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OPTIMIZATION AND VALIDATION OF A SPECIES-SPECIFIC *IN VIVO* APPROACH FOR STUDIES ON GROWTH AND PROGRESSION OF NEURAL CREST DERIVED TUMOURS

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“Per Aspera Ad Astra”

To my family

ABSTRACT

Background: Despite great advantages in cancer therapeutics during the last decades, therapy-resistance remains a problem in the majority of malignancies. Hence, development of more effective therapeutics is critical to enhance survival of patients with cancer. A big challenge for therapy development is low efficacy upon clinical translation; therapies proven highly effective in animal trials often exhibit a poor effect or even fail in the clinic. This indicates the need for more relevant preclinical *in vivo* model systems for cancer. Malignant melanoma and pediatric neuroblastoma, occurring in derivatives of the neural crest, are both exhibiting a high degree of therapy-resistance, with a high number of deaths as a consequence. These malignancies are characterized by a significant plastic developmental capacity with a tumour phenotype extensively depending on the surrounding microenvironment. Thus, they provide excellent opportunity for studies on interactions between the tumour and adjacent supportive tissue.

Objective: The overall aim of this thesis is to optimize and validate a human *in vivo* model system, based on human embryonic stem cell derived teratomas (hEST-model), for growth and progression of neural crest derived malignancies. Further on, I aim to elucidate whether human tumours grafted in such system are having a higher resemblance to clinical tumours, as compared to conventionally used xenografts.

Results: Based on a thorough kinetic study of development in hESC derived teratoma from the cell line HS181, we chose day 45 as the time point for tumour inoculation. At this time, mature embryonic tissues are formed and human vascularisation is initiated. Also, this allows further grafting of the tumour cells without affecting the animals well-being and occurrence of necrotic areas. The capacity for teratoma formation was also explored using a subline of HS181 with altered karyotype, i.e. trisomic for chromosome 12. Results revealed that these cells are capable of forming teratomas, however with a skewed tissue contribution with higher frequency of renal formation. Cell lines from malignant melanoma and neuroblastomas were grafted in the hEST-model and compared to the corresponding xenograft. For the melanomas, striking differences in expression of markers related to melanocytic differentiation was seen, indicative of induced differentiation from the xenograft-environment. Also, a dedifferentiated and invasive subpopulation of melanoma cells was detected, but not present in the xenograft, indicating species-specific interactions upon migration and invasion into surrounding stroma. Grafting of three different neuroblastoma cell lines in the hEST-model resulted in a general finding of higher histological heterogeneity and more resemblance to clinical neuroblastomas, as compared to the xenografts. Grafting of both tumour types in the hEST-model induced an extensive neo-vascularisation of human origin in the surrounding mesenchyme indicating species-specific effects.

Conclusions: The results presented in this thesis indicate that a human *in vivo* model system for cancer based on hESC-derived teratomas add significant importance for preclinical cancer studies. The embryonic environment of the teratoma is probably most relevant for grafting of embryonic tumours, indicated also by our results using pediatric neuroblastoma. Altogether, the hEST-model provides unique possibilities to study human tumours in a species-specific environment, and is therefore suggested a well-needed complement to current preclinical *in vivo* models.

LIST OF PUBLICATIONS

I. Gertow, K.*, **Cedervall, J.***, Jamil, S., Imreh, M.P., Ali, R., Gulyas, M., Sandstedt, B., Ährlund-Richter, L. **Early and late events in xenograft development from the human Embryonic Stem Cell line HS181 - Resemblance with an initial multiple epiblast formation followed by a chaotic embryonic organoid development.** *Submitted*

II. Gertow, K., **Cedervall, J.,** Unger, C., Szöke, K., Blennow, E., Imreh, M.P., Ährlund-Richter, L. **Trisomy 12 in hESC leads to no selective *in vivo* growth advantage in teratomas, but induces an increased abundance of renal development**. *J Cell Biochem* 2007; 100(6): 1518-1525.

III. **Cedervall, J.,** Jamil, S., Prasmickaite, L., Cheng, Y.F., Eskandarpour, M., Hansson, J., Maelandsmo, G.M., Ringborg, U., Gulyas, M., Suo, Z., Kanter, L., Ährlund-Richter, L. **Species-specific *in vivo* engraftment of the human BL melanoma cell line results in an invasive dedifferentiated phenotype not present in xenografts**. *Cancer Res* 2009; 69: 3746-3754.

IV. **Cedervall, J.,** Jamil, S., Hultman, I., Ali, R., Kanter, L., Orrego, A., Sandstedt, B., Sveinbjörnsson, B. , Johnsen, J.I., Kogner, P., Ährlund-Richter, L. **Modeling of neuroblastoma growth in a human embryonic micro-environment adds emphasis to clinical relevance.** *Submitted*

CONTENTS

LIST OF ABBREVIATIONS

αSMA	Alpha-Smooth Muscle Actin
BMP	Bone Morphogenetic Protein
CAF	Cancer Associated Fibroblasts
CEP	Chromosome Enumeration Probe
DAB	Di-Amino-Benzidine
EB	Embryoid Body
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
FISH	Fluorescence In Situ Hybridisation
GCT	Germ Cell Tumour
GEM	Genetically Engineered Mice
HE	Hematoxylin & Eosin
hECC	human Embryonic Carcinoma Cells
hESC	human Embryonic Stem Cells
hEST	human Embryonic Stem Cell derived Teratoma
HIF	Hypoxia Inducible Factor
ICC	Immunocytochemistry
IHC	Immunohistochemistry
iPSC	induced Pluripotent Stem Cells
LOH	Loss Of Heterozygosity
mESC	mouse Embryonic Stem Cells
RT-PCR	Reverse Transcriptase- Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
TGCT	Testicular Germ Cell Tumour

1 INTRODUCTION

Despite significant progress in cancer therapeutics, cancer is still the leading cause of death in the world. A majority of the malignancies strikes against the older part of the population; pediatric cancers represent only less than one percent of all cases. However, cancer remains the leading cause of death in Swedish children. Malignant melanoma and pediatric neuroblastoma, both derived from derivatives of the neural crest, are the malignancies in focus of this thesis. Despite their different target groups, they share some characteristics such as high probability to metastasize and a tendency to frequently develop therapy-resistance. Hence, this results in a high number of deaths and highlights the need for more effective treatment methods.

Development of more effective treatment methods for human cancer is highly dependent on evolvement of more relevant preclinical model systems. In this thesis, I present the use of human embryonic stem cell derived teratomas as *in vivo* model systems for growth and progression of human neural crest derived cancers. My hope is that the model will contribute to cancer research by taking preclinical testing one step further, offering new and unique possibilities to study the behavior of a tumour in a relevant biological context. Ultimately, this might result in more successful clinical translation of new therapies, and consequently a decreased number of deaths caused by various malignancies.

1.1 DEVELOPMENT OF THE NEURAL CREST

Knowledge about normal development of the neural crest and its derivatives, here especially the peripheral nervous system and the melanocytes of the skin, is essential for understanding the characteristics of both neuroblastoma and melanoma. The heterogeneity, plasticity and migrative behavior of these cancers, contributing to their malignancy, are well reflected by their developmental origin in the neural crest.

The neural crest is a population of cells present during embryogenesis, giving rise to a variety of different cell types in the body of vertebrates. It was first identified in the chick embryo already 1868, as a highly migrative cell population occurring at neural tube closure, contributing to formation of the dorsal root ganglia (His, 1868). However, it is presently known that its developmental potential reaches way beyond this. Cells of the neural crest are contributing to formation of several tissues and organs of the body, such as neurons and glia of the sensory, sympathetic and enteric systems, cartilage and bone of the facial area, endocrine cells of the adrenal medulla and melanocytes of the skin (Anderson et al., 1993; Unsicker et al., 1993; Baker et al., 1997; Le Dourain et al., 1999; Hall et al 1999).

Neural crest cells are derived from the ectodermal part of the embryo, arising from the dorsal part of the neural tube just upon or after its closure, i.e. during the fourth week of human development (Larsen W.J., Human embryology, 2001). Induction of the neural crest is regulated by signals from surrounding non-neural ectoderm and mesoderm, mainly exerted by interplay between BMP, Notch, Wnt and FGF-signaling (Meulemans et al., 2004; Monsoro-Burg et al., 2005). Cells destined to form the neural crest undergo an Epithelial-Mesenchymal Transition (EMT), involving disruption of tight cell to cell contacts with surrounding neuro-epithelial cells, reorganization of the cytoskeleton and altered gene activation, altogether resulting in cells with mesenchymal

phenotype. The neural crest cells are thereafter migrating out from the neural tube, home to the proper site to settle, and subsequently undergo differentiation into specified cell lineages.

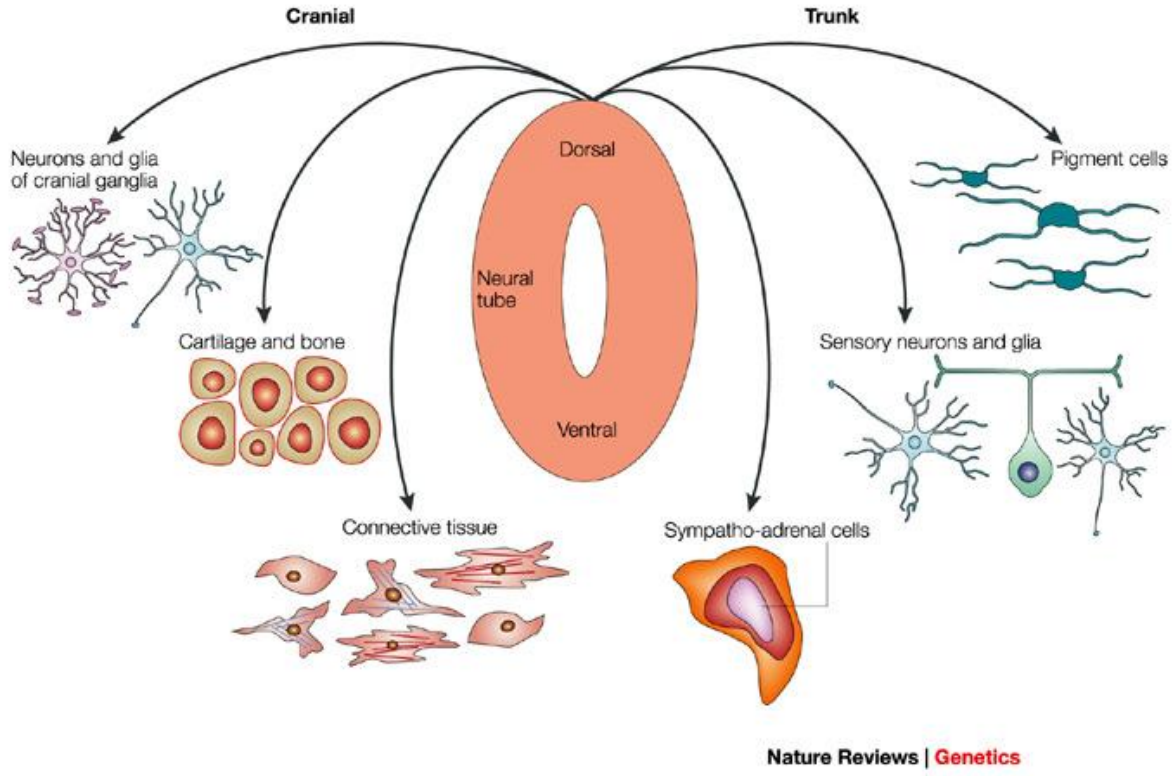

Figure 1: Migration and differential potential of the neural crest cells. (Adapted by permission from Macmillan Publishers Ltd; Nat Rev Genet, Knecht & Bronner-Fraser, 2002)

Most of the current knowledge about marker genes expressed during different stages of neural crest formation is based on experiments performed in animals, and despite species-discrepancies, some characteristics seem to be shared in between species. Transition of a cell from an epithelial to a mesenchymal, migrative state, is strongly linked to changes in adhesion potential. In the neural crest, this is revealed partly by changes in cadherin expression, in the mouse represented by a switch from N-cadherin to cadherin-6 expression (Pla et al., 2001). Some other genes considered to be upregulated by cells of the neural crest are Snail, Slug, Sox9 and FoxD3. Observations from neural crest formation in Zebra fish suggest that the neural crest induction, revealed by moderate levels of BMP-signaling in combination with Notch, Wnt and FGF, is transduced by factors such as Pax3 and Msx1/2, by confining the dorsal part of the neural tube (Meulemans et al., 2004).

The neurogenic and melanogenic neural crest, being the non-transformed ancestors of neuroblastoma and melanoma respectively, are migrating out from the closing neural tube in different manners. Neurogenic neural crest cells are migrating along a ventral route through the somites, while melanogenic neural crest cells mainly take a dorsolateral route between the somites and the ectoderm, and later invade the ectoderm where they complete their differentiation into melanocytes (Le Dourain and Kalcheim, 1999).

The choice of pathway taken is determined by differential patterns of gene expression and surface molecule expression (Harris and Erickson, 2007). Specification of the

neural crest cells is in most cases taking place already before migration, meaning that the neural crest already at this early time point is a heterogeneous population of cells, complicating characterization of the neural crest as an entity. Nevertheless, some neural crest cells seem to stay in a multipotent stage, even after migration. The neural crest has earlier been regarded as a transient cell population, solely present in early development. However, studies performed both *in vitro* and *in vivo* have reported presence of non-specified neural crest cells long after emergence from the neural tube, suggestive of a neural crest stem cell population, possibly persisting also in the adult (Delfino-Machín et al., 2007).

1.1.1 Development and specification of the Peripheral Nervous System

The peripheral nerves of the adrenal medulla and paraspinal sympathetic ganglia, being the main origin of neuroblastoma, are developing from the trunk neural crest cells, migrating out relatively early via the ventral pathway.

Development of the peripheral nervous system from the neural crest is regulated by surrounding tissues, inducing expression of transcription factors needed for neuronal differentiation. First, pan-neuronal markers are expressed, followed by up-regulation of catecholaminergic markers (Lo et al., 1991; Groves et al., 1995). A number of bone morphogenetic proteins, e.g. BMP-2, BMP-4 and BMP-6, are released during early neural crest development, orchestrating the formation of sympathetic ganglia (Reissmann et al., 1996; Shah et al., 1996). It has been shown that treating chick-embryos with the BMP-antagonist Noggin resulted in failed pan-neuronal and subsequent catecholaminergic differentiation into sympathetic ganglia, based on absent Tyrosine Hydroxylase (TH) and Dopamine β Hydroxylase (DBH) expression (Schneider et al., 1999). The BMP-signaling results in expression of a variety of different transcription factors essential for proper differentiation, such as basic helix-loop-helix (bHLH), mammalian achaete-scute homolog 1 (Mash-1), Hand-2, the paired homeodomain factors Phox2a and Phox2b, and the zinc-finger proteins Gata2 and Gata3 (Hirsh et al., 1998; Lo et al., 1998; Pattyn et al., 1999; Stanke et al., 1999; Lim et al., 2000; Tsarovina et al., 2004).

Apoptosis of neuronal precursor cells is a crucial part of the differentiation and maturation of the peripheral nervous system, mainly regulated by PI3K-signaling and transient MYCN activity (De Zio et al., 2005; Opel et al., 2007). By interacting with Mdm2, MYCN is capable of suppressing apoptosis in a p53-dependent manner.

1.1.2 Development and specification of the melanocytes

Melanocytes are pigmented cells, synthesizing and storing melanin, in the epidermal and dermal layers of the skin in vertebrates. These cells are derived from the neural crest, migrating out along the dorsal pathway somewhat later than the neural crest cells destined to form the peripheral nervous system.

The development of a melanocyte is a stepwise process, via a progenitor stage common for glia cells and melanocytes. These developmental lineages are then separated, resulting in a non-pigmented precursor cell, the melanoblast, destined to become a melanocyte (Cohen and Konigsberg, 1975; Ito et al., 1993). A vast majority of the current knowledge on melanocyte differentiation is based on studies performed in animals, giving a clue about the development taking place during human embryogenesis.

Proper expression of a number of genes during embryogenesis has been proven important for normal development of the melanocytic lineage. Mitf, Pax3 and Sox10, are all essential for melanogenesis, and defective function of any of these three transcription factors results in failure of melanoblast development. Not seldom, Mitf is considered the master regulator of melanogenesis, since it is crucial both for specification of the melanoblast and for the survival, and loss of Mitf leads to absence of melanocytes (Lister et al., 1999; Mochii et al., 1998; Steingrimsson et al., 2004). Mitf is also responsible for regulating genes involved in melanogenesis, such as tyrosinase (Aksan and Goding, 1998) and MelanA/MART1 (Du et al., 2003). Transcription of Mitf itself is driven primarily by Pax3, which is regulated by BMP-signaling and Sox10 (Bondurand et al., 2000; Potterf et al., 2000; Watanabe et al., 1998). Another critical factor regulating melanogenesis is the transcriptional repressor FoxD3, capable of inhibiting melanocytic differentiation by repressing Mitf (Ignatius et al., 2008). FoxD3 is expressed in the neural tube before its closure and in migrating neural crest cells destined to develop into the neuro- or glia lineages (Dottori et al., 2001; Kos et al., 2001; Lister et al., 2006; Yamagata and Noda, 1998). Down-regulation of FoxD3 is essential for neuroblast specification, enabling Mitf to exert transcription of genes responsible for melanocytic differentiation.

Two other factors known to be important for proper melanocytic differentiation and proliferation are c-kit and Endothelin-3, also being involved during melanomagenesis (Reid et al., 1996). Interestingly, c-kit has been proven essential also for melanocytic invasion into the dermis during normal development in mice, further reflecting its possible role for metastatic melanoma (Nishikawa et al., 1991).

1.2 TUMOURS OCCURING IN NEURAL CREST DERIVED TISSUES

Several different malignancies, both during early life and in adults, occur in cell types originating from the neural crest, e.g. neuroblastic tumours, schwannomas, certain sarcomas, melanomas etc. Two tumor types are specifically in focus in this thesis; embryonic neuroblastoma of the developing peripheral nervous system, and cutaneous malignant melanoma, the most aggressive form of skin cancer usually occurring later in life. Despite obvious differences between these tumours, they do have some traits in common.

Both neuroblastoma and malignant melanoma are tumour types with high degree of therapy resistance and high mortality rates, even though they target entirely different groups of the population. Contrary to most other tumour types, primary tumours of neural crest origin are usually not having mutations in the tumour suppressor gene p53 (Albino et al., 1992; Montano et al., 1994; Vogan et al., 1993). However, the p53 signaling pathway is often dys-regulated in other ways. On the genomic level, these tumours do often display loss of heterozygosity in the distal part of the short arm of chromosome 1, i.e.1p (Brodeur et al., 1981 and 1982; Gilbert et al., 1984; Walker et al., 1995). Especially neuroblastoma, but in rare cases also melanoma, have the capacity to undergo spontaneous regression (Everson et al., 1964). The mechanisms behind this ability is however probable to differ between the two tumour types. Importantly, both neuroblastomas and melanomas are considered highly plastic, meaning that they have great ability to adapt according to changes in their surrounding microenvironment. Hence, the environment supplied by preclinical models is of utmost importance to ensure tumour growth relevant for clinical comparisons.

1.2.1 Neuroblastoma

Neuroblastoma is the most common extra cranial solid tumour during childhood and the third most common pediatric cancer over all, after brain tumours and leukemias (Brodeur et al., 2003). It is occurring in the sympathetic peripheral nervous system, most often in the abdomen and especially the adrenal medulla, but occasionally also in peripheral nerves at other sites, such as the neck, chest and pelvis (Brodeur et al., 2003; Maris et al., 2007). Neuroblastoma is exhibiting a high clinical variation, spanning from a less aggressive phenotype with capacity to undergo spontaneous regression or differentiate into benign ganglioneuromas, to a highly aggressive type with widespread disease and high degree of therapy-resistance (Miller et al., 1994; Brodeur et al., 2001; Evans et al., 1976; DAngio et al., 1971; Haas et al., 1988; Brodeur et al., 1988).

1.2.1.1 Staging and clinical variation

An international staging system for neuroblastoma (INSS) was first presented more than two decades ago, and was later revised in 1993 (Shimada et al., 1984; Brodeur et al., 1993). INSS is dividing the tumours into stages from 1 up to 4, with 1 being a localized tumour with complete gross excision, and 4 being an advanced malignant stage with widespread disease. However, infants less than one year of age, with localized primary tumour and metastatic spread limited to skin, liver and/or bone marrow, are falling into a specific group, 4S. This group of tumours is characterized by a high frequency of spontaneous regression, contrasting remarkably to the other stage 4 tumours usually having a worse prognosis (Nickerson et al., 2000). Stage of disease is considered the most important prognostic factor for neuroblastoma. Age at diagnosis is also of importance, with children less than 18 months having a better prognosis (Evans et al., 1987). Further on, genetic aberrations, especially status of the MYCN gene, is closely correlated to clinical outcome.

1.2.1.2 Histology

Neuroblastic tumours are categorized, according to the International Neuroblastoma Pathology Classification (INPC), as favorable or unfavorable based on degree of differentiation, Schwannian stroma content, mitosis-karyorrhexis index (MKI) and age at diagnosis. The resulting types of tumours can be divided into four different groups; neuroblastoma (Schwannian stroma-poor), ganglioneuroblastoma intermixed (Schwannian stroma-rich), ganglioneuroma (Schwannian stroma-dominant) and ganglioneuroblastoma nodular (composite Schwannian stroma-rich/dominant and stroma-poor). Tumours considered favorable include ganglioneuroblastoma intermixed and ganglioneuroma, as well as poorly differentiated neuroblastoma if age at diagnosis is below 18 months, and differentiating neuroblastoma if age at diagnosis is less than five years. Undifferentiated phenotype is associated with unfavorable histology regardless of age. High MKI is considered unfavorable, as is also an intermediate MKI if age at diagnosis is more than 18 months (Shimada et al., 1999; Sano et al., 2006).

1.2.1.3 Genetics

DNA content has been correlated to prognosis of neuroblastoma in a number of published studies. This has in particular been shown for children diagnosed under the age of one year, where hyperdiploidy is associated with low-stage tumours and predictive of good outcome, while diploidy is on the other hand occurring in a high number of therapy-resistant tumours (Look et al., 1984 and 1991; Taylor et al., 1990).

On the genetic level, some specific aberrations are more frequently detected in neuroblastomas, and will be presented below.

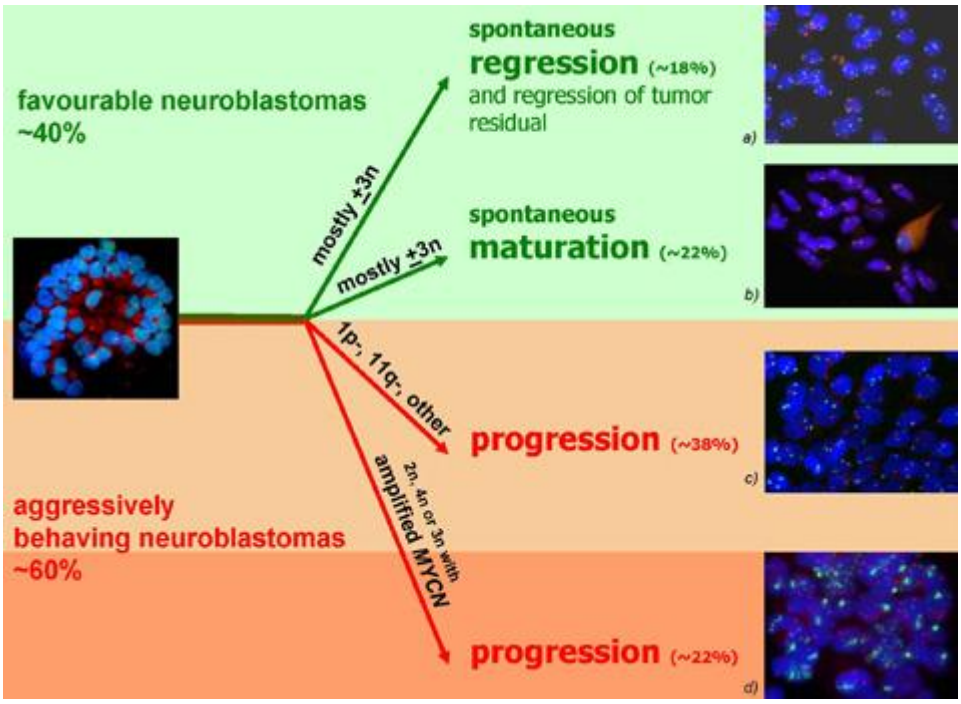

Figure 2: Overview of the clinical heterogeneity and correlation to genetics in neuroblastic tumours. (Adapted from the Atlas of Genetics and Cytogenetics in Oncology and Haematology, http://atlasgeneticsoncology.org)

A small subset (<5%) of human neuroblastomas are inherited in an autosomal dominant manner. They usually exhibit a broad clinical heterogeneity, with some having a low-malignant variant while others encounter an aggressive high-malignant disease. Two different genes have been found frequently altered in familial neuroblastoma. First, constitutional mutations of the PHOX2B gene have been detected in a small number of cases (Bordeaut et al., 2005; Mosse et al., 2008). The PHOX2B gene encodes a transcription factor regulating development of the autonomous nervous system. Aberrations of this gene is also involved in Hirschsprung´s disease and Congenital Central Hypoventilation Syndrome, both being caused by developmental disorders of the neural crest derived autonomous nervous system, and correlating with an increased risk of developing neuroblastoma (Raabe et al., 2008).

Second, mutations or amplifications of the Anaplastic Lymphoma Kinase (ALK) gene have been suggested to occur in a majority of patients with familial neuroblastoma (Chen et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). ALK is located on chromosome 2p23, centromeric to MYCN, and is frequently amplified also in sporadic cases of neuroblastomas. Therefore, it has been suggested as a promising therapeutic target (George et al., 2008).

A number of different genetic aberrations are frequently observed in sporadic neuroblastomas.

Amplification of MYCN, located on chromosome 2p24, is occurring in 20-25 % of sporadic neuroblastoma cases, and correlates with poor prognosis, independent of stage, age at diagnosis and other genetic aberrations (Seeger et al., 1985; Maris et al., 1999; Cohn et al., 2009). MYCN has the potential to function both as a transcriptional

activator and suppressor, affecting multiple physiological aspects such as proliferation, apoptosis and genomic instability (Grandori et al., 2000; Sakamuro et al., 1999; Nesbit et al., 1999; Felsher et al., 1999). It has been shown that expression of genes, transcribed by the oncogenic transcription factor MYCN, are characteristic for neuroblastoma progression, and MYCC has been suggested to exert a similar role in the absence of amplified MYCN (Fredlund et al., 2008; Westermann et al., 2008).

Deletions on chromosome 1p, particularly LOH of 1p36, is occurring in approximately 25 % of the neuroblastomas, and as mentioned, this is a trait suggested to be shared with other tumours derived from the neural crest (Brodeur et al., 1988). Chromosome 1 deletions are strongly associated with factors linked to poor prognosis, such as higher age at diagnosis, MYCN amplification and metastasis, and thus, predictive of bad clinical outcome (Fong et al., 1989; Caron et al., 1996; Maris et al 1995). However, more research is needed to clarify whether 1p aberrations are responsible for the poor prognostics of these patients, regardless of amplified MYCN.

Other genetic deletions frequently observed are located on chromosome 11q, most common being LOH of 11q23 (Guo et al., 1999; Luttikuis et al., 2001; Spitz et al., 2003). Contrary to 1p36 alterations, LOH of 11q23 is not commonly seen together with amplified MYCN (Plantaz et al., 2001). However, LOH of 11q23 has been suggested predictive of poor prognosis in patients with non-amplified MYCN (Carén et al., 2010).

Gain of chromosome 17q, a commonly occurring aberration detected in 70 % of the neuroblastomas, is also related to unfavorable outcome (Maris et al., 1999), especially in tumours without amplified MYCN and no deletion of chromosome 11q (Carén et al., 2010).

In addition to the genetic aberrations presented above, loss of copy number in chromosome 3p, 4p, 10q, 14q, 19p and 19q, is occasionally seen (Maris et al., 1999).

1.2.2 Malignant melanoma

Cutaneous malignant melanoma is a cancer arising from the neural crest derived melanocytes, being the most aggressive type of skin cancer with high metastatic capability and therapy-resistance. At metastatic disease the 10-year survival is appreciated to be less than 10% (reviewed in Bhatia et al., 2009). The incidence of malignant melanoma has rapidly increased, especially in the Caucasian population, where the increase during the last years has been higher than for any other cancer (Berwick et al., 2006).

It is appreciated that 10 % of the melanomas are occurring partly as a consequence of germ line mutations, preferentially in genes regulating apoptotic capability, as described below. Still, the cause of malignant melanoma is thought to be a complex combination of genetics and environmental factors. Epidemiological studies have shown that UV-irradiation is a causative agent for melanoma (Gandini et al., 2005), however, development of the cancer is thought to occur progressively involving a number of genetic alterations, affecting proliferation as well as apoptotic capacity.

1.2.2.1 Melanoma progression

A high amount of clinical and histological studies has contributed to the current knowledge about melanocytic transformation and melanoma progression. Based on this, a model for melanoma progression has been developed by Clark, Elder and

Guerry, describing a stepwise process starting with transformation of a melanocyte, development of common and dysplastic nevus, melanoma in situ, radial growth phase (RGP) and vertical growth phase (VGP) melanoma, and finally metastatic melanoma (Clark et al., 1984).

Each step is associated with a number a biological hallmarks, contributing to the progression. The first events are believed to involve altered adhesion characteristics, enabling the melanocyte to escape normal regulation by surrounding keratinocytes, forming nevus with a preference for homogenous melanocyte-melanocyte adhesions. Genetic alterations are probably needed for further progression into dysplastic nevi and RGP melanoma, where cytological atypia is obvious and contacts with the basement membrane can be disrupted without apoptotic consequences. Further progression into the VGF is characterized by proliferation and ability to invade dermis. These cells are considered highly aneuploid, with some being capable to metastasize and invade other tissues and organs. Metastatic cells are often having a highly plastic phenotype, offering possibilities to adjust in new environments (Clark et al., 1984; DeVita and Hellman, 2004).

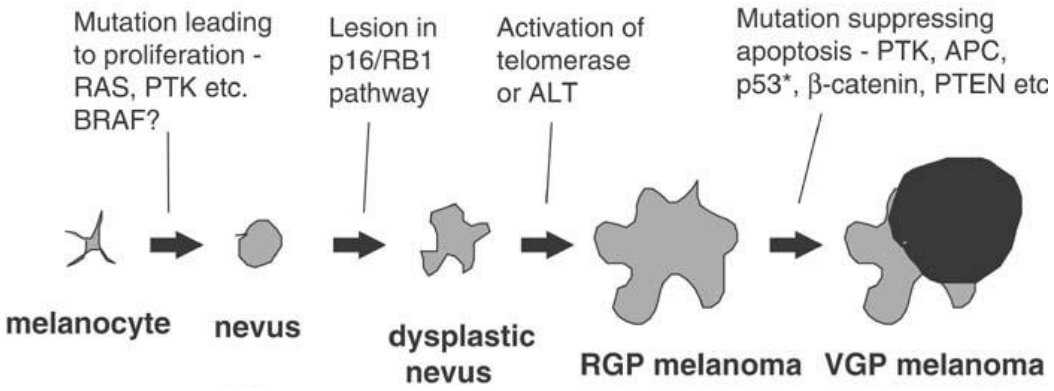

Figure 3: Suggested stepwise progression of a malignant melanoma. (Adapted, with permission, from Bennett, Oncogene 2003; 22: 3063-3069)

However, it is probable that some of these steps are not needed for development of a metastatic melanoma (www.wistar.org/herlyn). The role of possible stem cell populations in development of melanomas are under investigation, and will hopefully shed more light over the process of melanomagenesis and progression.

1.2.2.2 Genetics

Several main signaling pathways related to proliferation, migration and apoptosis are known to be dysregulated in malignant melanoma. This dysregulation is often depending on minor genetic alterations as described below.

The MAPK/ERK pathway, involved in major cellular functions such as cell growth, apoptosis and migration, is thought to be of importance for both development and progression of melanoma (Davies et al., 2002). High activity of the MAPK/ERK pathway in melanoma is conducted by alterations in either of two up-stream factors; BRAF or NRAS. These genetic alterations are mutually exclusive, hence, never occurring together in the same tumour (Sensi et al., 2006). Interestingly, tumours having the different mutations seem to preferentially occur in different body sites. NRAS mutated tumours are, in contradiction to BRAF mutated ones, usually developing in sun-exposed areas, suggesting a direct UV-linked induction of these melanomas (Jiveskog et al., 1998; El Shabrawi et al., 1999).

Mutations of BRAF have been detected in almost half of all cases of malignant melanoma (Palmieri et al., 2007). In the majority of these cases, a point mutation is causing an amino acid substitution, resulting in constitutively active MAPK/ERK signaling (Davies et al., 2002; Palmieri et al., 2007; Wan et al., 2004). Downstream, this result in expression of genes involved during developmental melanogenesis, e.g. MiTF, and cell cycle progression (Carreira et al., 2005). Presence of mutated BRAF has only been detected on somatic level. Furthermore, studies on primary melanomas reveal that this alteration is mainly observed in later stages of tumour progression (Casula et al., 2004; Pollock et al., 2003; Dong et al., 2003; Greene et al., 2009). Hence, BRAF mutations are not probable to be responsible for establishment of melanoma, but rather contribute to its progression during later phases.

Mutations in NRAS, upstream of BRAF in the MAPK/ERK pathway, are appearing in approximately 15 % of all malignant melanomas. The majority of alterations occur in codon 61, where an amino acid substitution results in keeping NRAS in a constitutively active GTP-bound stage, consequently leading to continuous MAPK/ERK signaling (Ball et al., 1994; Albino et al., 1989; Platz et al., 1994).

Another signaling cascade frequently altered in malignant melanoma is the PI3K-pathway. The major protein responsible for this is the tumour suppressor PTEN, located on chromosome 10q, where LOH has been detected for a numbers of cancers, including melanoma (Bastian et al., 2003; Wu et al., 2003). Allelic loss or changed expression of PTEN is detected in 60% of all melanomas, and a minor proportion exhibits other genetic alterations in the same locus (Goel et al., 2006). The tumour suppressor function of PTEN is exerted via inhibiting phosphorylation of the serin/threonin kinase AKT, located downstream of PTEN in the PI3K-pathway. Thereby, PTEN can promote apoptosis and inhibit growth and metastatic potential (Robertson et al., 1998; Stewart et al., 2002; Stahl et al., 2003). An inverse correlation between patient survival and phosphorylated AKT has been shown, further indicating the importance of PTEN (Dai et al., 2005).

Dysregulation of both MAPK/ERK and PI3K-signaling might also occur due to alterations in upstream receptor tyrosine-kinases (RTKs). Various alterations in c-kit, c-met and EGFR have been identified in melanomas, all resulting in changed cellular signaling. C-kit is known for promoting growth and proliferation also of non-transformed melanocytes during development, and genetic alterations have been detected in skin after chronic UV-exposure (Sviderskaya et al., 1995; Curtin et al., 2006). No mutations have been detected in the genes encoding c-met or EGFR, however, their activity is suggested to be altered in other ways (Bastian et al., 1998; Koprowski et al., 1985; Udart et al., 2001).

The CDKN2A locus (9p21) is probable to be of great importance for melanomagenesis. It has been shown that LOH or mutations in CDKN2A is frequent in families prone to develop melanoma, and these alterations are considered the major genetic inherited risk-factors (Hussussian et al., 1994; Kamb et al., 1994). CDKN2A is encoding two factors, $p16^{Ink4a}$ and $p14^{ARF}$, both implicated in cellular apoptosis, by regulating the p53- and Rb-pathways respectively (Chin et al., 2006). Hence, mutations in the CDKN2A locus is striking against the two major pathways leading to apoptosis, enabling apoptotic escape of transformed cells.

1.3 COMPLEXITY OF THE TUMOUR MICROENVIRONMENT

It has been increasingly accepted that cancer growth and progression is not only a matter of the tumour cells *per se*, but also depends on non-tumorigenic cells supporting these processes in different ways. The tumour is a complex entity containing a heterogeneous mass of tumour cells, but also cancer associated fibroblasts (CAFs), infiltrating immune cells being either tumour-inhibiting or tumour-promoting, and endothelial cells participating in neo-angiogenesis essential for sustained tumour growth. Indeed, all different cell types have their own purpose in helping the tumour to progress.

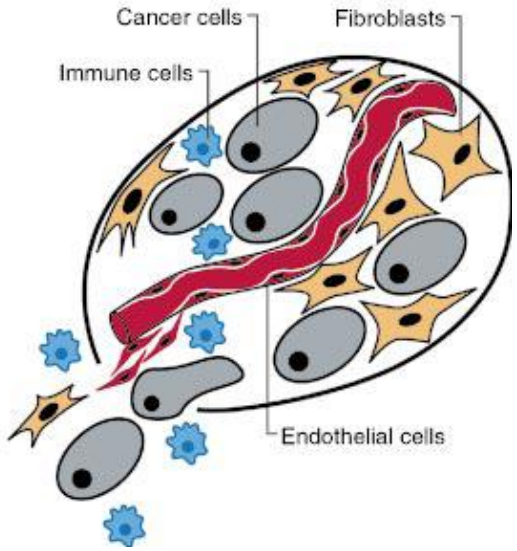

Figure 4: The complexity of a tumour illustrated by some of the main cell types present in the cancer environment; a heterogeneous population of cancer cells, supporting fibroblasts, endothelial cells participating in formation of the tumour vasculature and infiltrating immune cells. (Adapted from Hanahan & Weinberg, Cell 2000; 100: 57-70)

1.3.1 The heterogeneous tumour cell mass

The cellular heterogeneity of a tumour has been observed and discussed by scientists for more than a century (Virchow et al., 1860), and it is today accepted that cells in a tumour might have different genotype, phenotype, and display various degrees of differentiation. As a consequence, therapies have to target not only one specific cell type, but rather strike against various different cell types, to be effective.

Already a few decades ago, assays were developed to detect and identify stem cells within a tumour, suggestive of being the cell population responsible for therapeutic resistance and cancer recurrence (Salmon et al., 1978). The existence of cells in the tumour having characteristics similar to those of non-tumorigenic stem cells has now been validated in a number of cancers, e.g. melanoma and neuroblastoma. However, the importance and the origin of these cells are still debated and yet to be discovered.

1.3.2 Tumour angiogenesis

Recruitment of vasculature ensuring a continuous blood-supply, revealing delivery of oxygen and nutrients, is essential for a tumour to grow larger than 2-3 mm in diameter (Folkman et al., 1971). Up to this stage, the tumour is dependent on surrounding vessels and diffusion of oxygen. However, as the tumour grows bigger in size the oxygen

levels are decreasing in the central parts leading to hypoxia. The tumour is responding to the hypoxic environment by stabilization of Hypoxia Inducible Factors (HIFs), inducing transcription of pro-angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) and Angiopoetin-1 (Ang-1), enabling tumour vascularization to start off (Pugh et al., 2003; Carmeliet et al., 2003).

The blood cells forming the tumour vasculature are differing from endothelial cells of the normal tissue vasculature in several ways. They frequently lack associated pericytes and vascular smooth muscle cells, having thin and fragile vessel walls. Further on, the vessel density and blood flow rate is often highly variable. On the cellular level, endothelial cells of the tumour vasculature are usually immature, often having abnormal cell shapes or functions (reviewed by Abdelrahim et al., 2010).

Moreover, several publications are now supporting the fact that the actual tumour cells, including melanoma and neuroblastoma cells, can participate in forming new blood vessels by differentiating into endothelial cells, i.e. vasculogenic mimicry (Maniotis et al., 1999; Pezzolo et al., 2007; van der Scaft et al., 2005). This phenomenon is relatively rare in tumours, but occurrence of vasculogenic mimicry has been associated with an increased risk for metastasis and thus worse clinical prognosis (Sun et al., 2004). Whether this depends on high plasticity and potential stem cell characteristics in these tumours, or the actual vascularization *per se*, remains to be clarified. As mentioned above, oxygen deprivation due to limited vascularization is resulting in hypoxia, known to affect the tumour cells dramatically. Stabilization and transcriptional activation of HIFs is not only driving the expression of pro-angiogenic factors, but has also proven to be involved in favoring a stem cell like tumour phenotype (Jögi et al., 2002; Covello et al., 2006).

1.3.3 Cancer Associated Fibroblasts

Fibroblasts in the tumour microenvironment are producing a variety of pro-tumorigenic factors, such as EGF, HGF and TGF-β, thus having essential role for tumorigenesis (Kalluri et al., 2006). Tumour associated stroma, including the fibroblasts as the main cell type, has proven to be of prognostic importance in breast cancer, underlining the great impact of CAFs (Finak et al., 2008).

Except for being growth promoting, a novel role for CAFs has emerged, showing their importance also during metastasizing events. It has been shown that co-injection of breast cancer cells and mesenchymal stem cells significantly increase metastatic ability in animals, and similar observations were done using cell lines derived from pancreatic cancer (Karnoub et al., 2007; Hwang et al., 2008).

The origin of CAFs is still debated. Numerous studies are indicating that there are several sources of origin, including local infiltrating fibroblasts, endothelial cells or pericytes of the vascular system, or cancer cells themselves undergoing fibroblastic transformation (Kalluri et al., 2006; Zeisberg et al., 2007).

Based on the importance of CAFs, several therapeutic approaches are in use or under development targeting this cell population, mainly via RTKs, e.g. sutinib and imatinib.

1.3.4 Immune cells in the tumour microenvironment

The tumour microenvironment is also infiltrated by cells of the immune system, having diverse effects on the tumour progression, being either tumour promoting or tumour

suppressing. It is well known that tumours are able to induce immunologic responses, however, the tumour cells are frequently adapting, developing skills to avoid immune-surveillance (Zitvogel et al., 2006). Cancer therapies based on immunology is currently used for a variety of cancers, among them malignant melanomas, where extensive studies have shown that the immune system is highly important during various stages of disease (Dranoff et al., 2009).

1.4 PRECLINICAL TUMOUR MODELS

Development of effective cancer therapies is depending upon relevant preclinical model systems, mimicking the complex tumour environment as much as possible. For obvious reasons, modeling of cancer growth and progression is limited to *in vitro* cell culture systems or *in vivo* models based on animal experiments, which is not to the full extent recapitulating the behavior of a clinical tumour in a patient.

Lots of information about human cancer has been achieved using *in vitro* culture systems of cancer cell lines and primary tumours. However, these models have a clear drawback in lacking the supportive microenvironment of adjacent non-tumorigenic tissues, certainly present *in vivo*, as described above. Therefore, complex essential processes of tumour progression, such as metastasis or angiogenesis, would not be possible to study in such systems.

With the importance of the complex tumour microenvironment in mind, the need for proper animal models of cancer becomes obvious. Cancer models have during the last decades been developed using different species serving different purposes. However, the absolutely dominant species used have been *M.musculus*, providing different sorts of mouse models of both murine and human cancers.

1.4.1 Mouse models of cancer

The reasons for preferentially using mice for modeling of cancer are several. First, mice are small sized mammals, relatively easy to breed in captivity, and have a comparatively short life-span of approximately three years, enabling experiments spanning over a reasonable time. Second, and of high importance, the whole genome of the mouse has been sequenced, offering a well characterized model, which is also to large extent similar to human.

Development of xenograft models, i.e. human tumours propagated in immunocompromised mice, offered a way to rapid development of human tumour tissue *in vivo*. Human tumour cell lines, or primary biopsies, are in this case injected either orthotopically, i.e. at the cancer-type specific site of origin, or at an easily accessible and monitorable site, such as subcutaneous. However, there are discrepancies comparing these tumours with corresponding primary tumour from a patient. Obviously, the tumour surrounding and the normal tumour architecture are altered, meaning that interactions between the tumour and its microenvironment are disturbed. This is probably favoring an increased autonomous regulation of tumour growth, resulting in loss of cellular heterogeneity due to pressure from the surrounding, and though, selective clonal expansion (De Both et al., 1997, Staroselsky et al., 1992). These differences are essential to keep in mind when evaluating drug efficacy in such models. Probably, this selection can be reflected by the fact that numerous anti-cancer components proven effective in xenograft models have low effect or even fail when translated into the clinic (Johnson et al., 2001).

Later on, the development of genetically engineered mice (GEM) opened up the door for more sophisticated animal models, offering the possibilities to study effects of tumour specific genetic aberrations *in vivo*. GEMs are basically mice harboring and expressing mutated genes, for cancer mainly putative oncogenes or tumour suppressors, being either endogenous or exogenous. Mice with exogenous genetic aberrations are also referred to as transgenic, thus, harboring and expressing human gene variants of interest, while the endogenous models are entirely murine.

There has been a variety of different approaches to develop preclinical mouse-models for neuroblastoma and melanoma specifically, and these will now be further presented and discussed.

1.4.1.1 Mouse models of neuroblastoma

Xenograft models, orthotopic (adrenal) and heterotopic, has been widely used for studies on neuroblastomas. A comparison revealed that grafting of neuroblastoma cell lines orthotopically is to prefer, providing a more relevant tumour biology (Khanna et al., 2002). However, the limitation using xenografting for embryonic tumours such as neuroblastoma is the lack of an embryonic microenvironment.

The development of a number of GEMs modeling neuroblastoma establishment and progression has opened up for new possibilities, enabling studies on tumours arising spontaneously in genetically modified mice. A successful GEM developed for studies on neuroblastoma was based on MYCN over expression under control of the Tyrosine Hydroxylase promoter, specifically expressed in the neural crest and peripheral nerves of the adrenal medulla and parasympathetic ganglia. These mice do develop tumours with high resemblance to human neuroblastomas, concerning tumour site, morphology and further genomic alterations (Weiss et al., 1997). Other approaches have been used to develop GEMs giving rise to neuroblastomas mimicking those in human. Despite limitations set by the species-discrepancies between mice and man, these are offering possibilities to study up-come as well as preventive effects of neuroblastomas.

1.4.1.2 Mouse models of melanoma

Unfortunately, physiological differences between mice and man are causing difficulties for development of relevant mouse models for melanoma. Adult mice have, in contrast to human, their melanocytes mainly located in the hair follicles, and only a minor melanocytic contribution can be seen in the epidermis of hairless regions, such as the tail or the ear (Rosdahl et al., 1978). Also, melanocytes of murine origin show a high resistance to UV-irradiation, known to be a major risk factor inducing melanomas in human. Using chemical carcinogens only, or in combination with UV-irradiation, has proven more efficient for inducing melanoma in mice (Epstein et al., 1967; Berkelhammer et al., 1982; Kligman et al., 2001; Strickland et al., 2000; Broome et al., 1999).

However, a transgenic mouse model showing some response to UV-induction was developed by putting the SV40 T antigen under control of the tyrosinase promoter, enabling over expression of the polyoma virus-derived protein, specifically in melanocytes (Mintz et al., 1991). Results obtained using this model indicated a dose-dependent effect of SV40 T, capable of intervening with both the p53 and Rb-pathways, in combination with UV-irradiation. A high dose was mainly causing rapid evolvement of ocular melanomas, while a low dose showed a tendency of infrequently inducing cutaneous melanomas (Kelsall et al., 1998).

GEMs with mutations similar to those present in human melanomas have also been developed. For example, mice exhibiting both activated Ras and deletion of the CDKN2A locus have been developed mimicking some of the most frequently observed genotypes for malignant melanoma, and support the involvement of these genes in development and progression of melanoma (Chin et al., 1997).

In addition to the animal models described, some models based on xenografting of human skin have been developed. For example, Kunstfeld et al. established a model where human skin was transplanted between epidermis and dermis of CB17 SCID mice and NMRI nude mice. Subsequently, A-375 melanoma cells were injected into the transplant, and allowed successfully to form tumours in the resulting, partly human, environment (Kunstfeld et al., 2003). Another approach was presented by Berking et al. who showed that human skin transplanted to the back of SCID/Beige-mice, and exposed to UV-irradiation in combination with bFGF, developed pigmented lesions with hyperplastic melanocytes, and occasionally even high-grade atypia resembling malignant melanoma (Berking et al., 2001, 2001 and 2002).

1.4.1.3 Other animal models for melanoma

Development of melanoma, spontaneously or induced, has been reported in a number of animal species, such as horse, swine and goat (Fleury et al., 2000; www.cmv.tamu.edu; Mc Govern et al., 1973). However, it has experimentally been studied mainly in species more accessible to research, such as the non-mammalian *Xiphophorus* fish (Swordtail) and the mammalian *Monodelphis Domestica* (Opossum) (Walter et al., 2001; Kusewitt et al., 1991). To some extent, the melanomas appearing in these animals resemble the ones occurring in human. For example, an extended exposure to UV irradiation was shown to induce melanoma metastasizing to the lymph nodes in *Monodelphis Domestica* (Kusewitt et al., 1991). Further on, homologues to human genes known to be frequently altered in melanoma, such as CDKN2A and RAS, have been found, suggesting existing similarities also on genetic level (Sabourin et al., 1992; Sherburn et al., 1998). However, there are limited possibilities using *Monodelphis Domestica* in experimental genetic studies.

1.4.2 Limitations using mouse models

Certainly, the models described above do all have their advantages and have contributed a lot to our current knowledge about cancer development and progression. Nevertheless, there are a number of disadvantages using animal systems, which cannot be neglected.

When using xenografts of human tumours in animals, as previously mentioned, altered microenvironment is probable to cause clonal selection, resulting in a less heterogeneous tumour type more autonomously regulated than regulated by, or responsive to, microenvironmental signals (De Both et al., 1997; Staroselsky et al., 1992). Consequently, the developing tumour will lack sub-populations of cells, and any tested therapy will most probably show a diverse result compared to the one obtained in the primary, more heterogeneous tumour. Also, non-tumourigenic cells in the tumour environment contributing to tumour progression, such as endothelial cells of the vasculature, immune-cells and fibroblasts will be derived from a different species. This makes evaluations of therapies targeting partly non-tumorigenic cells, for example anti-angiogenic drugs, difficult, since cellular interactions might be altered.

The drawback of mixing species is overcome by using GEMs. Nevertheless, other limitations are encountered. Despite high genetic similarities between mouse and human, there are clear discrepancies, making it difficult to directly extrapolate results obtained in mouse to human. For example, development of a human cancer, at least in the adult, is considered a multistep process thought to involve independent mutations of at least five to six different genes (Peto et al., 1977). GEMs are often been based on mutations of one specific oncogene or tumour suppressor, thus producing tumours with a highly homogenous genotype. Compound mutant mice have been developed to serve the purpose to study synergistic effects of several mutations, e.g. for pancreatic cancer (Ijichi et al., 2006; Izeradjene et al., 2007). However, in this case, one should be aware of the fact that the mutations are occurring progressively and not all at once, to be able to recapitulate human tumour progression as much as possible. Tissue specific expression of an altered gene is often used in GEMs to induce a cancer specifically in the organ of interest. This results in a simultaneous transformation of practically all cells of that tissue *per se*, causing a homogenous tumour cell mass, extremely different to the cancers seen in the patients.

Some factors of great importance for cancer biology do also differ between human and mouse. One example is the telomere ends of the chromosomes, involved in cellular aging and essential for the tumour to escape senescence, and the expression of the telomere regulating enzyme telomerase. The human telomeres are normally 5-10 kb while the mouse counterparts are more than 40 kb (Kipling et al., 1990). Further on, human somatic cells are mainly lacking expression of telomerase, which is however expressed in transformed cells, while telomerase is expressed in somatic cells of the mouse (Harley et al., 1990; Prowse et al., 1995). Considering the importance of telomerase in human oncogenesis, this is a notable discrepancy comparing the two species.

The great advantage of mice having a short life-span, enabling rapid experimental results, is also a limitation. As mentioned, development of an adult human cancer is considered a long process involving multiple steps, probable to progress during years or even decades. This is not possible to recapitulate in mice, where the accessible experimental time is limited. However, this might be less of a problem considering embryonic tumours, which do often have a less complex genotype with fewer mutations, and certainly have developed during a shorter time (Scotting et al., 2005).

When it comes to neuroblastomas and melanomas, there are several limitations motivating the need for more relevant preclinical model systems. Neuroblastomas, originating from the early embryo, would probably be most suitable to study in an embryonic or fetal environment, and not in the adult mouse which is often the case. When it comes to melanomas, the obvious physiological differences between mouse and human melanocyte localization, is a drawback. Hence, additional approaches have to be developed to fulfill the needs for relevant *in vivo* models of these cancer types.

1.4.3 An alternative model based on stem cell derived teratomas

An alternative and species-specific *in vivo* model system for cancer, based on the use of embryonic stem cell derived teratomas, was first presented by Tzukerman et al. in 2003. They were injecting ovarian tumour cells into mature teratomas, allowing tumours to develop and be compared to the corresponding xenografts (intramuscular grafting) of the same cell line. Interestingly, the results revealed different tumour histology in the two microenvironments, and tumour invasion into surrounding tissues

was observed in the teratoma. In addition, an increased frequency of human vasculature was detected, surrounding and infiltrating the teratoma-grafted tumours (Tzukerman et al., 2003).

The initial study was followed up a few years later by the same research group, revealing further findings strengthening the power of the model system (Tzukerman et al., 2006). This time, the experiment was extended by using a panel of cancer cell lines of different origin (prostate, breast, lung, colorectal and glioblastoma) in the same model, and compare it to intramuscular injections. For all cell lines tested, they reported a higher survival of tumour cells after injection into the human teratoma environment. Furthermore, the study was exhibiting differential therapeutic effects comparing treatment of tumours in the different microenvironments. While the tested therapy was able to completely eradicate tumours grafted intramuscular, tumours grafted in the teratoma resisted full regression despite escalating therapeutic doses.

Further support for the model was generated recently when the same research group did show that the human teratoma microenvironment was less selective for sub-populations of primary ovarian tumour cells, as compared to grafting in xeno-environments (Katz et al., 2009).

Altogether, the results from the above described studies do all suggest that human teratomas might provide more relevant preclinical *in vivo* model systems for studies of human cancer. Their findings do also highlight the importance of the microenvironment for development and progression of tumours.

1.5 HUMAN EMBRYONIC STEM CELL DERIVED TERATOMAS

Enormous possibilities have followed the discovery of stem cells of different sorts. Depending on origin and potency, human stem cells are today used for experimental purposes in the laboratory as well as in the clinic. Despite some common basic traits, such as capacity for self-renewal and differentiation into mature cell types, human stem cells constitute a heterogeneous group of cells, having different origin and varying developmental potential.

The *in vivo* model system presented in this thesis is based on the use of pluripotent human Embryonic Stem Cells (hESC), which due to their characteristics offer a unique source of cells for generation of human tissues.

1.5.1 Human Embryonic Stem Cells

The first successful derivation of a permanently growing hESC line was performed more than a decade ago, and the production of new cell lines has since than practically exploded (Thomson et al., 1998). Before that, pluripotent ES cell lines of murine origin, i.e. mESCs, (Evans and Kaufman, 1981; Martin et al., 1981), as well as primate ES cells from Rhesus monkeys (Thomson et al., 1995), had been derived.

Human ES cells are derived from the inner cell mass (ICM) of the blastocyst prior to implantation, i.e. day 5-6 post fertilization. At this time, cells of the ICM are pluripotent, retaining the capability to develop into any cell of the human body. This feature is extremely valuable for research, providing ES cells with a developmental potential way beyond that of most other cell types.

1.5.1.1 In vitro propagation

Pluripotency of the cells in ICM is a transient condition, and maintenance of the undifferentiated state under *in vitro* propagation is highly artificial, demanding certain culturing conditions.

To avoid biological variations present in serum, a commercial available Serum Replacement has been developed, revealing stable cultures. Also, presence of the growth factor basic-FGF has proven essential for maintaining cells in the undifferentiated state, and is therefore added to the culturing media (Thomson et al., 1998; Amit et al., 2000).

In addition, provision of an extracellular matrix is needed for hESC culture. Hence, the first human ES cell line derived was cultured on a layer of murine fibroblast used as so called feeder-cells, providing matrix favorable for propagation (Thomson et al., 1998).

However, aiming to avoid animal products in the culture has led into the use of human fibroblasts as feeder-cells in many laboratories, including our own. At present, a number of feeder-free culturing alternatives have been developed, such as the use of Matrigel, offering hESC cultures without contamination of other cells (Xu et al., 2001; Klimanskaya et al., 2005). For the experiments in this thesis, hESC were cultured using human fibroblast feeder cells, the in our hands most stable and cost effective means to keep hESC proliferating in an undifferentiated state.

1.5.1.2 Genomic instability

Compared to most other mammalian cells in culture, hESCs have been considered karyotypically stable (Brimble et al., 2004; Carpenter et al., 2004; Rosler et al., 2004). However, several laboratories, including our own, have published occurrence of genomic alterations in their hESC cultures, frequently in chromosomes 12, 17 and X. In most cases, such alterations are likely results of culture-adaptations, where the genomically altered cells have a growth-advantage, causing favorable selection for this specific sub-population. The same karyotypic aberrations are also observed in a number of human malignancies, and are highly probable to affect the *in vivo* characteristics and developmental potential upon teratoma induction.

Gain of genetic material from chromosome 12 has been detected in two variant sub-lines of the hESC line HS181, derived and cultured by us and colleagues (Imreh et al., 2006; Gertow et al., 2007), but also by others (Herzfeld et al., 2006; Draper et al., 2004; Brimble et al., 2004; Cowan et al., 2004; Ludwig et al., 2006). Occasionally, the alteration is implying gain of a chromosome i12p only, a karyotype extremely prevalent in testicular germ cell tumours (TGCTs), thought to contribute to tumour progression by inducing sertoli cell independence (Looijenga et al., 2003; Reuter et al., 2005). Gain of chromosome 12, full or in part, is the single most common genetic change observed in our laboratory for the hESC line HS181. Interestingly, we found that selection for hESC growth directly on plastic, without supporting matrix, resulted in a gain of i12p. Despite similarities to malignant TGCT, this cell line was not forming teratomas *in vivo* (Imreh et al., 2006).

Gain of chromosome 17, as whole or as parts of the long arm q, has been reported both as a sole aberration and in combination with gain of chromosome 12 and X (Mitalipova et al., 2005; Maitra et al., 2005; Baker et al., 2007). Interestingly, gain of the distal part of chromosome 17q is occurring in several tumour types, including TGCTs, but also

neuroblastomas (Maris et al., 1999; Kraggerud et al., 2002). As previously described, 17q gain is a predictor of unfavorable outcome in neuroblastoma, and present in almost 70% of the cases (Maris et al., 1999). Possibly, this genotype is reflecting the poorly differentiated state of the cells of origin, valid also for pluripotent germ cell tumours.

Chromosome X gains have mainly been reported in combination with other genomic aberrations, such as gain of chromosome 12 or 17 (Mitalipova et al., 2006; Brimble et al., 2001; Ludwig et al., 2006; Inzunza et al., 2004). As for the genomic alterations mentioned above, gain of chromosome X is also common in TGCTs, and selection for this genotype in hESC cultures might be due to known oncogenes located in the amplified genomic area (Sandberg et al., 1996; Yang et al., 2003; Wu et al., 1996).

In addition to the genomic aberrations presented above, a number of others have been published. This indicates further the importance of keeping the cells under strict culture conditions, avoiding chromosomal alterations which are probably affecting the hESCs in several aspects.

1.5.1.3 Pluripotency and self-renewal

The great potential for expansion and differentiation of hESCs are due to their capability to self-renew and their pluripotent character. Self-renewal allows stem cells to divide while maintaining their undifferentiated state, which theoretically results in limitless proliferative potential. Pluripotency offers a wide developmental potential reflected by the fact that hESCs can differentiate into derivatives from all the three embryonic germ layers, which are ultimately responsible for forming all cells of the human body. Pluripotency and self-renewal are strictly regulated both on transcriptional- and cell cycle level.

Regulation of hESC on the transcriptional level is mainly directed by the Oct4-Sox2-Nanog network, forming a positive feed-back loop system inhibiting expression of genes leading to differentiation and thus favors pluripotency. These transcription factors are also involved in regulating processes during normal murine embryonic development; Oct4 is essential for pluripotency of cells in the inner cell mass of the embryo (Nichols et al., 1998), while maintenance of pluripotency in the embryo is dependent on Sox2 (Avilion et al., 2003) and Nanog (Mitsui et al., 2003). In addition to Oct4, Sox2 and Nanog, a number of other factors are involved in regulating this machinery. One example is the Polycomb family (PcG) of proteins, contributing on the epigenetic level by inhibiting differentiation and maintaining pluripotency (Schuettengruber et al., 2007).

The cell cycle regulatory machinery of hESCs is exhibiting remarkable differences compared to somatic human cells. In normal tissue cells, the p53- and Rb-pathways are providing protection against tumorigenesis by preventing cell cycle progression after DNA damage, ensuring either DNA repair or apoptosis. However, it has been shown that hESCs in culture have a very low and sometimes even absent expression of p53, and the same was observed for other genes known to cooperate with p53 in cell cycle regulation such as p16, p19 and p21 (Brandenberger et al., 2004, Miura et al., 2004). Expression of genes in the Rb-pathway has been suggested to follow a similar pattern (Miura et al., 2004). In addition to this it has been shown that hESCs have high telomerase activity (Thomson et al., 1998; Carpenter et al., 2004). Altogether, this might explain the capability of hESCs to escape cellular senescence and undergo un-limited self-renewal.

1.5.1.4 In vitro differentiation

As previously mentioned, maintenance of cells in a pluripotent state is highly artificial, and unless strict culture conditions are provided, cells will spontaneously differentiate into various cellular derivatives.

Growing hESCs as spherical embryoid bodies (EBs) in suspension is a common way to induce *in vitro* differentiation into mixed cell types. Theoretically, formation of EBs shall result in cell types belonging to all three germ layers of the developing embryo, i.e. ectoderm, endoderm and mesoderm (Itskovitz-Elder et al., 2000). EB formation can therefore be used as a method for evaluating the actual developmental potency of a hESC line, proving maintenance of pluripotency in the continuous hESC cultures.

For obvious reasons it is desirable to control also these differentiation events, enabling production of one or a few specific cell types only. Differentiation of hESC *in vitro* provides the advantage of using chemically defined culture conditions, allowing controlled experimental setups. Protocols for differentiation of hESCs into a wide variety of cell types, such as neurons and insulin producing β-cells of the pancreas have been established and are continuously optimized (Zhang et al., 2001; Liew et al., 2008).

1.5.1.5 In vivo differentiation

In vivo pluripotency and developmental capacity of mouse ES cells can be evaluated based on their ability to form chimeras after injection into the ICM of an epiblast. This method can obviously not be used for the same purpose in humans, thus, other methods have been established. *In vivo* pluripotency of hESCs is therefore commonly evaluated by induction of teratomas; benign growths containing a variety of ectoderm-, endoderm- and mesoderm derived embryonic tissues.

1.5.2 Teratomas

Benign tumours composed by a variety of tissue types were first described and illustrated already in the 17th century (Birch and Tyson, 1683). However, the term teratoma was first introduced by Rudolf Virchow in 1863 (Virchow, 1863). Naturally occurring teratomas are associated with pluripotent germ cells (Andrews, 1988; Askanazy 1907; Teilum, 1965) and are in the majority of cases exhibiting a well delimited benign tumour mass, containing different types of relatively mature tissues, which can be surgically removed without later recurrence (Isaacs, 2004). However, malignant variants occur, i.e. teratocarcinomas, with more immature tissues and remaining pluripotent cells (Andrews, 2002).

The use of experimentally induced teratomas for research purposes was initiated in the middle of the 20th century, using embryonic carcinoma cells (Stevens and Little, 1954). The rise of the embryonic stem cell field did however draw advantage of the methodology for teratoma formation from another aspect. Due to the ethical obstacles to evaluate pluripotency of hESC by chimera formation another approach was needed, and teratoma formation in immunodeficient mice was an alternative. Nowadays, induction of teratomas is used as a world-wide standard method for *in vivo* evaluation of hESC pluripotency (Brivanlou et al., 2003; Adewumi et al., 2007; Gertow et al., 2007). Teratomas induced by xenografting of hESC show high resemblance with the most mature clinical teratomas, thus reflecting their normal karyotype and non-

malignant characteristics (reviewed in Lensch et al., 2007). However, unlike clinical teratomas, those formed experimentally by hESC are derived from genotypically normal cells, and have been proven to be of multiclonal origin (Blum and Benvenisty, 2008).

1.5.2.1 Teratomas formed from human ES cells

It has long been known that embryonic cells, such as hESCs, but also the malignant counterpart human embryonic carcinoma cells (hECCs), are giving rise to complex teratomas when grafted *in vivo*. Despite this similarity, it is of great importance to distinguish between these two embryonic cell types. By definition, a teratoma is composed by a variety of more or less mature somatic tissue types, representing the three embryonic germ layers, as seen in ES cell induced teratomas (Gonzalez-Crussi et al., 1982; Ulbright et al., 2005). Similar tissues are also formed by EC cells, but with interspersed areas of remaining pluripotent cells, resulting in formation of a highly malignant teratocarcinoma. On the genomic level, this difference is reflected by an altered karyotype in EC cells (Ulbright et al., 2005; Oosterhuis et al., 2005; Pierce et al., 1960). Hence, it is important to keep in mind that ES cell derived teratomas from karyotypically normal cells are not malignant.

Teratoma formation can be achieved by injecting hESCs in various sites of a host animal, such as intratesticularly, intramuscularly, subcutaneously or under the kidney capsule (Gertow et al., 2004; Plaia et al., 2006; Cooke et al., 2006; Heins et al., 2004). It is reasonable to presume that the microenvironment of the implantation site is affecting the growth and differentiation of the hESCs, and hence, might result in discrepancies regarding teratoma formation efficacy and tissue composition. Indeed, a study comparing teratoma induction in different sites of SCID-Beige mice, indicate that some tissues are more supportive than other (Prokhorova et al., 2008). Their results suggest the kidney capsule to be most favorable, revealing a 100% success rate, followed by intratesticular injections resulting in teratomas in 60% of the cases. However, only 25-40% of the subcutaneous and 12% of the intramuscular injections resulted in teratoma formation. Subcutaneous injections were proven more effective in combination with Matrigel, increasing the success rate to approximately 100%. The same study did not report any difference in tissue composition comparing the different teratoma sites. Such differences have previously been suggested by Cooke et al., who did show that hESC grafted in the liver resulted in formation o more immature tissues, compared to teratomas induced subcutaneously (Cooke et al., 2006). Further investigations are probably needed to fully elucidate the power of the external microenvironment on teratoma formation.

1.5.2.2 Formation of the three germ layers during gastrulation

As previously mentioned, the tissue composition of a teratoma should represent derivatives of all three germ layers; ectoderm, endoderm and mesoderm. These embryonic cell populations constitute the origin of all cells of the adult organism, ensuring development of a diversity of cell types responsible for forming a complex, multi-cellular organism.

Even though the same type of cells formed in the normal embryo can be found in a teratoma, the development is differing in several ways. Nevertheless, understanding the

formation of early progenitor cells by establishment of the three germ layers might be helpful when exploring teratoma development.

During the first week of human development, the zygote undergoes cleavage producing the inner cell mass (ICM) and the outer cell mass, i.e. the trophoblast, giving rise to extraembryonic tissues. Early during the second week, ICM is further divided into two separate structures, the epiblast (primary ectoderm) and the hypoblast (primary endoderm), ensuring establishment of the dorsoventral axis of the embryo. Establishment of the three germ layers takes place in the beginning of the third week during human embryonic development, by a process called gastrulation. Briefly, this is resulting in formation of endoderm and mesoderm, leaving the reminiscent parts of the epiblast to form the ectoderm (Larsen, W.J., Human Embryology, 2001).

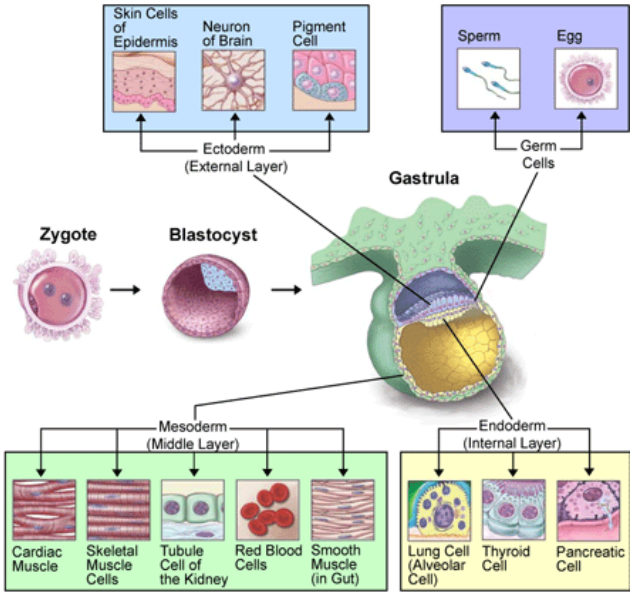

Figure 5: Gastrulation is resulting in formation of the three embryonic germ layers; ectoderm, mesoderm and endoderm, giving rise to the somatic cells of the human body. (Reproduced with permission from NCBI)

Subsequently, ectoderm is giving rise to external ectoderm (epidermis) and neuroectoderm, including the neural tube forming the central nervous sytsem (CNS) and the neural crest, capable of differentiating into various cell types, such as peripheral neurons and melanocytes, as described earlier.

Endoderm is giving rise to the major part of linings of the digestive tube, as well as linings of the pancreas, liver, bronchi and urinary bladder, and parts of the auditory system.

Further development of the mesodermal derivatives can be divided into paraxial, intermediate and lateral plate mesoderm. Paraxial mesoderm is located centrally in the embryo forming somites, which are giving rise to the axial skeleton, voluntary musculature and parts of dermis of the skin. The intermediate mesoderm, located in between the paraxial and lateral mesoderm, will develop into parts of the urogenital system. Lateral part mesoderm will subdivide into a ventral layer associating with the endoderm, forming mesothelial linings of visceral organs, and a dorsal layer associating with ectoderm, giving inner linings of body wall, part of the limbs and most of the dermis (Larsen, W.J., Human Embryology, 2001).

1.5.2.3 Development in a teratoma

Despite their ability to form embryonic tissues, development of hESCs grafted *in vivo* differs from normal embryonic development in several ways. One important aspect is the lack of developmental axis, resulting in a mixture of tissues which are spatially chaotically arranged. Another aspect is the polyclonal origin of the teratoma, possible to reveal numerous start points of development in different time points (Blum et al., 2007; and paper I). Nevertheless, human tissues of more or less mature origin, similar to those of a human embryo, are formed in the hESC derived teratomas.

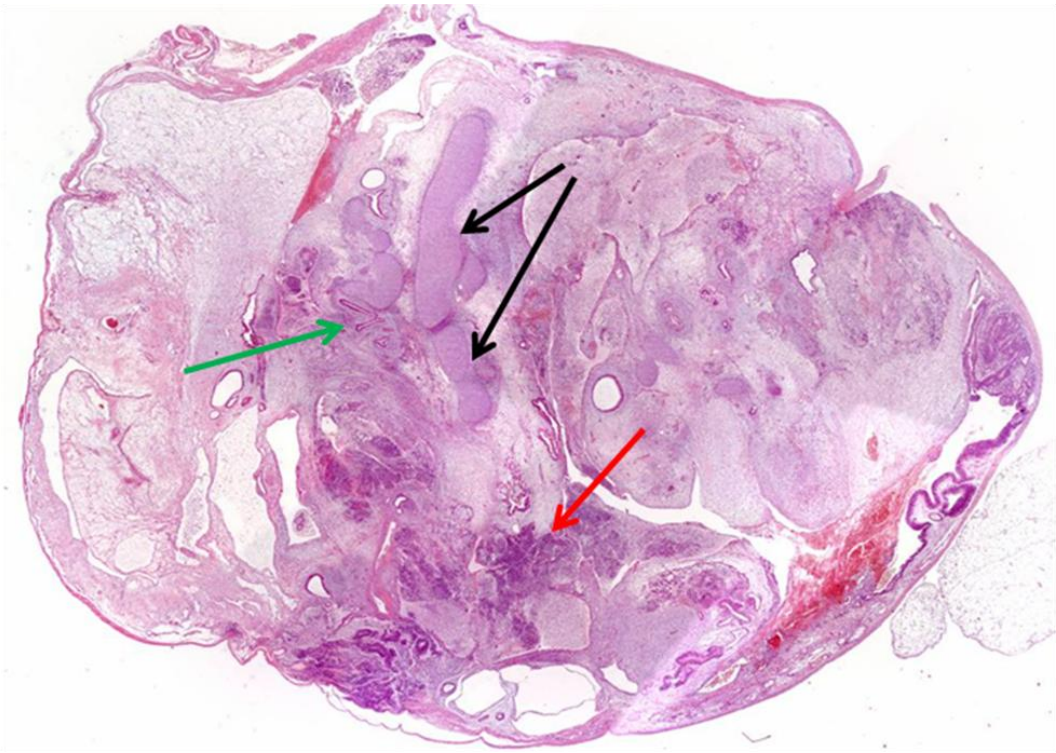

Figure 6: Cross section of a hESC-derived teratoma containing a variety of tissues representing the three germ layers. Black arrows indicate cartilage formation representing mesoderm, red arrow indicates neuro-ectodermal development and green arrow indicates epithelium of endodermal origin.

Human ES cell derived teratomas are containing a variety of tissues of ectodermal, endodermal and mesodermal origin. The first thorough analysis of the tissues in a hESC teratoma was presented by Gertow et al. in 2004, using the cell line HS181 for teratoma generation. Their results indicated that tissues derived from ectoderm were dominating, with a high degree of neuronal development of different grade. Rosette formations of presumptive neural epithelium were detected, expressing markers associated with early neural development. More mature neuronal areas and ganglion like structures were also observed, as well as epithelium resembling retinal formation. Tissues of mesodermal origin, such as cartilage but also bone, formed both by intra-membranous and endochondral ossification, were detected. Further on, endoderm derived epithelial structures with expression of markers suggesting development of bronchi- and intestine could be found. Formation of renal structures with expression profile indicating primitive kidney formation was also reported, representing a more complex organoid development (Gertow et al., 2004). The same study, together with several others, did not reveal any remaining pluripotent cells in the mature teratoma (Thomson et al., 1998; Reubinoff et al., 2000).

Due to the common use of the teratoma model as an *in vivo* pluripotency assay, numerous studies have now been published revealing similar tissue formations in mature teratomas.

1.5.2.4 Vascularization in a teratoma

Vasculogenesis, a prerequisite for proper embryo formation, begins during the third week of human development (Larsen, W.J., Human Embryology, 2001). Cells of the mesoderm are undergoing differentiation into endothelial progenitors, induced by the adjacent endoderm, and thereafter unite to form vessels invading the embryonic tissues (Risau et al., 1995).

Several studies have confirmed that hESCs are able to differentiate into endothelial cells *in vitro* as well as *in vivo* (Levenberg et al., 2002; Gerecht-Nir et al., 2003; Gertow et al., 2004; Gerecht-Nir et al., 2004). However, the hESC contribution to the vasculogenesis in teratomas seems to be of minor extent, with the majority of endothelial cells provided by the host animal (Gerecht-Nir et al., 2004). The endothelial cells of human origin do though appear capable of connecting to the invading murine vasculature, resulting in partly chimeric vessel structures (Gertow et al., 2004).

Low expression of genes involved in human lymphangiogensis, such as LYVE-1, has also been detected, but actual presence of lymphatic vessels has not been verified on the histological level (Gerecht-Nir et al., 2004).

1.5.3 Other sources of pluripotent stem cells

Derivation of the first hESC lines was revolutionary due to the great potential of these pluripotent cells. Theoretically, hESCs could provide an unlimited source of human cells, possible to use for various types of biological research as well as for clinical purposes. With no doubt, emergence of the stem cell field has broaden our understanding about basic cellular mechanisms, which resulted in the rise of yet another pluripotent stem cell type; induced Pluripotent Stem Cells (iPSC), forming teratomas in a similar way as hESCs.

1.5.3.1 Induced Pluripotent Stem Cells

Reprogramming of somatic cells into a pluripotent embryonic-like state was first described in murine cells, using retro-viral mediated introduction of Oct4, Sox2, c-myc and Klf4 (Takahashi et al., 2006). Since then, numerous publications have presented similar findings using also human somatic cells, and the methods have been refined (Takahashi et al., 2007; Yu et al., 2007; Park et al., 2008). Theoretically, iPSCs would allow biological studies on an individual basis, offering unique opportunities when it comes to development of patient-specific therapies in numbers of diseases. However, the field of induced pluripotency is still young, and the full characteristics of these cells are still not explored.

2 AIMS

The overall aim of this thesis was to evaluate and optimize a novel *in vivo* model system for the use in preclinical testing of growth and progression of neural crest derived tumours.

The following aims were initially set:

- To test kinetics of early and late differentiation in hESC derived teratomas, with the ultimate purpose to optimize the model system for use as a surrogate human environment for cancer

- To test whether a spontaneous mutation, trisomy of chromosome 12, commonly occurring in the hESC line used in the study could affect the capacity for teratoma formation

- To apply the above gained knowledge for studies on tumour growth and progression of neuroblastomas and melanomas. Specifically;

    - To determine whether tumours exhibit differential histopathological characteristics upon grafting in a species-specific versus xenoenvironment *in vivo*

    - To elucidate whether a human microenvironment supports growth of tumours histologically more resembling clinical tumours, and thus, are more clinically relevant than xenografts from the same cell lines

3 MATERIAL AND METHODS

3.1 TUMOUR CELLS

3.1.1 Cell lines

A number of human tumour cell lines, all of male origin, were used in paper III and IV.

In paper III, the malignant melanoma cell line **BL**, derived from a lymph-node metastasis of a male melanoma patient in Karolinska University Hospital, Solna, has been used (Salazar-Onfray et al., 1997). On the genetic level, this line is known to have an amino acid substitution in codon 61 of the NRAS gene, resulting in a constitutively active Ras-MEK-ERK pathway (Eskandarpour et al., 2005).

Four different human neuroblastoma cell lines, IMR32, Kelly, SK-N-BE(2) and SK-N-FI, have been used in paper IV (all from ATCC, Manassas, VA).

IMR32 is originating from an abdominal mass of a 13 month old boy. Genetically, it has an amplification of the MYCN gene, in combination with partial chromosome 1 deletion.

The **Kelly** line is derived from a neuroblastoma brain metastasis. The genetic upset is complex with MYCN amplification, gain of chromosome 17q, and deletion of 11q as the major aberrations. In addition, numerical minor genetic alterations have been identified.

SK-N-BE(2) has been derived from a bone marrow metastasis of a two year old patient, after several rounds of chemotherapy and radiation. This line has a genetic amplification of MYCN, as well as mutations in the tumour suppressor gene p53.

The fourth line, **SK-N-FI**, is originating from a non-treated bone marrow metastasis of an eleven year old patient. No major genetic aberrations have been reported, however, high expression of multi-drug resistance gene 1 (MDR1) and autocrine TNF-signaling have been detected.

3.1.2 Culture conditions for tumour cell lines

All tumour cell lines were cultured in Iscove´s Modified Dulbecco´s Medium (IMDM) supplemented with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin, at 37°C, 6.8% CO_2 and high humidity. Splitting was performed either mechanically or enzymatically using 1x trypsin for 5 minutes (all products described are obtained from Invitrogen, Carlsbad, CA).

3.1.3 Verification of genetic markers for later *in vivo* identification

Identification of tumours in the teratomas was performed using FISH for either the y-chromosome or for amplified MYCN. Presence of a y-chromosome possible to identify with FISH was verified using a CEP XY probe (CEP XY, Vysis Inc., Downers Grove, IL) on tumour cells in culture for BL, IMR32, and SK-N-FI. However, due to lack of detectable y-chromosomes, both Kelly and SK-N-BE(2) had to be detected using a probe against amplified MYCN, which was verified to give positive signals in the cells

in culture. For a detailed description of the FISH procedure, see "Fluorescent In Situ Hybridization" below.

3.2 HUMAN EMBRYONIC STEM CELLS

3.2.1 Cell lines

Two hESC lines have been used throughout the projects included in this thesis; the H9 cell line derived by Thomson et al. in 1998, and the HS181 line derived in Karolinska Institutet by Hovatta et al. in 2003. Both these cell lines are of normal female genotype, i.e. 46[XX]. However, a spontaneously arising subline derived from HS181, with aberrant karyotype, has been used and studied in paper II.

3.2.2 Culture conditions for human ES cells

Human ES cells were cultured using human foreskin fibroblasts, hFS (CRL-2429; ATCC, Manassas, VA), as feeder cells. The feeder cells were mitotically inactivated using two different methods; irradiation with 35 Gy before seeding (paper I and II) or treatment with mitomycin C (10 µg/ml, 3 hours) (paper III and IV). The fibroblasts were then seeded at a density of approximately $2x10^4$ cells /cm^2 in 6-well plates (Corning Inc., NY) and ready to use for hESC culture.

The human ES cells were maintained in a culture medium containing 80% Knock-Out Dulbecco´s Modified Eagle Medium (KO-DMEM), 20% KnockOut-Serum Replacement (KO-SR), 2 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercaptoethanol, 4 ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen, Carlsbad, CA) at 37^{o}C, 6.8% CO_2 and high humidity (95%).

Splitting was performed enzymatically using dispase (10 mg/ml) (Invitrogen, Carlsbad, CA) for 5-7 min at 37^{o}C, and by mild mechanical separation.

3.2.3 Karyotyping

The karyotype of the human ES cells was regularly verified using Q-banding, Spectral Karyotyping (SKY) and Fluorescent In Situ Hybridization (FISH) for individual chromosomes.

3.2.4 Characterization

Characterization of the human ES cells has been performed regularly using RT-PCR and immunocytochemistry (ICC) for marker analysis, and EB- and teratoma formation as pluripotency assays *in vitro* and *in vivo*, respectively.

RNA expression of genes characteristic for human ES cells such as Oct4 and Nanog, and for genes related to differentiation into various lineages, has been performed according to the protocol described below (RT-PCR section). Also, expression of genes related to a differentiated phenotype has been analyzed to reveal an undifferentiated state of human ES cells in culture, and to verify differentiation into all three germ layers upon EB formation. For primer sequences and conditions used for RT-PCR, see table 2 below.

ICC was used to verify expression of Oct4 and Nanog on the protein-level, and expression of the hESC characteristic glyco-antigens TRA1-60, TRA1-81 and SSEA-4.

For antibodies and conditions used for ICC during hESC characterization, see table 1 below.

	Dilution	Source
Oct4	1:50	Chemicon, MAB4401
Nanog	1:50	R&D, AF1997
TRA1-81*	1:50	Chemicon, ab 90233
TRA1-60*	1:50	Chemicon, ab 90232
SSEA-4*	1:50	Chemicon, ab 90230

Table 1: Antibodies used for hESC characterization
For antibodies used for experimental analysis, see the individual papers.
*Antibodies included in ES cell characterization kit (SCR001), Chemicon.

3.3 *IN VIVO* EXPERIMENTAL SETUP

3.3.1 Animals

SCID-Beige male mice at the age of 6-8 weeks were used for teratoma- and tumour inductions. Genetically, the SCID (Severe Combined Immunodeficiency) mutation results in lack of both B- and T-lymphocytes, due to failure in the V(D)J recombination, while the beige mutation causes impaired NK-cell function and macrophage defects (Croy et al., 1990; MacDougal et al., 1990).

The animals were housed according to recommendations, i.e. at 20°C to 24°C, 50% relative humidity, and a 14 to 10 hour light-dark cycle with food and water *ad libitum*.

All animal experiments were performed in accordance with local ethical permits S-99/06, N105/07, N112/09.

3.3.2 Teratoma induction

Teratomas were induced by injection of $1x10^4$-$1x10^5$ human ES cells beneath the testicular capsule of anaesthetized (3% isofluran) SCID-Beige mice. The cells were allowed to engraft during eight weeks, resulting in formation of a mature teratoma, or for a shorter time depending on experimental setup (see individual papers). Animals were euthanized by cervical dislocation at the experimental endpoint, and teratomas were dissected, fixed (4% paraformaldehyde), paraffin-embedded, and finally cut in serial sections of 5µm thickness.

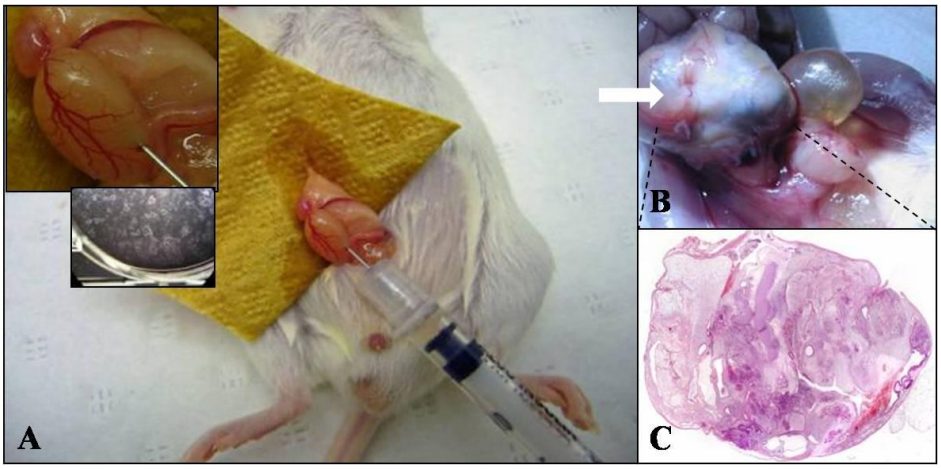

Figure 7: Teratoma induction. Human ES cell colonies are harvested mechanically and immediately injected under the testicular capsule of SCID-Beige mice (A). After 6-8 weeks a mature teratoma is formed (B), containing tissues representing the three germ layers (C).

In paper IV, the same procedure was used for induction of teratomas from mouse embryonic stem cells (mESC) and induced pluripotent stem cells (iPSC).

For pluripotency assay and histological evaluation, tissue sections were stained using the standard procedure with Hematoxylin & Eosin.

3.3.3 Tumour engraftment

In paper III and IV, 1-2x10^6 tumour cells were injected into 6-7 weeks old teratomas, and xenografted by subcutaneous (paper III) and intratesticular injections (paper III and IV). After two weeks of engraftment, the animals were euthanized and tissues prepared according to the description above.

3.4 METHODS USED FOR ANALYSIS

3.4.1 Fluorescence In Situ Hybridization

FISH has been included as a method in all papers. However, the purpose and the binding specificity of the probe used are varying.

In paper I, a human specific probe (Red labeled total human genomic DNA, Vysis Inc., Downers Grove, IL) was used to verify the human origin of tissues in the teratomas.

A chromosome enumeration probe specific for chromosome 12 (CEP SO 12, Vysis Inc., Downers Grove, IL) was used in paper II, enabling us to differ disomic cells from cells being trisomic for chromosome 12.

In paper III and IV, tumours were detected in the teratomas using a probe against the sex-chromosomes (CEP XY, Vysis Inc., Downers Grove, IL). However, in the absence of detectable y-chromosomes, neuroblastoma cells were identified by the use of a probe detecting amplified MYCN (LSI n-myc, 2p24, Vysis Inc, Downers Grove, IL).

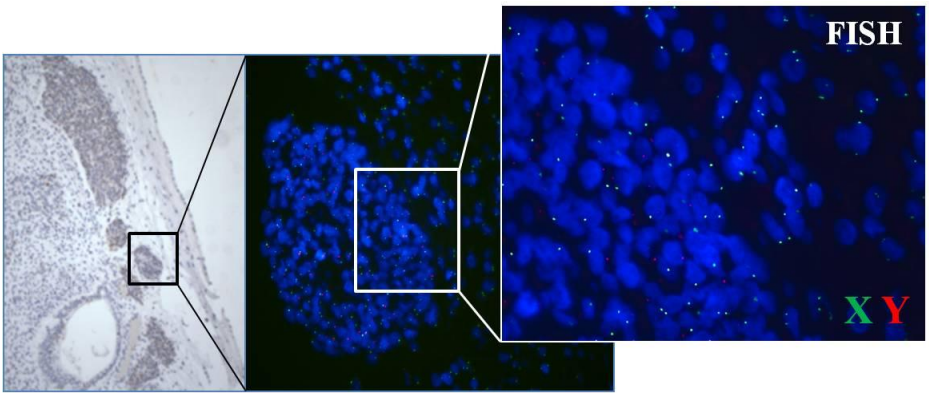

Figure 8: Verification of a tumour in a teratoma using xy-specific FISH, enabling positive detection of y-chromosomes only present in the male tumour cells. Probable tumour areas were detected in H&E stained section and consecutive sections were used for FISH, as illustrated above.

The protocol recommended by the manufacturer was followed for all probes, i.e. an identical procedure was used, with different probe-specific hybridization temperatures as the only varying factor. Briefly, tissue sections were de-paraffinized in xylene and rehydrated through a graded series of ethanol and water. The slides were thereafter pre-treated by boiling in citrate buffer (pH 6.0) and pepsin at 37°C. Denaturation of double stranded DNA and probe was performed by heating at 74°C for 5 minutes, and probe hybridization was allowed by over night incubation in recommended temperatures (varying from 37°C to 42°C depending on probe). The tissue sections were mounted with Vectashield containing DAPI, and analyzed using a Zeiss Axiovert 200M microscope and Openlab 5.0 as the software.

As described earlier, FISH was also performed on cells in culture to verify presence of chromosome y or amplified MYCN. For this purpose, cells were seeded on gelatine coated glass chamber slides, fixed for 5 minutes in ice-cold methanol, and thereafter immediately treated for denaturation and hybridization, according to the same procedure described for FISH on tissue sections.

3.4.2 Immunocytochemistry

ICC has been used in papers II, III and IV for characterization of cells *in vitro*. For this, cells were fixed using 4% paraformaldehyde, permeabilized by incubation in 0.25% Triton X, and unspecific binding was blocked using 3% Bovine Serum Albumin (BSA). Incubation with the primary antibody was performed either in room temperature for 1 hour, or at 4°C over night, followed by 30 minutes incubation with the secondary antibody, in room temperature. The cells were counterstained using DAPI and analysis was performed using a Zeiss Axiovert 200M microscope and Openlab 5.0 as the software. Specific antibodies used are presented in table 1 (only markers related to self-renewal and pluripotency) and in the individual paper.

3.4.3 Immunohistochemistry

A broad panel of antibodies has been used for analyzing the tumours and the tissues formed in the teratomas (see individual papers and table 1). This has been performed on 5µm thick paraffin-sections, which were pre-treated in a way optimized for that specific antibody and antigen, i.e. low pH buffer treatment using Citrate, high pH buffer treatment using Tris-EDTA, or in some cases, no pre-treatment was needed. For further information about conditions and secondary detection systems used see the individual papers (I-IV).

The sections were analyzed using a Zeiss Axiovert 200M microscope with Q-Imaging as the main software.

3.4.4 RT-PCR

RT-PCR was used in paper I for analyzing expression on the RNA level. RNA was extracted from teratoma tissues using TRIzol (Invitrogen, Carlsbad, CA), following the protocol recommended by the manufacturer. Thereafter, RNA was DNA-treated to avoid contamination of genomic DNA, and diluted in DEPC treated water to a final concentration of 0.1µg RNA/µl.

Complementary DNA (cDNA) was synthesized using Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the recommended protocol. Platinum Taq-polymerase was used in the following PCR reaction containing cDNA synthesized from 20 ng total RNA. Primers (Cybergene, Stockholm, Sweden) and conditions used are listed in table 2.

RT-PCR has also been used as a routine method for characterizing hESC. For this purpose, RNA was extracted from *in vitro* cultured cells or EBs using the Micro-to-Midi kit (Invitrogen, Carlsbad, CA). Preparation of cDNA and subsequent PCR was performed as described above.

Gene	Primer sequences	Size (bp)	Annealing Temp.°C	mM [$MgCl_2$]
β-actin	F:5´-GACATTAAGGAGAAGCRGTGCTATGTT-3´ R:5´-GCCTAGAAGCATTTGCGGTGGACGA-3´	497	58	3,0
NANOG	F:5´-CGGCTTCCTCCTCTTCCTCTATAC-3´ R:5´-ATCGATTTCACTCATCTTCACACGTC-3´	960	57	1,5
Oct4	F:5´-AGGATCACCCTGGGATATACACA-3´ R:5´-AAGCTAAGCTGCAGAGCCTCA-3´	113	55	3,0
Klf4	F:5´-CTGCGGCAAAACCTACACAA -3´ R:5´-GGTCGCATTTTTGGCACTG -3´	182	51	3,0
hTERT	F:5´-CGGAAGAGTGTCTGGAGCAA-3´ R:5´-GGATGAAGCGGAGTCTGGA-3´	147	51	1,0
Shh	F:5´-GAAAGCAGAGAACTCGGTGG-3´ R:5´-GGAAAGTGAGGAAGTCGCTG-3´	170	51	5,0
Brachyury	F:5´-GTGACCAAGAACGGCAGGAGG-3´ R:5´-TGTTCCGATAGCATAGGGGC -3´	706	52	1,0
Wnt3	F:5´-ACTTCGGCGTGTTAGTGTCC-3´ R:5´-ATTTTTCCTTCCGCTTCTCC-3´	501	51	1,0
Wnt5a	F:5´-CAGTTCAAGACCGTGCAGAC-3´ R:5´-TGGAACCTACCCATCCCATA-3´	501	51	1,0
BMP-4	F:5´-GCTGAAGTCCACATAGAGCGAGTG-3´ R:5´-ACTTCGAGGCGACACTTCTGC-3´	785	53	1,0
Nodal	F:5´-CATGAAAGCTATAGGTGACTTCAT-3´ R:5´-TGTAAATGAAGGGCTCAGTGGA-3´	250	56	1,0
Fgf9	F:5´-AGCCCGGTTTTGTTAAGTG-3´ R:5´-AGTATCGCCTTCCAGTGTC-3´	401	49	2,0
CD90	F:5´-ATGAACCTGGCCATCAGCATCGC-3´ R:5´-TCACAGGGACATGAAATCCGTGG-3´	525	55	2,0
Flt-1	F:5´-ATCAGAGATCAGGAAGCACC-3´ R:5´-GGAACTTCATCTGGGTCCAT-3´	441	51	3,0
CD31	F:5´-CAACGAGAAAATGTCAGA-3´ R:5´-GGAGCCTTCCGTTCTAGAGT-3´	260	49	4,0
CD34	F:5´-TGAAGCCTAGCCTGTCACCT-3´ R:5´-CGCACAGCTGGAGGTCTTAT-3´	200	51	4,0
CD45	F:5´-AACCTGAAGTGATGATTGCTG-3´ R:5´-TACCTCTTCTGTTTCCGCAC-3´	499	51	3,0
VE-cadherin	F:5´-ACGGGATGACCAAGTACAGC-3´ R:5´-ACACACTTTGGGCTGGTAGG-3´	596	51	1,0
Osterix	F:5´-TGCCCAGTGTCTACACCTCTC-3´ R:5- AGTGTCCCTTGCAGCCCATC -3´	181	59	3,0

Osteocalcin	F:5´-TGGTCCCTCAGTCTCATTCCC-3´ R:5- AGCCATTGATACAGGTAGCGC -3´	191	54	3,0
Osteopontin	F:5´-CGGAGGAGACAATGGAGAAG-3´ R:5- GACGCCCGTGTATTCGTACT-3´	226	54	3,0
MyoD	F:5´-GGG AAG AGT GCG GCG GTG TCG AG-3´ R:5´-TCCCAGAAGGGTGCTGCGTGGAA-3´	445	57	1,0
AFP	F:5´-TGCAGCCAAAGTGAAGAGGGAA-3´ R:5´-ATA GCG AGC AGC CCA AAG AAG A -3´	215	54	4,0
C/EBPα	F:5´-CAC GAA GCA CGA TCA GTC CAT-3´ R:5´-CGC ACA TTC ACA TTG CAC AAG-3´	135	53	1,0
GATA4	F:5´-CAT CAA GAC GGA GCC TGG CC-3´ R:5´-TGA CTG TCG GCC AAG ACC AG-3´	216	52	1,0
Sox17	F:5´-TGC AAC TAT CCT GAC GTG TGA C-3´ R:5´-TAGTGTGACAGAGGTACTAGTAG-3´	429	54	1,0
Ipf1	F:5´-CCC ATG GAT GAA GTC TAC C-3´ R:5´-GTCCTCCTCCTTTTTCCAC-3´	261	49	1,0
Pax2	F:5´-GCA CTT GCG AGC TGA CAC CT-3´ R:5´-CGT TGT AGG CCG TGT ACT GG-3´	480	51	2,0
Wnt2	F:5´-TGA TCC AAA GAA GAT GGG AAG-3´ R:5´-TGT AGC GGT TGT CCA GTC AG-3´	670	52	1,0
NESTIN	F:5´-CAGCTGGCGCACCTCAAGATG-3´ R:5´-AGGGAAGTTGGGCTCAGGACTGG-3´	208	55	2,0
E-Cadherin	F:5´-GAC GCG GAC GAT GAT GTG AAC-3´ R:5´-TTGTACGTGGTGGGATTGAAGA-3´	280	53	1,0
Pax6	F:5´-AACAGACACAGCCCTCACAAACA-3´ R:5´-CGGGAACTTGAACTGGAACTGAC-3´	274	55	1,0
hCG	F:5´-CTACTGCCCCACCATGACCC-3´ R:5´-GGACTCGAAGCGCACATC-3´	105	48	1,0

Table 2: Primers and conditions used for hESC characterization and analysis using RT-PCR.

4 RESULTS AND DISCUSSION

4.1 DEVELOPMENT OF HESC-DERIVED TERATOMAS: PAPERS I AND II

The *in vivo* developmental potential of hESCs upon xenografting, i.e. teratoma formation, was thoroughly explored in papers I and II. It has long been known that embryonic stem cells have the capacity to differentiate into derivatives of the three germ layers after grafting *in vivo,* and that more or less mature tissue and organoid structures are formed. However, the events leading the path, from the inoculated pluripotent cells to the mature teratoma several weeks later, are largely unknown.

In paper I, we aimed to explore the developmental processes leading to the mature teratoma, and investigate how well this is correlating with human embryonic development. Ultimately, this would indicate how relevant the teratoma would be as an *in vivo* model system for studies on growth and progression of human cancers.

Teratomas were induced by injection of hESC (HS181) under the testicular capsule, as described in Material and Methods, and harvested at various time points; day 5, 10, 20, 30, 45 and 60, post-injection. Engraftment with active proliferation of the hESC-derived cellular progeny was detected already at the first observation point at day 5. This growth was mainly represented by single or multilayered pseudo-stratified columnar to cylindrical epithelium, resembling formation of primitive ectoderm, and the histology correlated well with Thieler Stage (TS) 8 in mouse and Carnegie Stage 5 in human embryonic development. Immunohistochemistry revealed expression of markers related to pluripotency, but also early neuronal markers such as Nestin, suggestive of an early neuro-ectodermal characteristic. The early development of hESC after grafting *in vivo* is of interest also concerning the aspect of what hESCs really are, i.e. which naturally occurring *in vivo* cell type a hESC can be comparable to. As previously mentioned, hESCs are highly artificial in the sense that cells of the ICM are not retaining prolonged pluripotency, but differentiate into cell types contributing to formation of the embryonic tissues. Our results from day 5 suggest that the specific cell line HS181 at this time point represents a stage similar to primitive ectoderm (PE) or the epiblast, in line with its origin, derived from a day 6 blastocyst, and consecutive *in vitro* culture.

Development up to day 30 was dominated by a gradual increasing amount of multilayered epithelia, with a neural dominance. However, occasionally other structures were observed, e.g. a formation resembling an early limb bud, with a condensing mesenchyme expressing CD56, delimited by a double-layered epithelium expressing p63 and E-cadherin, as well as CK18 in the peripheral layer.

Evidence for more mature tissues and organoid structures representing the three germ layers were observed in the more mature teratomas between day 45 and 60, however in a disorganized manner and lacking developmental axis. Still, a high frequency of neuro-ectodermal structures was present, but also ectodermal tissues of non-neuronal character. Endoderm-derived tissues resembling early gut and bronchi-epithelium were detected, as well as renal structures representing presence of intermediate mesoderm, and cartilage of mesodermal or eventually ectodermal neural crest origin. Despite

presence of tissues derived from all germ layers, the development was showing some restriction. For example, no somites, i.e. early representatives of paraxial mesoderm, were detected. The somites represent an important part of the mesoderm development, giving rise to cartilage of the vertebrae, ribs and musculature of the back etc, but also influence the formation of other parts of the embryo, such as development of the neural crest. Hence, the chaotic tissue distribution seen in the teratomas might partly depend on absence of somites. The neural dominance present from the earliest time points, and partly lasting in the later stages as well, might also contribute to the somewhat restricted developmental potential. It cannot be excluded that the neural character is influenced by a predisposition for the neuro-ectodermal faith caused already in the *in vitro* culture stage, i.e. before injection. Presence of derivatives of the three germ layers at later time points does nevertheless indicate a retained pluripotency of the cells.

Vascularization of human origin, as detected by human specific immunohistochemistry for CD34 and CD31, could be observed at later time points. However, as previously reported vasculature from the host was dominating, with only a minor contribution from the human ES-cell derivatives. The human vessels were shown capable to connect with the murine vasculature, and appeared functional.

The awareness of culture adaptation and concomitant risk for karyotypic aberrations in hESCs has increased, and sub-lines with genetic abnormalities have been reported from a number of research groups. In our own laboratory gain of chromosome 12 has been the most frequent karyotypic alteration. In paper II, *in vivo* developmental potential of a sub-line from HS181 with the karyotype 47, XX(+12) was evaluated. Hence, teratomas derived from hESCs with different degree of cells trisomic for chromosome 12 were compared on the histological level with teratomas formed from HS181 with a normal diploid karyotype. Results from this study revealed that cells trisomic for chromosome 12 are not favored upon xenografting, but has the capacity to grow and participate in formation of tissues from all three germ layers.

Interestingly, analysis of tissue distribution in the resulting teratomas indicated a higher frequency of renal formation in teratomas derived from the karyotypically altered cell line. Thorough analysis did not suggest a higher frequency of trisomic cells especially in these structures, why indirect mechanisms are probable to cause this phenomenon. Trisomy for chromosome 12 has also been reported to occur in pediatric renal tumours, such as Wilm´s Tumour (WT) and especially in cystic partially differentiated nephroblastoma (CPDN) (Austruy et al., 1995; de Chadarévian et al., 1996). CPND is a rare variant of pediatric renal malignancy with an often favorable outcome, and it has been suggested that trisomy of chromosome 12 is a common trait present in a majority of these tumours (de Chadarévian et al., 1996). This supports the notion that genes on chromosome 12 might be involved, directly or indirectly, in regulating growth of renal structures during development.

Most likely, karyotypic changes affect the developmental capacity of hESCs upon teratoma formation. As described here, such cells might still be capable of forming benign teratomas, but the tissue distribution might be skewed. In other cases, hESCs with abnormal karyotype have proven incapable of producing teratomas *in vivo*. This has been shown by our research group using a culture adapted cell line with the

karyotype 47, XX, del(7) (q11.2),+i(12)(p10), which failed to form teratomas in SCID-Beige mice (Imreh et al., 2006).

Hence, considering the risk for mutations and subsequent consequences, karyotyping should be performed regularly to ensure the stability of the genome. Stable culture conditions for hESC are also of great importance, avoiding selection of altered clones in the culture. This would minimize the risk for inadvertent genomic alterations, affecting the ability of hESCs to form teratomas.

Day 45 represented a time point with occurrence of mature teratoma and an initiated human vascularization process. Therefore, we have chosen day 45 as the most advantageous time for injection of tumour cells, thus providing optimal conditions for engraftment and an experimental time window allowing development before occurrence of necrosis, and before bringing unnecessary harm to the recipient animal.

4.2 HEST AS A MODEL SYSTEM FOR MALIGNANT MELANOMA: PAPER III

The cutaneous malignant melanoma cell line BL was grafted in hESC derived teratomas (i.e. the hEST-model) and compared to corresponding xenograft tumours (subcutaneous and intra-testicular), all allowed to engraft for two weeks. Areas of melanoma growth were verified using FISH (as described in Material and Methods) and the tumor histology was thoroughly examined and compared to the xenograft tumours. No obvious histological difference could be observed comparing the bulk-tumours of the different systems; the tumours appeared highly malignant and pleomorphic, growing with high cellular density and low stroma contribution. On the cytological level, melanoma cells exhibited large irregular nuclei, distinct nucleoli and less prominent cytoplasm. Nevertheless, immunohistochemistry revealed striking differences concerning differentiation status of tumours in the different systems. While xenografts in both systems were homogeneously expressing genes related to melanocytic differentiation, such as HMB45, MelanA and Tyrsinase, these were mainly absent in melanoma grafted in the hEST-model. Immunocytochemistry for HMB45 and MelanA on BL cells *in vitro* showed that these markers were absent already at this stage, suggesting that the xeno-environment *per se* induced differentiation of BL melanoma cells. The embryonic environment in the teratoma did not exert a similar effect, but allowed the tumour cells to stay in a more undifferentiated state.

Further on, an additional population of melanoma cells, with a spindle-like morphology indicative of a desmoplastic tumour type, could be observed exclusively in peripheral tumour parts in the hEST-model. These cells formed what appeared to be fronts, invading into the surrounding mesenchymal stroma supplied by the teratoma. The migrative and invasive character of these cells was further supported by expression of the metastasis related marker S100A4 (Garrett et al., 2006), which was almost exclusively present in cells of the peripheral parts or invasive fronts. Cells expressing S100A4 were detected also in the xenografts; however, these were dispersed in the tumour cells mass and did not form organized fronts invading adjacent tissues.

Invading melanoma cells were also secreting the embryonic morphogen Nodal, not expressed in the other parts of the tumours. Expression of Nodal has previously been correlated to an invasive phenotype in melanoma (Topczewska et al., 2006), thus supporting our suggestion that these cells are more invasive than cells of the bulk-tumour and tumour cells present in the xenografts.

Melanoma engraftment did induce extensive vasculogenesis in the adjacent stroma which was almost exclusively of human origin, as proved by immunohistochemistry for human CD31. As previously described, vascularization in hESC derived teratomas are mostly ensured by the host vasculature, with only a minor contribution from hESCs differentiating into endothelial cells (Gerecht-Nir et al., 2004; Gertow et al., 2004). However, our results did clearly indicate that the proportion of human vessels was dramatically increasing upon tumour grafting, and especially in tissues facing invading melanoma fronts. Tumours are known to produce angiogenic factors, such as VEGF, to recruit vasculature and ensure supply of oxygen and nutrients enabling further expansion (reviewed in Carmeliet, 2003). The human-specific response observed here suggests that this induction might be partly species-specific. At least, it indicates that there are discrepancies between species making surrounding cells more or less responsive to this induction. Considering the importance of tumour vasculature for the characteristics of a cancer, this could cause striking differences in tumour histology upon xenografting of human tumours. Malignant melanoma was the first tumour type shown to be capable of so called vasculogenic mimicry, meaning that the actual tumour cells undergo differentiation into an endothelial cell type, participating in formation of the tumour vasculature (Maniotis et al., 1999). This capability indicates how important proper vascularization is for melanoma biology, but as well proves the fantastic plasticity of tumour cells in general and melanoma cells *per se*. Our results did not reveal any presence of vasculogenic mimicry from the BL melanoma cells grafted. Sex-specific FISH indicated that all human cells participating in formation of the vasculature was of female origin, i.e. derived from the hESCs. However, it is probable that the developing embryonic teratoma environment exclude any need for the melanoma cells to undergo endothelial differentiation; the surrounding cells were still immature and highly prone to respond to any inductive stimuli from the surrounding. Hence, the formation of new vessels could be satisfactory even without melanoma cell participation.

The plasticity of malignant melanoma, as proved by reported differentiation into a wide variety of lineages such as myofibroblastic, schwannian, smooth muscle, osteocartilaginous, ganglionic, neuroendocrine and epithelial, might cause confusion and problems for diagnostics (reviewed in Banarjee et al., 2008). This broad potential of melanoma cells is well reflecting the origin of melanocytes in the embryonic neural crest, which is giving rise to a variety of quite different cell types, such as chondrocytes, neurons and melanocytes. Additional knowledge about regulatory mechanisms during neural crest formation and subsequent lineage specification would probably contribute also to a better understanding in the field of melanoma biology. A number of studies have been performed to elucidate how an embryonic microenvironment is affecting the behavior of malignant melanoma cells. Surprisingly, it has been shown that injection of aggressive melanoma cells into chick and zebrafish embryos do not result in tumour formation. Instead, the tumour cells seemed to undergo

a normalization process, participating in formation of the neural crest and its lineage derivatives, suggesting that the embryonic environment is able to epigenetically reprogram aggressive tumour cells (Lee et al., 2005; Kulesa et al., 2006; Haldi et al., 2006). This is in part showing the opposite effect compared to the more invasive melanoma growth observed in the hEST-model. However, an explanation for this might be that our model represents an embryonic state dominated by tissues in a post-neural crest stage, i.e. specification of lineage derivatives has already occurred. The other model systems all use earlier embryos representing a stage before or at neural crest emergence, more probable to exert signals regulating development of the neural crest cells and therefore also the melanoma cells.

On the other hand, a certain tropism of engraftment was observed since melanoma cells were exclusively integrated into tissues of mesenchymal stroma character. This resembles the behavior of normal neural crest cells which during development are migrating out from the neural tube via the surrounding mesenchyme to their final sites, where they upon induction settle to accomplish the development into functional derivatives.

Altogether, the results indicate that tumour histology upon xenografting of melanoma cell lines cannot always be presumed to reflect the histology of the same tumour cell line grafted in a human microenvironment. This is important to keep in mind when using such xenograft-systems for therapeutic evaluations; the results obtained in a xenograft might not reflect the outcome in a human environment. However, it is not clear which effects are consequences of the species-specificity, and which are rather caused by the embryonic nature of the teratoma. It is very probable that both these factors influence the histopathological picture. Also, one has to remember that the hEST-model is not representing the correct developmental stage, but a tumour type occurring in the adult has been grafted in embryonic tissues, certainly differing a lot from its more mature counterparts. Nevertheless, our data is clearly indicating the powerful impact of the tumour surrounding as well as the plasticity and fantastic capability of malignant melanomas to adjust to the environment. Definitely, this underscores the need for optimizing preclinical tumour models aiming towards a higher clinical relevance, ultimately leading to development of more effective therapeutics.

4.3 HEST AS A MODEL SYSTEM FOR NEUROBLASTOMA: PAPER IV

Four different neuroblastoma cell lines, IMR32, Kelly, SK-N-BE and SK-N-FI, were grafted in the hEST-model, and three of them (IMR32, Kelly and SK-N-BE) were extensively studied using a panel of antibodies. The same cell lines were also grafted as intra-testicular xenografts and in teratomas derived from mouse embryonic stem cells (mESCs) and histologically compared to tumours in the hEST-model.

A general finding was that tumours grafted in the hEST-model displayed higher cellular heterogeneity with histology more resembling neuroblastomas seen in the clinic, as compared to xenograft tumours. This histological observation was often supported by focal expression of genes associated with differentiation into various cellular lineages, as proven by immunohistochemical analysis. This was most obvious for IMR32 exhibiting focal expression of tyrosinase, indicative of melanogenic

differentiation, exclusively in the hEST-model but not in the xenograft. In addition, expression of Neuron Specific Enolase (NSE) was detected only in IMR32 grafted in the hEST-model, further suggesting a partly neurogenic phenotype not present in the corresponding xenografts. Similar findings were obtained also from grafting of the Kelly cell line, exhibiting higher expression of NSE as well as synaptophysin, indicating a focal neuronal- and neuroendocrine phenotype in the hEST-model. The SK-N-BE(2) line was exhibiting the most aggressive tumour growth comparing the different cell lines used. Also, the histology of the SK-N-BE(2) tumours grown in the hEST-model and as xenografts did not differ to the same extent as observed for previous lines. Possibly, this reflects the origin of the cell line; it is derived from a bone marrow metastasis after several rounds of both chemo- and radio-therapy. As a consequence, the cell line is extremely selected containing only the most malignant and therapy-resistant cell populations of the original tumour cell mass, as indicated by the presence of a p53 mutation. As previously described, p53 mutations are relatively rare in primary neuroblastomas, but more common in metastases, recurring tumours and in reminiscent cell populations after treatment (Vogan et al., 1993; Brodeur, 2003; Keshelava et al., 2001; Tweddle et al., 2001). It is reasonable to presume that such aggressive cell lines are less dependent on support from the surrounding microenvironment and rather grow in an auto-regulatory manner, independent of stimuli from outside. In this way, the tumour is capable of growing in almost any environment, with less selection pressure on specific sub-populations of cells. However, results from immunohistochemistry did indicate some impact on the differentiation pattern, but with a reversed pattern compared to previous lines, i.e. higher tendency for differentiation into neurogenic or neuroendocrine lineages in the xenografts.

Heterogeneity in neuroblastomas has been correlated both with poor prognosis and therapy-resistance (Shimada et al., 2001). Therefore, maintenance of heterogeneity also after *in vivo* grafting in preclinical model systems is of utmost importance for relevant tumour histology. Our results confirm that the tumour microenvironment indeed has a powerful impact on the differentiation and heterogeneity of the tumour cells, further emphasizing the need for optimized *in vivo* model systems. Hopefully, this would be a clue to achieve more successful treatments and a decline in numbers of deaths caused by neuroblastoma.

A striking difference observed for all cell lines used was a higher frequency of cells of mesenchymal phenotype in the xenografts, as indicated by expression of vimentin. It has previously been suggested that low oxygen tension, i.e. hypoxia, pushes neuroblastoma cells into an immature neural crest like phenotype (Jögi et al., 2002). Hence, the hypoxic condition in the testis (Setchell et al., 1964; Free et al., 1976), as compared to the teratoma environment with active neo-vascularization taking place, may be a possible reason for this. A neural crest like phenotype with stemness characteristics has also been linked to up-regulation of HIF2α (Nilsson et al., 2005; Pietras et al., 2008). We did show that the sub-cellular localization of HIF2α was significantly altered in the different model systems. Considering the role of HIF2α as a transcription factor, the cellular localization would be of great importance, only allowing HIF2α to exert its transcriptional function upon nuclear localization. For Kelly, a higher frequency of nuclear HIF2α is in line with those for vimentin,

supporting the notion of a more neural crest cell like tumour in the testis. On the other hand, HIF2α expression was entirely cytoplasmic in Kelly grafted in hEST, possibly due to richer vascularization and higher oxygen levels, and consequently resulted in a less immature phenotype.

It is clear that the interaction between a tumour and its surrounding is not a one-way communication affecting only the tumour cells, but is rather a complex cross-talk influencing also the behavior of the non-tumorigenic tissues in the surrounding. This was obvious when looking into the supportive vascularzation and fibroblast-infiltration taking place.

Similar to previous findings using other tumour types, grafting of all neuroblastoma cell lines induced an extensive human neo-vascularization in the tumour-adjacent hESC-derived stroma. This effect was most striking using SK-N-BE(2), were human vasculature was frequently recruited and infiltrated the tumour nodules. These observations are in line with previously published results, showing that vasculogenic induction by neuroblastoma is significantly correlating to aggressive tumour types and therapy-resistance (Michaelis et al., 2009). Immunohistochemistry for human specific CD31 did also reveal that the tumour cells from SK-N-BE(2) occasionally underwent vasculogenic mimicry, i.e. differentiated into an endothelial phenotype participating in formation of new blood vessels. Vasculogenic mimicry has been reported in clinical neuroblastomas and suggested to be specifically frequent in high grade tumours, i.e. stage 3 and 4. Interestingly, the same study did show that the proportion of tumour derived endothelial cells was correlating with worse prognosis and stated that as much as 70% of the endothelial cells in some stage 4 tumours were of tumour origin (Pezzolo et al., 2007). Hence, a number of traits of SK-N-BE(2), linked to highly aggressive malignancy, might explain the differential response from this cell line compared to the others.

Cancer Associated Fibroblasts (CAFs), a heterogeneous population of tumour stroma cells, has been associated with metastasis and poor clinical outcome in a number of adult cancers, e.g. carcinoma of the breast and melanoma (Orimo et al., 2005; Yazhou et al., 2004; Huber et al., 2003). Recently, CAFs were also suggested to have a role in neuroblastoma progression, mainly by stimulating angiogenesis (Zeine et al., 2009). Results presented in that study showed that the number of infiltrating CAFs was inversely correlating to the presence of schwann-cells. A high degree of schwannian-stroma in neuroblastoma is generally linked to a favourable prognosis (Nagoshi et al., 1992; Hachitanda et al., 1992), and it has been suggested to depend on angiogenic inhibition exerted by the schwann-cells (Peddinti et al., 2007). Hence, it is probable that schwann-cells inhibit tumour angiogenesis by restraining activation of fibroblasts in the surrounding. Our results did exhibit cells within the tumours from Kelly and SK-N-BE(2) grafted in hEST expressing human αSMA, thus indicating presence of infiltrating hESC derived fibroblast. Further on, immunohistochemistry for calretinin was negative for both tumour cell lines, suggesting absence of schwann-cells in the surrounding. As described above, these tumours, and especially SK-N-BE(2), were rich in vasculature mainly of human origin, all in line with the above suggested hypothesis. However, it can not be excluded that the αSMA positive cells detected in tumours grafted in the hEST are of tumour origin. Expression of human αSMA in xenografts

from IMR32 indicated that this cell line do have the capability to undergo such differentiation, possibly as a way to support its own growth and progression. On the other hand, no αSMA positive cells were detected in the IMR32 tumours grafted in hEST. This further emphasized the differential characteristics of the tumours grafted in the different *in vivo* systems. A possible explanation for the absence of CAFs in IMR32 hEST is the limited tumour size; IMR32 tended to grow as small groups of tumour cells rather then larger tumour nodules, and adjacent stroma might be capable of exerting its effect without infiltrating the tumour *per se*.

Cox2, a protein supposed to have growth promoting as well as anti-apoptotic effect in neuroblastomas, is considered a promising therapeutic target (Johnsen et al., 2004). We detected differences in Cox2 expression levels comparing the different preclinical models. Taking the Kelly cell line as an example, Cox2 tended to decrease in the xenograft compared to the hEST. These variations are essential to keep in mind, since therapeutic efficacy is highly probable to depend on expression levels of the target gene.

Conclusively, the results obtained in this study show differential characteristics of neuroblastoma cell lines grafted in a human embryonic environment compared to a xeno-environment represented here by the testis of SCID-Beige mice. Preliminary histological observations indicate though, that some of the differences observed might be due to developmental cues present specifically in an embryonic environment, and are not necessarily caused by species-specific impact. The effects induced by the microenvironment do seem to vary between cell lines, probably depending on several factors, e.g. genetics of the cell line, derivation site etc. This in turn, suggests a strong need for even more specific preclinical tumour model systems.

4.4 HEST AS A MODEL SYSTEM

As described above, a teratoma derived from human pluripotent stem cells is providing an environment rich in a variety of developing human tissues. Hence, it is allowing unique opportunities to study human biological processes in an *in vivo* system. However, some limitations of the system are obvious. Due to animal ethics, the size of the teratoma and therefore also the experimental time window is limited. As a consequence the microenvironment is embryonic rather than adult. Results from paper I indicate that the kinetics of the teratoma is quite similar to human development, resulting in 60 day old teratomas corresponding approximately to week 9 in human embryogenesis, which is representing an active environment with high levels of growth-promoting factors and ongoing organogenesis. This is important to consider when grafting adult tumours in the teratoma model, such as malignant melanoma studied in paper III. It is possible that the embryonic surrounding *per se* is contributing to dedifferentiation of melanoma cells in the system, not necessarily meaning that the resulting tumour is more clinically relevant. Nevertheless, it certainly high-lights the need for proper preclinical *in vivo* models for these types of cancers. On the other hand, the embryonic teratoma environment more resembles that of the originating neuroblastoma, and one would therefore presume the model to be more suitable for pediatric malignancies. Results from paper III show that growth of neuroblastoma cell lines is well supported by the hEST-model and do suggest that the resulting tumours

exhibit a more clinically relevant histology. However, tumours in the hEST were compared with xenografts growing directly in the testis of an adult mouse, meaning that the model system differs in at least two major aspects; the hEST-model is offering a species-specific and a developmental-specific, i.e. embryonic, environment. In an attempt to differ between effects caused by species-specific versus developmental-specific cues, the neuroblastoma cell lines were grafted in teratomas derived from mouse ES-cells in parallel. Histological observations revealed that the resulting tumours had some similarities with both xenografts and tumours grafted in hEST, indicating influences depending on both factors. Nevertheless, some results did clearly show the species-specific impact, such as the massive human neo-vascularization induced both by neuroblastoma and melanoma. A more thorough analysis is needed to definitely separate consequences according to these different aspects.

The reasons for choosing testis as the site for implantation are several; testis is not a vital organ, it is relatively easy to access, and the teratoma growth can at least partially be monitored by external examination (Gertow et al., 2007). However, it is probable that the local environment (hypoxic and hormonal) in the testis is affecting the transplanted cells, particularly at early time points when mouse testis is dominating the microenvironment. It should be noted however, that at day 45, the time chosen for exogenous tumour injection, the mouse testis structure has been fully replaced with hESC-derived tissue (Paper I). Thus, no hormonal influences from the testis are likely affecting the experimental outcome.

As previously described, it has not been proven that the developmental capacity or tissue distribution in a teratoma is differing depending on grafting site (Prokhorova et al., 2008), but such effects cannot be excluded. The testis has also been used for xenografting of the tumour cell lines in paper III and IV. In paper III, the BL melanoma cell line was grafted subcutaneously in parallel, and the results did not show any difference in histology or immunohistochemical profile comparing xenografts from the two sites. This indicates that the local environment in the testis *per se* might not have a significant impact on the melanoma growth and development. In paper IV, testis was used as the only direct site for neuroblastoma xenograft induction, paralleled with injections into mESC-derived teratomas. This complicates the elucidation of eventual hormonal effects. It is nowadays well known that estrogen is produced by Leydig cells and germ cells in the adult testis of several species, including the mouse (Nitta et al., 1993; Janulius et al., 1996; Wang et al., 2001; Bilinska et al., 2003; Catalano et al., 2003; Golovine et al. 2003). Estrogen signaling is implicated in normal development and differentiation of the nervous system and has also been suggested to play an important role in progression of neuroblastoma (Ma et al., 1993; Lovén et al., 2010). Thus, it is possible that the neuroblastomas grafted in the testis are affected by estrogen or linked signal pathways.

The immunodeficiency of the model system is important to keep in mind. It is partly reducing the complexity of the tumour and contributes to simplified interpretations of other effects. However, interactions with the immune system are of importance for growth and progression of several cancers, e.g. malignant melanoma. The capability of melanoma to induce activity of the immune system has been well studied. Primary melanomas in early phases are often eliciting reactions of dermal lymphocytes resulting

in focal tumour destruction, and disseminated melanoma may in rare cases undergo spontaneous regression upon activation of immune responses (Clark et al., 1969; Oble et al., 2009; Dranoff et al., 2009).

With no doubts, studies on the clinical tumour cell mass would be desirable for evaluating maintenance of cellular heterogeneity. Even short term *in vitro* culture of tumour cell lines unavoidably leads to adaptation and selection of sub-populations of cells. However, it has been shown that many tumours, especially neuroblastoma, exhibit some cellular heterogeneity even after establishment of cell lines *in vitro*. Three major different cell types have been described; a dominating neuroblast with neurites growing as poorly attached aggregates (N-type), a non-neuronal substrate-adherent fibroblast-like cell (S-type), and an intermediate plastic cell type with stemness characteristics (I-type), capable of differentiation into either of the two other cell types (Ross et al., 1983; Ross et al., 1995). Our results do also suggest certain heterogeneity of several cell lines after engraftment in the proposed *in vivo* model. Nevertheless, further studies on clinical material which is not pre-selected and though not affected by *in vitro* culture conditions would add significant knowledge regarding the question of cellular heterogeneity upon grafting of neuroblastoma in preclinical model systems.

A tropism of engraftment was observed using both melanoma and neuroblastoma cell lines; tumour cells preferentially resided in areas of hESC-derived loose mesenchyme. The result is not surprising considering the similarity with the embryonic mesenchyme used for migration of emerging neural crest cells during development. However, the highly aggressive SK-N-BE(2) neuroblastoma cell line did also integrate into other tissues of the teratoma, exhibiting a less restrictive demand on a specific surrounding. This indicates that the rich variety of tissue derivatives present in the teratoma can be a valuable feature of the model system. Again, using a more heterogeneous clinical tumour material for similar studies would allow elucidation of eventual preferences for certain metastatic sites in different sub-populations of cells. This might be of importance for predicting metastatic behaviour of tumours, and also for designing therapies targeting different subpopulations of cells as well as metastatic tumours in different sites.

Two different hESC lines, HS181 and H9, have been used throughout these studies. We did not see that the cell line used for teratoma induction was affecting the outcome of the experiment, i.e. the tumours grafted in teratomas from the different hESC lines had similar histology and immunohistochemical profiles. However, based on previous reports and considering that developmental restrictions were observed for HS181, it is possible that tissue composition and distribution in teratomas can be varying between different lines, and this might have effects on the experimental outcome (Allegrucci et al., 2007). To fully exclude hESC line specific effects, future parallel experiments in teratomas derived from several cell lines would therefore be preferred.

The observation of vasculogenic mimicry of IMR32, as well as the altered expression of markers linked to differentiation for all tested tumour cell lines, illustrates the plasticity of these tumour types. The fact that the resulting tumours are differing significantly in differentiation grade comparing the different model systems indicates

that a relevant non-tumorigenic surrounding is needed to achieve tumours with clinically relevant histopathology.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

The following conclusions can be drawn based on the findings of this thesis:

- The analysis of *in vivo* differentiation from hESC injected into experimental animals (teratoma formation) showed a differentiation resembling, but not identical to, human embryonic development. Kinetic studies revealed an optimal time point for an exogenous tumour inoculation into the developing hESC-teratoma at day 45; by that time point highly organized and advanced embryonic structures were observed together with an initial human vasculogenesis. Also, injections at this time point allowed a further observation period of the exogenously added tumour for 2-4 weeks while not interfering with the well-being of the host animal.

- Teratoma formation using two separate hESC lines, HS181 and H9, showed progressive development of mature teratoma, exhibiting no gross morphological differences between these two hESC lines. Analysis of mutant HS181 cells, carrying a spontaneous appearance of chromosome 12 trisomy, revealed similar development with no abrogation of mature teratoma formation, or observations of a selective *in vivo* growth advantage, of the mutant. However, a partly skewed tissue composition was observed with higher frequency of renal formation.

- When utilizing hESC induced teratomas as a surrogate human environment for cancer we could demonstrate additional emphasis to clinical relevance, i.e. compared to xenografts the human embryonic microenvironment provided tumours with additional resemblance to clinical observations.

- Importantly, the study illustrated a functional impact from the microenvironment for the occurrence of tumour phenotype variations, as noted in the reproducible development of heterogeneous tumour histology, or in the growth support of tumour phenotypes that could not be detected in xenografts.

Altogether, the results presented indicate that the tested novel *in vivo* model system can add significant knowledge to the field of human cancers. The model provides unique possibilities to study interactions between the tumour and its surrounding non-tumorigenic tissues in a species-specific environment. We showed that individual human tumour cell lines responded differently to microenvironments of human or murine origins. Considering the since long known clinical correlation between tumour heterogeneity and prognosis, maintenance of a heterogeneous histology upon grafting into preclinical model systems is of great importance.

For future studies the model could be used for analysis of also clinical tumour samples. As previously discussed this could give important information on clinical tumour cellular heterogeneity; an essential aspect for relevant preclinical *in vivo* cancer models.

The recent progress in generating induced pluripotent stem cells is opening doors for even further optimization including patient-specific teratoma-based *in vivo* model systems. This would give the opportunity to study unique patient-specific interactions, which might offer additional clinical relevance to the system.

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