type neuroblastoma cells might play a role in the acquired resistance towards RA treatment in the clinic. We finally demonstrate that hypoxia-inducible factor (HIF)-2α, a transcription factor involved in cellular adaption to low oxygen levels, is highly expressed at oxygenated conditions both *in vitro* an *in vivo* in neuroblastoma, particularly in the cytoplasmic fraction. We further show that treatment with the HIF-2α transcriptional inhibitor PT2385 had no effects on HIF-2 downstream targets, in contrast to HIF-2α protein knockdown, suggesting that HIF-2α possesses additional, non-canonical functions in neuroblastoma.

Key words: Cancer, Neuroblastoma, Hypoxia, Preclinical cancer models, ALDH, HIF-2α, PT2385

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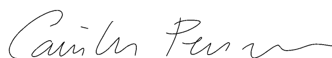
Signature  Date 2019-10-16

Hypoxia and Stem Cells in Normal and Tumor Development

Camilla Persson

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List of Papers

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

I. Neuroblastoma patient-derived xenograft cells cultured in stem-cell promoting medium retain tumorigenic and metastatic capacities but differentiate in serum
Persson CU, von Stedingk K, Bexell D, Merselius M, Braekeveldt N, Gisselsson D, Arsenian-Henriksson M, Påhlman S, Wigerup C.
Scientific Reports. 2017, 7(1):10274

II. Maintaining multipotent trunk neural crest stem cells as self-renewing crestospheres
Mohlin S, Kunttas E, **Persson CU**, Abdel-Haq R, Castillo A, Murko C, Bronner ME, Kerosuo L.
Developmental Biology. 2019, 447(2):137-146

III. Immature neuroblastoma cells are resistant to retinoic acid and synthesize this drug
Van Groningen T*, **Persson CU***, Chan A, Akogul N, Westerhout E, von Stedingk K, Hamdi M, Valentijn L, Mohlin S, Stroeken P, Hasselt N, Haneveld F, Lakeman A, Zwijnenburg D, van Sluis P, Bexell D, Adameyko I, Wigerup C, Påhlman S, Koster J, Versteeg R, van Nes J.
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IV. HIF-2 transcriptional activity is not sufficient to regulate downstream target genes in neuroblastoma suggesting a non-transcriptional role of HIF-2α
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I. Neuroblastoma associated genes are enriched in trunk neural crest.
Persson CU and Mohlin S.
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II. Promoter-associated proteins of EPAS1 identified by enChIP-MS – a putative role of HDX as a negative regulator
Hamidian A, Vaapil M, von Stedingk K, Fujita T, **Persson CU**, Eriksson P, Veerla S, De Preter K, Speleman F, Fujii H, Påhlman S, Mohlin S.
Biochemical and Biophysical Research Communication. 2018, 499(2):291-298

III. Combined BET bromodomain and CDK2 inhibition in MYC-driven medulloblastoma.
Bolin S, Borgenvik A, **Persson CU**, Sundström A, Qi J, Bradner JE, Weiss WA, Cho YJ, Weishaupt H, Swartling FJ.
Oncogene. 2018, 37(21):2850-2862

IV. HIF2alpha contributes to antiestrogen resistance via positive bilateral crosstalk with EGFR in breast cancer cells.
Alam MW, **Persson CU**, Reinbothe S, Kazi JU, Rönnstrand L, Wigerup C, Ditzel HJ, Lykkesfeldt AE, Påhlman S, Jögi A.
Oncotarget. 2016, 7(19):11238-50.

Abbreviations

ADH	Alcohol dehydrogenase
ADRN	Adrenergic
ALDH	Aldehyde dehydrogenase
ALK	Anaplastic lymphoma kinase
ALT	Alternative lengthening of telomere
AML	Acute myeloid leukemia
ARNT	Aryl hydrocarbon receptor nuclear translocator
ATRX	α-thalassemia/mental retardation syndrome X-linked
bFGF	basic fibroblast growth factor
bHLH-PAS	basic-helix-loop-helix-Per-Arnt-Sim
BMP	Bone morphogenetic protein
CAIX	Carbonic anhydrase 9
CBP	CREB binding protein
CCHS	Congenital hypoventilation syndrome
ccRCC	Clear cell renal cell carcinoma
CHGA	Chromogranin A
CNS	Central nervous system
CRC	Core regulatory circuitries
C-TAD	Transactivating domain, C-terminal
DBH	Dopamine β-hydroxylase
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transition
ENS	Enteric nervous system
EPO	Erythropoietin
GEMMs	Genetically engineered mouse models
GM-CSF	Granulocyte macrophage colony stimulating factor
HIF	Hypoxia-inducible factor
HRE	Hypoxia response elements
IL-2	Interleukin-2
INSS	International Neuroblastoma Staging System
INRG	International Neuroblastoma Risk Group
LDHA	Lactate dehydrogenase A
MES	Mesenchymal
NCAM	Neural cell adhesion molecule
NF	Neurofilament
NGF	Nerve growth factor
NSCLC	Non-small cell lung carcinoma
NSE	Neuron specific enolase
NT-3	Neurotrophin 3
N-TAD	Transactivating domain, N-terminal
ODD	Oxygen-dependent degradation domain
p300	300-kilodalton co-activator protein
PDK1	Pyruvate dehydrogenase 1
PDX	Patient-derived xenograft
PHOX2B	Paired–like homeobox 2B
PKM	Pyruvate kinase
PNMT	Phenylethanolamine N-methyltransferase
PNS	Peripheral nervous system
RA	Retinoic acid
RAR	Retinoic acid receptor
RAREs	Retinoic acid response elements
ROS	Reactive oxygen species
RXR	Retinoid x receptor
SCID	Severe combined immunodeficiency
SCG2	Secretogranin II
SCG10	Secretogranin 10
SCP	Schwann cell precursor
SIF	Small intensely fluorescent
SNS	Sympathetic nervous system
TERT	Telomerase reverse transcriptase
TH	Tyrosine hydroxylase
TrkA/B/C	Tropomyosin receptor kinase A/B/C
VEGF	Vascular endothelial growth factor
VHL	von Hippel-Lindau
VIM	Vimentin

Abstract

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Chapter 1.

Tumor Development

Overview

Cancer is a broad term used for a group of diseases characterized by uncontrolled growth of abnormal cells within the blood or organs, causing tumor masses in the latter. In some cases, tumor cells will detach from the tumor and spread to other organs of the body to form metastasis (Hanahan and Weinberg, 2000).

As the second leading cause of death globally in 2018, it was estimated that there would be 18.1 million new cancer cases and 9.6 million cancer death in 2018 (Bray et al., 2018). The cancer incidence and mortality are increasing rapidly worldwide and cancer is expected to become the leading cause of death in every country of the world in the 21st century (Bray et al., 2018). This escalation is both due to aging and growth of the human population as well as an increase in the prevalence and distribution of the main risk factors for cancer (Gersten, 2002; Omran, 2005)

Cancer is a multistep process where the transformation of a normal cell into a malignant cell is the consequence of accumulated genetic alterations overtime within the cellular DNA that, in the end, promote survival advantages. In this way, the restricted growth potential is lost and malignant cells acquire limitless number of cell divisions along with the ability to spread and invade into distant organs. In order to support their rapid growth, tumors cells also need to ensure adequate oxygen and energy supply, which is acquired by the formation of new blood vessels. Shifting the energy metabolism along with changes in the immune system have also been shown to be important requirements for survival and progression of tumors. Together, these tumor requirements are nowadays summarized as the *Hallmarks of cancer*. The current hallmarks are: (1) sustaining proliferative signalling, (2) evading growth suppressors, (3) evading cell death, (4) replicative immortality, (5) inducing angiogenesis, (6) tissue invasion and metastasis, (7) genome instability, (8) evading immune destruction, (9) reprogramming metabolism and (10) tumor-promoting inflammation (Hanahan and Weinberg, 2000, 2011).

It is crucial to devise more effective cancer therapies since both the cancer incidence and mortality increases worldwide. One way of improving current treatment protocols is to define which tumor cell, or population of tumor cells, that have the

capacity to initiate and/or drive the disease. Theories on the origin of cancer have been discussed since the 19th century, and during the years, two main models have emerged; *clonal evolution model* and *cancer stem cell model* (Figure 1).

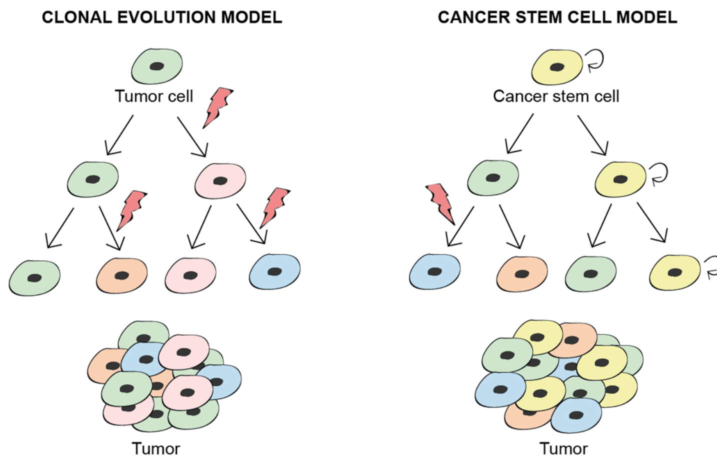

Figure 1. Clonal evolution model vs Cancer stem cell model.
Illustration of tumor development by clonal evolution model or cancer stem cell model. The clonal evolution model proposes that any cell can to transform into a malignat cell and give rise to a tumor through stepwise accumulation of genetic alterations. In contrast, the cancer stem cell model suggests that tumors are formed and progressed by a subpopulation of cells that have, or have acquired, stem-like properties.

Clonal Evolution Model

The clonal evolution model is the classical model of tumor development. The term *clonal evolution* was first used in 1976 when Peter Nowell tried to summarize emerging data suggesting that cancer was a result of malignant transformation of a single cell (Nowell, 1976). The clonal evolution model proposes that tumors arise when any cell, independent of developmental status, acquire genetic alterations that result in growth advantages (e.g. by making cells more prone to proliferate or less responsive to cell death cues) over the adjacent cell (Greaves and Maley, 2012). Individual tumor cells will stepwise acquire additional mutations due to genetic instability, resulting in subclones displaying unique genetic profiles with different traits of advantages, including the ability to metastasize and altered sensitivity towards radio- and chemotherapy. These subclones will seed different parts of the tumor and are constantly going through an adaption process to select for the fittest clones as the different clonal advantages may differ during the tumor development. As a result of the branching evolution along with the of natural selection of clones, tumors display significant intratumor heterogeneity (Burrell et al., 2013; Gerlinger et al., 2012; Greaves and Maley, 2012; Prasetyanti and Medema, 2017), a feature known to contribute to both treatment failure and disease progression.

Cancer Stem Cell Model

A model that have emerged and gained a lot of attention lately is the cancer stem cell model. An early version of this model was put together already in 1876 by Julius Cohnheim (Cohnheim, 1875). In his work, he observed similarities between tumor cells and embryonic cells and suggested that cancers arise from remnants of the embryonic development.

The cancer stem cell model proposes that tumors are maintained and propagated by a subpopulation of cells within the tumor that have, or have acquired, stem-like properties (Kreso and Dick, 2014; Plaks et al., 2015). This subpopulation of cells is the cancer stem cells. The first experimental support (although unethical) for the cancer stem cell model was demonstrated in the 1960s (Southam, 1961). By transplanting patient-derived cancer cells back to patients subcutaneously after surgery, Southam and Brunschwig observed that at least 1,000,000 injected tumor cells were required in order to form a new tumor, suggesting that the ability to initiate tumor growth is not equal among all tumor cells. Successful isolation of a cell population with traits of cancer stem cells was first performed in John Dick's laboratory in 1994 (Lapidot et al., 1994). By transplanting cells from human acute myeloid leukemia (AML) into NOD/SCID mice, they found that it was only the $CD34^+/CD38^-$ subpopulation of cells that was able to engraft and form leukemia in mice. Since then, expression of distinct surface markers has been used for cell purifications followed by transplantation in immunodeficient mice as 'the golden standard' to identify functional cancer stem cell populations. With this method, subpopulations of tumor cells with stem-like traits have been described in numerous cancers including breast ($CD44^+/CD24^-$, (Al-Hajj et al., 2003)), brain ($CD133^+$, (Singh et al., 2004)), head and neck ($CD44^+$, (Prince et al., 2007)) and colon ($CD133^+$, (Ricci-Vitiani et al., 2007). However, in some tumor types, it has not been possible to distinguish cancer stem cells from bulk tumor cells as most cells possess these stem-like features (Kreso and Dick, 2014). Moreover, the interpretation of these studies has been further complicated by the finding that the efficiency of tumor formation among the purified cell populations can be very much affected by the host animal, e.g. the level of immunodeficiency (Quintana et al., 2008).

The origin and characteristics of a cancer stem cell

The cancer stem cell model does not stipulate how the stem cell-like properties have been acquired and at the moment two hypothesize exists. The first hypothesis suggests that the cancer stem cells originate from normal stem cells that have undergone malignant transformation (Plaks et al., 2015). However, the second hypothesis instead proposes that a more mature transformed cell acquires these features via dedifferentiation as a result of cellular plasticity or via mutations that

activates e.g. the self-renewal machinery (Kreso and Dick, 2014). The notion about cellular plasticity is a likely explanation since lineage-tracing studies have showed that committed cells can move up and down in the hierarchy of differentiation in normal tissues (Buczacki et al., 2013; Kusaba et al., 2014; Tata et al., 2013; Tetteh et al., 2016; Tian et al., 2011a; van Es et al., 2012).

By definition, both normal tissue stem cells and cancer stem cells are long-lived and possess the capacity to self-renew and differentiate into multiple cell lineages, i.e. multipotency (Batlle and Clevers, 2017). Self-renewal is a key biological process for stem cells in order to maintain the stem cell pool, and in the asymmetric division, which produces one stem cell and one progenitor cell, the progenitor cell can differentiate into a more mature cell. Thus, while tissue-specific stem cells give rise to the different cell types within an organ, the cancer stem cells are believed to give rise to all different tumor cell types within a tumor (Batlle and Clevers, 2017; Kreso and Dick, 2014; Plaks et al., 2015). Furthermore, as adult stem cells are not necessarily quiescent, but instead can divide actively throughout life (Barker et al., 2010; Barker et al., 2007), it is assumed that cancer stem cells possess the same ability. For this reason, the maintenance and metastatic spread of a tumor is thought to be more dependent on the cancer stem cells since the bulk tumor cells are more short-lived (Kreso and Dick, 2014).

The cancer stem cells are also believed to confer therapy resistance (Kreso and Dick, 2014; Plaks et al., 2015). The resistance towards radio- and chemotherapy was initially regarded as an intrinsic property of cancer stem cells, acquired through, for example, upregulation of drug efflux pumps (e.g. ABC transporters), increased expression of anti-apoptotic proteins, enhanced DNA repair system or increased protection against reactive oxygen species (ROS) (Bao et al., 2006; Borst, 2012; Diehn et al., 2009; Holohan et al., 2013; Li et al., 2008b). However, emerging data have shown that cellular plasticity is an extremely important driver of therapy resistance. The ability of quiescent or slow-growing cancer stem cells to resist treatment and to later enter the cell cycle and cause tumor relapse have been observed in both leukaemia and solid tumors (Batlle and Clevers, 2017; Cronkite, 1970; Kreso et al., 2013; Kurtova et al., 2015; Oshimori et al., 2015). Moreover, it has also been shown that cancer stem cells can enter a slow proliferative state in order to evade anti-proliferative therapies (Liau et al., 2017).

Cancer stem cell vs Tumor-initiating cell

The definition of a cancer stem cell has evolved during the last years. It was originally thought that cancer stem cells constituted a minor subpopulation of immature, self-renewing and multipotent cells within the tumor that had the capacity to initiate tumor growth. However, recent data suggests that the cancer stem cells can comprise a larger proportion of the tumor (Johnston et al., 2010). Moreover, as

several studies have reported a dramatic difference in the initiating capacities of proposed cancer stem cells, e.g. in melanoma (Quintana et al., 2008), it is no longer assumed that the tumor-initiating capacity is only limited to the cancer stem cells. As such, some researchers are now using the term *tumor-initiating cell* when discussing tumor cells that possesses the capacity to initiate tumor growth (Batlle and Clevers, 2017). But, this has caused confusion in the cancer field since many researchers are using the terms *cancer stem cells* and *tumor-initiating cell* interchangeably. Although the tumor-initiating cells and the cancer stem cells are two cell populations with the capacity to self-renew and give rise to more differentiated tumor cells, they do not necessarily refer to the same cell *per se*. Recent data suggests that the tumor-initiating cell is the cell-of-origin and is responsible for the initial growth of the tumor. On the other hand, the cancer stem cells are instead thought to be responsible for the maintenance and sustained growth of the tumor as well as the metastatic spread. They are also thought to promote tumor heterogeneity through their ability to give rise to more differentiated tumor cells and to confer multidrug resistance, which in turn is associated with tumor relapse (Baccelli and Trumpp, 2012; Batlle and Clevers, 2017; Rycaj and Tang, 2015; Visvader, 2011). This means that traits like metastatic potential and intrinsic multidrug resistance has been added later on to the 'original' definition of a cancer stem cell.

Aldehyde dehydrogenase: A marker of stemness

It is not only the expression of distinct surface markers that have been used for isolation of cancer stem cells, the presence of enzymes crucial for stemness has also been utilized. One such enzymatic stem cell marker is aldehyde dehydrogenase (ALDH). ALDH consists of 19 genes that is subdivided into 11 families and their normal function is to convert a wide range of endogenous and exogenous aldehydes to their corresponding carboxylic acid (Ma and Allan, 2011). Aldehydes are highly reactive compounds that can cause damage to both cellular DNA and protein. In addition to their detoxifying property, ALDHs are also important for the biosynthesis of retinoic acid (RA), a metabolite of vitamin A_1. RA induces transcription of gene and regulates numerous cellular processes like cell proliferation, differentiation, cell cycle arrest and apoptosis (Marcato et al., 2011; Xu et al., 2015). In the RA signaling pathways, retinol (vitamin A_1) is taken up by the cell, and once inside, it is oxidized by alcohol dehydrogenase (ADH) to retinal. Retinal is further oxidized by cytoplasmic ALDH enzymes to RA. By diffusing into the nucleus, RA binds to heterodimers of retinoic acid receptor (RAR) and retinoid x receptor (RXR). To induce gene transcription, the activated receptor complex binds to regulatory sequences in target genes called retinoic acid response elements (RAREs) (Figure 2).

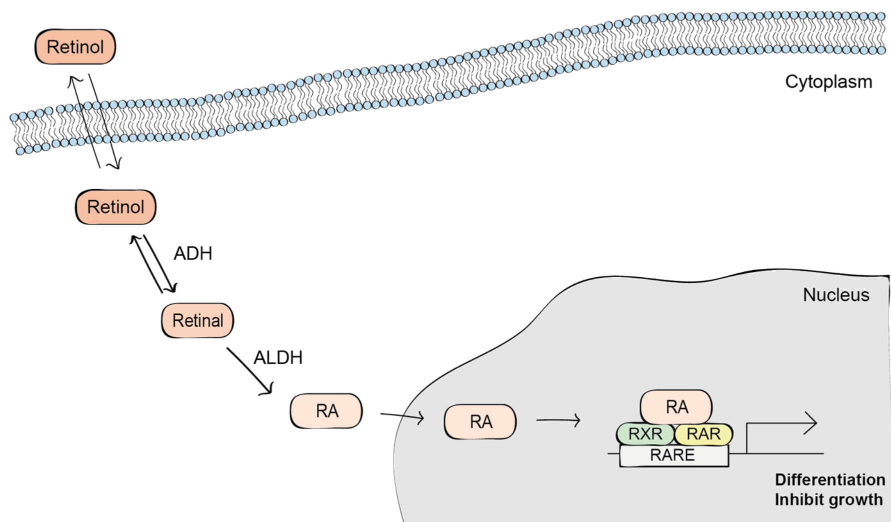

Figure 2. Schematic illustation of the RA signalling pathway.
Retinol (Vitamin A_1) is absorbed by cells and oxidized to retinal in the cytoplasm by ADH. Retinal is further oxidized to RA by cytoplasmic ALDH enzymes, and after diffusing into the nucleus, RA binds to heterodimers of RAR and RXR to induce expression of downstream targets at specific regulatory sequences known as RAREs. ADH, alcohol dehydrogenas; ALDH, aldehyde dehydrogenase; RAR, retinoic acid receptor; RA, retinoic acid; RXR, retinoid x receptor; RAREs, retinoic acid response elements.

The development of the Aldefluor flow cytometry assay facilitated the usage of ALDH as a stem cell marker (Jones et al., 1995; Storms et al., 1999), and early studies revealed that elevated ALDH activity could be used for isolating murine and human hematopoietic, neural stem and neural progenitor cells (Armstrong et al., 2004; Hess et al., 2004; Hess et al., 2006; Matsui et al., 2004; Pearce and Bonnet, 2007; Storms et al., 2005; Storms et al., 1999). ALDH has been used as a cancer stem cell marker numerous cancers, including leukemia, breast, liver, lung, prostate and pancreas, where high ALDH activity has been associated with self-renewal, clonogenic growth, tumor-initiating capacity, poor clinical outcome as well as drug resistance (Al-Hajj et al., 2003; Ginestier et al., 2007; Jimeno et al., 2009; Lapidot et al., 1994; Li et al., 2010; Ma et al., 2008; Matsui et al., 2004; Moreb et al., 2008). Resistance towards radio- and chemotherapy have been observed in ALDH positive cells in both leukemia and solid tumors (Hilton, 1984; Honoki et al., 2010; Magni et al., 1996; Raha et al., 2014; Sladek et al., 2002; Sun et al., 2011; Tanei et al., 2009), and might, to some extent, be explained by the metabolic activity of the ALDH enzymes (Ma and Allan, 2011). For example, in hematopoietic stem cells, it has been shown that high ALDH enzymatic activity have the capacity to metabolize and detoxify cytotoxic drugs through oxidation of a specific aldehyde group of the drug (Magni et al., 1996). Collectively, these findings support a central role for ALDH in tumor initiation and progression.

The role of ALDHs has not been studied extensively in childhood tumors. As of today, only a few studies have reported a functional role of ALDH enzymes in neuroblastoma, which is the childhood malignancy this thesis will focus on. Specifically, increased expression of *ALDH1A2* and *ALDH1A3* has been identified in a subpopulation of neuroblastoma cells (Coulon et al., 2011; Flahaut et al., 2016; Hartomo et al., 2015). Expression of *ALDH1A2* has been found to be upregulated in spheres derived from patient bone marrow metastatic neuroblastoma cells that had been serially passaged *in vitro*. Further analysis showed that *ALDH1A2* played an important role for the self-renewal and stemness traits of these cells (Coulon et al., 2011). *ALDH1A2* has also been shown to promote *in vivo* tumor growth as well as an undifferentiated tumor phenotype in neuroblastoma and to correlate with poor prognosis (Hartomo et al., 2015). Similarly, expression of *ALDH1A3* has been found to be overexpressed in patients with worse clinical outcome and to play a role in tumor progression and chemo-resistance (Flahaut et al., 2016). However, as the amount of data on ALDH in neuroblastoma is limited, and also somewhat questioned in regards to performance and data interpretation, further studies are needed to elucidate if ALDH play an important role in neuroblastom and can be used as a treatment target for high-risk patients.

Chapter 2.

Sympathetic Nervous System Development

Overview

The human nervous system can be divided into two parts, the central nervous system (CNS), which is comprised of the brain and spinal cord, and the peripheral nervous system (PNS), which consists of all of the nerves and ganglia outside the CNS. The PNS can in turn be divided into the somatic and the autonomic nervous system. As part of the autonomic nervous system, the sympathetic nervous system (SNS) is triggered by stress and mediates the stress-induced "fight-or-flight" response. The main cell types of the SNS are the sympathetic neurons (known as neuroblasts during development), small intensely fluorescent (SIF) cells and chromaffin cells, and they all originate from a common neural crest-derived sympathoadrenal progenitor cell. While the function of SIF cells is unknown, sympathetic neurons and chromaffin cells have an important role of regulating most organs by transmitting catecholamines such as adrenaline and noradrenaline. Inaccurate or disturbed differentiation of SNS cells can result in various medical conditions like Hirschsprung disease, but also malignant transformation followed by development of for example the childhood malignancy neuroblastoma (Vega-Lopez et al., 2018).

Neural Crest

The neural crest is a transient embryonic structure that is unique to vertebrates and was first described in the chick embryo by Wilhem His in 1868 (His, 1868). This cell population arises at the border of the neural plate as a result of inducing signals during gastrulation (Figure 3A). These signals are timely and spatially coordinated for proper establishment of the neural crest and include Wnts, RA, fibroblast growth factor (FGF) and bone morphogenetic proteins (BMPs) (Lewis et al., 2004; Mayor and Aybar, 2001; Villanueva et al., 2002). Together, these factors upregulate expression of a set of genes, known as the neural plate border specifiers, which are

crucial for establishment of neural crest identity and include *PAX3*, *PAX7* and *TFAP2B* (Sauka-Spengler et al., 2007).

In the following process, known as neurulation, the neural crest emerges as a structure at the dorsal region of the neural tube after invagination and closure of the neural plate (Bronner and LeDouarin, 2012). In this premigratory phase, the neural crest precursors reside as a cell population within the neural tube and are characterized by the expression of various transcription factors such as *FOXD3*, *SOX10*, *SNAIL2*, *SOX9* and *MYC*. Together, these transcription factors are known as the neural crest specifier genes and their function is to modulate effector genes that regulate adhesive properties, shape, motility, differentiation as well as the signalling machinery of the neural crest progenitors for proper fate specification (Khudyakov and Bronner-Fraser, 2009; Sauka-Spengler et al., 2007).

Once induced by neural crest specifiers, the premigratory precursors undergo epithelial to mesenchymal transition (EMT) to enable delamination from the neural tube followed by migration throughout the developing embryo (Bronner and LeDouarin, 2012). Activation of BMP signaling in combination with upregulation of the Wnt pathway is essential for the transition into this migratory phase (Ahlstrom and Erickson, 2009). Single cell analysis of early migrating neural crest cells *in vivo* (Baggiolini et al., 2015; Bronner-Fraser and Fraser, 1988) and clonal analysis i*n vitro* (Calloni et al., 2009) has shown that the majority of cells are multipotent at this stage. However, as neural crest cells migrate along well-defined routes, their potential to differentiate into various cell types become more and more restricted via cues in the microenvironment. These cues, which are highly coordinated, involve both cell-cell and cell-environment interactions and are crucial for proper neural crest cell guidance, differentiation and cell-lineage specification (Kasemeier-Kulesa et al., 2005; Kulesa et al., 2000; McLennan and Kulesa, 2007; Teddy and Kulesa, 2004). Finally, after reaching their pre-determined destination, neural crest cells differentiate into a wide range of derivatives.

Neural crest derivatives

Derivatives from the neural crest originates from four distinct segments on the rostro-caudal embryonic axis. These segments of the neural tube are referred to as cranial, vagal, trunk and sacral (see Figure 3B, (Bronner and LeDouarin, 2012)). The cranial neural crest is located in the anterior part of the embryo and is essential for the formation of bone and cartilage of the head, the carotid body, different eye tissues and the cranial meninges surrounding the brain (Dupin and Coelho-Aguiar, 2013). The vagal crest includes the level of somites 1-7 (Bronner and LeDouarin, 2012) and contributes to the development of structures such as the heart, enteric ganglia of the gut and PNS (Lajiness et al., 2014; Vega-Lopez et al., 2018; Verberne et al., 2000). The trunk neural crest is the largest segment and comprises the level

of somites 8-28 (Bronner and LeDouarin, 2012), and contributes to chromaffin cells, Schwann cells, melanocytes, neurons of the enteric nervous system (ENS) as well as neurons and glia cells of the dorsal root ganglia and the sympathetic ganglia (Fontaine-Perus et al., 1982; Lallier and Bronner-Fraser, 1988; Teillet et al., 1987; Weston, 1963). Finally, sacral neural crest is the most posterior segment and innervates the lower urogenital tract and generates neurons of the ENS (Vega-Lopez et al., 2018). Thus, depending on the axial level of origin in the developing embryo, the neural crest cells follow different migratory pathways and give rise to a unique set of derivates.

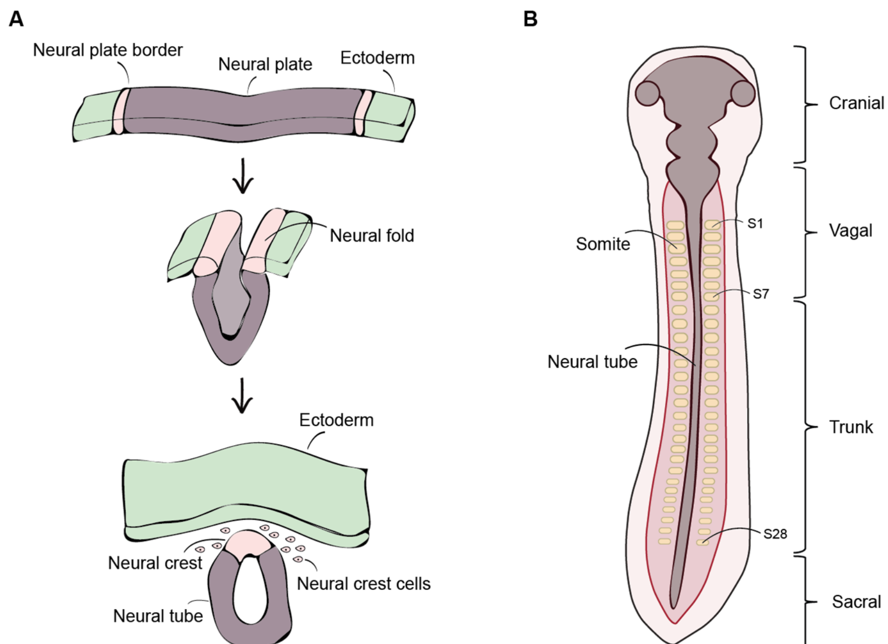

Figure 3. Development of neural crest.

(A) The neural crest is a multipotent and highly migratory cell population that forms transiently in the developing embryo. It arises at the border of the neural plate, the region in between the neural plate and the adjacent non-neural ectoderm. Neural crest cells migrate along defined routes to differentiate into a wide range of neural crest derivates. (B) Schematic view of the four distinct neural crest segments along the rostro-caudal embryonic axis illustarted in a chick embryo: cranial, vagal, trunk and sacral. S1, S7 and S28 refers to somite numbers.

Sympathoadrenal Cell Lineage

Neural crest cells from the trunk region can undertake three major migratory routes to their pre-determined destination from the neural tube. The first wave of trunk neural crest cells will follow the ventromedial pathway. Upon somite maturation, the second wave of cells undertake the ventrolateral route through the somites to

give rise to Schwann cells and dorsal root ganglia, whereas the last wave of trunk neural crest cells will migrate along the dorsolateral pathway and contribute to the formation of melanocytes (Gammill and Roffers-Agarwal, 2010; Krispin et al., 2010). The subpopulation of trunk neural crest cells leaving in the first wave, receives signals from the somites, ventral neural tube and notochord to aggregate by the dorsal aorta (Gammill and Roffers-Agarwal, 2010; Loring and Erickson, 1987). This aggregated cell population constitute the sympathoadrenal progenitor cells (Anderson and Axel, 1986; Anderson et al., 1991) and they contribute to sympathetic neurons, neuroendocrine chromaffin cells and SIF cells (Figure 4).

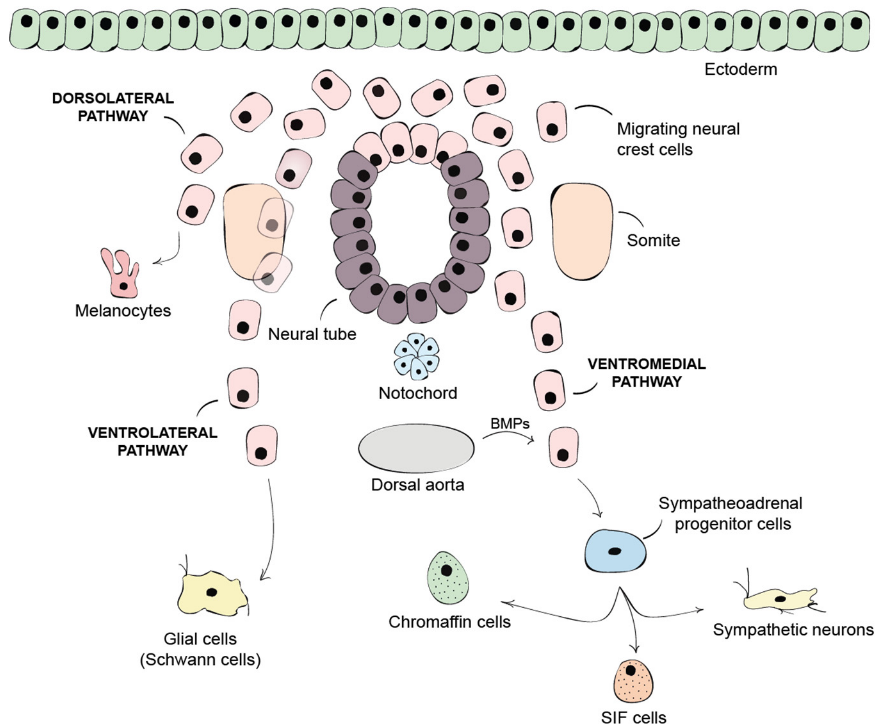

Figure 4. Migration and derivatives of trunk neural crest cells.
Schematic illustation of migrating trunk neural crest cells in the developing embryo. In the ventromedial pathway, trunk neural crest cells migrate in the interspace between the neural tube and the developing somites to aggregate by the dorsal aorta. At the dorsal aorta, trunk neural crest cells commit to the sympathoadrenal lineage by being exposed to BMPs. Fate-restricted sympathoadrenal cells contribute to chromaffin cells, SIF cells and sympathetic neurons. The second wave of trunk nerual crest cells leaving the neural tube will follow the ventrolateral pathway where cells will pass through the mature somites and contribute to Schwann cells and dorsal root ganglia. The remaining trunk nerual crest cells will undertake the dorsolateral pathway to differentiate into melanocytes. BMP, bone morphogenetic protein.

The lineage specification of the sympathoadrenal cells is highly dependent on BMP signaling, and by aggregating next to the BMP-producing dorsal aorta, the sympathoadrenal cells are exposed to high levels of BMPs (McPherson et al., 2000; Reissmann et al., 1996; Schneider et al., 1999; Shah et al., 1996). As a result, a

network consisting of multiple transcription factors is induced and together they control the development of sympathetic neurons and chromaffin cells. This network includes *ASCL1* (encoding human Hash-1), *PHOX2A*, *PHOX2B*, *HAND2*, *GATA2* and *GATA3* (Guillemot et al., 1993; Howard et al., 2000; Huber et al., 2005; Pattyn et al., 1999). Of these transcription factors, Phox2b is most likely the most important for the generation of both sympathetic neurons and chromaffin cells. Phox2b has the capacity to induce expression of all the other transcription factors in this network, except for Hash-1. However, Phox2b is still required for maintenance of Hash-1. Knockout studies in mice have shown that Phox2b regulates very early step in chromaffin cell development as loss of *PHOX2B* prevent chromaffin progenitor cells to undergo further differentiation (Huber et al., 2005). *ASCL1* has also been shown to be crucial for the development of chromaffin cells, and together with Phox2b, they induce expression of tyrosine hydroxylase (*TH*) and dopamine β-hydroxylase (*DBH*), two enzymes required for synthesis of noradrenaline (Huber, 2006; Huber et al., 2002). *TH* and *DBH* expression are observed early on in sympathoadrenal cells (Cochard et al., 1978; Ernsberger et al., 1995; Ernsberger et al., 2000), together with several neuronal markers including secretogranin 10 (*SCG10*) and neurofilament (*NF*) (Cochard and Paulin, 1984; Groves et al., 1995; Schneider et al., 1999; Sommer et al., 1995).

Fate-restricted sympathoadrenal cells then enter a second migratory phase to ultimately end up at their final destination, which is the adrenal medulla and paraganglia for differentiation into chromaffin cells or the definite sympathetic ganglia for differentiation into sympathetic neurons (Anderson and Axel, 1986; Anderson et al., 1991). During chromaffin cell differentiation, expression of neuronal markers is downregulated, and although the exact mechanism of chromaffin specification is yet undefined, it has been shown that expression of phenylethanolamine N-methyltransferase (*PNMT*, an adrenaline synthesizing enzyme) and secretogranin II (*SCG2*) is important for this process (Finotto et al., 1999). For the sympathetic neuroblast, proteins such as the BHLH transcription factor N-MYC act as cues for continued differentiation, while late stage and terminal differentiation into sympathetic neurons requires neurotrophic signaling (Huber, 2006). This is primarily regulated via neurotrophin 3 (NT-3) and nerve growth factor (NGF). When committed neuroblasts are instructed for continued differentiation, tropomyosin receptor kinase C (TrkC), the receptor for NT-3, is upregulated, which results in TrkC-NT-3 cell signaling. This stimulates upregulation of tropomyosin receptor kinase A (TrkA), the receptor for NGF, and neuroblasts become dependent on NGF for their survival and differentiation. As a result, the Trk receptors are expressed in a sequential manner on neuroblasts and patterning of the SNS is dependent on secretion of NT-3 and NGF as it will attract the Trk-expressing neuroblast accordingly (Birren et al., 1993; Hoehner et al., 1995; Verdi et al., 1996).

Chapter 3.
Neuroblastoma

Overview

Neuroblastoma is a SNS-derived malignancy that almost exclusively occurs in early childhood. It is a relative rare disease, accounting for around 7% of all cancer in patients younger than 15 years of age. However, 15% of all cancer-related deaths in children are caused by neuroblastoma (Brodeur, 2003; Maris, 2010). Neuroblastoma is regarded as a unique malignancy where some patients display a highly metastatic disease that regress spontaneously even without treatment while other children succumb to the disease despite being treated with intense combinatory therapy for years (Brodeur and Nakagawara, 1992; D'Angio et al., 1971; Matthay et al., 2016). Given this heterogeneity in clinical outcome, patients are stratified into different risk group based on various prognostic factors such as age, disease stage, amplification of the *MYCN* gene and segmental chromosomal aberrations in order to decide appropriate treatment protocol. While neuroblastoma is mainly regarded as a copy-number driven disease, recurrent mutations are observed in some cases, but mostly in relapsed or familial neuroblastoma (Matthay et al., 2016).

Neuroblastoma Origin

Neuroblastoma is a malignancy of the sympathetic ganglia and adrenal medulla, structures derived from the trunk neural crest cells in the developing SNS (Brodeur, 2003). The precise cell of origin of neuroblastoma remains unknown, but based on the location of primary tumors it has long been assumed that neuroblastoma is a developmental disease of the neural crest and derives from cells of the sympathoadrenal lineage of the trunk neural crest (Brodeur, 2003). This hypothesis has been further supported by gene and protein expression studies in neuroblastoma where comparison of normal neuroblasts and neuroblastoma cells revealed striking similarities in their expression profiles (De Preter et al., 2006; Hoehner et al., 1996; Hoehner et al., 1998; Nakagawara and Ohira, 2004). Immunohistochemical and in situ hybridization studies of normal human fetal tissue (obtained from week 8 to 24) and clinical neuroblastoma samples demonstrated that the expression of sympathetic

neuronal differentiation markers, such as TrkA, TrkC and TH, cohered between more differentiated, often extra-adrenal and favourable neuroblastomas as well as fetal extra-adrenal chromaffin cells (Hoehner et al., 1996). Moreover, analysis of neuroblastomas with an undifferentiated phenotype, unfavourable prognosis and adrenal origin displayed an expression profile more similar to early fetal sympathetic neuroblasts (Hoehner et al., 1996; Hoehner et al., 1998). Through technological advances, it is nowadays possible to perform gene expression profiling in a more unbiased approach via whole genome sequencing. In 2006, De Preter et al compared the gene expression profile of isolated human fetal sympathetic neuroblast, adjacent cortex cells and patient-derived neuroblastoma cells (De Preter et al., 2006). They found that the profiles of sympathetic neuroblasts and neuroblastoma cells overlapped significantly, while the fetal cortex cells clustered far away from neuroblasts and neuroblastoma cells. Further, comparison of neural stem cells with neuroblastoma cells and normal neuroblasts showed that neural stem cells have more genes in common with neuroblastoma cells than with fetal neuroblasts (De Preter et al., 2006). Together, these data provide some evidence for a neuroblast origin of neuroblastoma.

According to the classical view of the neural crest and its derivates, neural crest cells of the sympathoadrenal lineage give rise to sympathetic ganglia and the adrenal medulla. However, this dogma was recently revisited by Furlan et al (Furlan et al., 2017). The authors found that the majority of chromaffin cells that form the adrenal medulla stems from peripheral glia stem cells called Schwann cell precursors (SCPs, (Furlan and Adameyko, 2018)). By performing genetic cell lineage tracing studies in mice, they showed that early-migrating neural crest cells differentiate into sympathetic neurons and give rise to the sympathetic ganglia and only a small part of the adrenal medulla. On the other hand, late-migrating neural crest cells differentiate into SCPs and migrate along axons of preganglionic neurons towards the forming adrenal gland where they will detach and give rise to neuroendocrine chromaffin cells (Furlan et al., 2017). Thus, these findings suggest that there are at least two possible origins of neuroblastoma: the sympathoadrenal progenitor cell destined to become sympathetic neurons and chromaffin cells of the developing SNS and the SCPs destined to become chromaffin cells of the adrenal medulla.

Clinical Manifestation and Prognosis

Neuroblastoma is the most common and deadliest extracranial solid tumor of childhood. Most primary tumors arise in the medulla of the adrenal glands and symptoms vary from patient to patient depending on tumor location and metastasis status (see Figure 5, (Johnsen et al., 2019; Maris et al., 2007; Vo et al., 2014). The most common sites for metastasis are bone, bone marrow, lung and liver The median

age at diagnosis is 17-18 months and around 40% of all patients are younger than 1 year of age at diagnosis while less than 10% of patients are older than 10 years (Brodeur, 2003; London et al., 2005; Maris, 2010; Park et al., 2010). Thus, neuroblastoma rarely occurs in adolescents and young adults, but when it does, the disease tends to be much more aggressive and lethal (Mosse et al., 2014).

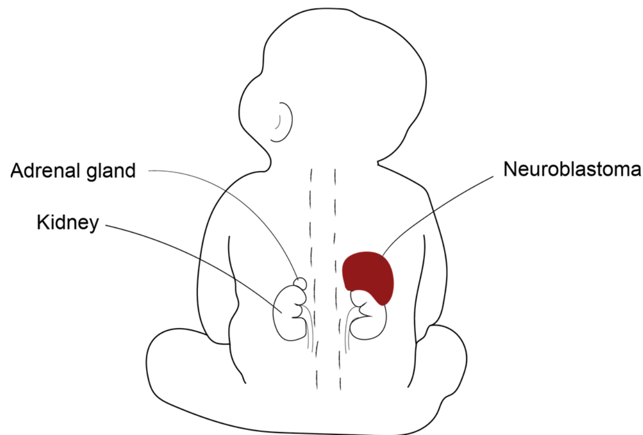

Figure 5. Schematic illustartion of a child with neuroblastoma.
Neuroblastoma is heterogeneous tumor of infancy and youth. The majority of all neuroblastomas arise in the adrenal gland, which is situated ontop of the kidney.

Prognostic markers in neuroblastoma

Several clinical and biological markers confer prognostic values in neuroblastoma. Important clinical markers are age at diagnosis (Breslow and McCann, 1971; Evans et al., 1987), where children younger than 18 months have a better clinical outcome than older patients (George et al., 2005; London et al., 2005; Schmidt et al., 2005), and the disease stage (Evans et al., 1987). Neuroblastoma is clinically classified into five different stages according to the International Neuroblastoma Staging System (INSS), specifically stage 1 to 4 and 4S (S for special, (Brodeur et al., 1993)). Several different classification systems are used in neuroblastoma, e.g. the disease staging system International Neuroblastoma Risk Group (INRG) that will be discussed later on, but INSS is the most widely accepted system and is based on the extent of surgical excision at diagnosis and the presence of metastasis. Low stage neuroblastomas belong to stages 1 and 2, these tumors are more easily treated since they reside only locally. However, high stage neuroblastomas, i.e. stage 3 and 4, are highly metastatic and are associated with therapy resistance and poor prognosis. The fifth class of neuroblastomas, 4S, is only observed in patients younger than 12 months and are unique in their capacity to spontaneously regress, despite the

presence of metastasis to skin, liver and/or bone marrow, even without or only limited therapy intervention (D'Angio et al., 1971; Evans et al., 1971).

Biological prognostic factors in neuroblastoma include tumor histology, grade of tumor differentiation, *MYCN* amplification and segmental chromosomal alterations. Tumor histology is defined by the relative proportion of neuronal cells and stromal Schwannian cells within the tumor (Shimada et al., 1984; Shimada et al., 2001). Further, the tumor differentiation stage is defined by the expression of a set of markers for sympathetic neuronal differentiation like *TRKA*, *SCG10* and chromogranin A (*CHGA*) and correlates to prognosis and outcome in neuroblastoma (Fredlund et al., 2008). More specifically, an undifferentiated tumor stage, i.e. low expression of these sympathetic neuronal associated markers, correlates to an aggressive disease and these tumor cells are characterized by increased expression of neural crest and stem cell-like markers. Finally, chromosomal damages are commonly observed in neuroblastoma and are frequently associated with an aggressive disease and poor prognosis (Park et al., 2010). These damages include *MYCN* amplification (Brodeur et al., 1984) and segmental chromosomal aberrations like 1p deletion, 11q deletion or 17q gain (Bown et al., 1999; Fong et al., 1989; Gilbert et al., 1984; Park et al., 2010; White et al., 1994; White et al., 2005).

Heterogeneity in neuroblastoma

Neuroblastoma is characterized as being an extremely heterogeneous disease, ranging from spontaneous regression of either localized tumors or metastatic tumor with no or only limited therapy to highly aggressive tumors with widespread metastases already at diagnosis and fatal relapse despite intensive multimodal treatment (Brodeur and Nakagawara, 1992; D'Angio et al., 1971; Matthay et al., 2016). Tumor heterogeneity has been described at multiple levels, including tumor location and histology. Several studies have shown that the origin of the primary tumor can affect patient outcome, where adrenal tumors often display features associated with a more aggressive disease, such as *MYCN* amplification (Brisse et al., 2017; Vo et al., 2014). One possible explanation for the particularly poor outcome of adrenal neuroblastoma, compared to non-adrenal tumors, could be the cell-of-origin as recent data suggest that SCPs give rise to the majority of chromaffin cells of the adrenal gland whereas the sympathoadrenal progenitor cells mainly give rise to the sympathetic ganglia (Furlan et al., 2017; Vo et al., 2014).

The tumor heterogeneity can also be described at a cellular and molecular level, i.e. intratumoral heterogeneity (Boeva et al., 2017; van Groningen et al., 2017). Early studies by Dr. Biedler and coworkers showed that cultured neuroblastoma cells contained at least three distinguishable and interconvertible cell types: the moderately malignant immature sympathetic neurons (N-type), the non-malignant surface adherent Schwann cells (S-type) and the highly malignant intermediate

stem-like cells (I-type) (Ciccarone et al., 1989; Rettig et al., 1987; Ross et al., 1983). This phenotypic difference has also been observed in human neuroblastoma specimens where neuroblastic tumor cells and those with a more mesenchymal phenotype are found within the same tumor (Pietras et al., 2008). More recently, van Groningen et al discovered two neuroblastoma cell types within the same tumor, the undifferentiated mesenchymal (MES) cells and committed adrenergic (ADRN) cells that displayed divergent transcriptomic profiles and super-enhancer-associated transcription factor network (van Groningen et al., 2017). The ADRN cells expressed transcription factors of the adrenergic lineage, e.g. *PHOX2A*, *PHOX2B* and *GATA3*, as well as enzymes required for synthesis of catecholamine like *TH* and *DBH*. By stark contrast, the minor subpopulation of MES cells lacked expression of these adrenergic markers but instead displayed a neural crest cell-like gene signature and expressed mesenchymal marker genes such as vimentin (*VIM*) and *SNAI2* (van Groningen et al., 2017). The authors also showed that these two cell types can interconvert and that MES-type cells displayed reduced sensitivity towards chemotherapy and are enriched in post-therapy and relapsed tumors (van Groningen et al., 2017). In a similar study, Boeva et al investigated the super-enhancers and core regulatory circuitries controlling the transcriptional program in neuroblastoma cell lines (Boeva et al., 2017). The authors observed two cell types with distinct identities (sympathetic noradrenergic-like cells and neural crest-like cells) and a third group with mixed identity. Thus, together these data provide evidence for the existence of phenotypically different neuroblastoma cells within the same tumor.

INRG staging system

As a result of this extreme heterogeneity, efforts have been focused on trying to predict the outcome for neuroblastoma patients at the time of diagnosis to ensure optimal treatment strategies. For this reason, the disease staging system INRG was established that uses clinical and biological prognostic factors to classify neuroblastoma into very low-, low-, intermediate- and high-risk disease (Cohn et al., 2009; Monclair et al., 2009). In this way, the INRG system offers pretreatment risk stratification of patients in contrast to the INSS classification, which is a postsurgical staging system. Around 40% of all patients are diagnosed with high-risk neuroblastoma, and due to the aggressiveness of this disease in combination with treatment resistance, the survival rate is less than 50%. On the other hand, children diagnosed with low- and intermediate-risk disease do quite well and 85-90% can be cured (Ladenstein et al., 2017; Matthay et al., 2009; Mosse et al., 2014; Valteau-Couanet et al., 2014). By establishing the INRG staging system, it is nowadays also possible to compare the result of clinical trials performed worldwide.

Genomics of Neuroblastoma

The understanding of genomic events underlying neuroblastoma has significantly improved during the last decades but the number of recurrent somatic alterations identified in newly diagnosed neuroblastomas have been scare (Cheung et al., 2012; Molenaar et al., 2012b; Pugh et al., 2013; Sausen et al., 2013), which has impeded the effort of developing targeted therapy. However, it has been shown that relapsed tumors display an enrichment in genes predicted to activate signaling pathways, e.g. RAS-MAPK and Hippo-YAP pathway (Eleveld et al., 2015; Schramm et al., 2015).

Familial neuroblastoma

Familial neuroblastoma is rare, accounting for only 1-2% of all neuroblastoma cases, and is inherited in an autosomal-dominant manner (Knudson and Strong, 1972; Kushner et al., 1986). Among affected families, the median age at diagnosis is around 9 months and there is also an increased risk of having multiple primary tumors (Knudson and Strong, 1972; Maris et al., 2002).

The first gene identified to predispose to hereditary neuroblastoma was *PHOX2B* (Mosse et al., 2004; Trochet et al., 2004). *PHOX2B* is located on chromosome 4p12 and is induced by BMP signaling at the dorsal aorta to promote proper development of the neural crest and its derivates (Pattyn et al., 1999). The interest for *PHOX2B* in neuroblastoma emerged when it was identified as one of the main disease-causing genes of congenital hypoventilation syndrome (CCHS) (Amiel et al., 2003). Patients with CCHS have a predisposition for developing SNS tumors, e.g. neuroblastoma (Rohrer et al., 2002). Shortly after the discovery of *PHOX2B* in CCHS, heterozygous missense and frame-shift mutations were identified in *PHOX2B* in both hereditary and sporadic neuroblastoma (Bourdeaut et al., 2005; Mosse et al., 2004; Trochet et al., 2004; van Limpt et al., 2004). *PHOX2B* loss-of-function mutations account for ~10% of all familial neuroblastoma cases (Raabe et al., 2008).

In 2008, gain-of-function mutations in the anaplastic lymphoma kinase (*ALK*) gene was demonstrated as the major cause of familial neuroblastoma, accounting for ~80% of all cases (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). *ALK* is located on chromosome 2p23 and encodes a receptor tyrosine kinase that regulates proliferation and differentiation of neural crest cells (Iwahara et al., 1997). In familial neuroblastoma, constitutive ALK activation is mainly achieved through point mutations in the kinase domain, but activating deletions and translocations have also been described. Many of these germline mutations can be found in sporadic neuroblastomas, where 14% of patients carry somatic *ALK* mutations (Bresler et al., 2014; Chen et al., 2008; Fransson et al., 2015; Hallberg and Palmer, 2016; Janoueix-Lerosey et al., 2008; Mosse et al., 2008; Okubo et al., 2012).

Sporadic neuroblastoma

In 1983, *MYCN* was identified as an amplified gene in neuroblastoma (Kohl et al., 1983; Schwab et al., 1983). *MYCN* amplification is one of the most prominent indicators of poor prognosis in neuroblastoma and is detected in 20-30% of all tumors (Brodeur et al., 1984; Matthay et al., 2016; Thompson et al., 2016). *MYCN* is located on chromosome 2p24 and encodes the N-MYC transcription factor, which is a master regulator of transcription involved in several cellular processes like neural crest development (Grimmer and Weiss, 2006; Park et al., 2010). Several studies have shown that *MYCN* amplification is associated with a more invasive and metastatic disease, treatment failure and rapid disease progression (Brodeur, 2003; Brodeur et al., 1984; Seeger et al., 1985).

Whole and segmental chromosomal gains and losses are commonly observed in neuroblastoma, where whole chromosomal aberrations associate with favourable outcome while segmental aberrations instead predict advanced stage disease and poor outcome (Park et al., 2010). Almost all high stage tumors show recurrent segmental chromosomal aberrations; loss of chromosome 1p has been observed in 30% of patients (Attiyeh et al., 2005; Fong et al., 1989; Gilbert et al., 1984) and gain of chromosome 17q is found in more than half of all neuroblastoma cases (Bown et al., 1999). Both loss of 1p and gain of 17q associates with poor prognosis and *MYCN* amplification. Further, loss of chromosome 11q is found in a third group of high stage neuroblastoma and is also associated with poor outcome, but is instead inversely correlated with amplification of *MYCN* (Attiyeh et al., 2005).

Chromothripsis has been identified in 18% of all high stage neuroblastomas and is associated with poor prognosis (Molenaar et al., 2012b). Whole genome sequencing of primary neuroblastomas has identified genomic rearrangements of the telomerase reverse transcriptase (*TERT*) gene on chromosome 5p. These rearrangements affect only high stage tumors and occur in a mutually exclusive fashion with *MYCN* amplification (Peifer et al., 2015; Valentijn et al., 2015). The end result of *TERT* rearrangement is epigenetic remodelling and overexpression of *TERT*, which in turn induces maintenance and lengthening of the telomeres that enables neuroblastoma cells to proliferate indefinitely. This mechanism is also present in *MYCN* amplified tumors since *TERT* is a downstream target of N-MYC, resulting in *TERT* overexpression (Peifer et al., 2015; Valentijn et al., 2015). Inactivating mutations of the chromatin-remodelling gene α-thalassemia/mental retardation syndrome X-linked (*ATRX*) has also been identified in ~10% of patients (Valentijn et al., 2015). Loss-of-function genetic alterations in *ATRX* induces the alternative lengthening of telomere (ALT) pathways to maintain the telomeres through homologous recombination and occurs in a mutually exclusive fashion with *MYCN* amplification and *TERT* rearrangements (Molenaar et al., 2012b; Valentijn et al., 2015). Thus, high-risk neuroblastoma can be divided into three distinct groups with mutually

exclusive genetic alterations (*MYCN*, *TERT* and *ATRX*) that can induce telomere maintenance and lengthening. The presence of telomere maintenance program has been shown to correlate to poor patient outcome, whereas patients whose tumors lacked this mechanism had an excellent outcome (Ackermann et al., 2018).

Relapsed neuroblastoma

Patients with high-risk neuroblastoma have a survival rate of less than 50% (Park et al., 2013), and despite an initial response to the given therapy, up to 60% of these patients subsequently relapse with highly aggressive and treatment-resistant tumors (Cohn et al., 2009; Maris, 2010; Simon et al., 2011). Recent whole-genome sequencing of paired primary and relapsed neuroblastomas has shown that relapsed tumors have a higher mutational burden as compared to the primary tumors (Eleveld et al., 2015; Padovan-Merhar et al., 2016; Schleiermacher et al., 2014; Schramm et al., 2015).

Sequencing of the *ALK* locus in neuroblastoma at the time of relapse, showed that 26% of cases harboured *ALK* inactivating mutations (Schleiermacher et al., 2014). Eleveld et al compared paired primary and relapsed neuroblastoma and showed that 78% of relapsed tumors carried mutations in e.g. *ALK*, *NF1* and *KRAS,* all predicted to hyperactivate the RAS-MAPK signaling pathway (Eleveld et al., 2015). In a similar study, Schramm et al identified recurrent mutations in *CHD5*, *DOCK8* and *PTPN14* at relapse, which suggests that the Hippo-YAP signaling pathway is involved in neuroblastoma relapse (Schramm et al., 2015). This enrichment of recurrent alterations in relapsed neuroblastoma have been further supported in a more recent study (Padovan-Merhar et al., 2016). Together these data suggest that neuroblastoma undergo considerable mutational evolution in during therapy.

Current Treatments of Neuroblastoma

Treatment of neuroblastoma patients deviates extensively and depends on the respective INRG stage. The number one treatment for low-risk neuroblastoma is surgery, and if necessary, minimal chemotherapy. Some patients are just placed under observation due to spontaneous regression of the disease. Patients belonging to the intermediate-risk group are given milder chemotherapy followed by surgical resection of the remaining tumor. In case of unresectable tumors, chemotherapy is usually followed up with radiotherapy to increase the chance of survival (Baker et al., 2010; Matthay et al., 2016; Shohet and Foster, 2017; Strother et al., 2012). On the other hand, the treatment protocol for high-risk patients involves intense induction chemotherapy that includes cisplatin, vincristine, carboplatin, etoposide

and cyclophosphamide (COJEC, (Ladenstein et al., 2017; Pearson et al., 2008)), which is followed up by surgery and myeloablative therapy combined with reinfusion of hematopoietic stem cells as well as local radiotherapy (Matthay et al., 2016; Shohet and Foster, 2017). To improve the event-free survival of high-risk patients, maintenance treatment is given and include 13-cis-retinoic acid aiming to differentiate the remaining tumor cells (Matthay et al., 1999) and immunotherapy using monoclonal antibodies targeting the disialogangliodise GD2 on neuroblastoma cells in combination with granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-2 (IL-2) (Matthay et al., 2016; Yang and Sondel, 2010; Yu et al., 2010). However, even though the prognosis for high-risk neuroblastoma patients have improved during the last years, a substantial proportion still relapse and have a fatal outcome. To improve the clinical outcome even further for these patients, we have to identify biomarkers that can be used both for diagnostics and drug discovery. For this we need more clinically relevant preclinical models of neuroblastoma. A general discussion about preclinical cancer models is presented in Chapter 5, whereas *Paper I* and *Paper II* elaborate on the usage of neuroblastoma patient-derived xenograft (PDX) cells and the chick embryo model in cancer research, respectively.

Neuroblastoma Stemness

The three cell types of neuroblastoma

The search of a more undifferentiated, immature and stem-like neuroblastoma cell has been going on since the 1980s when the N-type, S-type and I-type neuroblastoma cell types were identified (Ciccarone et al., 1989; Rettig et al., 1987; Ross et al., 1983). These cell types have also been shown to be present in human neuroblastoma tumors (Ross et al., 2003). The I-type neuroblastoma cells have an intermediate phenotype and are characterized by their ability to self-renew and differentiate into either N- or S-type cells (Ross et al., 1995). Compared to the other two cell types, the I-type cells are highly tumorigenic. Studies have shown that they are six times more likely to form tumors *in vivo*, irrespective of the *MYCN* amplification status (Walton et al., 2004). Moreover, gene expression analysis has also revealed a distinct I-type signature, consisting of e.g. *CD133*, *KIT*, *NOTCH1* and *TRKB* (Ross et al., 2015). Thus, the I-type cells are thought of as a more primitive multipotent embryonic precursor cell with the ability to differentiate and has therefore been suggested to be a potential neuroblastoma stem cell.

Potential neuroblastoma stem cell markers and signatures

Extensive studies exploring distinct molecular signatures of potential neuroblastoma stem cells have been performed during the last 30 years. This has resulted in a long list of markers associated with increased tumorigenic capacity, self-renewal, clonogenicity, poor prognosis and co-expression of typical stem cell-like markers. One of these markers is the ALDH which was discussed in Chapter 2 (Coulon et al., 2011; Flahaut et al., 2016; Hartomo et al., 2015), and their role in neuroblastoma as well as association with the MES-type neuroblastoma cells will be further elaborated on in *Paper III*. Other markers that have been reported as potential neuroblastoma stem cell markers are CD44 (Jensen et al., 2015; Mehrazma et al., 2013), CD133 (Cournoyer et al., 2012; Sartelet et al., 2012; Tong et al., 2008), C-KIT (CD117) (Ross and Spengler, 2007; Ross et al., 2015; Walton et al., 2004), ABCG2 (Hirschmann-Jax et al., 2004) and *LGR5* (Vieira et al., 2017). However, contradicting results have been reported for many of these markers, for example CD44 where increased expression has been associated with metastatic behavior in one study (Jensen et al., 2015), whereas others have reported that lack of CD44 expression correlates with aggressiveness, widespread metastasis and poor outcome (Munchar et al., 2003; Rabadan et al., 2013; Siapati et al., 2011).

Finally, low oxygen level, or hypoxia, is associated with tumor aggressiveness. All cells, including tumor cells, respond to hypoxia by activating a transcriptional program mainly driven by hypoxia-inducible factors (HIF)-1 and HIF-2 (Semenza, 2014). The expression levels of both HIF-1α and HIF-2α have been correlated to patient outcome in several human tumors. In neuroblastoma, several studies have suggested that high levels of HIF-2α in perivascular located tumor cells is a marker of a putative neuroblastoma stem cells as these HIF-2α positive cells present with an immature, neural crest-like phenotype and are associated with a high stage disease and metastasis (Holmquist-Mengelbier et al., 2006; Noguera et al., 2009; Pietras et al., 2009; Zhang et al., 2014). The role of hypoxia and HIF-2α in cancer will be discussed further in Chapter 4 and elaborated on in *Paper IV*.

Chapter 4. Hypoxia

Overview

The term hypoxia refers to a reduction in the physiological oxygen level in any given tissue, resulting in altered phenotype. Tissue hypoxia is commonly observed in pathophysiological conditions, including vascular- and pulmonary diseases as well as cancer, but can also be observed in individuals located at high altitude due to reduced oxygen tensions. Despite extensive research on tissue oxygen, it has proven difficult to set a specific oxygen level that defines hypoxia or normoxia (a term normally used in *in vitro* cultures, corresponding to ambient oxygen levels), as tissues display different sensitivity to reduced oxygen tension. Even though the definition of the different oxygen conditions varies depending on for example CO_2 concentration and temperature, a general approximation has been set for *in vitro* experimental settings. In the tissue culture flask, the atmospheric oxygen pressure of 160 mmHg, which corresponds to 20-21% oxygen, has been used to describe normoxia. Similarly, pathological low oxygen tension occurs at 8-10 mmHg *in vitro*, and for this reason, 1% oxygen is most commonly used to describe hypoxia. However, the oxygen levels used in experimental settings to define normoxia is far from the physiological oxygen tension found in normal peripheral tissues, which is termed *physoxia*. Physoxia varies depending on tissue type, but the percentage used *in vitro* to describe "physiological and end-capillary oxygen tension" is 5% oxygen (Hockel and Vaupel, 2001; McKeown, 2014).

Hypoxia is commonly observed in solid tumors and occurs when tumors expand and outgrow their vascular supply. The diffusion of oxygen is limited to 5-10 cell layers (100-150 µm) and since the formation of new blood vessels is both inadequate and dysfunctional, some tumor areas will experience little or no access to oxygen. Hypoxia is a feature known to be associated with tumor aggressiveness as limitation to oxygen has proven to influence several aspects of the biology of the tumor, including mutation frequency rate, regulation of gene transcription and response to radio- and chemotherapy (Bedford and Mitchell, 1974; Dachs and Chaplin, 1998; Harris, 2002; Sutherland, 1998). Both normal and tumor cells have the ability to adapt to low oxygen levels by activating a transcriptional program that changes the metabolism of the cell to move away from energy consuming processes. This transcriptional program is mainly regulated via hypoxia-inducible factors (HIFs).

Hypoxia-Inducible Factors

The HIF proteins are members of the basic helix loop helix and Per-Arnt-Sim (bHLH-PAS) family and function as heterodimers, consisting of an α- and β-subunit. Whereas the β-subunit, known as HIF-1β or aryl hydrocarbon receptor nuclear translocator (ARNT), is oxygen-insensitive and continuously expressed, the α-subunits are oxygen-sensitive and degraded under oxygenated conditions (Kaelin and Ratcliffe, 2008). Three HIF-α subunits have been identified: HIF-1α (Semenza and Wang, 1992; Wang et al., 1995; Wang and Semenza, 1995), HIF-2α (Tian et al., 1997; Wiesener et al., 1998) and HIF-3α (Makino et al., 2001). HIF-1α and HIF-2α are both essential for adaption to low oxygen levels and they share a 48% primary amino acid sequence homology (Tian et al., 1997), with the highest similarity within the bHLH and PAS (PAS-A and PAS-B) domains, which are responsible for the interaction with ARNT. The bHLH domain is also responsible for binding to the DNA at specific locations termed hypoxia response elements (HRE). The two α-subunits also contain an oxygen-dependent degradation domain (ODD), that is involved in HIF-α stability, and two transactivating domains (CTAD and NTAD; N-terminal and C-terminal), where CTAD is important for the interaction with co-activators like CBP and p300 (CREB binding protein and 300-kilodalton co-activator protein, respectively) (see Figure 6, (Kaelin and Ratcliffe, 2008; Semenza, 2014)). The third α-subunit, HIF-3α, is less studied and exists in multiple splice variants that all lacks CTAD. The exact functions of all splice variants are not yet known, but HIF-3α is mainly described as a negative regulator of HIF-1 and HIF-2 (Duan, 2016; Makino et al., 2001; Maynard et al., 2007).

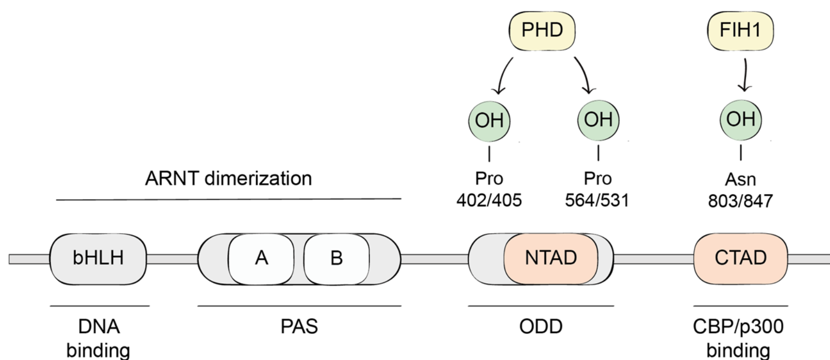

Figure 6. Illustration of the HIF-alpha domain structure.

The HIF-1α and HIF-2α subunits contain a bHLH and PAS domain important for ARNT dimerization and DNA binding as indicated, an ODD domain and two transactivating domains known as NTAD and CTAD. At normoxia, the HIF-α subunits are hydrolyxated by PHDs at conserved proline residues (Pro402/564 in HIF-1α and Pro405/531 in HIF2α) or by FIH at conserved asparagine residues (Asn803 in HIF-1α and Asn847 in HIF-2α). At hypoxia, the co-activators CBP/p300 bind to CTAD in the HIF-α subunit to promote transcriptional activation. ARNT, aryl hydrocarbon receptor nuclear translocator; bHLH, basic helix loop helix; CTAD, C-terminal transactivating domain; FIH1, factor inhibiting HIF1; NTAD, N-terminal transactivating domain; OH, hydroxylation group; PAS, Per-Arnt-Sim; PHD, prolyl hydroxylase domain.

Oxygen-dependent regulation of the HIFs

The oxygen-dependent regulation of HIF-1α and HIF-2α has traditionally been viewed to be mediated at a post-translational level. At oxygenated conditions, HIF-α is hydroxylated at conserved proline residues (Pro402/564 in HIF-1α and Pro405/531 in HIF-2α) within ODD by prolyl hydroxylase domain (PHD) proteins encoded by the *EGLN* family (Figure 6, (Bruick and McKnight, 2001; Epstein et al., 2001; Huang et al., 1998; Ivan et al., 2001; Jaakkola et al., 2001)). PHD-mediated hydroxylation results in a high-affinity binding site for the von Hippel-Lindau (VHL) ligase complex that promotes ubiquitination and proteasomal degradation of HIF-α (Huang et al., 1998; Maxwell et al., 1999; Tanimoto et al., 2000).

Multiple differences have been described among the PHD isoforms, including their affinity for oxygen as well as preference for hydroxylation of a certain proline residue or specific HIF-α isoform (Ivan and Kaelin, 2017). PHD2 is regarded as the main oxygen sensor, mostly likely because it has the lowest oxygen affinity and exhibit a preference for HIF-1α (Berra et al., 2003). In contrast, both PHD1 and PHD3 have a preferred binding for HIF-2α (Appelhoff et al., 2004). It has also been shown that all three PHD isoforms can hydroxylate Pro564 in HIF-1α, a highly conserved proline residue, while the recently evolved Pro402 can only be hydroxylated by PHD1 and PHD2 (Berra et al., 2003; Chowdhury et al., 2016). There is also evidence that the PHD isoforms have other targets than HIFs (Guo et al., 2016a) and that they are involved in a HIF regulatory feedback loop as both PHD2 and PHD3 are induced at hypoxia (Ivan and Kaelin, 2017).

The HIF-α subunit can also be regulated through hydroxylation of an asparagine residue (Asn803 in HIF-1α and Asn847 in HIF-2α) located in CTAD by factor inhibiting HIF1 (FIH1, Figure 6). FIH1-mediated hydroxylation does not regulate stability of the α-subunit, as the proline hydroxylation, but instead transcriptional activity by blocking interaction between HIF-α and the co-activators CBP and p300 (Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001). Although FIH1 is able to target both HIF-1α and HIF-2α, it has been reported that FIH1 is more prone to regulate HIF-1α via asparagine hydroxylation. This can, at least in part, be explained by a single amino acid substitution within CTAD just immediate of the asparagine hydroxylation site in HIF-2α. This substitution seems to make HIF-2α less sensitive for FIH1-mediated hydroxylation (Bracken et al., 2006).

In order to hydroxylate HIF-α, PHD and FIH1 require oxygen, iron and α-ketoglutarate to function, which in turn generate succinate and carbon dioxide as waste products (Thompson, 2016). Thus, at hypoxia, neither PHD nor FIH1 can mediate HIF-α hydroxylation, resulting in stabilization and rapid accumulation of the α-subunit followed by dimerization with nuclear ARNT and binding to HRE located near HIF targets. Subsequent interaction with CBP and p300 results in transcriptional activation (Figure 7, (Kaelin and Ratcliffe, 2008; Semenza, 2014)).

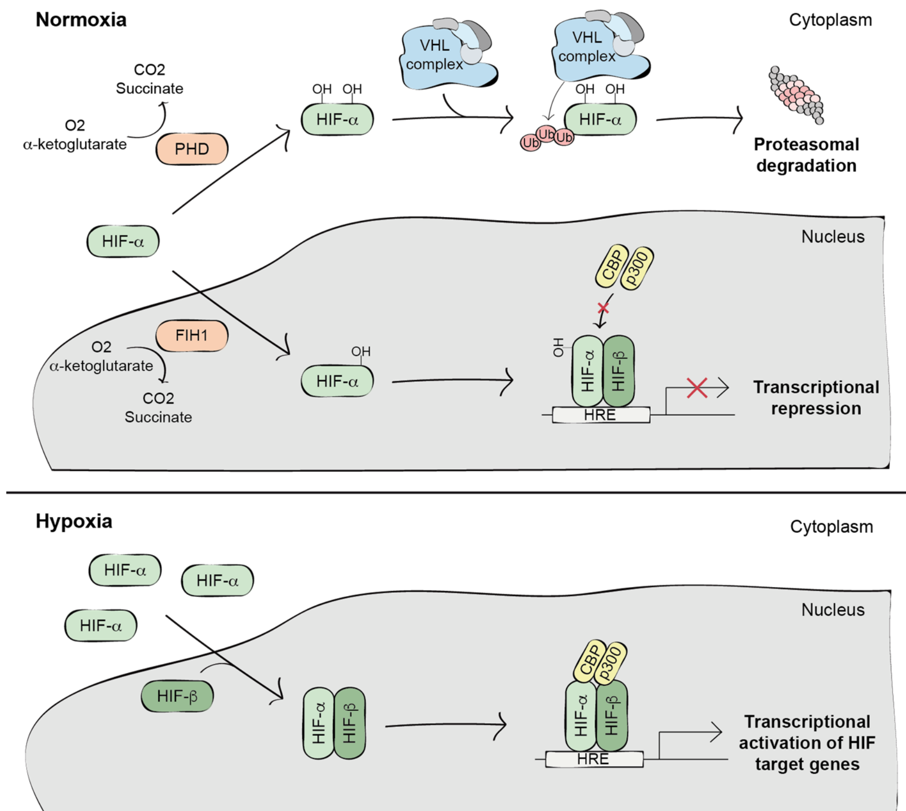

Figure 7. Oxygen-dependent regulation of HIFs.
At normoxia (top), HIF-α is hydroxylated by PHD or FIH1 at highly conserved proline or asparagine residues, respectively. These hydroxylations require oxygen (O_2) and α-ketoglutarate as substrates and generate carbon dioxide (CO_2) and succinate as byproducts. Whereas the PHD-mediated proline hydroxylation promoted pVHL-mediated proteasomal degradation of HIF-α, the FIH1-mediated asparagine hyroxylation blocks binding of CBP/p300 and results in transcriptional repression. At hypoxia (bottom), the PHD proteins and FIH1 are unable to hydroxylate HIF-α, which leads to stabilization of HIF-α followed by nuclear translocation and ARNT dimerization. The resulting heterodimer then drives HRE-dependent transcription of target genes together with CBP/p300. CBP, CREB binding protein; FIH1, factor inhibiting HIF1; HIF, hypoxia-inducible factor; HRE, hypoxia respone element; OH, hydroxylation group; Ub, ubiquitin; VHL, von Hippel-Lindau.

Differential regulation of HIF-1α and HIF-2α

In response to hypoxia, the HIF-α proteins are stabilized to transcriptionally regulate several hundreds of genes in order to maintain the cellular function by balancing the oxygen supply and energy consumption (Semenza, 2017). Although both HIF-1α and HIF-2α bind to the same HRE motifs in the DNA, they are fundamentally different in many aspects and their downstream targets are distinctly regulated by either both or just one of the α-subunits. This distinct regulation may in part be explained by the expression pattern of the HIF-α subunits over time and oxygen concentration. *In vitro*, HIF-1α mediates the immediate and acute response

to hypoxia and is active under the first 24 hours, while HIF-2α is stabilized over time to slowly degrade after 72 hours of hypoxia and thereby mediates the late and chronic response to hypoxia. In this way, genes that are driven by hypoxia, such as *VEGF*, is primary regulated by HIF-1α in the acute phase, while being induced by HIF-2α during later phases of hypoxia (Holmquist-Mengelbier et al., 2006). Moreover, in contrast to HIF-1α, HIF-2α is also stabilized at higher oxygen levels, i.e. physoxia, suggesting a different oxygen sensing mechanism (Holmquist-Mengelbier et al., 2006). This could partly be due to differential regulation of HIF-1α and HIF-2α by FIH1, since it has been demonstrated that FIH1 has a higher oxygen affinity than the PHDs and is more prone to hydroxylate HIF-1α (Tian et al., 2011b). Another explanation for this distinct regulation of downstream targets may be attributed to the chromatin landscape at HRE motifs. The presence or absence of chromatin remodelers, co-regulators and other transcription factors can influence and affect which α-subunit that is mostly likely to bind and activate transcription of target genes (Ivan and Kaelin, 2017).

Target gene expression of HIF-1α and HIF-2α

HIF-1α and HIF-2α share many downstream targets involved in various cellular processes such as angiogenesis, growth, survival, apoptosis, genomic instability and invasion (Lofstedt et al., 2007). However, as a result of the differential regulation of the HIF isoforms, they also induce expression of a unique set of genes.

Overall, HIF-1α is mostly described as a driver of the metabolic response to hypoxia. It controls transcription of various glycolytic proteins like lactate dehydrogenase A (*LDHA*) and pyruvate kinase (*PKM*) to increase conversion of glucose to pyruvate and subsequently lactate. Furthermore, HIF-1α also regulates and maintains intracellular pH by inducing expression of carbonic anhydrase 9 (*CAIX)* and suppress genes involved in mitochondrial respiration by increasing the expression of genes that limit the oxygen consumption, e.g. pyruvate dehydrogenase 1 (*PDK1*) (Kim et al., 2006; Semenza, 2017).

HIF-2α, on the other hand, seems to be more important in later phases of hypoxia and has been shown to specifically regulate for example erythropoietin (*EPO*), which stimulates erythropoiesis (Rankin et al., 2007). In solid tumors, HIF-2α promotes tumor progression by regulating angiogenesis (through vascular endothelial growth factor, *VEGF*) as well as invasion and metastasis by up-regulating the expression of matrix modulating enzymes (*MMPs* and *LOX)* (Holmquist-Mengelbier et al., 2006; Hu et al., 2003; Raval et al., 2005; Wiesener et al., 1998). Interestingly, HIF-2α has also been shown to specifically drive expression of the stem cell marker Oct-4 (Covello et al., 2006), supporting a role for HIF-2α in early development and stem cells.

Hypoxia and HIFs in Normal and Tumor Tissue

HIFs in normal development

Both HIF-1α and ARNT are ubiquitously expressed throughout the murine development (Jain et al., 1998), whereas HIF-2α expression is mainly observed in the endothelial cells of the vasculature system (Jain et al., 1998). Expression of HIF-2α can also be observed in cells of the sympathoadrenal cell lineage at discrete time points during human and mouse development (Jögi et al., 2002; Mohlin et al., 2013; Nilsson et al., 2005; Tian et al., 1998; Tian et al., 1997). For example, HIF-2α is expressed in the organ of Zuckerkandl during human fetal development, whose main function is to produce catecholamines (Nilsson et al., 2005; Tian et al., 1998).

Homozygous deletion of HIF-1α in mice is associated with embryonic lethality as a result of abnormal vascularization, reduced number of somites and aberrant neural fold formation (Iyer et al., 1998; Ryan et al., 1998). However, the embryonic phenotype of HIF-2α null mice is less clear. As of today, four different mouse models of homozygous deletion of HIF-2α has been published with slightly divergent phenotypes, possibly partly due to differences in the strain background (Compernolle et al., 2002; Peng et al., 2000; Scortegagna et al., 2003; Tian et al., 1998). The overall conclusion from these studies is that HIF-2α is essential for proper fetal SNS development by regulating genes involved in the catecholamine synthesis and vascularization. Lack of enzymes required for adrenaline and noradrenaline synthesis during fetal development has long been known to be incompatible with life, probably as a result of abnormal cardiovascular function (Kobayashi et al., 1995; Thomas et al., 1995).

Hypoxia and HIFs in tumors

Hypoxia and expression of HIF-1α and HIF-2α is frequently observed in solid tumors (Talks et al., 2000), and hypoxia is an independent factor predicting poor outcome in numerous tumors, including breast cancer, glioblastoma, clear cell renal cell carcinoma, non-small cell lung carcinoma and neuroblastoma (Giatromanolaki et al., 2001; Giatromanolaki et al., 2006; Helczynska et al., 2008; Hockel and Vaupel, 2001; Holmquist-Mengelbier et al., 2006; Jögi et al., 2002; Li et al., 2009; Mandriota et al., 2002; Talks et al., 2000; Tan et al., 2007; Yoshimura et al., 2004). This is most likely due to a combination of factors, such as therapy resistance, increased vascularization, an undifferentiated phenotype as well as increased tumor aggressiveness and metastatic potential (Lofstedt et al., 2007; Semenza, 2003). The role of HIF-1α and HIF-2α and their association to patient outcome in solid tumors is still debated, due to discrepant data, but seems to be tumor specific.

Breast cancer

There are conflicting data about the prognostic value of HIF-1α in breast cancer, but in more recent studies, it has been proposed that expression of HIF-1α is associated with a favourable patient outcome (Helczynska et al., 2008; Tan et al., 2007). The role of HIF-2α has been less studied in breast cancer, but high expression has been associated with distant metastasis and poor outcome in two unrelated studies (Giatromanolaki et al., 2006; Helczynska et al., 2008).

Glioblastoma

Hypoxia has been shown to promote stemness in glioblastoma by increasing the clonogenic capacity of cells along with increased percentage of the stem cell-like populations (Bar et al., 2010). However, even though several studies have shown that both HIF-1α and HIF-2α are expressed in glioblastoma (Jensen, 2006; Li et al., 2009), their contribution to the disease is still unclear. Some studies propose that HIF-1α is important for maintenance and self-renewal of glioma stem cells (Bar et al., 2010; Soeda et al., 2009), whereas others have reported that HIF-1α is expressed in both glioma stem cells and progenitor cells and that *HIF1A* is not associated with poor outcome (Li et al., 2009). Data on HIF-2α in glioblastoma is also conflicting as HIF-2α has been attributed a tumor suppressor role (Acker et al., 2005) as well as being a marker for poor clinical outcome (Li et al., 2009). Investigation of putative markers for glioma stem cells, such as CD133, have shown that HIF-2α is highly expressed in the CD133 positive glioma stem cells (McCord et al., 2009) and co-localize with expression of cancer stem cell markers in tumor specimens (Li et al., 2009). Interestingly, in both glioblastoma and neuroblastoma, a fraction of HIF-2α positive cells are located adjacent to blood vessels, i.e. in perivascular niches (Li et al., 2009; Pietras et al., 2008; Pietras et al., 2009), leading to a pseudo-hypoxic phenotype. Further, downregulation of HIF-2α in glioma results in reduced tumor initiating capacity and glial differentiation. Thus, HIF-2α has been suggested to be a marker of glioma stem cells (Heddleston et al., 2009; Li et al., 2009).

Clear cell renal cell carcinoma (ccRCC)

Loss of pVHL expression is observed in the majority of all ccRCC patients and is caused by mutation or hypermethylation of the *VHL* gene, resulting in impaired degradation of HIF-α and increased vascularization (Gnarra et al., 1994; Herman et al., 1994). However, even though pVHL targets both HIF-1α and HIF-2α for proteasomal degradation, most pVHL-deficient ccRCC tumor and cell lines express HIF-2α exclusively (Krieg et al., 2000; Maxwell et al., 1999). Moreover, several studies have also showed that HIF-2α promotes tumor growth in pVHL-deficient ccRCC (Kondo et al., 2003; Raval et al., 2005; Zimmer et al., 2004). Thus, pVHL-deficient ccRCC seems to be mostly driven by HIF-2α. In contrast, HIF-1α has been associated with a more favourable patient outcome (Lidgren et al., 2005). HIF-1α

is located on chromosome 14q, which is often deleted in ccRCC, and most ccRCC cell lines used in cancer research have a homozygous deletion that specifically inactivates HIF-1α (Cho and Kaelin, 2016). Numerous *in vivo* studies have shown that overexpression of HIF-1α represses tumor formation while elimination of one wild type HIF-1α allele (most 14q-deleted ccRCCs retain one wild type HIF-1α allele) enhances tumor growth (Raval et al., 2005; Shen et al., 2011).

Non-small cell lung carcinoma (NSCLC)

Intratumoral hypoxia is associated with decreased overall survival in lung cancers (Le et al., 2006; Swinson et al., 2003), and expression of both HIF-1α and HIF-2α is often observed in NSCLC, even at early disease stages (Giatromanolaki et al., 2001). While HIF-2α has been shown to be a marker of poor prognosis (Giatromanolaki et al., 2001), the prognostic role of HIF-1α is still debated. Some reports claim that HIF-1α has no influence on overall survival in patients (Giatromanolaki et al., 2001; Kim et al., 2005), whereas there are contradicting reports claiming the opposite (Volm and Koomagi, 2000; Yohena et al., 2009). Mutations in the *KRAS* gene are commonly observed in lung adenocarcinoma and predict poor clinical outcome (Huncharek et al., 1999). Mice expressing both a nondegradable variant of HIF-2α and mutated *KRAS* showed increased tumor burden, developed larger and more invasive tumors and displayed decreased survival compared to mice only expressing mutated *KRAS* (Kim et al., 2009). These results implicate that HIF-2α promotes tumor growth in lung cancer.

EPAS1 mutations in pheochromocytoma and paraganglioma

Mutations in the gene encoding HIF-2α, *EPAS1*, was identified for the first time in cancer in 2012. Two somatic gain-of-function mutations were reported in two patients with paraganglioma (Zhuang et al., 2012). Both mutations resulted in an amino acid substitution adjacent to the PHD hydroxylation site in HIF-2α, leading to increased protein half-life and HIF-2α activity (Zhuang et al., 2012). As of today, an array of activating mutations in the HIF-2α subunit have been identified in both pheochromocytoma and paraganglioma (Comino-Mendez et al., 2013; Fishbein et al., 2017; Toledo et al., 2013) and HIF-2α is considered as one of the main drivers in both tumor forms (Favier et al., 2012; Rathmell et al., 2004). Intriguingly, both paragangliomas and pheochromocytomas are believed to derive from the sympathoadrenal progenitor cell lineage, in similarity to neuroblastoma, thereby highlighting the importance for proper HIF-2α regulation during SNS development.

The role of HIF-2 in neuroblastoma

HIF-2α was early on identified to be expressed at discrete time points during the development of SNS in both mice (Jögi et al., 2002; Tian et al., 1998) and humans

(Mohlin et al., 2013; Nilsson et al., 2005). Since neuroblastoma is a SNS-derived malignancy, the interest of studying the effects of hypoxia on neuroblastoma cells emerged. Hypoxic culturing of neuroblastoma cell lines resulted in stabilization and accumulation of both HIF-1α and HIF-2α as well as activation of downstream target genes, such as *VEGF* (Jögi et al., 2002; Jögi et al., 2004; Lofstedt et al., 2004; Nilsson et al., 2005). Unexpectedly, hypoxia downregulated expression of genes associated with a more mature sympathetic neuronal phenotype, e.g. *ASCL1* and *dHAND*, while inducing expression of neural crest and early developmental associated genes like *NOTCH1*, *HES1*, *KIT* and *ID2* (Jögi et al., 2002; Jögi et al., 2004; Lofstedt et al., 2004). These data suggest that hypoxia induces a phenotypic shift of the neuroblastoma cells, resulting in an undifferentiated phenotype with stem-like features. Apart from neuroblastoma, hypoxia has also been shown to induce dedifferentiation of glioma, breast and prostate cancer cells (Ghafar et al., 2003; Heddleston et al., 2009; Helczynska et al., 2003) and to block differentiation of non-malignant cells (D'Ippolito et al., 2006; Lin et al., 2006). The underlying mechanisms of this dedifferentiation in neuroblastoma cells is still unclear, but numerous explanations have emerged. Hypoxic culturing of neuroblastoma cells activates the Notch signaling pathway (Jögi et al., 2002; Pahlman et al., 2004) and studies have shown that hypoxia requires Notch signaling in order to block myogenic and neuronal differentiation (Gustafsson et al., 2005). It is also possible that this phenotype is a result of induced expression of the stem cell marker *OCT4*, since Covello et al showed that HIF-2α-induced *OCT4* expression blocks differentiation of stem cells (Covello et al., 2006). Moreover, downregulation of pro-neural specification genes and upregulation of genes associated with inhibition of differentiation (e.g. *ID2*, which is HIF-regulated) might also explain the observed immature neuroblastoma cell phenotype (Jögi et al., 2004; Lofstedt et al., 2004).

Immunohistochemical stainings of neuroblastoma specimens showed that HIF-2α positive cells are frequently located in perivascular niches, suggesting that HIF-2α, in contrast to HIF-1α, is not completely degraded in well-oxygenated areas in neuroblastoma (Holmquist-Mengelbier et al., 2006). When culturing neuroblastoma cells *in vitro* at different oxygen levels, it was clear that HIF-1α is only transiently stabilized in the acute phase of hypoxia at 1% O_2, whereas the expression of HIF-2α is stabilized over-time at 1% O_2 but also detected at end-capillary, near-physiological oxygen conditions, i.e. 5% O_2 *in vitro* (Holmquist-Mengelbier et al., 2006). A closer investigation revealed a number of genes, previously shown to be upregulated at low oxygen, also to be induced at near-physiological oxygen levels. Of these genes, *SERPINB9* and *VEGF* were shown to be regulated exclusively by HIF-2α at 5% O_2 (Holmquist-Mengelbier et al., 2006), indicating that HIF-2α is able to create a pseudo-hypoxic phenotype under oxygenated conditions. However, emerging data in neuroblastoma indicate that HIF-2α exclusivity in regards to driving target genes might not be universally specific, at either 1% or 5% O_2 *in vitro*,

which will be further discussed in *Paper IV*. Importantly, Holmquist-Mengelbier et al also showed that the presence of these highly positive HIF-2α cells located in perivascular niches correlates with aggressive disease and poor outcome in neuroblastoma (Holmquist-Mengelbier et al., 2006). The tumor promoting role of HIF-2α was further supported when subcutaneous injection of neuroblastoma cells transfected with siRNA targeting HIF-2α resulted in smaller and more slow-growing tumors as opposed to tumors formed by wild-type cells (Holmquist-Mengelbier et al., 2006).

Further immunohistochemical characterization of this subset of perivascular HIF-2α positive neuroblastoma cells revealed that they are neural crest- and stem cell like with high expression of Notch-1, Hes-1 and Vimentin, while lacking expression of SNS markers like TH and neuron specific enolase (NSE), otherwise expressed by the bulk tumor cells (Pietras et al., 2008). These cells are indeed tumor cells as determined by the presence of *MYCN* amplification (Pietras et al., 2008). This distinction is important to make, since HIF-2α positive tumor-associated macrophages are frequently observed in perivascular areas. Based on all of these findings, it has been postulated that these immature stem-like HIF-2α positive cells are neuroblastoma stem cells. This hypothesis is strengthened by the fact that knockdown of HIF-2α results in a more differentiated tumor phenotype, indicating that HIF-2α is important to maintain neuroblastoma cells in the immature, stem cell-like state (Pietras et al., 2008; Pietras et al., 2009).

Non-transcriptional, ARNT-independent function of HIF-2a

Based on years of research, it is nowadays well accepted that HIF-1α and HIF-2α are non-redundant as well as distinctly regulated, both temporally and spatially, during normal fetal development and in solid tumors. However, the knowledge about the underlying mechanisms for this differential regulation is still limited. In the attempt to unravel these mechanisms, researchers have identified that both HIF-1α and HIF-2α potentially have non-transcriptional roles, independent of ARNT dimerization (Hubbi et al., 2013; Uniacke et al., 2012). HIF-1α has been shown to cause cell cycle arrest by acting as an inhibitor of DNA replication in response to hypoxia (Hubbi et al., 2013), and HIF-2α has been reported to be part of a cytoplasmic hypoxia-regulated translational initiation complex together with RBM4 and eIF4E2 to promote cap-dependent translation at polysomes (Uniacke et al., 2012). Interestingly, cytoplasmic HIF-2α has been reported in *in vitro*-cultured neuroblastoma cells at normoxia (Holmquist-Mengelbier et al., 2006) and the role of cytoplasmic HIF-2α at oxygenated conditions will be further discussed in *Paper IV*. Thus, unravelling transcriptional as well as non-transcriptional, possibly ARNT-independent roles of HIFs, and understand how these processes are regulated, may prove important in order to identify new treatment strategies in HIF-driven tumors.

The Pseudo-hypoxic Niche and HIF-2α: Targets for Novel Tumor Treatment

It is still not known whether the pseudo-hypoxic niche created by HIF-2α differs from the hypoxic niche, however, there is a concordant expression of *EPAS1* and *VEGF* expression in pseudo-hypoxic areas in neuroblastoma, suggesting that HIF-2α drives tumor angiogenesis at physiological oxygen tensions (Holmquist-Mengelbier et al., 2006). Thus, the correlation between the presence of HIF-2α positive neuroblastoma cells and unfavourable prognosis could be explained by increased *VEGF* expression since there is a relationship between the number of blood vessels and tumor aggressiveness (Carmeliet, 2005). Together these data strongly suggest that HIF-2α, and/or the pseudo-hypoxic niche, is an attractive treatment target, not least in neuroblastoma.

Pharmacological inhibition of HIF-2α: PT2385 and PT2399

Based on structural studies on the HIF-2α-ARNT heterodimer, a hydrophobic pocket was identified in the PAS-B domain of HIF-2α (Scheuermann et al., 2009). This finding allowed for identification of small ligands binding to this pocket to inhibit HIF-2 transcriptional activation of downstream targets by preventing dimerization between HIF-2α and ARNT (Rogers et al., 2013; Scheuermann et al., 2013). This inhibition did not affect HIF-2α mRNA or protein level, indicating that ARNT-independent functions of HIF-2α, such as translational activation (Uniacke et al., 2012), will not be affected. Not long after the initial discovery of the hydrophobic pocket, Peloton Therapeutics (Dallas, Texas) performed an extensive screen of small-molecules libraries and developed the PT2385 inhibitor and the related compound PT2399 (Chen et al., 2016; Cho et al., 2016; Wallace et al., 2016). Both compounds were found to be highly specific, only inhibiting the HIF-2-dependent transcription while having no effect on the HIF-1α/ARNT dimerization or HIF-1 downstream targets, and also displayed good anti-tumor effects in ccRCC PDX models (Chen et al., 2016; Cho et al., 2016; Wallace et al., 2016). Some PDX models and patients have, however, developed resistance towards PT2399. This resistance could, to some extent, be explained by variability in the HIF-2α dependence as resistant models generally displayed lower levels of HIF-2α as well as p53 mutations, indicating that biomarkers has to be identified in order to predict treatment response in patients (Chen et al., 2016; Cho et al., 2016).

PT2385 is now being tested in clinical trials against ccRCC (clinical trial: NCT02293980) and recurrent glioblastoma (clinical trial: NCT03216499). The effect of HIF-2 transcriptional inhibition via PT2385 versus knockdown of the HIF-2α protein via siRNA in neuroblastoma will be further discussed in *Paper IV*.

Chapter 5. Cancer Models

Overview

Cancer research is, like any other research field, greatly dependent on reliable and representative model systems. However, cancer is a highly complex disease characterized by inter- and intratumoral heterogeneity meaning that no single model is good enough to recapitulate all aspects of the disease. For this reason, several tumor model systems have been developed throughout the years with the aim of identifying new biomarkers or treatment targets, perform drug screenings, evaluate toxicity or predict treatment response in the clinic. Examples of model systems that are currently in use include *in vitro*-cultured cell models, various *in vivo* models and computational cancer models. All of these preclinical models have, in one way or another, contributed extensively to the field of cancer research, which in turn have led to improved treatment strategies and better patient outcome. The aim of this chapter is to present a selection of cancer model system along with their advantages and disadvantages, with a special focus on preclinical models of neuroblastoma.

In vitro Models

Cell based *in vitro* models of solid tumors have been used for years as preclinical models to study molecular mechanism of tumor biology. The complexity of current *in vitro* models varies drastically from cancer cell lines that have been cultured in serum-containing medium for decades to 3D models of the tumor microenvironment (Greshock et al., 2007; Sharma et al., 2010). The aim of these models is to provide an understanding of for example tumor proliferation, invasion, migration, drug response, intra- and extravasation. Advances of *in vitro* models are rapidly emerging and new models are characterized by increased complexity through e.g. co-culture of cell types (Hulkower and Herber, 2011; Katt et al., 2016; Vidi et al., 2013).

Cancer cell lines

Human cancer cell lines have been used for decades in cancer research to provide insight into various tumor biological questions and to serve as a model system for preclinical drug testing (Sharma et al., 2010). The first cancer cell line to ever be cultured *in vitro* was HeLa, which was derived from Henrietta Lacks in 1951 who suffered from cervical cancer (Scherer et al., 1953). As of today, cancer cell lines are the most frequently used model system in cancer research (Klinghammer et al., 2017), however, their relevance as a representative and reliable model has been brought into question. First of all, it is uncertain how well they actually represent the primary tumor since it has becoming increasingly clear that cancer cell lines acquire both reversible and irreversible phenotypic and genotypic aberrations during prolonged *in vitro* culture, like gain and loss of genetic information, alteration in growth and invasion properties as well as loss of heterogeneity (Ben-David et al., 2018; Byrne et al., 2017; Gillet et al., 2011; Greshock et al., 2007; Hausser and Brenner, 2005; Li et al., 2008a; Nelson-Rees et al., 1976). Secondly, cross-contamination of cell lines is another major problem that was already discovered back in the 1950s as a result of interspecies contamination, but is equally relevant today (Allen et al., 2016; Capes-Davis et al., 2010; Gillet et al., 2013).

Cancer cell lines, including cell lines used in neuroblastoma research, have traditionally been established in serum-containing medium and serum is without doubt the most common supplement in cell culture media (Baker, 2016; Thiele, 1998). However, serum has been shown to induces irreversible differentiation of neural stem cells (Gage et al., 1995; McKay, 1997; Reynolds et al., 1992). The effect of serum on cancer cell lines was investigated in 2006 by Lee et al and they showed that serum induces phenotypical and genotypical changes of glioma cells (Lee et al., 2006). By culturing freshly isolated glioma cells in either neural stem cell conditions (serum free media supplemented with epidermal growth factor (EGF) and bFGF) or serum-containing media, they found that cells cultured in serum free media grew as neurospheres and displayed tumorigenic capacity when injected orthotopically into immunodeficient mice along with invasive growth pattern. These neurospheres also had the ability to self-renew and differentiate terminally. By stark contrast, serum-cultured cells grew adherently and had lost the capacity to self-renew and differentiate terminally. Moreover, only late passaged serum-cultured cells had the capacity to form tumors *in vivo* and displayed limited tumor cell infiltration into the surrounding normal brain (Lee et al., 2006).

Further studies in neuroblastoma, breast and prostate cancer has also showed that *in vitro* propagation of tumor cells in serum free media maintain patient tumor characteristics as well as the ability of the tumor cells to self-renew, differentiate and initiate tumor growth *in vivo* (Bate-Eya et al., 2014; Ponti et al., 2005; Ricci-

Vitiani et al., 2007). This topic will be further discussed in *Paper I*, where we have optimized culture conditions for our neuroblastoma PDX cells.

In vivo Models

Using animals in cancer research goes back to more than 100 years ago when inbred mouse strains were used for transplantation of syngeneic tumors (Ehrlich, 1905), but it was not until the 1960s that mouse models started to be used more frequently in anticancer drug screening programs (Suggitt and Bibby, 2005). This resulted in improved clinical outcome for both human leukemias and lymphomas, however, the development of treatment for solid tumors were less successful (Suggitt and Bibby, 2005). This resulted in the development of the athymic nude mouse model (Flanagan, 1966) that could be used for growth of human tumor xenografts based on implantation of solid tumor material (Rygaard and Povlsen, 1969). The subsequent development of another immune-deficient mouse strain, the severe combined immunodeficiency (SCID) mouse, rapidly improved testing of cancer drugs in mice and enabled a widespread opportunity of growing both solid tumor material and cell lines as xenografts in the mid-1980s (Klinghammer et al., 2017; Sausville and Burger, 2006). As of today, the mouse is the most widely used and validated model system in cancer research, but the usage of complementary *in vivo* models has emerged and include the zebrafish and avian models.

Cell line-derived xenografts models

Cancer cell lines can either be cultured *in vitro* as a monolayer culture or propagated *in vivo* as xenografts in mice (Mattern et al., 1988). For *in vivo* propagation, cells are most commonly injected subcutaneously or orthotopically. In orthotopic xenografts, tumor cells or explants are implanted into the "proper" organ or tissue (i.e. the tissue of origin most commonly observed for primary tumors of respective tumor form) for tumor growth to recapitulate the microenvironmental cues and cellular interactions that the patient primary tumor experience (Ruggeri et al., 2014).

Subcutaneous xenografts, which is also known as ectopic xenografts, has been widely used in cancer research to study tumor growth and for drug screening (Klinghammer et al., 2017; Sausville and Burger, 2006). The advantages with ectopic xenografts are that treatment responses as well as tolerability of many tumor types can easily be monitored *in vivo*. They are also fairly cost- and time efficient (Ruggeri et al., 2014). Despite these favorable attributes, the cell line-derived ectopic xenograft model is associated with numerous limitations. The major disadvantage is the non-physiological growth location, which prevents cellular

interaction with the host stromal compartment. As a result, ectopic xenografts often display inadequate formation of blood vessels and rarely metastasize to distant organs. Moreover, as cell lines usually have been propagated *in vitro* for years before injection, the established tumors often lack patient specific histological and genetic features and display limited intratumor heterogeneity (Abate-Shen, 2006; Becher and Holland, 2006; Gillet et al., 2011; Gillet et al., 2013; Kung, 2007; McMillin et al., 2013; Voskoglou-Nomikos et al., 2003). Thus, the clinical relevance of the ectopic xenograft models is often questioned.

In orthotopic xenografts, tumor cells or explants are transplanted into an organ or tissue, meaning that the tumor cells are surrounded by host stromal cells and tissues, which enables microenvironmental interaction and signaling. As such, orthotopic xenografts are often highly vascularized and metastasize to clinically relevant sites and can therefore be used to study the microenvironmental role in tumor development, response to anticancer drugs, tumor invasion as well as metastatic behavior (Gao et al., 2012; Loi et al., 2011; McMillin et al., 2013; Smith et al., 2011). Although orthotopic xenografts are more clinically predicative then ectopic models (Bibby, 2004; Killion et al., 1998), they are still derived from cancer cell lines that may have acquired numerous phenotypical and genotypical aberrations while being cultured *in vitro* (Gillet et al., 2011). Moreover, orthotopic xenografts are also quite often technically challenging and not very cost- and time-efficient.

In 2002, Khanna et al developed orthotopic xenograft models of neuroblastoma based on established neuroblastoma cell lines and compared these to subcutaneous xenografts (Khanna et al., 2002). They showed that orthotopic xenograft tumors displayed invasive growth pattern and were highly vascularized, a common feature in neuroblastoma (Meitar et al., 1996). They also metastasized spontaneously to for example liver, lung and bone marrow. By stark contrast, subcutaneous tumors displayed minimal invasiveness, were poorly vascularized and did not metastasize (Khanna et al., 2002). This data was further supported in a similar study by Patterson et al (Patterson et al., 2011). Together, these data support the importance of orthotopic models in cancer research.

Patient-derived xenograft (PDX) models

To avoid *in vitro*-induced selection and adaptation of cells, human tumor material from patients can directly be ectopically or orthotopically transplanted into immunodeficient mice, which generate so-called PDXs (Bleijs et al., 2019; Hidalgo et al., 2014; Tentler et al., 2012). Studies have shown that PDXs recapitulate patient specific tumor properties, like tumor histopathology, metastatic behavior as well as the genomic landscape and proteomic profiles of the corresponding patient tumor in several cancer types, including melanoma, breast cancer, head and neck cancer, NSCLC, colorectal cancer, medulloblastoma and neuroblastoma (Braekeveldt et al.,

2015; DeRose et al., 2011; Dong et al., 2010; Fichtner et al., 2008; Gao et al., 2015; Guo et al., 2016b; Hidalgo et al., 2014; Huang et al., 2017; Julien et al., 2012; Klinghammer et al., 2015; Li et al., 2016; Reyal et al., 2012; Zhao et al., 2012). Moreover, it has also been shown that established PDX tumors can be serially passaged *in vivo* with retained phenotypic and genotypic features (Hidalgo et al., 2014; Tentler et al., 2012). Hence, PDXs have been extensively used in drug screening programs, biomarker discovery as well as in therapy resistance studies and have displayed good ability to predict clinical outcomes in patients (Malaney et al., 2014; Rosfjord et al., 2014). Despite all of the advantageous, PDXs also have limitations. First, the host mice lack a fully functional immune system, which prevents e.g. immunotherapy testing. One solution for this problem is the development of humanized mice and different attempts are being made to reconstitute the human immune system in mice (Allen et al., 2019; Morton et al., 2016; Walsh et al., 2017). Secondly, the human stromal components are gradually replaced by murine stroma in PDX tumors, meaning that the extracellular matrix, tumor-associated macrophages, endothelial cells as well as cancer-associated fibroblasts will all be of murine origin (Braekeveldt et al., 2016; Julien et al., 2012; Peng et al., 2013). As of today, it is however not known if species-to-species differences could alter the tumor growth (Langenau et al., 2015). Third, loss of tumor heterogeneity and/or subclones with important driver mutations may be observed as a single tumor piece is implanted into the mouse from the whole patient tumor. Moreover, further selection of subclones is likely to occur during the engraftment process of the tumor piece where more aggressive subclones have a higher tumor take rate (DeRose et al., 2011; Garrido-Laguna et al., 2011; Kemper et al., 2015; Morgan et al., 2017; Sivanand et al., 2012; Smith et al., 2010). As such, multiple biopsies from several different regions of the tumor should be collected and engrafted in to better reflect the tumor heterogeneity observed in corresponding patient tumors (Braekeveldt et al., 2018).

We and collaborators recently established and characterized orthotopic neuroblastoma PDXs from highly aggressive and metastatic neuroblastomas through implantation of tumor pieces into the adrenal gland of immunodeficient mice (Braekeveldt et al., 2015). These PDX tumors recapitulated expression pattern of typical neuroblastoma markers and retained patient-specific chromosomal aberrations. In addition, the established PDX tumors also displayed infiltrative growth and metastatic spread to liver, lung and bone marrow (Braekeveldt et al., 2015). Further analysis showed that PDX tumors retained important stromal hallmarks observed in aggressive neuroblastoma (Braekeveldt et al., 2016) and preserved essential genetic and phenotypic characteristics after being serially passaged *in vivo* for more than 2 years (Braekeveldt et al., 2018). Cells isolated from PDX tumors expressed typical neuroblastoma markers and could be cultured *in vitro* with retained tumorigenic capacity (Braekeveldt et al., 2015). A thorough

characterization of these *in vitro*-cultured PDX cells is presented in *Paper I*, which have enabled further investigation using the PDX cells to e.g. assay the anti-tumor effect of a PIM/PI3K/mTOR triple kinase inhibitor in neuroblastoma (Mohlin et al., 2019), identify a mesenchymal, RA resistant neuroblastoma cell populations (*Paper III*) and investigate the role of HIF-2α in neuroblastoma (*Paper IV*).

Genetically engineered mouse models (GEMMs)

GEMMs have been important in cancer research to elucidate specific roles of various oncogenes and tumor-suppressor genes in tumor development and treatment response. The first transgenic mouse models were established in the 1980s through overexpression of oncogenes (Adams et al., 1985; Hanahan, 1989), and with technological advancements, mouse embryonic stem cells could be modified through gene-targeting to overexpress oncogenes or knockdown expression of tumor-suppressor genes (Becher and Holland, 2006). Both germline and conditionally regulated GEMMs exist, where the conditional models allow for both spatial and temporal regulation of gene expression. The advantageous with GEMMs, in contrast to xenograft models, is that the tumor develops spontaneously *in situ* in the appropriate tissue or organ and frequently display histological and genetic features that are representative of the original patient tumor. The tumor also consist of mouse tumor cells, .i.e. no interspecies differences between tumor and stromal cells (Becher and Holland, 2006). In addition, as the GEMMs have an intact immune system, they can be used to study the response to immunotherapy (Frese and Tuveson, 2007). Nevertheless, as with any other model system, there are also drawbacks with GEMMs, which include latency, penetrance and frequency of the tumor formation. The asynchronous tumor development is a problem when setting up treatment studies. Also, most GEMMs are based on a single oncogenic event that drive the tumor formation and progression, thereby lacking the genomic setup displayed in patient tumors (Kucherlapati, 2012; Olive and Tuveson, 2006).

Genetically engineered mouse models (GEMMs) of neuroblastoma

The most well-known and widely used GEMM in neuroblastoma is the TH-MYCN mouse model (Weiss et al., 1997). In this model, there is a targeted mis-expression of *MYCN* to peripheral neural crest cells via the rat *TH* promotor and hence tumors are developed in the SNS. Weiss et al showed for the first time that N-MYC contributes to tumor formation in neuroblastoma. As aforementioned, *MYCN* amplification is associated with an aggressive disease, metastasis and poor prognosis (Benard, 1995; Brodeur et al., 1984; Goodman et al., 1997; Seeger et al., 1985; Zaizen et al., 1993). Metastatic spread could be observed to various organs in the TH-MYCN model, including lung and liver, but only one mouse displayed bone marrow metastasis (Weiss et al., 1997). The TH-MYCN mouse model has been

important for preclinical evaluation of new anticancer therapies and for identification of genes that cooperate with *MYCN* during neuroblastoma initiation, progression and dissemination. However, a drawback with this model is that it is driven by *TH*, which is considered to be a late SNS marker. Other transgenic mouse models of neuroblastoma include mis-expression of *ALK* and *LIN28B* that have helped to further elucidate underlying mechanism of tumor formation in neuroblastoma (Heukamp et al., 2012; Molenaar et al., 2012a).

Transgenic zebrafish model

The zebrafish is a tropical fish frequently used in cancer research to study tumorigenesis and treatment response, thereby providing researchers with a model that complements the mouse and human model systems. The favorable attributes of the zebrafish are its small size, high fertility, their highly conserved formation of the SNS and that they are visually transparent (Corallo et al., 2016; Morrison et al., 2016). The transparency of zebrafish allows for real-time imaging and direct visualization of e.g. tumor development, tumor growth, metastatic spread and angiogenic patterning over time by using fluorescently tagged DNA vectors or cancer cells (Ignatius et al., 2012; Zhu et al., 2012; Zhu et al., 2017). Limitations with the zebrafish model is that not all signaling pathways are conserved in the zebrafish and there is also a need for more species-specific antibodies (Langenau et al., 2015). Moreover, injection of human cancer cells is challenged by immune rejection as well as the number of cells that can be injected into the zebrafish (10-100 cells/injection (Langenau et al., 2003; Smith et al., 2010)).

Transgenic models of neuroblastoma

As the development of the SNS is highly similar in humans and in zebrafish (Morrison et al., 2016), the zebrafish is an excellent model system to exploit when studying cellular and genetic mechanisms that might play a role in neuroblastoma formation and progression. In 2012, Zhu et al developed the first transgenic zebrafish model of neuroblastoma by driving the expression of *MYCN-EGFP* in the peripheral SNS and interrenal gland, the analogous to the adrenal medulla of mammals, via the *dβh* gene promotor (Zhu et al., 2012). However, *DBH* is considered to be a late SNS marker, making this model less predicative. The sustained *MYCN* expression in zebrafish prevented differentiation of chromaffin cells and blocked development of sympathoadrenal precursor cells, resulting in widespread cell death. However, some cells were able to escape this N-MYC-induced apoptosis and generated tumor masses in the interrenal gland that were histologically and structurally comparable to human neuroblastomas (Zhu et al., 2012). Activating mutations in *ALK* is the most common mutation in high-risk neuroblastoma and *ALK* is often mutated in *MYCN* amplified tumors (Chen et al.,

2008; Janoueix-Lerosey et al., 2008). To understand the role of *ALK* in *MYCN* amplified neuroblastoma, Zhu et al also developed a *dβh* promotor-mediated zebrafish model of wild type and mutated *ALK* (Zhu et al., 2012). They found that neither wild type nor mutated *ALK* induced tumor formation in, however, combined injection of mutated *ALK* and amplified *MYCN* resulted in earlier tumor onset compared to overexpression of *MYCN* alone. Thus, mutated *ALK* seems to counteract the N-MYC-induced apoptosis in sympathoadrenal precursor cells to promote neuroblastoma formation (Zhu et al., 2012). The cooperative role of *ALK* and *MYCN* was further supported in two TH-MYCN transgenic mouse models co-expressing amplified *MYCN* and mutated *ALK* (Berry et al., 2012; Heukamp et al., 2012).

Numerous transgenic zebrafish models of neuroblastoma are available today (Casey and Stewart, 2018) and many of these demonstrated for the first time the involvement of a certain proteins in neuroblastoma formation and progression, like mutated *ALK* (Zhu et al., 2012).

Avian model

The avian/chick embryo model has been used for research purposes since the 1600s and has been absolutely crucial for our understanding of the human development, including the migration and fate of the neural crest cells (His, 1868; Le Douarin, 2004; Stern, 2004, 2005). Since the mechanisms governing the survival and motility of cells during the development are similar to those involved in cancer formation and metastasis in humans, the chick embryo model has also been used in cancer research (Bader et al., 2006; Fergelot et al., 2013; Nieto et al., 1994; Palmer et al., 2011). The chick embryo development from the laid egg to the hatched chick is divided into 46 morphological distinct stages, known as Hamburger Hamilton (HH) stages, and these stages provide standardisation among researchers (Hamburger and Hamilton, 1951). Other advantageous attributes with the chick embryo model is their accessibility, the ethical acceptability, their time- and cost-efficiency features as well as that many early developmental processes are comparable to that of the human embryo like the neural crest (Stern, 2005). The main advantage is, however, that chick embryos are easily manipulated both *in ovo* and *ex ovo*. Through technological advancements, *in ovo* electroporation of chick embryos have enabled both gain-of-function and loss-of-function experiments. Knockdown of genes can be mediated via morpholinos or siRNA, and gene editing can also be performed by using CRISPR/Cas9 (Gandhi et al., 2017; Hou et al., 2011; Itasaki et al., 1999; Nakamura et al., 2004; Veron et al., 2015; Yokota et al., 2011). Moreover, isolation of cells from the developing chick embryo for *in vitro* culturing is yet another favourable attribute of the chick embryo model, which will be discussed further in *Paper II*. The limitations of the chick embryo model are similar to that of the

zebrafish, as probably not all signaling pathways are conserved between chick and humans and there is also a desire for more species-specific antibodies.

The chick embryonic model in neuroblastoma

One of the challenges with paediatric cancers, like neuroblastoma, is to recapitulate the early tumorigenic events due the paucity of embryonic models. For this reason, the chick embryo model is an excellent *in vivo* model to use when aiming to elucidate the cellular and genetic mechanisms putatively underlying the events that prime and transform neural crest cells. Recently, Delloye-Bourgeois et al developed a chick embryonic model driving formation of neuroblastoma in sympathetic ganglia and adrenal glands by injecting established serum-cultured and PDX-derived neuroblastoma cell lines (Delloye-Bourgeois et al., 2017). They observed that grafted neuroblastoma cells migrated along the neural crest cell migratory routes as chains or clusters, thereby mimicking the migratory pattern of neural crest cells (Huber, 2006; Kulesa and Fraser, 1998; Li et al., 2019; Theveneau and Mayor, 2012). Dissemination of neuroblastoma cells via peripheral nerves and aorta could also be observed (Delloye-Bourgeois et al., 2017). Together, these data highlight the relevance of using the chick embryo as a model system in neuroblastoma, especially since it recapitulates the embryonic microenvironment in which the neural crest cells reside in and neuroblastoma emerges from and this will be further discussed in *Paper II*.

Chapter 6.

The Present Investigation

Paper I: Neuroblastoma patient-derived xenograft cells cultured in stem-cell promoting medium retain tumorigenic and metastatic capacities but differentiate in serum

Overall Aims

The general aim of *Paper I* was to characterize *in vitro* cultures of neuroblastoma patient-derived xenograft (PDX) cells and optimize culture conditions that would maintain their immature phenotype as well as their tumorigenic and metastatic capacity.

Summary

In this paper, we isolated and characterized two *MYCN* amplified cell lines from orthotopic neuroblastoma PDXs. These PDXs had been established through implantation of tumor pieces from high-risk patients into immunosuppressed mice and serially passaged *in vivo*. The PDX-derived cell lines, named LU-NB-2 and LU-NB-3, were established in serum free media supplemented with EGF and bFGF and grew as free-floating spheres. While the expression of typical neuroblastoma markers in LU-NB-2 and LU-NB-3 cells, like neural cell adhesion molecule (*NCAM*) and *NSE*, were in parity with those of the serum-cultured neuroblastoma cell line SK-N-BE(2)c, the PDX cells displayed an increased expression of the stem cell associated markers *NOTCH1*, *NOTCH3* and *C-KIT*. Further characterization of the PDX cells revealed that they are genomically stable and maintained patient-specific genomic aberrations, even after 30 passages *in vitro*. Orthotopic re-injection of *in vitro*-cultured PDX cells resulted in tumor growth and metastatic spread to lung, liver and importantly bone marrow in all tumor bearing animals.

Culturing PDX cells in serum-containing conditions resulted in overt morphological changes, including adherent growth, neurite outgrowth and reduced cell

proliferation. Addition of serum was also followed by increased expression of differentiation markers (e.g. *TH*, *SYP* and *SCG2*), while the stem cell associated markers *NOTCH1* and *NOTCH3* were downregulated under serum culture conditions. Both EGF and bFGF seems to be important for the proliferation of PDX cells and prevented, to some extent, the serum-induced differentiation. Stimulation with NT-3 and/or NGF did not induce terminal differentiation of PDX cells and transferring serum grown cells back to serum free conditions resulted in a phenotypic switch with recovered proliferative rate and decreased expression of differentiation markers.

Growing PDX cells adherently on human recombinant laminin (LN-521) resulted only in slight morphological differentiation, but did not affect growth rate or the tumorigenic and metastatic capacity of the PDX cells. The response to cytostatic drugs was also preserved. We conclude that maintaining our PDX cells in serum free culture conditions, either as spheres or adherent on laminin, is critical to retain the immature, tumorigenic and metastatic phenotype observed in patient tumors.

Discussion

As discussed in Chapter 4, cell lines are the most widely used preclinical model system in cancer research. Nevertheless, an increasing number of studies are reporting that phenotypic characteristics and genetic aberrations observed in *in vitro*-cultured cancer cell lines often deviates from the corresponding human patient tumor. For instance, although most established cancer cell lines have been derived from highly aggressive and metastatic tumors, they often display limited infiltrative growth in xenografts and lack metastatic spread to clinically relevant organs (Ben-David et al., 2018; Gillet et al., 2011; Greshock et al., 2007; Hausser and Brenner, 2005; Li et al., 2008a; Nelson-Rees et al., 1976). These observations could, in part, explain why both *in vitro* and cell line-derived xenograft models used in drug screening programs poorly predict the therapeutic response in patients and why reproducibility in drug sensitivity is an issue as different cell batches may have acquired different aberrations (Ben-David et al., 2018; Borrell, 2010; Freedman et al., 2015; Gillet et al., 2011; Johnson et al., 2001; Prinz et al., 2011; Sharma et al., 2010; Simon, 2008). Thus, the clinical relevance of the classical *in vitro* models in drug discoveries is controversial, and as of today, the success rate in clinical trials of anticancer drugs is less than 10% (Sharpless and Depinho, 2006).

These insights have led cancer researchers to search for more clinically relevant cancer models, leading to the establishment of PDXs (Bleijs et al., 2019; Hidalgo et al., 2014; Tentler et al., 2012). Technically, the first PDX model was established already in 1953 (Toolan, 1953), but reemerged during the 2000s as it was shown to reflect phenotypic and genotypic heterogeneity found in patient tumors (Bleijs et al., 2019). We and collaborators recently established orthotopic neuroblastoma

PDXs from high-risk patients and demonstrated that these PDXs maintain and recapitulate patient tumor characteristics (Braekeveldt et al., 2015; Braekeveldt et al., 2016). Based on recently established protocols (Bate-Eya et al., 2014; Lee et al., 2006; Ponti et al., 2005; Ricci-Vitiani et al., 2007), we cultured isolated PDX-derived cells as spheres in serum free conditions and could in this way promote maintenance of neuroblastoma cells with an immature phenotype with characteristics of the corresponding patient tumor during prolonged *in vitro* culture. Based on our observations, avoiding serum seems to be a key strategy in order to preserve the phenotypic origin of these cells. Our data also indicate that adherent culture on human recombinant laminin (LN-521) did not alter essential patient tumor characteristics. Culture on LN-521 can be employed for short term culture to for example facilitate the performance and data interpretation of future drug screenings as sphere culturing may be associated with increased cell death and spontaneous differentiation due to reduced access of oxygen and growth factors in the sphere center (Reynolds and Rietze, 2005). However, since some differentiation markers were upregulated to some extent at both mRNA level (*TH*, *CHGA* and *SYP*) and protein level (TH and GAP43) after 72 hours of laminin culture, our data indicate that neuroblastoma PDX cells should routinely be propagated as spheres to maintain the immature tumor phenotype.

Cancer cell lines have traditionally been propagated as two-dimensional (2D) cultures *in vitro*, but with increased knowledge about the drawbacks with these models and improved technology, three-dimensional (3D) cultures have been established during the last decade. One type of 3D culture is the suspension grown tumor spheres, also known as tumor spheroids, microtumors or tumor organoids (Katt et al., 2016; Sant and Johnston, 2017; Schutgens and Clevers, 2019). The many names and broad definitions have led to confusions about the 3D suspension cultures in the cancer research field. The most recent coined term is *tumor organoid*. The organoid field was initiated by the groups of Hans Clevers (Sato et al., 2009) and Yoskiki Sasai (Eiraku et al., 2011) and was initially mostly applied in the stem cell field. Organoid means ‘similar to organ’ and are structures derived from e.g. stem cells or organ-specific progenitor cells that self-organize into 3D structures that display both functional and structural similarities to the tissue of origin (Clevers, 2016). Lately, organoids have also been established from human tumor material derived from various solid tumors such as breast, lung, liver, kidney and colon cancer to form tumor organoids (Bleijs et al., 2019; Sato et al., 2011). Importantly, these tumor organoids reflect the histopathology of patient tumors and display essential patient tumor characteristics, including intratumor heterogeneity (Huang et al., 2015; Roerink et al., 2018; Sachs et al., 2018; van de Wetering et al., 2015; Yan et al., 2018). Tumor organoids can be cultured *in vitro* for an extended time period and cryopreserved, which enables the generation of tumor organoid biobanks (Drost and Clevers, 2018). Through technological advancements, it is also possible

to generate tumor organoids from normal epithelial organoids via CRISPR/Cas9 to study the role of specific mutations in tumor formation (Dekkers et al., 2019; Drost et al., 2015; Matano et al., 2015). Orthotopic transplantation of patient-derived or genetically engineered tumor organoids into mice enables modeling of metastatic spread (Schutgens and Clevers, 2019).

Based on the published features of the tumor organoids, it would be possible to say that our neuroblastoma PDX cells indeed are tumor organoids as they display cellular heterogeneity and have tumorigenic as well as metastatic capacitates when re-injected orthotopically into mice. However, since the definition of *tumor organoids* is still a bit unclear and often confounded with the definition or meaning with organoids derived from embryonic and adult stem cells, it should be used with consideration to avoid confusion within the cancer research field.

Paper II: Maintaining multipotent trunk neural crest stem cells as self-renewing crestospheres

Overall Aims

The general aim of *Paper II* was to optimize *in vitro* culture condition for maintenance of chick-derived trunk neural crest stem cells.

Summary

In this paper, we isolated and established primary *in vitro* cultures from neural tubes at trunk axial level derived from chick embryos at HH13/14 (Hamburger and Hamilton, 1951), a developmental stage when trunk neural crest cells are premigratory and hence retained within the neural tube. These cultures are termed crestospheres based on their neural crest origin and spheroid growth pattern. Crestospheres derived from the cranial axial level of the neural tube was recently established with retained self-renewal capacity and multipotency for up to seven weeks in culture (Kerosuo et al., 2015).

By optimizing the culture conditions established for cranial crestospheres via increased RA concentration, decreased starting culture volume and addition of BMP-4, trunk crestospheres could be expanded rapidly *in vitro* and be kept in culture for six to seven weeks with retained proliferative capacity. Comparing the expression of trunk neural crest associated genes (e.g. *HES6*, *AGPAT4*, *RASL11B* and *FMN2* (Murko et al., 2018)) in cranial and trunk crestospheres demonstrated that trunk-derived crestospheres have enriched expression levels of these genes. Further characterization revealed that trunk crestospheres are enriched for neural crest cells, with high expression of neural crest markers *FOXD3*, *B3GAT1*, *SOX9* and *SOX10* and low expression of mesodermal markers *MYOD1* and *BRACHYURY* as compared to wild type embryos. The trunk crestospheres also demonstrated important stem cell associated traits by committing to several cellular linages when cultured under differentiation conditions, i.e. multipotency, and self-renewal capacity after single cell seeding. Finally, trunk crestospheres can efficiently be transduced using lentiviral vectors.

Discussion

Neuroblastoma is a childhood malignancy that since long has been considered to arise from the neural crest cells of the sympathoadrenal cell lineage. Priming events targeting migrating neural crest cells for neuroblastoma transformation most likely

occur during the normal fetal development (as recently suggested by Kerosuo et al by using the chick embryo model (Kerosuo et al., 2018)), however, the actual timing and nature of the events leading to the onset of neuroblastoma are still unknown. To increase our understanding of neuroblastoma initiation, we need models that can recapitulate the embryonic microenvironment in which these priming events occur and neuroblastoma emerges from, such as the chick embryo model. The chick model has been used in research for hundreds of years and has laid the foundation for our understanding of the human embryonic development (Stern, 2004, 2005).

A favourable feature with the chick embryo, especially in research purposes, is that the embryos are easily manipulated in a time- and tissue-specific manner and can be cultured both *in ovo* and *ex ovo*. Based on our data, we can isolate trunk neural stem cells from chick neural tubes and maintain these trunk crestospheres for several weeks *in vitro* with maintained multipotency and self-renewing capacities. As trunk crestospheres are comprised of both neural crest stem and progenitor cells, they will provide an excellent tool in the future to study various aspects related to neural crest and neural crest-derived diseases, including neuroblastoma.

Recently, Delloye-Bourgeois et al modelled neuroblastoma in chick embryos by injecting established serum-cultured and PDX-derived neuroblastoma cell lines at the trunk neural crest level of stage HH14 chick embryos (Delloye-Bourgeois et al., 2017). They observed that grafted neuroblastoma cells migrated along neural crest migratory routes to collect in sympathetic ganglia and adrenal glands. Secondary dissemination of neuroblastoma cells via peripheral nerves and aorta could also be observed (Delloye-Bourgeois et al., 2017). Thus, engrafted neuroblastoma cells mimic essential cellular properties of neural crest cells (Huber, 2006; Kulesa and Fraser, 1998; Li et al., 2019; Theveneau and Mayor, 2012), thereby providing evidence that the chick embryo is a relevant model system in neuroblastoma.

Two major disadvantages with the above-mentioned neuroblastoma chick model are that it is based on already transformed neuroblastoma cells, and even though neuroblastoma cells collected in proper developmental organs, interspecies differences might negatively affect the model (Delloye-Bourgeois et al., 2017). These interspecies differences could be overcome by engrafting chick-derived trunk crestospheres. Neuroblastoma associated genes like *PHOX2B*, *TH* and *TRKC* are enriched in trunk crestospheres (Persson and Mohlin, 2019) and ongoing projects in our lab are currently investigating the potential of applying this approach to the chick embryo model. In *Paper II* we showed that trunk crestospheres could be transduced with a GFP-tagged vector using lentiviral-based methods. This method could be used as a future tool to knockdown or overexpress a gene of interest (for example a disease-causing gene or newly identified gene) in these multipotent and self-renewing trunk crestospheres to study the role of this gene in relation to stemness, proliferation, differentiation as well as its potential to induce neural crest-

derived diseases, like neuroblastoma. Further, by engrafting manipulated trunk crestospheres into chick embryos, it would also be possible to investigate how this specific gene is involved in neural crest migration, patterning of the SNS system and formation of organs. Thus, in neuroblastoma, this type of engraftment would allow us to investigate the involvement of genes at an appropriate developmental stage in an embryonic environment using immature cells with the same origin as the recipient. This is in contrast to xenografts in mice, where human neuroblastoma cells are injected into mature recipients of another species.

The mice and zebrafish models are well-established *in vivo* model systems in neuroblastoma and have been crucial for our understanding of the underlying biology of neuroblastoma and vital for drug screening studies. Both models display high tumor penetrance and have truly demonstrated the involvement of *MYCN* amplification and activated *ALK* in neuroblastoma formation and progression (Berry et al., 2012; Heukamp et al., 2012; Weiss et al., 1997; Zhu et al., 2012). However, as neuroblastoma is a highly complex disease characterized by tumor heterogeneity, no single model will be able to represent all aspects of the disease. For this reason, the chick embryo model will be an appealing complement to current neuroblastoma models, especially since it can mimic the embryonic microenvironment from which neuroblastoma emerges. The chick embryo model has a number of favourable advantageous compared to the mouse and zebrafish models, including the accessibility of fertilized eggs, their ethical acceptability, a rapid embryonic development and a 60% sequence homology when comparing to the human genome (International Chicken Genome Sequencing, 2004; Stern, 2005). It is also a valid model system to use when studying the normal human development and development-associated diseases as the early embryonic chick and human morphology are highly similar.

The main disadvantages with the transgenic mouse and zebrafish models are that they are driven by *TH* and *dβh* promoters, respectively. TH and DBH are enzymes required for the synthesis of noradrenaline (Huber et al., 2002) and are considered to be late SNS/neural crest markers as they are expressed for the first time in early stages of the sympathoadrenal cell (Cochard et al., 1978; Ernsberger et al., 1995; Ernsberger et al., 2000). Since neuroblastoma is thought to arise at earlier developmental stages, it is uncertain how well the mouse and zebrafish models actually represent and recapitulate the human patient tumor. This limitation can be approached with the chick embryo model as any gene can be manipulated, knocked down or overexpressed via *in ovo* electroporation or by engrafting modified chick-derived crestospheres in a time- and site-specific manner.

Paper III: Immature neuroblastoma cells are resistant to retinoic acid and synthesize this drug

Overall Aims

The general aim of *Paper III* was to investigate why mesenchymal-type neuroblastoma cells display increased resistance to retinoic acid (RA), a component used to combat minimal residual disease in high-risk neuroblastoma patients.

Summary

In this paper, we investigated the treatment response to RA in isogenic pairs of undifferentiated mesenchymal (MES) neuroblastoma cells and lineage committed adrenergic (ADRN) neuroblastoma cells to unravel the underlying RA resistance mechanism of MES-type cells. Each pair of isogenic MES and ADRN cell lines has been derived from a single patient tumor and previously characterized to have a unique set of lineage-specific super-enhancers and associated core regulatory circuitries (van Groningen et al., 2017).

Treating MES- and ADRN-type neuroblastoma cells with RA resulted in a clear inhibition of proliferation of ADRN cells, whereas MES cells were unaffected by the treatment and continued to proliferate and preserve their cellular morphology. Further investigation showed that MES cells have an endogenous synthesis of RA, as determined by the MES-type specific expression of enzymes required for RA synthesis, like *ALDH1A3*, *ALDH1A1* and *RDH10*, and activation of retinoic acid response elements (RAREs) in gene promoters. Blockage of the RA synthesis pathway resulted in reduced proliferation and migration of MES-type cells, which was not observed in their isogenic ADRN counterpart. Cluster analysis revealed that MES-type neuroblastoma cells resemble the recently identified Schwann Cell Precursors (SCPs, (Furlan et al., 2017)), where both MES and SCP cells are highly migratory and display similar expression pattern of RA target genes (Furlan et al., 2017; van Groningen et al., 2017), suggesting that the MES cells have 'inherited' their RA synthesis, motility and RA resistance from an earlier developmental stage. Finally, qRT-PCR analysis of neuroblastoma PDX cells (*Paper I*), confirmed *ALDH1A3* expression in both LU-NB-2 and LU-NB-3 cells. Based on ALDH activity, LU-NB-2 cells were FACS-sorted and analyzed *in vitro* and *in vivo*. *In vitro* culture of ALDH^{Pos} and ALDH^{Neg} sorted cells revealed spontaneous and bidirectional transdifferentiation of both cell types. Orthotopic re-injection into immunocompromised mice demonstrated that ALDH^{Pos} and ALDH^{Neg} cells have tumorigenic and metastatic capacity *in vivo* and gave rise to heterogeneous tumors, indicating that ALDH^{Pos} constitute a fully malignant neuroblastoma cell type.

Discussion

There is an increasing number of studies reporting that solid tumors are comprised of a fraction of immature tumor cells that lack expression of specific lineage differentiation markers (Boshuizen et al., 2018; Hovestadt et al., 2019; Lim et al., 2017; Neftel et al., 2019; Patel et al., 2014; Pietras et al., 2008; Sanchez-Danes et al., 2018; Tirosh et al., 2016; van Groningen et al., 2017). These immature tumor cells can be present already from the beginning in the treatment-naïve tumor and are frequently treatment resistant. It has therefore been suggested that these immature cells are causing the tumor relapse. This notion is supported by recent studies where combination therapy targeting both immature tumor cells and lineage-differentiated tumor cells significantly improved the survival of mice (Biehs et al., 2018; Boshuizen et al., 2018; Sanchez-Danes et al., 2018).

The existence of phenotypically divergent neuroblastoma cells has been known for many years through pioneering work by Dr Biedler and co-workers. They showed that *in vitro*-cultured neuroblastoma cells contained at least three distinguishable and interconvertible cell types (Ciccarone et al., 1989; Rettig et al., 1987; Ross et al., 1983), and this phenotypic divergency has also been observed in human neuroblastoma specimens (Pietras et al., 2008). Further, in 2017 two independent groups reported that neuroblastoma is composed of at least two phenotypically divergent tumor cell types: the lineage-committed adrenergic-like ADRN cells and the mesenchymal-like MES cells (Boeva et al., 2017; van Groningen et al., 2017). van Groningen et al showed that ADRN-type neuroblastoma cells expressed transcription factors of the adrenergic lineage as well as late SNS markers. By stark contrast, MES-type neuroblastoma cells lacked expression of these markers but instead showed a neural crest-like gene signature and expressed mesenchymal marker genes. The expression profile displayed by ADRN and MES cells is driven by a unique set of lineage-specific super-enhancers and associated core regulatory circuitries (CRCs) (van Groningen et al., 2017). Super-enhancers have been implicated to play a key role in determining the cellular identify of mammalian cells (Hnisz et al., 2013; Whyte et al., 2013; Young, 2011), and interestingly, expressing MES-type CRCs transcription factors in ADRN cells enabled ADRN cells to transdifferentiate into MES-type cells through reprogramming of the ADRN super-enhancer and gene expression profile (van Groningen et al., 2019; van Groningen et al., 2017). Moreover, *in vitro* studies of isogenic MES and ADRN neuroblastoma cells demonstrate that MES cells are highly migratory and display reduced sensitivity to a number of chemotherapeutic drugs as compared to their ADRN counterpart, which is in line with that MES-type neuroblastoma cells are enriched in post-therapy and relapsed tumors (van Groningen et al., 2017).

RA has been used for anticancer purposes as a differentiating agent in human tumors, including high-risk neuroblastoma, for many years (de The, 2018). As

outlined in the “Prognostic markers of neuroblastoma” section in Chapter 2, the tumor differentiation status in neuroblastoma is correlated to prognosis and outcome (Fredlund et al., 2008). Based on evidence that *in vitro*-cultured neuroblastoma cells differentiated in response to chemical agents (Pahlman et al., 1981; Sidell, 1982) and that spontaneous differentiation of highly aggressive and metastatic neuroblastomas is a common feature in stage 4S diseases (Matthay et al., 2016), raised the hope that high-risk neuroblastoma could be treated with differentiating agents to improve their prognosis. Early studies showed that 13-cis-retinoic acid, or isotretinoin, induced differentiation and growth arrest of neuroblastoma cells *in vitro* (Reynolds et al., 1991). Isotretinoin is currently used in the clinic to combat minimal residual disease in high-risk patients (Matthay et al., 1999). However, the prognostic value of using isotretinoin in high-risk neuroblastoma is debated due to contradicting reports but, overall, it seems like the strong effects observed *in vitro* when using differentiation agents cannot be translated into the clinic (Matthay, 2013; Matthay and Reynolds, 2000; Matthay et al., 1999; Peinemann et al., 2017).

Our data indicate that the presence of phenotypically divergent neuroblastoma cells have major impact on the therapeutic response towards RA as ADRN-type neuroblastoma cells differentiated, while the MES-type cells were RA resistant. We also showed that MES-type cells have an endogenous synthesis of RA and require RA for their proliferation and migration, meaning that this subset of cells is dependent on an “anticancer drug” for their propagation. The question is why?

Furlan et al recently performed genetic cell lineage tracing and RNA-sequencing of adrenergic cells of mouse embryos and identified three main cell types, including the SCPs (Furlan et al., 2017). The authors found that the majority of chromaffin cells of the adrenal medulla stems from neural crest-derived SCPs. Based on our data, the cluster of mouse-derived SCPs was strongly positive for the human MES signature established by van Groningen et al in 2017, but negative for the ADRN signature. The SCP cells also expressed Aldh1a3 as well as RA target genes induced in MES cells. Thus, the MES-type neuroblastoma cells resemble the SCP cells, meaning that an intriguing answer to why MES cells endogenously synthesize RA is that MES-type cells might have ‘inherited’ their RA synthesis, motility and RA treatment resistance from a precursor cell of the adrenergic lineage during the embryonic development. This resemblance between tumor cells and embryonic cell populations has also been observed in other childhood tumors, i.e. medulloblastoma, ependymoma and cerebellar pilocytic astrocytomas (Vladoiu et al., 2019). Thus, together these data suggest that each of the above-mentioned childhood tumors arises in a particular cell type at a specific developmental time point as most of these cells are only present for a restricted time period during the embryonic development. However, extensive *in vitro* and *in vivo* studies have to be performed to exclude that these childhood tumor cells have not undergone de-differentiation or trans-differentiation during the transformation process.

Paper IV: HIF-2 transcriptional activity is not sufficient to regulate downstream target genes in neuroblastoma suggesting a non-transcriptional role of HIF-2α

Overall Aims

The general aim of *Paper IV* was to study the effects of HIF-2 inhibition and the roles of HIF-2α in neuroblastoma by using the novel HIF-2 specific inhibitor PT2385.

Summary

In this paper, we detected high protein levels of hypoxia-inducible factor (HIF)-2α in perivascular niches in tumors established from re-injected *in vitro*-cultured neuroblastoma PDX cells (*Paper I*), which is in coherence with observations in neuroblastoma patient tumors (Pietras et al., 2008). We also detected high levels of HIF-2α protein during *in vitro* culture of neuroblastoma PDX cells at oxygenated conditions, particularly in the cytoplasmic fraction. Treating PDX cells with the specific HIF-2α inhibitor PT2385 resulted in reduced nuclear HIF-2α protein levels. Surprisingly, virtually no effects were observed on *in vivo* tumor growth or expression of the canonical HIF-2 downstream targets *VEGFA* and *BHLHE40* (also known as DEC1) *in vitro* following PT2385 treatment in PDX cells, despite complete ablation of HIF-2α/ARNT dimerization. Treating the conventional serum-cultured neuroblastoma cell line SK-N-BE(2)c with PT2385 *in vitro* resulted in similar result, indicating that this lack of transcriptional effect is not restricted to the neuroblastoma PDX cells.

RNA sequencing data obtained from PT2385-treated PDX cells confirmed *in vitro* experiments as it revealed a virtually unaffected transcriptome. By stark contrast, knockdown of HIF-2α protein using siRNA demonstrated a profound downregulation of both *VEGFA* and *BHLHE40*. Further investigation revealed that the lack of PT2385-mediated effects on cellular transcription can, to some extent, be explained by compensatory mechanism of HIF-1.

Discussion

HIFs are heterodimeric proteins composed of an oxygen-regulated HIF-α subunit and a constitutively expressed ARNT (also known as HIF-1β) subunit that directly or indirectly control the expression of several thousands of genes (Semenza, 2010). The main function of the HIF family proteins is to transcriptionally change the

metabolism of the cell to move away from energy consuming processes when normal or tumor cells experience low oxygen levels. This cellular machinery is essential for life and our understanding on how cells sense and adapt to low oxygen levels was initially unravelled in the 1990s by Gregg L. Semenza, Sir Peter J Ratcliffe and William G Kaelin Jr who will be awarded the *2019 Nobel Prize in Physiology or Medicine* for their discoveries.

Professor Gregg Semenza noticed binding of hypoxia-inducible nuclear factors to a specific enhancer element located 3' to the human *EPO* gene when hepatocytes were cultured under low oxygen condition, now known as hypoxia response elements (HREs) (Semenza et al., 1991; Semenza and Wang, 1992). In an effort to identify these hypoxia-inducible nuclear factors that bind to HRE sequences in an oxygen-dependent manner, Semenza discovered a complex that he decided to call *hypoxia-inducible factor* or *HIF* that consisted of two transcription factors: HIF-1α and ARNT (Wang et al., 1995; Wang and Semenza, 1993). Peter J Ratcliffe was also interested in the oxygen-dependent regulation of the *EPO* gene and discovered that the cellular pathway responsible for adaption to low oxygen was present in multiple cell types and not only the liver and kidney where cells respond to hypoxia by producing EPO (Maxwell et al., 1993). At the same time, William Kaelin was researching on the VHL disease, which is an inherited syndrome characterized by increased risk of developing for example ccRCC (Maher and Kaelin, 1997). Kaelin discovered that ccRCC cells with defective pVHL displayed constitutive upregulation of hypoxia-regulated genes and that reintroduction of wild type pVHL into pVHL-defective ccRCC cell lines suppressed the formation of tumors *in vivo* and reduced the expression of hypoxia-regulated genes (Iliopoulos et al., 1995; Iliopoulos et al., 1996; Kaelin and Maher, 1998). With these findings, Ratcliffe and his group could demonstrate that pVHL is required for the degradation of HIF-1α under oxygenated conditions (Maxwell et al., 1999). However, at this time point, it was not known exactly how pVHL could promote degradation of HIF-1α. But in 2001, Kaelin and Ratcliffe showed that under oxygenated conditions, PHD proteins hydroxylate HIF-α at conserved proline residues on HIF-1α, which results in a high-affinity binding site for pVHL (Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). Together, their discoveries have led to new promising strategies to combat many human diseases, including cancer.

Hypoxia is an independent factor predicting poor patient outcome in numerous solid tumors. However, the prognostic value and tumorigenic role of HIF-1α and HIF-2α in human cancer has been shown to be tumor type specific. In both glioma and neuroblastoma, a subset of immature tumor cells are located in perivascular niches and stains intensively for HIF-2α despite their well-oxygenated microenvironment, thereby creating a pseudo-hypoxic niche. The presence of these immature, neural crest-like HIF-2α positive cells correlates to poor prognosis and tumor aggressiveness (Holmquist-Mengelbier et al., 2006; Li et al., 2009; Pietras et al.,

2008). Moreover, there are also indication that HIF-2α is not only associated with, but also appear to promote, an undifferentiated phenotype in both neuroblastoma cells and pluripotent stem cells (Covello et al., 2006; Holmquist-Mengelbier et al., 2006; Pietras et al., 2009). For these reasons, HIF-2α has been suggested to be a marker of stem cells. The mechanism behind this non-hypoxic stabilization of HIF-2α is poorly understood and is currently under investigating in our lab by using both neuroblastoma PDX cells and the chick embryo model. We know that HIF-2α is transiently expressed during human embryonic stages correlating to time points when neuroblastoma is thought to arise (Mohlin et al., 2013; Nilsson et al., 2005), but little is known about its function at these early time points.

Since expression of HIF-2α is associated with poor prognosis, tumor aggressiveness and metastasis in several solid tumors, efforts have been made to target the transcriptional activity of HIF-2α (Semenza, 2019). Transcription factors have historically been challenging to target therapeutically due to their nuclear localization, and they have even been considered as undruggable (Koehler, 2010). In 2009 however, Bruick and Gardner identified a hydrophobic pocket in the HIF-2α PAS-B domain, which enabled identification of allosteric inhibitors that prevented dimerization of HIF-2α and ARNT (Rogers et al., 2013; Scheuermann et al., 2013; Scheuermann et al., 2009). These discoveries led to the development of the two structurally similar PT2385 and PT2399 inhibitors by Peloton Therapeutics (Chen et al., 2016; Cho et al., 2016; Wallace et al., 2016). Both compounds were found to be highly specific, they only inhibited HIF-2α dependent transcription while having no effect on HIF-1α and displayed very good antitumor effect *in vivo* (Chen et al., 2016; Cho et al., 2016; Wallace et al., 2016). Based on this data, we synthesized PT2385 to test it in a neuroblastoma setting. Although PT2385 was proven to be effective in cellular systems where *bona fide* HIF-2 targets genes have been identified (*EPO* in hepatocellular carcinoma Hep3B cells) or in settings where the HIF-2 transcriptional-driven pseudo-hypoxic phenotype is the main driver of the disease (786-O ccRCC cells), cellular responses to PT2385 in neuroblastoma was virtually absent. This could, in part, be explained by overlapping functions of HIF-1α and HIF-2α, as discussed in more detail below. In addition, a more intriguing explanation could be that HIF-2α possesses additional functions solely than acting as a transcription factor in the nucleus. This notion is supported by the data presented in this study. First, we observe high expression of HIF-2α protein at oxygenated conditions both *in vitro* and *in vivo*, particularly in the cytoplasmic cell fraction, which is in conjunction with previous data (Holmquist-Mengelbier et al., 2006; Pietras et al., 2008). Moreover, PT2385 treatment completely prevents dimerization between HIF-2α and ARNT and reduced nuclear HIF-2α protein levels in our neuroblastoma cells, however, virtually no effects were observed on expression of classical HIF-2 downstream targets, cellular proliferation *in vitro* or tumor growth *in vivo*. Based on our data, we hypothesize that it is plausible that

HIF-2α possesses an almost unexplored function in the cytoplasm, possibly independent of ARNT, that can regulate e.g. gene transcription and cell proliferation. HIF-2α has previously been described to promote cap-dependent translation of mRNAs in the cytoplasm under hypoxic oxygen conditions together with RBM4 and eIF4E2 (Uniacke et al., 2012), thereby supporting our hypothesis. However, how this possible ARNT-independent function of HIF-2α is regulated in the cytoplasm at oxygenated conditions and the downstream effects of it is yet to be discovered. Dissecting the role of subcellular localization of HIF-2α during the SNS development and identifying novel binding partners in the cytoplasm at oxygenated conditions will be of high importance to uncover non-canonical HIF-2α protein function in neuroblastoma. The importance of investigating the HIF-2α interactome to identify novel nuclear protein binding partners has been demonstrated in melanoma (Steunou et al., 2013), and by employing the same type of experimental idea to neuroblastoma, but by also extending it to the cytoplasmic interactome, might unravel putative functions of HIF-2α.

HIF-1α and HIF-2α are closely related and both activate HRE-dependent transcription of target genes (Wenger, 2002). Nevertheless, as described in the "HIFs in normal development" section in Chapter 4, knockout studies in mice have demonstrated that HIF-1α and HIF-2α are nonredundant and that inactivation of either one of them results in distinctly different phenotypes. This could, in part, be explained by cell- and tissue-specific expression of HIF-1α and HIF-2α, or when the two isoforms are induced and expressed in a cell, i.e. temporal induction and regulation, (Holmquist-Mengelbier et al., 2006; Rosenberger et al., 2002; Wiesener et al., 2003), but it could partly also be due to distinct transcriptional targets of HIF-1α and HIF-2α (Hu et al., 2003; Rankin et al., 2007). For example, *BNIP3* and *PGK1* are mainly thought of as HIF-1α transcriptional target genes in many mammalian cells. However, during the progression of ccRCC, expression of HIF-1α is commonly lost, meaning that HIF-2α assumes the transcriptional control of otherwise HIF-1 regulated genes (Lau et al., 2007). Moreover, the less studied HIF-3α isoform has also been shown to regulate the expression levels of for example *EPO* and *GLUT1*, i.e. known HIF-2 and HIF-1 genes, respectively, in human Hep3B hepatocellular carcinoma cells (Heikkila et al., 2011). Thus, the transcriptional response to hypoxia is strictly regulated in mammalian cells by the HIF proteins, but determining exactly which of the three HIF-α isoform that activates a specific target gene in a certain condition could be extremely difficult. For this reason, it is of importance that an array of HIF-1α, HIF-2α and HIF-3α target genes are used in the experimental setting to facilitate data interpretation as most target genes can be transcriptionally activated by at least one HIF-α isoform depending on current conditions. Based on our data, HIF-1α seems to partly compensate for the lack of PT2385-mediated effects on cellular transcription in neuroblastoma, as showed by increased downregulation of both *VEGFA* and *BHLHE40* in cells where PT2385

treatment was combined with siRNA targeting HIF-1α. Thus, genes commonly used as HIF-2α downstream targets in neuroblastoma are partly also regulated by HIF-1α, at least in this setting.

Finally, there are emerging contradicting reports on the oncogenic role of HIF-2α in neuroblastoma. Westerlund et al recently concluded that HIF-2α correlates to good patient outcome, a more differentiated phenotype and neuroblastoma suppression (Westerlund et al., 2019; Westerlund et al., 2017b). The authors also suggest that HIF-2α is not a suitable target in neuroblastoma as they observe that PT2385 did not prevent cellular proliferation *in vitro* nor xenograft growth *in vivo* (Westerlund et al., 2019). However, based on the fact that HIF-2α is crucial for proper SNS development (Compernolle et al., 2002; Peng et al., 2000; Scortegagna et al., 2003; Tian et al., 1998), is expressed at discrete time points in the developing human and mouse SNS (Jögi et al., 2002; Mohlin et al., 2013; Nilsson et al., 2005; Tian et al., 1998; Tian et al., 1997), gain-of-function mutations have been observed in the closely related SNS-derived tumors paraganglioma and pheochromocytoma (Comino-Mendez et al., 2013; Fishbein et al., 2017; Toledo et al., 2013; Zhuang et al., 2012) and previously published data on the possibly oncogenic role of HIF-2α in neuroblastoma (Holmquist-Mengelbier et al., 2006; Mohlin et al., 2013; Mohlin et al., 2015; Noguera et al., 2009; Pietras et al., 2008; Pietras et al., 2009), it seems likely that HIF-2α remains to be an important player in neuroblastoma initiation and/or progression. The data interpretation by Westerlund et al., was also discussed in a follow-up Letter to the editor published in the same journal ((Mohlin et al., 2017), and further response by (Westerlund et al., 2017a)).

Chapter 7.
Conclusion and Future Perspective

Neuroblastoma is a childhood malignancy of the SNS that is characterized by high inter- and intratumoral heterogeneity (Matthay et al., 2016). Although pediatric tumors are distinct from adult tumors in many aspects, including cellular origin, response to therapy, mutation frequency and driving events (Grobner et al., 2018; Jones et al., 2019; Ma et al., 2018), one of the key factors contributing to treatment failures, therapy resistance and fatal outcomes in both pediatric and adult cancer is tumor heterogeneity (McGranahan and Swanton, 2017). The cure rates have increased for childhood cancers, but it is still the leading cause of death by disease among children above one year of age in the developed world (Pui et al., 2011; Siegel et al., 2019). To improve the outcome for childhood cancers, including high-risk neuroblastoma, we need reliable preclinical models to identify new rigorous biomarkers and validated treatment targets. However, emerging data have demonstrated that many of the widely used model systems in cancer research do not recapitulate essential patient tumor characteristics, such as the tumor heterogeneity observed in corresponding patient tumors, thereby resulting in poor prediction of treatment responses in patients (Ben-David et al., 2017; Ben-David et al., 2018; Gillet et al., 2011; Gillet et al., 2013; Greshock et al., 2007).

The overall aim with this thesis was to we established new preclinical models of neuroblastoma that can be used to understand the biology behind neuroblastoma and clarify timing of events preceding neuroblastoma initiation. In *Paper I* we reported a comprehensive characterization of two *MYCN* amplified PDX-derived cell lines. We showed that PDX cells are genetically stable and can be propagated *in vitro* under serum free conditions as spheres or adherently on laminin with retained patient tumor characteristics and maintained capacity to form tumors and metastasis *in vivo* when re-injected orthotopically. We also found that serum should be avoided during *in vitro* culture since it induced neuronal differentiation. In *Paper II* we isolated trunk neural tubes from developing chick embryos to establish *in vitro* cultures of chick-derived trunk crestospheres. Here we optimized the culture conditions for maintenance of trunk crestospheres, which are comprised of both neural crest stem and progenitor cells and can be maintained in culture for up to seven weeks with retained capacity to self-renew and differentiate into numerous

lineages, including smooth muscle, peripheral neurons and glial cells. Trunk crestospheres could also be efficiently manipulated using lentiviral vectors.

Phenotypically divergent cell types have been reported in numerous solid tumors where a fraction of tumor cells have been shown to lack expression of specific lineage differentiation markers and display increased resistant to therapy (Hovestadt et al., 2019; Neftel et al., 2019; Pietras et al., 2008; van Groningen et al., 2017). In *Paper III* we demonstrate that immature MES-type neuroblastoma cells are resistant to RA, a differentiating agent that is given to high-risk neuroblastoma patient to combat minimal residual disease. We also showed that MES-type cells endogenously synthesize RA and is dependent on RA for their propagation as inhibition of RA synthesis resulted in reduced proliferation and migration. Cluster analysis revealed that MES-type cells resemble SCP (Furlan et al., 2017), suggesting that the MES cells might have acquired their RA dependency from a precursor cell during embryonic development. In *Paper IV* we have studied the effect of HIF-2 transcriptional inhibition in neuroblastoma by comparing the HIF-2α inhibitor PT2385 with siRNA-mediated knockdown of the HIF-2α protein. HIF-2α has previously been shown to be marker of neural crest- and mesenchymal-like neuroblastoma cells within the perivascular niche in patient tumors, where the presence of these cells associate with metastatic disease and poor clinical outcome (Holmquist-Mengelbier et al., 2006; Noguera et al., 2009; Pietras et al., 2008). Here we demonstrate that PT2385 effectively inhibited the dimerization between HIF-2α and ARNT as well as reduced nuclear HIF-2α proteins level in lower oxygen concentrations. However, we observed virtually no effect on the transcriptome in neuroblastoma cells following PT2385 treatment and PT2385 did not block neuroblastoma cell proliferation *in vitro* nor xenograft growth *in vivo*. By stark contrast, downregulation of HIF-2α protein resulted in profound suppression of classical HIF-2 gene targets, highlighting the need to further elucidate transcriptional as well as non-transcriptional, ARNT-independent roles of HIF-2α.

Collectively, the work included in this thesis highlight the importance of optimizing and establishing culture conditions that promote maintenance of phenotypically and genotypically divergent cells. The sphere-cultured neuroblastoma PDX cells, or neuroblastoma PDX tumor organoids, have already in this thesis demonstrated their potential of being used as a clinically relevant model of neuroblastoma in *Paper III* and *Paper IV*, and have been or are currently used in a wide range of projects trying to identify new treatment targets or biomarkers and for uncovering the biology behind neuroblastoma (Hamidian et al., 2018; Mohlin et al., 2015; Mohlin et al., 2019). The scope and clinical impact of our neuroblastoma PDX cells is likely to increase by for example generating co-cultures with immune cells or cancer-associated stromal cells *in vitro*, or xenografting cells orthotopically into humanized mouse models. Furthermore, if manipulated chick-derived crestospheres can be successfully xenografted into chick embryos, this model can be used not only to

study the role of genes in relation to stemness and differentiation *in vitro*, but also *in vivo*. This would enable a wide range of experiments, which in turn could result in identification of signaling pathways, protein complexes or microenvironmental cues that are involved in neuroblastoma initiation. Thus, *in vitro* models have been, and will remain to be, an extremely valuable and powerful tool in cancer research to uncover the biology behind tumors and for treatment response studies. However, as mentioned throughout this thesis, cancer is a highly complex disease and no single model will be able to recapitulate all aspects of the disease. It is therefore of importance to combine multiple preclinical cancer models in research to get the best possible prediction of tumor sensitivity to anticancer therapies, which in turn will increase the translatability from bench to bedside.

The finding that MES-type neuroblastoma cells synthesize RA endogenously and resemble normal mouse-derived SCP cells could have several clinical implications. For example, as RA is currently used in the clinic to combat minimal residual disease, it will be of importance to find alternative treatment options to target the MES-type cells. Technically, the current usage of RA in high-risk patients might just enrich for more aggressive neuroblastoma cells, and for this reason, the RA treatment protocol might need to be carefully re-evaluated.

We still have to unravel the true function of HIF-2α in neuroblastoma and determine if it indeed have non-transcriptional roles independent of ARNT in the cytoplasm at oxygenated conditions. A first step towards this goal would be to immunoprecipitate HIF-2α at different conditions and when located in different cellular compartments to study potentially novel protein complexes of HIF-2α in the cytoplasm. However, due to technical difficulties this has not yet been successful, but when it does, our findings might lead to a better understanding for the priming/initial events of neuroblastoma and improved treatment protocol for high-risk patients.

Populärvetenskaplig Sammanfattning

Neuroblastom är en cancersjukdom som nästan uteslutande drabbar barn och uppstår i det icke-viljestyrda sympatiska nervsystemet. Cancer är en mycket komplex sjukdom och uppstår på grund av att cancerceller växer på ett okontrollerbart sätt. Tillväxten resulterar slutligen i en klump av cancerceller och den här ”klumpen” kallas för tumör.

Ett problem i cancerforskningen är bristen på relevanta system för att kunna studera hur cancerceller växer. Vi måste alltså utveckla nya modeller som återspeglar patienternas tumörer på ett trovärdigt sätt. Det övergripande målet med den här avhandlingen var att utveckla och använda nya metoder för att på ett så bra sätt som möjligt kunna studera neuroblastom samt därmed utveckla nya behandlingsmetoder.

I första delen av avhandlingen har vi etablerat två nya system för att studera neuroblastom. I *Delarbete I* studerade vi hur olika miljöer påverkar våra cancerceller. Vi visar att vi kan använda oss av cancerceller som kommer från patienter och att dessa celler behåller sina egenskaper även på laboratoriet. Barncancer skiljer sig från cancerformer som drabbar vuxna i det avseendet att uppkomsten av tumören sker på grund av onormala förändringar under fosterutvecklingen. Det är inte etiskt möjligt att använda sig av humana foster för att studera dessa förändringar och därför måste man använda sig av andra alternativ. I *Delarbete II* valde vi att använda oss av celler från kycklingembryon för att kunna studera vad som händer under fosterutvecklingen.

I andra delen av avhandlingen har vi använt oss av de cancerceller som vi kartlade i *Delarbete I*. Vi har på så sätt kunnat visa i *Delarbete III* att en viss typ av cancerceller är resistenta mot retinolsyra, vilket idag används som behandling av snabbväxande neuroblastom för att minska risken för återfall. Med hjälp av denna upptäckt kan vi nu försöka hitta en annan behandling som just dessa cancerceller är känsliga mot och på så sätt minska risken för återfall. I *Delarbete IV* testade vi ett nytt läkemedel som visat sig lovande i kliniska prövningar för vissa typer av vuxencancer. Det här läkemedlet riktar in sig på ett protein som heter HIF-2α som är sammankopplat med just neuroblastom. HIF-2α ett av kroppens viktigaste protein när vi utsätts för lågt syretryck, och 2019 års Nobelpris i Fysiologi eller Medicin belönade upptäckten av hur kroppens celler kan överleva olika syrenivåer. Vi upptäckte att läkemedlet inte hade den önskade effekten i neuroblastom och att det således krävs nya strategier för att förstå och behandla HIF-2α.

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