

FROM THE DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH

Karolinska Institutet, Stockholm, Sweden

**A PRECLINICAL THERAPY MODEL FOR CHILDHOOD
NEUROECTODERMAL TUMOURS**

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**Karolinska
Institutet**

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"It's not what you look at that matters, it's what you see."
- Henry David Thoreau

To my mother

ABSTRACT

Childhood cancers show fundamental differences to most common adult solid tumours in their cancer-causing genetics, cell biology and their local tissue microenvironment. Effective treatments will be attainable when the molecular events that are specific to childhood tumorigenesis are better understood. However, it is in this context critical to consider both species and developmental aspects when looking for the relevant signalling. An influence from the microenvironment on clonal dominance is likely contributing to the disparity between primary and metastatic tumours seen in many patients, as well as inter-tumour heterogeneity between patients with the same tumour type.

In this thesis a novel concept is presented for preclinical studies of embryonic tumours in a recently described humanised model. Benign human experimental teratoma was generated in NOD SCID gamma (NSG) mice from diploid bona fide pluripotent stem cells (the PSCT model). An abundant presence of developing early neural components suggests this human embryonic model to be uniquely suited for *in vivo* transplantation studies of primitive neuroectodermal tumours originating early in life.

Following transplantation of tumour cell lines (**Paper I**), or patient biopsy material (**Paper II**) the initial microcolonisation showed a tumour type specific tissue tropism in that growth was instigated into specific embryonic tissue compartments, constituting the preferred cellular context/niche supporting initial colonisation and expansion. The phenomenon of microcolonisation reflects in this context an ability of tumour cell clones to comply or adapt to new environments, a feature which has great impact on metastasis and clinical prognosis.

Chemotherapy is widely accepted as part of first-line therapy for high-risk paediatric neuroblastoma. In a third study (**Paper III**) we have examined chemotherapy responsiveness/resistance of neuroblastoma in the PSCT model. Two well-characterized neuroblastoma cell lines were subjected to chemotherapy using the anthracycline drug doxorubicin and we report for the first time *in situ* studies performed in a human homologous embryonic *in vivo* microenvironment. Here we combined classical histopathology with high throughput single cell screening for preclinical drug evaluation. Using laser scanning cytometry for screening of chemotherapy-induced changes of nuclear DNA index (sub G1 fraction), in combination with immunohistochemistry and cytology for proliferation, apoptosis and cell death, we could demonstrate dose dependent and diversified responses to single-dose regimens of doxorubicin. Following a recurrent doxorubicin regimen (repeated administration with 48h interval), we observed the presence of an asynchronous response within individual tumour colonies, with cells exhibiting cytotoxic effects or enhanced proliferative index.

The results illustrate the feasibility of the approach and are encouraging for clinically relevant studies of patient material regarding intra tumour heterogeneity and asynchronous response to therapy. Furthermore, the findings demonstrate several important advantages using the PSCT model compared to employing conventional preclinical *in vivo* models and lead us to propose that the use of this human embryonic microenvironment is a well-needed complement for preclinical *in vivo* studies of primitive neuroectodermal tumours originating early in life.

LIST OF PUBLICATIONS

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Manuscript

Appendix. Related review (not included in thesis)

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LIST OF ABBREVIATIONS

ALK	Anaplastic Lymphoma Kinase
ANOVA	Analysis Of Variance
BSA	Bovine Serum Albumin
CS	Carnegie Stage
CNS	Central Nervous System
EB	Embryoid Body
ESC	Embryonic Stem Cells
ENCODE	Encyclopedia of DNA elements
FBS	Fetal Bovine Serum
FISH	Fluorescent In Situ Hybridization
GEM	Genetically Engineered Model
HE	Hematoxylin & Eosin
hESC	Human Embryonic Stem Cells
hFS	Human Foreskin (cells)
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IMDM	Iscove's Modified Dulbecco's Medium
INGRSS	International Neuroblastoma Risk Group Staging System
INPC	International Pathology Classification System
INSS	International Neuroblastoma Staging System
IVF	In vitro fertilization
iPSC	Induced Pluripotent Stem Cells
KO-DMEM	Knock-Out Dulbecco's Modified Eagle Medium
KO-SR	Knock-Out Serum Replacement
LOH	Loss of Heterozygosity
LSC	Laser scanner cytometry
mESC	Mouse Embryonic Stem Cells
NB	Neuroblastoma
NK	Natural Killer (cell)
NSG	NOD SCID Gamma
MOCK	Treatment with not active solution
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDX	Patient derived xenograft
PFA	Paraformaldehyde
pPNET	Peripheral Primitive Neuroectodermal Tumour
PSC	Pluripotent Stem Cell
PSCT	Pluripotent Stem Cell derived-Teratoma
RT-PCR	Reverse Transcriptase-Polymerase Chain Reaction
RTKs	Receptor Tyrosine Kinases
SCID	Severe Combined Immunodeficiency
SNS	Sympathetic Nervous System
STR	Short tandem repeats
sPNET	Supratentorial Primitive Neuroectodermal Tumour

SR	Serum Replacement
SSEA	Stage-Specific Embryonic Antigen
Treg	Regulatory T cell
WHO	World Health Organization

1 INTRODUCTION

1.1 Cancer

The word cancer originates from the Greek word *Καρκίνοϋ* (crab) and was initially used by the famous physician Hippocrates (460–370 B.C) the “father” of medicine. Cancer is today used to denote tumours derived from epithelial tissues.

The conceptual definition of a tumour is an abnormal growth of cells, dysregulated in the balance of cell proliferation and cell death. This is commonly due to multiple genomic or epigenomic changes resulting in populations of cells with acquired properties beyond dysregulated growth, including ability to invade, migrate and metastasise at distant sites. The tumour will need to evade the host control system and induced cell death. In 2000 Hanahan and Weinberg published a review describing how cancer can acquire these capabilities through various strategies; the 6 hallmarks of cancer; 1: Self-sufficiency in growth signals, 2: Insensitivity to growth signals, 3: Tissue invasion and metastasis, 4: Limitless replicative potential, 5: Sustained angiogenesis and 6: Evading apoptosis (Hanahan and Weinberg 2000). In 2011 these authors further developed the theory and added two emerging hallmarks; 7: Avoiding immune destruction and 8: Reprogramming energy metabolism (Hanahan and Weinberg 2011 - Figure 1). They also described two enabling cancer characteristics; 9: Tumour promoting inflammation and 10: Genome instability and mutation. The assumption is that “most cancer has acquired the same set of function capabilities during their development, albeit through various mechanistic strategies“.

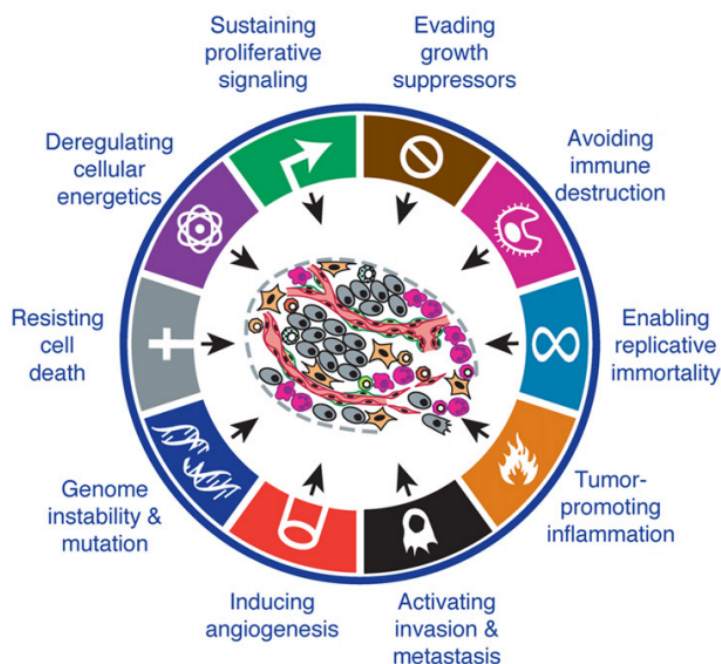


Figure 1. Hallmarks of cancer – Next generation.

Reproduced from Hanahan and Weinberg 2011, with permission from publisher.

Tumours are often categorised by site of origin, but can also be separated by histo-, or cytopathology and more recently by molecular characteristics. Tumours are furthermore divided into malignant and benign phenotypes. Cells in a malignant tumour can invade the surrounding tissue or spread as metastases while cells in a benign tumour do not.

The many aspects of cancer vary strongly between categories. Individual variations seen in cancers with similar origin strongly affect the outcome and the possibilities or difficulties for treatments. A personalized approach has been proposed both for diagnosis and treatments. The story of cancer is a complex case, one can speculate that perhaps the search for origin and treatment will continue as an unsolved dilemma only flavoured by further layers of complexity. Nonetheless, crucial breakthroughs have been made and the field of cancer therapy has flourished the last 50 years.

According to the WHO cancer report 2014 cancer is among the “leading cause of morbidity and mortality world wide with approximately 14 million cases and 8.2 million cancer related death in 2012”, these numbers are expected to rise over the next two decades with about 70% (<http://www.who.int/topics/cancer>; Accessed Feb 2015)

1.2 Childhood cancer

Although less common than in adults, only approximately 300 children are diagnosed with a malignant disease every year in Sweden, cancer is the primary cause of death in children 1-15 years of age in Sweden (Gustafsson et al. 2013). Apart from differences in age and incidence there are fundamental differences between childhood and adult cancer with regard to cell of origin, genetic aberrations, epigenetic features and the tumour environment (Scotting et al. 2005). Adult cancer is dominated by carcinomas, whereas >50% of all childhood cancers consist of haematological or CNS malignancies (Fig 2, from Gustafsson et al. 2013, see also Stiller 1994). The cancers of childhood frequently arise in immature tissue environments and the progenitor cell resulting in cancer may be at another developmental state indicating that childhood cancer may be considered a developmental disorder. Considering the time aspect, the causing events and triggering of cancer are supposedly different between childhood and adult cancer. The childhood cancer cell has been proposed to develop with fewer defects in its cell regulatory process compared to adults. (Scotting et al. 2005) and recently sequencing data have shown that pediatric malignancies carry significantly less mutations than adult malignancies (Alexandrov and Stratton 2014).

During the prenatal period of human postnatal development there are strongly regulated processes for tissue growth and differentiation, involving complex cellular processes to time the precision of mitosis and apoptosis, where and when. This suggests a close link between organogenesis and susceptibility to oncogenic transformation (Scotting et al. 2005).

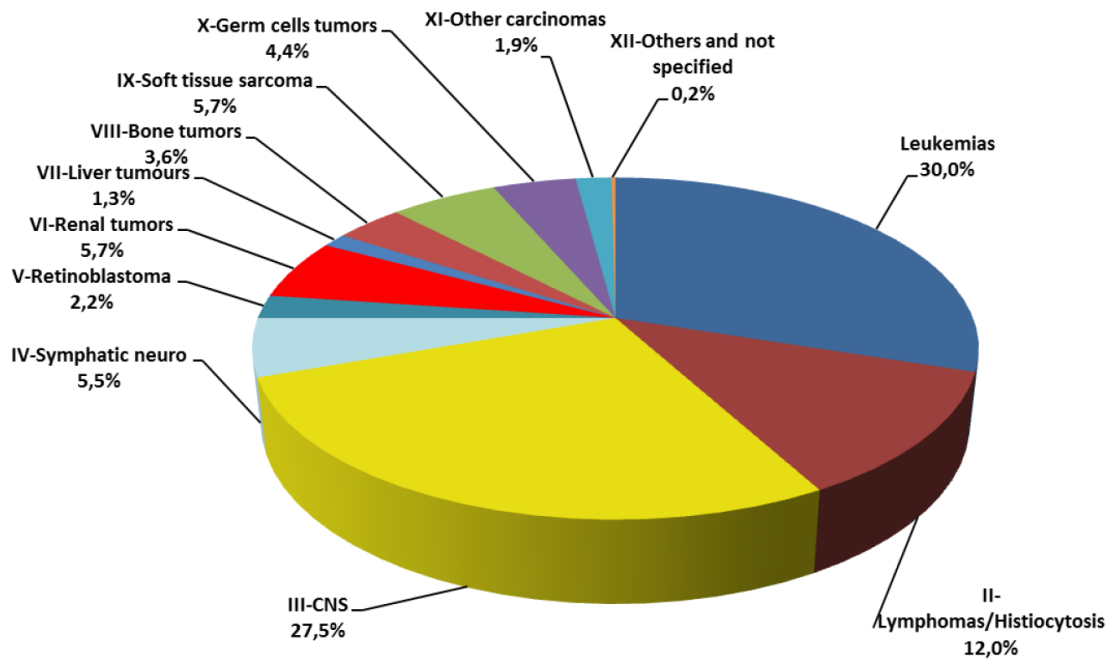


Figure 2. Distribution of childhood malignancies in Sweden diagnosed 1984-2010 <15 years of age at diagnosis (n=7065). Diagnoses are stratified according to the WHO ICC3 classification from 2005. Leukemias, lymphomas and CNS tumours constitute 70% of the children, and the other nine diagnoses account for 30%, with carcinomas only ~2%. Reproduced from Gustafsson et al. 2013, with permission from the publisher.

The genetically unique features for childhood cancer may be due to, or linked to their developmental phase and they may inherit similar immature properties, thus possibly providing a shorter route for tumour development. This is in contrast to the adult where progeny of mature tissues are set for differentiation and sensitivity for apoptosis. Thus the “multi hit” on DNA damage is key in adult cancer (Knudson et al. 1992). It is important to keep this in mind when treating cancer and developing cancer therapy for the child.

The differences in biology with childhood cancers being more sensitive to treatment, and children being less sensitive to side effects, in particular short term toxicity from chemotherapy, has allowed the development of better treatment and improved survival for children with cancer during the last decades (Figure 3).

Estimated
5 yrs Survival (%)

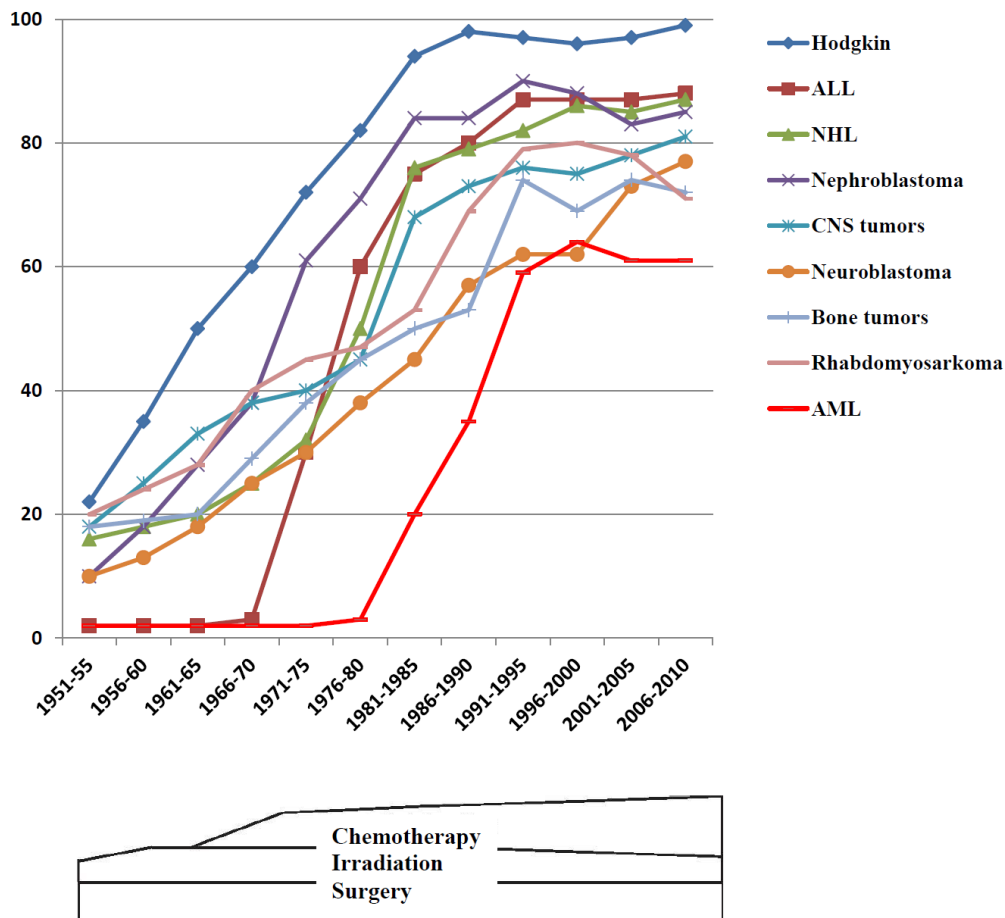


Figure 3. Childhood cancer prognosis in Sweden (5-year survival) over time for different, selected diagnostic groups. Prognosis improved considerably during 1970-1995. The results during the last decades seem to have reached a plateau, although survival for neuroblastoma and CNS-tumours have continued to improve. Reproduced from Gustafsson 2013, with permission from the publisher.

1.3 Pluripotency in normal and abnormal development

Pluripotent stem cells (PSC) are unique in their capacity for self-renewal and to sustain an undifferentiated state, but also in that they maintain a capacity to differentiate into any cell derived from the three germ layers of the embryo, endoderm, mesoderm and ectoderm. (Thomson et al.1998). Pluripotency in mouse stem cells has been divided into two separate states; naive and primed (Nichols et al. 2009). Interestingly, recent studies of the signalling pathways to induce pluripotency have revealed separate principles between mice and humans (Tesar et al. 2007; Schnerch et al. 2010) Such findings are intriguing considering the historical impact of mouse studies for our understanding of human early development. Furthermore, these findings are of vital importance also for cancer research since stem cell-like characteristics have been convincingly linked to drug resistance and metastasis in both mouse and human malignancies (Riggs et al 2013; Lee et al. 2013) This emphasizes the huge significance of complementing current *in vivo* mouse studies of human cancer with models adapted to homologous cell-cell interactions for studies on signalling pathways.

Following the initial report on pluripotency of cultured human embryonic stem cells (hESC), (Thomson et al. 1998), standardized protocols for quantitative *in vitro* assessment has been an important objective (Muller et al. 2011). For the full biological understanding of pluripotency complementary assessment *in vivo* is also necessary, e.g. for safety in regenerative medicine, or to quality assure new PSC lines. For this analysis the formation of experimental teratoma following xeno-transplantation into immunosuppressed animals is so far the only feasible option, often considered as a “golden standard” (Brivanlou et al. 2003; Gertow et al. 2007; Adewumi et al 2007). In 2004 our research group published the first more detailed analysis of experimental teratoma (Gertow et al. 2004), later followed by a kinetic analysis showing the embryonic process of experimental teratoma from the human embryonic stem cell line HS181 (Gertow et al. 2011).

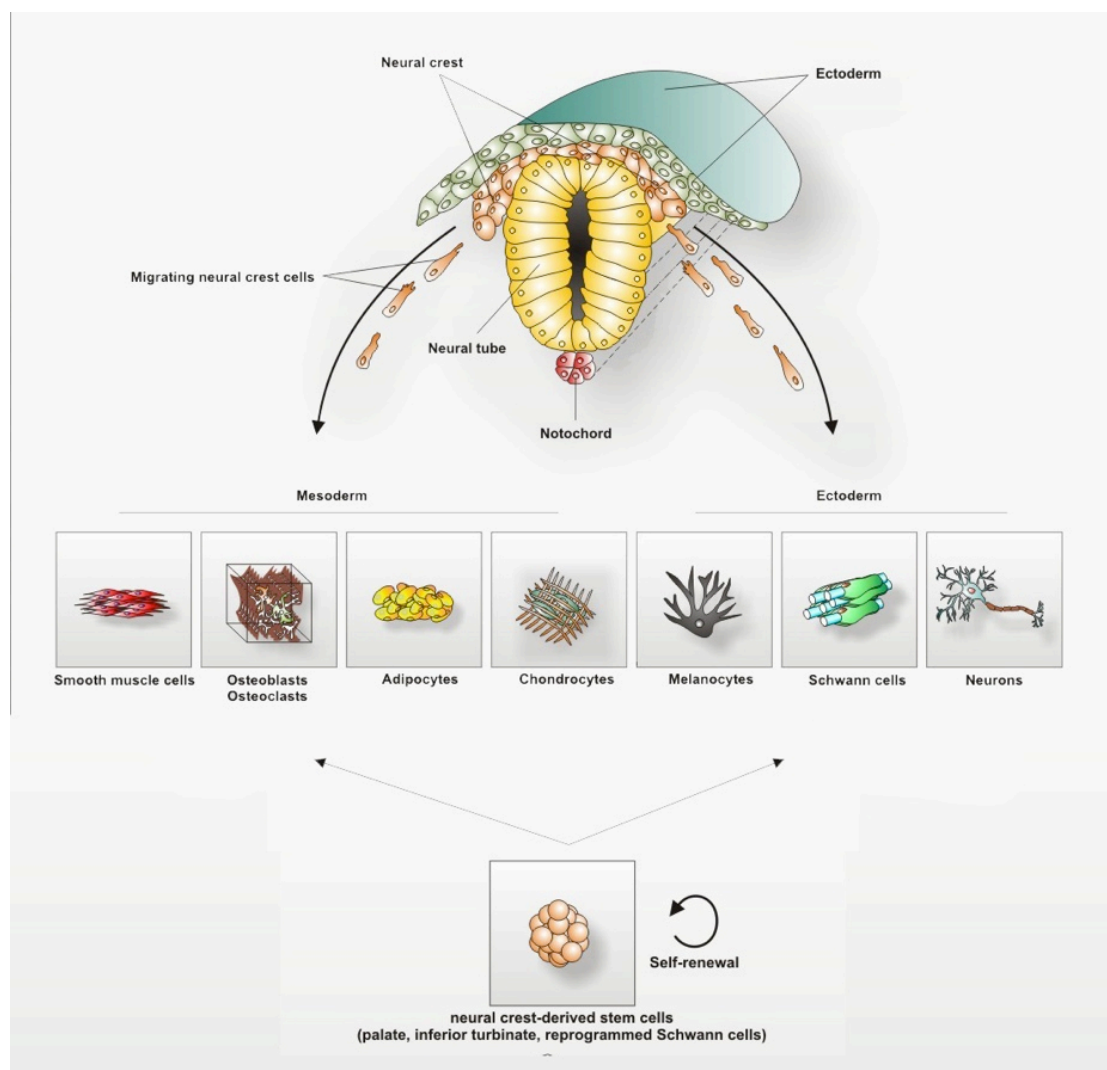


Figure 4. Neural crest cells and derivates in mesoderm/ectoderm. During early development after the process of neurulation the neural crest cells gain motile property and migrate out and differentiate into a variety of cell types, including neurons and progenitors of sympathetic nervous system. Here a subpopulation may gain potential to drive NB formation - or other tumours of neuroectodermal origin. Reproduced with the permission of D. Widera.
<http://web.biologie.unibielefeld.de/cellbiology/index.php/research/neural-crest-derived-stem-cells>

1.4 Neuroectodermal tumours

Embryonic tumours occurring in the central or peripheral nervous system, here collectively denoted embryonic neuroectodermal tumours, are known to be heterogenic between individuals and for intra-tumour subpopulations of cells with biological features responsible for aggressive metastatic behaviour and resistance to therapy (Brodeur 2003; Gilbertson et al 2004).

The PSCT embryonic microenvironment with an abundant presence of developing neural components, overlapping with gestation stages immediately preceding the positioning of adrenal sympathetic progenitors in embryonic mesenchyme, suggests the PSCT model to be uniquely suited for *in vivo* studies of primitive neuroectodermal tumours originating early in life (Gertow et al 2011; Hultman et al 2014).

The group of embryonic neuroectodermal tumours includes among other tumours neuroblastoma (NB) (studied in Papers I and II) and the pPNET and sPNET (peripheral and supratentorial primitive neuroectodermal tumours – included in Paper II). In this thesis there is focus on NB, mainly for practical reasons, with the availability of adequate cell lines (Paper I and III) and due to access of patient tumour material (Paper II).

1.4.1 Neuroblastoma

Neuroblastoma (NB) was first described in 1864 by Rudolf Virchow as an extra cranial solid embryonal tumour called “glioma”, first in 1910 it was called neuroblastoma when the similarities of tumour cells with embryonic adrenal medulla and migrating sympathetic neuroblasts were described (Wright 1910). Subsequently both biological and clinical features have been investigated and described in detail (as reviewed by Brodeur 2003 and Maris 2010). NB is the most common malignancy in the neonatal period when genetic predisposition is most significant (Orbach et al. 2013) and accounts for >20% of neonatal cancers and in total 5.5% of childhood cancers and 9.2% of childhood cancer deaths (Johnsen et al. 2009).

Half of the NB cases occur in children under 18 months of age, in support of the notion that this is a tumour of embryonic origin. NB is considered to stem from precursors of the sympathetic nervous system of the ganglionic lineage, with tumours arising in sympathetic ganglia or adrenal gland (Grimmer and Weiss 2006).

NBs are highly heterogenic between individuals with regard to genetics and histology, with a unique ability to spontaneously regress or differentiate (Bénard J et al. 2008; Brodeur 2003; Brodeur and Bagatell 2014). Spontaneous regression is however mainly observed in patients under one year of age (Schwab et al. 2003), interestingly mimicking the developmental process where prenatal neurons undergo programmed cell death at the prenatal stage. This indicates that the NB genetic profile is linear specific and developmentally regulated with links between NB and embryonic neuronal development at molecular level (Grimmer and Weiss 2006).

High-risk NB may on the other hand adapt to the microenvironment leading to aggressive metastatic tumours (bone marrow and bone are most common) (Maris 2010, Brodeur 2003). The majority of primary NB arises in the abdomen, along the sympathetic ganglia and in the medulla of adrenal glands (Maris et al. 2007) but

tumours have also been observed in the neck, chest and abdominal or pelvic regions. The figure below illustrates the metastatic preferences of NB (from Maris 2010).

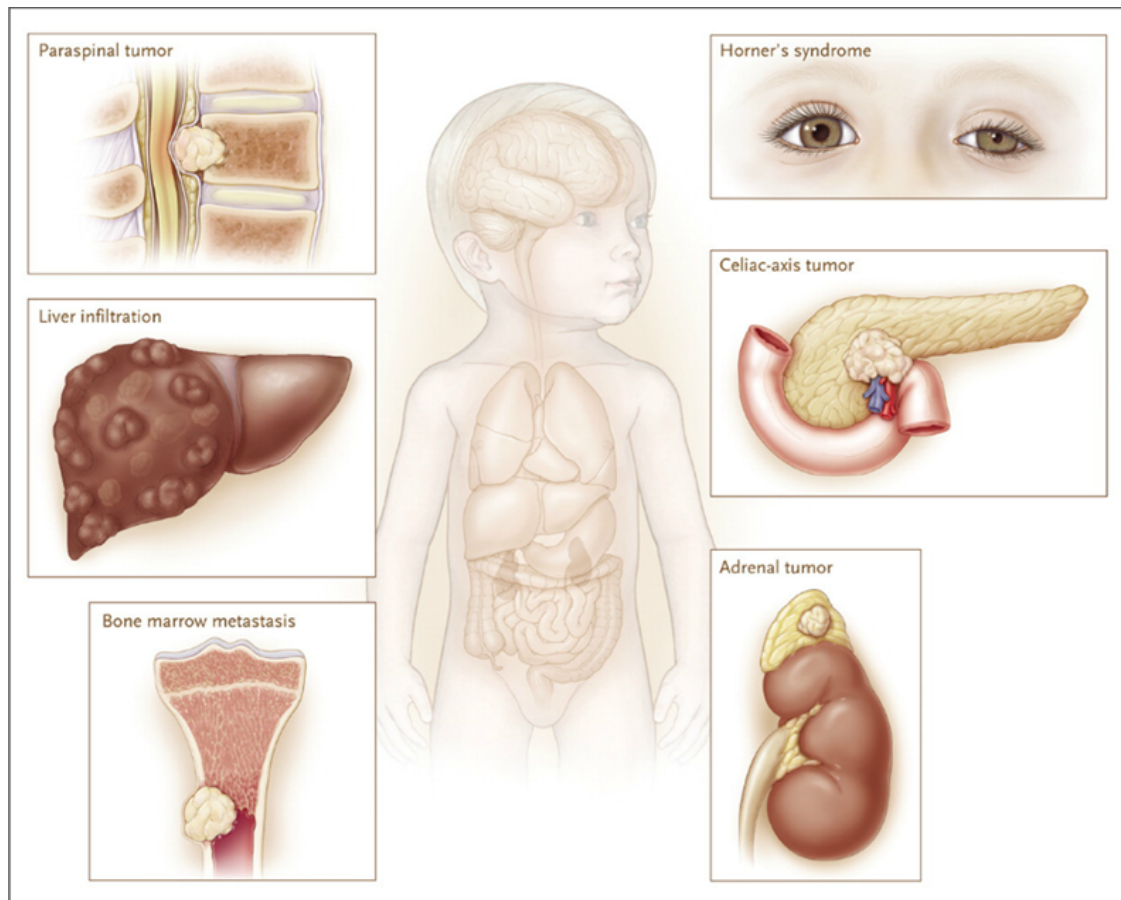


Figure 5. Illustration of neuroblastoma and potential locations of tumour lesions. From Maris 2010. Reproduced with permission from Massachusetts Medical Society.

1.4.2 Neuroblastoma staging and classification

The International Neuroblastoma Staging system (INSS) was presented in 1988 to unify intentional criteria in confirmation of diagnosis and response to treatment, defined as the International NB response criteria (INRC) (Brodeur 1993).

According to the INSS system NB is categorized into stages 1, 2, 3, 4 and 4S tumours (Brodeur et al.1993). Ranging from the less aggressive NB stage 1 tumours which are localized without metastasis and can be surgically removed to the highly aggressive stage 4 tumours commonly found in older children that exhibit metastatic spread and often show treatment resistance to chemotherapy. Stage 4S NB have rather unique tumour features that are manifested in infants under one year of age having a localized tumour with e.g. metastasis in skin, liver or bone marrow, but with a possibility to spontaneously regress completely or respond very well after minimal therapy. (Brodeur, 2003; Maris 2010, 2007).

Since the INSS system is based on post surgical evaluations there is a risk for over staging patients towards high-risk staging, thus there is a dependency on surgical assessment and skill. In 2009, this drawback was addressed, by the international NB risk group INRG who presented an alternative system based on Image defined risk factors (IDRF). This system categorizes patients in different stages depending on presence of metastatic spread (M) or the tumour being localised without (L1) or with IDRF (L2), also with a special metastatic stage MS – previously called 4S limited to children below 18 months of age at diagnosis having a favourable pattern of spread (excluding bone, lung and CNS) (Monclair et al. 2009). Together with other prognostic factors this renders a pre-treatment risk stratification system. To establish complete classification several additional parameters are needed including patient age, tumour genetics and tumour histology, separating NB patients into very low, low, intermediate and high-risk groups (Cohn et al. 2009). This will guide the treatment based on the evaluation if the cancer, if it is of favourable or an unfavourable nature and the child at risk of treatment response or treatment resistance and relapse.

1.4.3 Neuroblastoma genetic profiling

Neuroblastoma clinical heterogeneity is due to biologic features, mainly a wide heterogeneity of tumour genetics. However, there are still many uncertainties regarding the genetic basis of NB. Embryonic neural tumours have so far not been shown to entail any single dominant or shared essential genetic aberration within the tumour group (reviewed by Johnsen et al 2009 and references therein). However, for children with high-risk NB and poor prognosis several patient specific mutations and gene amplifications have been identified, such as amplification or overexpression of MYCN (Brodeur 2003), deletions of parts of chromosome arms 11q (Caren et al. 2010), or mutations in the anaplastic lymphoma kinase (ALK) gene (Mosse et al. 2008; Maris 2010).

MYCN is a transcription factor and a member of the MYC family of proto-oncogenes that regulates expression of several target genes (reviewed by Adhikary and Eilers 2005). This regulation is involved in monitoring of diverse fundamental cellular processes, e.g. dedifferentiation, cell growth, proliferation, protein synthesis, metabolism and apoptosis. This provides a dual nature, being able to drive both cellular proliferation as well as sensitizing cells to programmed cell death (Westermarck et al. 2011; Albihn et al. 2010). Transgenic expression of MYCN has been shown to result in neuroblastoma (Weiss et al. 1997). MYCN is developmentally restricted during the early stages of ontogeny and is important for proliferation and migration of the neuroblast of neural crest (Lu et al. 2003). In tumours with amplified MYCN, the amplification may be 3 to 300-fold. This amplification frequently leads to an overload of transcribed MYCN and increased cellular proliferation and decreased apoptosis. This leads to a poor differentiation of the cancer cells, as well as triggered vascularity driving the tumour progression. The amplification may be identified as both homologous and heterologous spread in the patient and may vary from primary site to metastatic areas (Schwab 1993; Brodeur et al 1988). MYCN amplification is found at all stages of NB, but dominates in the M stage and is strongly associated with bad prognosis of high-risk NB patients (Brodeur 2003).

The transmembrane receptor tyrosine kinase (ALK) is expressed in the central and peripheral nervous system (Chiarle et al. 2008). ALK mutations are today known to be a cause for hereditary NB, identified in NB pedigrees (Janoueix-Lerosey et al. 2008; Mosse et al. 2008) and also found to be mutated in sporadic NB up to 10% (Fisher and Tweddle 2012). Interestingly, besides ALK-mutations present in most cases of familial NB also PHOX2B mutations have been found in families with neuroblastoma and linked to the presence of "neurocristopathies" such as congenital central hypoventilation syndrome (CCHS) and Hirschsprung's disease caused by neural crest cell migrative failure (Mosse et al. 2004; Trochet et al. 2004).

The loss of sequences on chromosome 11q is a predictive factor for the outcome of MYCN non amplified L stage NB tumours (Brodeur 2003; Maris et al 2007) and also defining a group of high-risk tumours with genetic instability distinct from the MYCN-amplified tumours (Caren et al. 2010).

In summary, most NB tumours show mutations or segmental genetic aberrations usually linked to poor outcome including MYCN-amplification, ALK-mutations and 11q-deletions as well as other common aberrations including 17q gain or 1p deletions and a large variety of other oncogenic changes (Maris 2010; Caren et al 2010). NB with favorable prognosis usually show only numerical chromosomal aberrations (Maris 2010; Caren et al. 2010; Brodeur and Bagatell 2014).

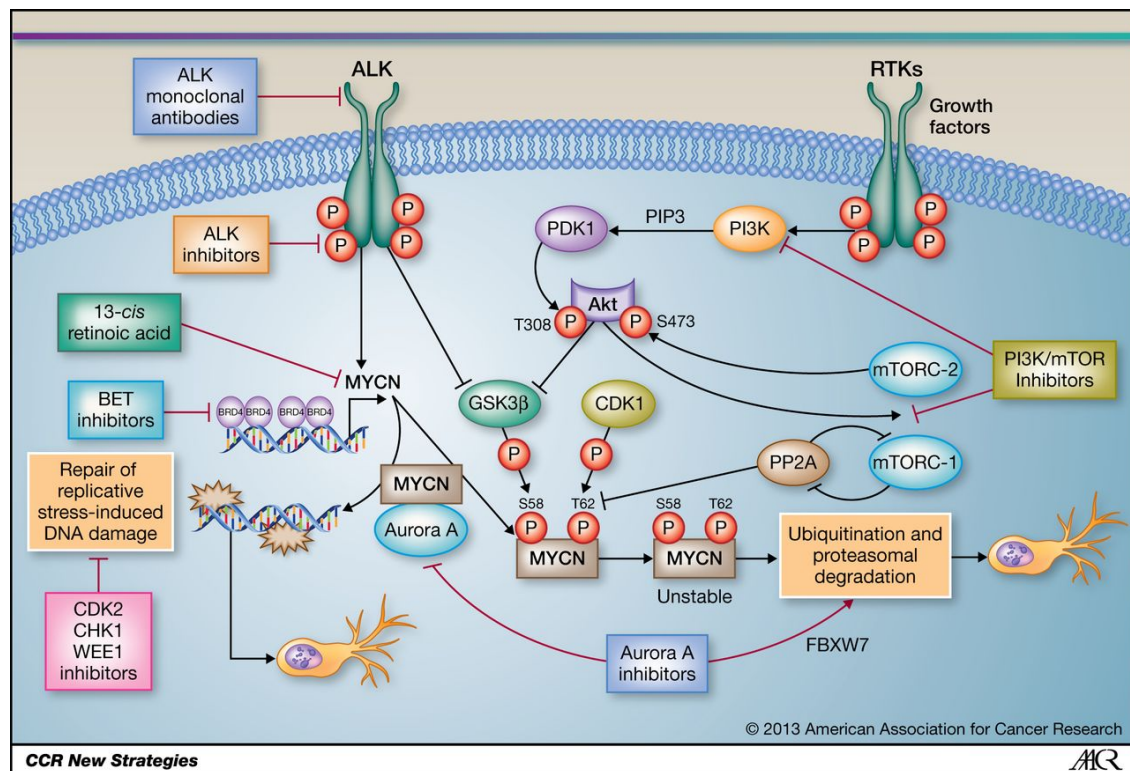


Figure 6. Schematic overview of MYCN interplay and therapy target strategies for MYCN and ALK, relevant in neuroblastoma. Reproduced from Barone et al 2013 with permission from the publisher.

1.5 Preclinical tumour models

The choice of model depends primarily on the scientific question, available resources and time.

The most widely used model of human cancer - *in vitro* studies (from the Latin word “in glass”), is a convenient and manageable approach but limited by the rudimentary mimicry of the full context in the host. The addition of 3D-cultures is an improvement (reviewed by Tanner and Gottesman 2015). *In vitro* models are still widely used and a relevant base for tumour research, but with obvious limitations e.g. due to the lack of context from the host influence as the metabolic system, vascularization and microenvironment, etc.

In silico studies (computer based research and modelling) is today gaining ground in step with the technological advancement, nonetheless *in vivo* studies are essential.

Animal models have been widely used in biological and medical research. In the mid 1950' it was documented that tests *in vivo* were more predictive for clinical response than *in vitro* tests (Suggitt and Bibby 2005). Animal research (dominated by studies in rodents), have lead to possibilities to assess advantages and side effects of drugs *in vivo* before applying them to human patients. The clinical track record from this type of analysis, i.e. attrition rate in clinical trials in oncology, is however disappointing (Hutchinson and Kirk 2011; Norris and Adamson 2012).

Recent findings have revealed differences between human and mouse species for the signalling pathways controlling the induction of cellular potency (Nichols and Smith 2009; Brons et al. 2007; Tesar et al. 2007). Since the processes of cancer progression have been linked to induction of cellular potency (Nguyen et al. 2012; Easwaran et al 2014), the use of mice for studies of human cancer could be challenged, and emphasize the importance of using humanized models.

1.5.1 Syngeneic models

Syngeneic mouse tumour models were first introduced in the 1930th with the use of, at the time, newly introduced inbred mouse strains. In syngeneic tumour models the host and the tumour are histocompatible and the host immune system is not necessarily compromised, although many models rely on reducing the innate immune system.

1.5.2 Xenograft models

The definition of xenografts originates from the Greek word ξένος (“foreign”) and relates to e.g. transplantation of a human tumour to an animal, i.e. over the species barrier. In contrast to the syngeneic models, a xeno-transplantation relies on compromising the immune system to avoid rejection in the host animal. This model system is well established and the access to severely immunodeficient animals permits reducing interference with the immune system allowing for transplantation resistance/acceptance.

Heterotopic xenografts

This indicates that the tumour graft is placed in an abnormal location, for example sub cutaneous injection of neuroblastoma (s.c).

Orthotopic xenografts

This defines engraftment at the tumour type specific site of origin. Except for species differences, this allows for an organ specific local environmental influence. This approach is generally regarded to provide a more relevant environment compared to heterotopic transplantation (Kanna et al. 2002). The first orthotopic NB model was described in 1994 (Flickinger et al. 1994).

Patient derived Xenograft models

Patient-derived tumour xenograft (PDX) models are generated by xenotransplantation of fresh human tumour material and are considered clinically predictive (Das Thakur et al. 2014). An alternative to conventional xenograft models using cell lines, or PDX models using fresh tumour, is to use low passage numbers of patient-derived tumour tissue (for example removed from the patient and passaged as xenograft or *in vitro* as cell cultures, for a very short period of time). The low passage is believed to conserve original tumour characteristics.

1.5.3 Genetically engineered models (GEM's)

Rudolf Jaenisch demonstrated the first genetic engineering of mice in 1974 by inserting viral DNA into an early stage mouse embryo (Jaenisch and Mintz 1974). Later, the classical transgenic mouse model was constructed by pronuclear injection of cDNA constructs containing promoter elements this was followed by research and collaborations from a large number of research groups (Gordon and Ruddle 1981; Costantini et al. 1981; Brinster et al. 1981) and in transgenic mouse models of today, the genetic modification may be an insertion of a new gene sequence or deletion/alteration stimulating the induction and growth of the cancers of interest, ideally confined to the relevant tissue environment (Teitz et al. 2011). The first report of a GEM model for NB came in 1990, showing pre-neoplastic lesions in the adrenal medulla and NB like tumours from a transgene insertion of polyoma virus T antigen under the control of the thymidine kinase promoter (Aguzzi et al. 1990). As mentioned above, MYCN amplification is associated with high-risk NB and poor prognosis (Brodeur et al. 1988). In 1997 a transgenic mouse model was generated with overexpression of MYCN from the tyrosine hydroxylase promoter providing a neuronal preference specifically expressed in neural crest and peripheral nerves of parasympathetic ganglia and adrenal medulla (Weiss et al. 1997). Although these models will develop NB with high resemblance to the human counterpart regarding morphology and site of origin, they do not fully mimic the human clinical picture due to species differences in the genome and crucial divergences of regulatory elements, metabolic processes that vary between humans and mice, e.g. described in the "ENCODE" project (encyclopedia of DNA elements -The mouse ENCODE consortium; 2014). Thus there is a need for new models also specifically for NB.

1.5.4 Humanised models for childhood cancer

To be straightforward – the best place to study *murine* tumours and interactions with murine stroma is in a mouse, and the best model for studies on *human* tumours and interaction with human stroma is hypothetically in humans. The latter presents of course a challenge for experimental applications, and new models are needed, since in the end it is human patients we strive to treat, not mice.

The design of GEM models may include the transgenic expression of selected entire human genes, in order to humanize metabolic or glycosylation enzymes, telomere structure, or the immune system (Frese and Tuveson 2007).

Humanised mice with regard to the hematopoietic system, for reconstitution of a human immune response to the tumour, have been obtained by injection of human peripheral blood, or human CD34⁺ hematopoietic stem cells from human umbilical cord blood (Shcultz et al. 2007, 2012). The methodology for hematologic reconstitution in humanized mice is still under perfection and reconstituted immune cells are quantitatively/qualitatively weak and also fail to be maintained over long-term, but improvements are reported (Bernard et al. 2008; Shultz et al. 2012).

1.5.5 The pluripotent stem cell teratoma (PSCT) model

This thesis explores the use of a human embryonic *in vivo* model based on experimental benign teratoma, here applied to studies of primitive neuroectodermal tumours originating early in life. Experimental teratoma has emerged as an alternative approach for *in vivo* modelling of intercellular interactions between developing tissues and cancer cells (Tzukerman et al. 2003, 2006; Cedervall et al. 2009; Katz et al. 2009; Burgos and Ojeda 2013; Abelson et al. 2013; Jamil et al. 2013, 2014). The pluripotent stem cell teratoma (PSCT) model belongs to a new generation of models, complementing conventional xenografts, in that it provides a human embryonic microenvironment (Tzukerman et al 2003, reviewed by Hultman et al. 2014). Similar to PDX models the PSCT platform requires immunodeficient hosts, but with the vital difference of supplying interaction with human microenvironment.

Protocols for the generation of human experimental teratoma by the injection of pluripotent stem cells have been described in detail for various heterotopic sites; e.g. intramuscularly, subcutaneously, under the kidney or testis capsule (Gertow et al. 2007). In this thesis, the route of intra testicular injection was used. This choice was selected due to several reasons – testis is immune-privileged, not vital for sustaining health of the mice, encapsulated to keep the graft in place and it is easily accessible and thus convenient for palpation. For ethical reasons, teratoma development was limited to a total size of 1,5 cm in order not to negatively affect the mouse host.

1.5.5.1 Embryonic and oncofetal nature of experimental teratoma

Mature benign experimental teratomas, derived from pluripotent stem cells with normal karyotype (the PSCT model), represent a benign growth that can be described as a chaotic embryonic process, lacking structural axis (Gertow et al. 2004, 2011; Lensch et al. 2007). Under standardized conditions such experimental growths consist of a reproducible microenvironment with a rich blend of embryonic tissues including early organoid development, as well as immature neural components (FIGURE 5 - Gertow et al. 2004, 2011). These benign immature components exhibit strong morphological resemblance with tumours of embryonic neuroectodermal origin (Cedervall et al. 2011), and when similar histopathology is present in patient samples they are considered potentially malignant. Nevertheless, in the embryonic model such findings are part of early development and the shared histology is the basis to our study of neuroectodermal tumours implanted into matching PSCT microenvironments.

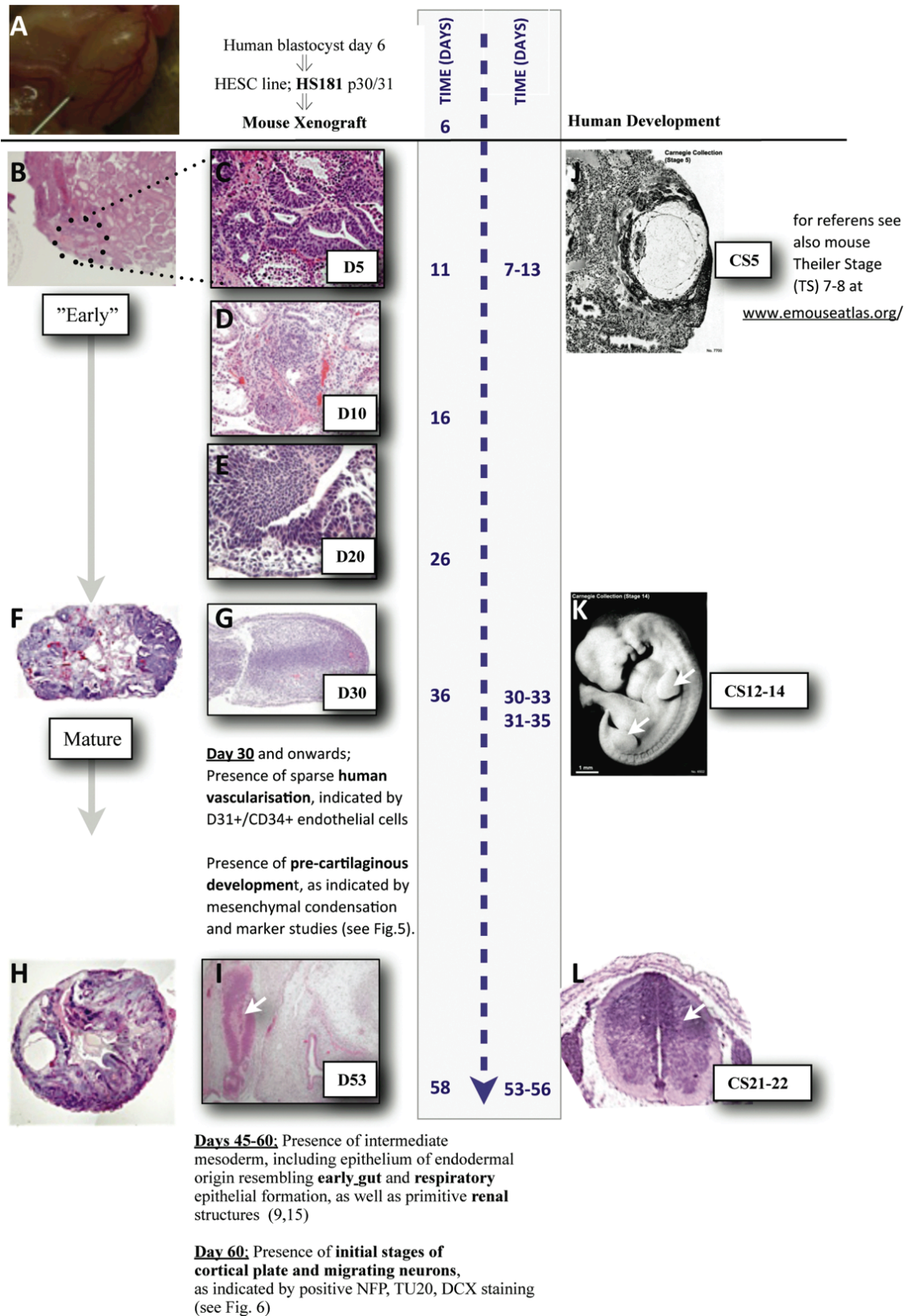


Figure 7. Experimental teratoma time alignment with human development. Reproduced from Gertow et al. 2011 with permission from the publisher.

1.5.5.2 Propagation of human tumours in the PSCT model

Tzukerman and collaborators were first to report the use of PSCT as a transplantation model for human tumours (Tzukerman et al. 2003). In a series of studies they studied the growth from a panel of different cancer cell lines originating from; glioma, prostate, breast, lung and colorectal cancer, but more importantly also from primary ovarian cancer, as well as short term cell lines from these types of cancer. Comparing transplantation into teratomas with conventional xenografts, they reported an improved and increased survival rate of the human tumours in the human model system. They also found differences in growth, invasion, and angiogenic responses. This research group also used the model to test effects of immunotherapy with a recombinant immunotoxin antibody against A431 directed against epidermoid carcinoma cell-related epitopes (Tzukerman et al. 2006). Intriguingly, while this therapy was effective in a conventional immunosuppressed murine xeno-model, the same immunotherapy failed to eradicate the tumour in a hESC growth environment (Tzukerman et al. 2006). This indicated differences between xenografts and the PSCT model in tumour behaviour and therapy response.

Cedervall et al studied a human cutaneous malignant melanoma cell line in the PSCT model (Cedervall et al. 2009). While the resulting histology was generally analogous compared to parallel xenografts, an additional de-differentiated melanoma phenotype was observed in PSCT model, not detected in xenografts This finding together with observations of abundant early neural development in experimental teratoma raised to us the prospect that the microenvironment in PSCT could be better matched for tumours originating early in life, and in particular tumours of neural origin. This hypothesis was further explored in Paper I with a study of neuroblastoma cell lines transplanted to the PSCT model.

1.6 The Tumour microenvironment

The development of malignant solid tumours requires a microenvironment that supports the uncontrolled proliferation and spread of cancer cells and also conditions that avoid destruction from the various arms of the immune system must be present. The tumour microenvironment (TME) consists, in addition to tumour cells, of non-cancerous cells that include endothelial cells and pericytes to support the growth of blood vessels, fibroblasts, lymphoid cells and cells of the myeloid lineage as well as signalling molecules and an extracellular matrix that support tumour cell growth (Quail and Joyce 2013; Hanahan and Coussens 2012; Hanahan and Weinberg 2011).

During tissue injury residential fibroblast differentiate into myofibroblasts. These cells also called cancer associated fibroblasts (CAFs) are abundantly presented in the TME (Sugimoto et al. 2006). CAFs secrete various growth factors important for cancer cell proliferation including TGF- β that may induce epithelial-mesenchymal transition (EMT) of tumour cells and contribute to the immunosuppressive conditions seen within TMEs (Erez et al. 2010). CAFs also produce chemokines, including CXCL12 that stimulate malignant cell growth and function as a chemoattractant to stimulate migration of other stromal cell types and their progenitors into the TME (Orimo et al.

2005). CAFs have fundamental functions for the growth of solid tumours since depletion of these cells in mouse tumour models induce tumour necrosis mediated by TNF- α and INF- γ (Kraman et al. 2010).

Tumour cells as well as other cells presented in the TME produce factors like VEGF, PDGF, FGF, cytokines and chemokines that stimulates neovascularization by inducing endothelial cells and pericytes to produce new blood vessels a process called angiogenesis (Folkman 1971). An angiogenic switch in which the TME becomes dependent on nutrition and O₂ from the formation of new blood vessels is an absolute requirement for continuous tumour expansion. The vessels sprout produced in this process shows chaotic branching and leakiness resulting in increased tumour interstitial fluid pressure. Increased fluid pressure triggers unstable blood flow, oxygenation and distribution of nutrition and waste compounds in the TME. This, in turn, increases hypoxia and facilitates metastasis (Eltzschig and Carmeliet 2011).

Reduced O₂ levels seen during hypoxia induce a dramatic change in gene expression mediated by hypoxia-inducible factors (HIFs), which are composed of an O₂-regulated HIF- α subunit (HIF-1 α , HIF-2 α , or HIF-3 α) and a constitutively expressed HIF-1 β subunit (Semenza 2012). During hypoxia the proteosomal degradation of HIF α subunits, mediated by O₂-dependent prolyl hydroxylation, are inhibited leading to stabilization and rapid accumulation of HIF proteins (Kaelin and Ratcliffe 2008). Activation of HIF proteins results in altered transcription and expression of more than 1500 genes that can induce angiogenesis, tumour cell aggressiveness, invasion and metastasis (Semeza 2012).

1.7 Interactions with the immune system

The immune system represents an important tool for the destruction of cancer cells and precancerous conditions in the human body. Malignant growing tumours have in most cases developed immune evasion strategies to avoid destruction by immune cells. As a result, immune cells infiltrating into the tumour sites are generally disabled to eliminate tumour cells. This ability to evade destruction by anti-tumour specific T cells has been accepted as an emerging hallmark of cancer (Hanahan & Weinberg 2011). Apart from suppression of cytotoxic immune cells, tumour-derived factors can induce the accumulation of inhibitory and regulatory immune cells including regulatory T cells (Treg) and suppressive cells derived from the myeloid lineage. These myeloid-derived suppressor cells (MDSC) comprise a heterogeneous group of immature cells, i.e. progenitors of macrophages, dendritic cells and granulocytes that are known to suppress T and NK cell functions (Gabrilovich and Nagaraj 2009). One essential immune evasion strategy that can be induced or applied by tumour cells is the formation of an inflammatory microenvironment. Tumour cells can induce inflammation directly through oncogenes that induce transcriptional programs responsible for the production of pro-inflammatory eicosanoids, cytokines and chemokines that attract different cells of the immune system to the microenvironment (Johnsen et al. 2009). Also chronic inflammation caused by viral or microbial infections, autoimmune diseases, dietary products or inflammatory conditions caused by unknown reasons can create an inflammatory microenvironment that supports tumour growth (Mantovani et al. 2008).

It should be stressed that mouse immune xeno-recognition of human tumour grafts is fundamentally different from the patient immune recognition of the autologous tumour, and provides little information for the patient situation.

The PSCT model is designed to avoid interference from the immune system using severely immunodeficient strains for the experiments. This allows for studies separate from most influence of the host immune xenoreactive immune system. To include interactions with effector arms of the human immune system in the PSCT analysis, a future option could be to reconstitute NSG mice with a human cellular immune system or specific immune components (Shultz et al. 2005). The methodology for hematologic reconstitution in humanized mice is however still under perfection and reconstituted immune cells are quantitatively/qualitatively weak failing to be maintained over long-term, but improvements are reported

1.8 Metastasis

Metastasis, defined as the spread of cells from the primary neoplasm to distant organs, is known to be the main cause of death from cancer. Cancer, primary tumour and secondary lesions, consists of a heterogeneous population with a variety of genetic and epigenetic properties, some of these have the potential to gain a more aggressive profile and ability to metastasize (Chaffer et al. 2007). They may disseminate through EMT-driven motility and angiogenesis mediated transportation to colonize a distant site. Thus the metastatic property is determined by capacity of individuals in a heterogeneous tumour population. (Fidler 2003; Heppner 1984) In xenografts models only as few as 0.01% obtain this capacity and develops into metastasis. (Luzzi et al. 1998; Chambers et al. 2002)

It was in 1929 proposed that metastatic dissemination occurs purely by mechanical factors as a result of the vascular system's anatomical structure and that the vasculature system is key to metastatic transport and spread (Ewing 1928). A later publication by Folkman proposed that all tumours are angiogenesis dependent, this has strongly influenced the cancer field (Folkman 1971).

The "seed and soil" concept (presented by Paget 1889) is a plausible explanation to tumour metastatic site selectivity, tropism. It has been defined that the tumour cells potential to metastasize depends on its interactions with the homeostatic factors that can promote survival, growth progression, the metastatic invasion and local angiogenesis. (Quail and Joyce 2013). The theory of "seed and soil" proposes that metastasis depends on cross-talk between selected cancer cells (the 'seeds') and specific organ microenvironments (the 'soil') the conclusion is that metastases will only form when the seed and soil are compatible. Components of the microenvironment appear to play a significant role in the metastatic process in several phases, ex, by altering MET/EMT at the pre-metastatic phase (Gao et al. 2012; Chao et al. 2012) or by preparing a pre-metastatic niche. To render the target metastatic-niche receptive to tumour colonisation several components of the microenvironment are involved, increased numbers of fibroblasts (and potentially CAF), growth abundance of BM-derived cell types and secreted cytokines. According to this concept (Kaplan et al. 2005) published

that BM-derived VEGFR1 positive cells pre colonize the pre-metastatic-niche prior to tumour cell arriving at the site, this indicates a possible interaction and communication between the primary and secondary sites and their microenvironment actors.

Thus it is crucial to achieve better understanding on systemic, cellular and molecular level. For clinical relevance it is in this context critical to consider both species and developmental aspects when looking for the relevant signalling.

1.9 Cancer stem cells

A small subpopulation of cells in tumours, so called cancer stem cells (CSC), have been described to drive malignant progression and repopulate the tumour after therapy. The concept of CSC is in many aspects overlapping with the definition of Tumour-Initiating Cells (TICs), i.e. cell subtype with potential to initiate xenograft growth in animals (Easwaran et al 20014). It has been suggested that CSC/TICs are responsible for metastatic processes and therapy resistance/recurrence (Nyguyen et al. 2012, Beck and Blanpain 2013).

The origin of the CSC/TIC is debated but has been suggested to arise in two alternative ways. Either from a normal stem cell with accumulated mutations becoming “cancerogenic” and keeping the stem cell properties. The other suggested path is that an adult differentiated cell accumulate genetic changes until it becomes tumourigenic and then furthermore acquires stem cell characteristics for immortalisation.

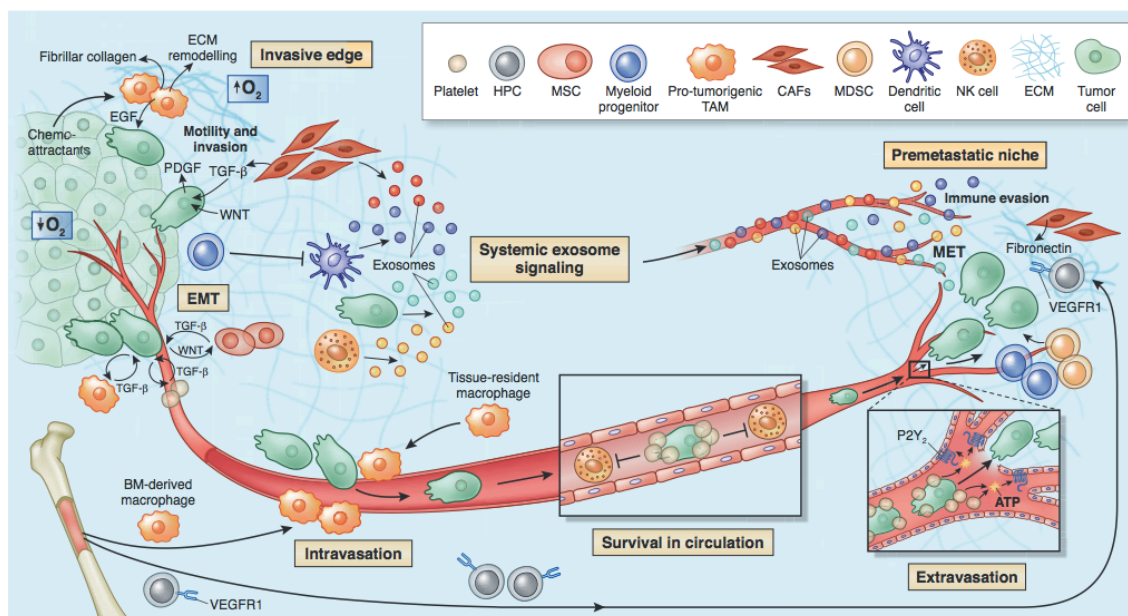


Figure 8. Illustration of the interplay between the cancer cells and the tumour microenvironment components at metastasis. Reproduced from Quail and Joyce 2013 with permission from the publisher.

1.10 Treating cancer

Today, and from the earliest historical documentations, surgery to remove tumours is a key component in cancer therapy. Access to appropriate anaesthetic agents is a prerequisite for more complex surgery to invasively remove tumours (Gilman 1963). The German physicist Wilhelm Röntgen made the discovery of the x-ray in 1895 leading way to the idea to combine surgery with radiation. The era of cancer chemotherapy began in the 1940s with Studies by Louis Goodman and Alfred Gilman (Goodman et al. 1946; Gilman 1963) The fascinating history of cancer therapy development has been comprehensively described in “The Emperor of All Maladies; A Biography of Cancer” (Mukherjee 2011). New approaches and candidate drugs are rapidly emerging. Predicting the individual response to therapy is a challenge in the clinic. Reliable assays for this prediction is however sparse. There are several reasons for this; The heterogeneity of tumours and a lack of predictive markers for therapy response. This may be reflected in the high failure rate in clinical trials of new drug candidates in the field of oncology, in spite of preceding excellence in pre-clinical studies. Although the background for this is multifactorial, existing pre-clinical assessments have many drawbacks.

1.10.1 Treating child cancer

Treating child cancer is challenging given that childhood malignancies present differences in clinic and biology and that children presents challenges of the full biologic and physiologic development from the fetus to the adult. The majority of todays cancer therapies have a primary focus targeting proliferating cells resulting in severe effect on immature organs and developing structures, in particularly tumours located in CNS and next to rapidly growing normal tissue (Duffner et al. 1988, Jannoun and Bloom 1990)

Dramatic improvement for pediatric malignancy in survival has been achieved in previous years (Figure 3) although advancements seems to be halted (Gustafsson et al. 2013), However, toxicities are high and a significant proportion of patients remain therapy resistant. Young children are at particular risk for adverse side effects from treatment and survivors present a wide range of specific health problems (Oeffinger and Hudson 2004). Therefore improved targeting may prove to be beneficial for the patient both through better therapeutic efficacy, as well as the putative limitation of adverse long-term neurological side effects.

1.10.2 Treating NB

Although significant progress has been made with multi-modal therapies, children with high-risk neuroblastoma still have a 5- year survival rate around 50% (Maris 2010). Predicting favourable outcome is difficult, and improved stratification is clearly required to avoid both under- and overtreatment and better understanding chancer. Young children are at particular risk for irreversible adverse side effects.

Neuroblastoma patients are treated according to their risk-group to avoid and decrease excessive side effects and long term late effect (Cohn et al. 2009; Maris 2010). The low and intermediate risk group have a good 10-year event free survival. Unfortunately poor survival in high risk NB is motivating a more aggressive treatment regime with high dose and multimodal therapy, chemo before and after surgery including stem cell rescue and radiation.

These patients may get a long follow-up therapy that includes immunotherapy combined with the retinoic acid. For the high risk (metastatic) NB group available data motivate an increased intensity of induction therapy to improve outcome and response, particularly improved median progression free survival (Cheung and Heller 1991).

On the other hand there has been a trend to reduce therapeutic intensity for tumours with favourable biological profile. (Maris 2010) The trick is to at an early stage make a good categorization and prediction of the cancer, to prepare the optimal treatment strategy at start.

Currently, different combinations of cytostatics are used as induction therapy, followed by attempts of radical surgery, high-dose myeloablative chemotherapy and autologous stem cell reinfusion, irradiation and maintenance therapy with differentiating retinoids and emerging immunotherapy as performed in novel protocols both in North America and Europe (Matthay 1999; Maris 2010; Ladenstein 2010). The current high-risk NB protocol in Europe, HR-NBL1/SIOPEN (Ladenstein 2010) is using a chemotherapy induction combining cisplatin, carboplatin, cyclophosphamide, vincristine and etoposide (COJEC) that is further combined with topotecan, vincristine and doxorubicin in case of lack of complete clinical response.

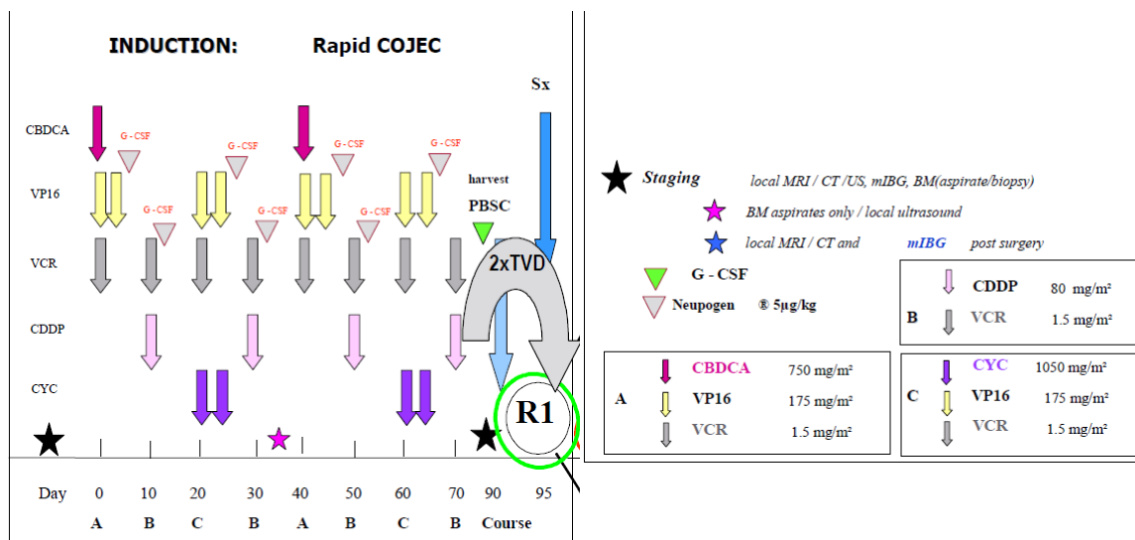


Figure 9. Chemotherapy induction for high-risk neuroblastoma in Europe (HR-NBL1/SIOPEN) using the COJEC regimen (cisplatin, carboplatin, vincristine, etoposide, cyclophosphamide) combined with TVD (topotecan, vincristine, doxorubicin) to obtain clinical remission for the randomization to high-dose therapy (depicted “R1” in the figure). Reproduced from Ladenstein et al. 2010 with permission from the publisher

Variable	Prognostic Category*			
	Low Risk	Intermediate Risk	High Risk	Tumor Stage 4S
Pattern of disease	Localized tumor	Localized tumor with locoregional lymph-node extension; metastases to bone marrow and bone in infants	Metastases to bone marrow and bone (except in infants)	Metastases to liver and skin (with minimal bone marrow involvement) in infants
Tumor genomics	Whole-chromosome gains	Whole-chromosome gains	Segmental chromosomal aberrations	Whole-chromosome gains
Treatment	Surgery [†]	Moderate-intensity chemotherapy; surgery [†]	Dose-intensive chemotherapy, surgery, and external-beam radiotherapy to primary tumor and resistant metastatic sites; myeloablative chemotherapy with autologous hematopoietic stem-cell rescue; isotretinoin with anti-GD2 immunotherapy	Supportive care [†]
Survival rate (%)	>98	90 to 95	40 to 50	>90

Table 1. Overview of survival of NB patients. Reproduced with permission from Maris 2010. Copyright Massachusetts Medical Society

1.10.3 Doxorubicin

Doxorubicin is a hydroxyl derivative of daunorubicin (Arcamone et al. 1969) discovered in the 1950's. Daunomycin (daunorubicin) was the first anthracycline initially isolated from a strain of *Streptomyces peucetius* bacteria that produced the red pigmented antibiotic later named Doxorubicin (Arcamone et al. 1969). This agent proved to be quite efficient, following research have brought forward an amount of other anthracycline antibiotics and analogues, as today there are over 2000s analogues of the prototype doxorubicin (Weiss et al. 1992). Doxorubicin is well-studied but the exact mechanism(s) of action has been debated. Doxorubicin may act through binding DNA-associated enzymes and interact with DNA base pairs, it has a range of molecular targets hence the multiple cytotoxic effects. Doxorubicin is known to effectively combat tumours with rapidly dividing cells and slow progressing cancer. Doxorubicin is included in the high-risk neuroblastoma treatment TVD combined with topotecan and vincristine (Garaventa et al. 2003; Ladenstein et al. 2010). Unfortunately the usage is dose limited due to toxicity, affecting most organs, inducing severe cardio toxicity (Oktay Tacar et al. 2012).

2 AIM

Translational studies towards the understanding of tumour-host interactions are of outmost importance for developing enhanced therapies. For the most aggressive forms of tumours in children effective treatments will be attainable when the molecular events that are specific to childhood tumourigenesis are better understood.

A central hypothesis behind the here presented approach is that compared to current animal models, the introduction of a homologous human tissue environment provides a more favourable setting for studying human tumours and evaluation of chemotherapy responsiveness/resistance.

This doctoral thesis focuses on exploring the complex *in vivo* microenvironment of compartmentalised human embryonic tissues in the human pluripotent stem cell induced experimental teratoma (PSCT) model, and addresses the following questions:

1. Does the PSCT milieu provide a relevant neoplastic niche for support of embryonic neuroectodermal tumours?

In Paper I we have explored the model for *in vivo* growth support of NB cell lines.

In Paper II the same questions were addressed for biopsy material from tumours of neural embryonic origin obtained directly from patients.

With the ultimate aim to add further clinical relevance to pre-clinical modelling and assessment of therapy compared to current preclinical *in vivo* models, we asked the question:

2. Can the PSCT milieu be used for a preclinical assessment of therapy response?

In Paper III we test the PSCT milieu for assessing doxorubicin chemotherapy response towards neuroblastoma.

3 MATERIALS AND METHODS

3.1. Flowchart of the experimental set up

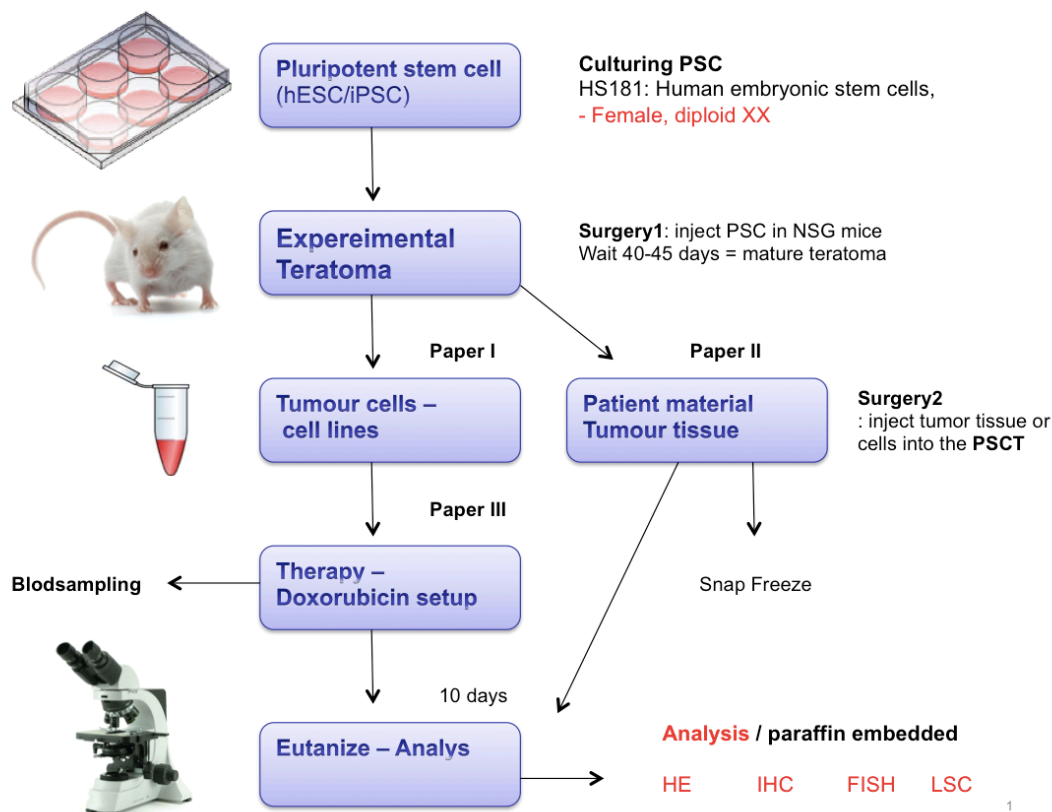


Figure 10. Schematic illustration of the interacting procedures in the study, providing an overview of the experimental set up.

3.2 Patient tumour material

Specimens from patients undergoing surgery at the Karolinska University Hospital, departments of neurosurgery or paediatric surgery were used. An inclusion criterion was presence of a tumour genetic marker enabling a positive identification of cells, e.g. MYCN amplification, or Y-chromosome (the PSCT model is generated from female origin). Information from the national tumour bank allowed precise molecular fingerprint markers on most tumours entering the study. Fresh tumour material was aliquoted, cryo-preserved, and thawed at chosen time points, aiding the logistics of the analysis. The logistics for transportation of tumour specimens from the clinic to the *in vivo* model and analysis is described in Paper 1.

Fresh tumour material were aliquoted, cryo-preserved, and thawed at chosen time points, aiding the logistics of the analysis.

3.3 Tumour cell lines

Neuroblastoma (NB) cell lines: [Papers I and III] Three Neuroblastoma cell lines IMR-32, SK-N-BE(2) and Kelly were used. The NB cell lines were obtained from ATCC.

IMR-32: was derived from the abdominal tumour mass of a 13-month old Caucasian male neuroblastoma patient. The tumour line has an amplification of the MYCN gene and ALK, together with partial deletion of chromosome 1 (Tumilowicz et al 1970; Caren 2008; Kryh 2011).

SK-N-BE (2): was derived from the bone marrow metastasis of a disseminated NB after repeated cycles of chemotherapy and radiotherapy from a two year old child in 1972. The tumour has an amplification of MYCN and a mutation (C135F) in the tumour suppressor gene p53.

Kelly: was derived from a brain metastasis of NB. It has MYCN amplification, gain of chromosome 17q, and deletion of 11q as major aberrations (Kryh 2011).

All tumour cell lines were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Invitrogen).

The cell line identities were all confirmed using STR profiling at the Cancer Center Karolinska Core Facility service, Karolinska Institutet.

Experiments were performed on Mycoplasma free cell cultures. Cells were regularly controlled EZ-PCR Mycoplasma Test Kit (Biological Industries). The cell culture supernatant was used for screening of cells that had stayed in same media without changing for 3 days. The PCR reaction mix was used according to the attached instructions, a positive control was provided by the kit.

3.4 Pluripotent stem cells

The human embryonic stem cell line HS181 was used for the experimental setup in papers I-III. It was originally derived from ICM (inner cell mass of a IVF blastocyst). The selected stem cells were initially cultured on Feeder cells (hFS / human fore skin fibroblasts) CRL-2429; ATCC – the feeder cells were mitotically inactivated by treating with mitomycin C (10µg/ml, 3hours). The hFS cells were seeded on 6 well plates (2x10⁴ cells/cm²) from BD Falcon and cultured in IMDM (Iscove's Modified Dulbecco Medium) supplemented with 10% foetal bovine serum (FBS) from Invitrogen with 1% PST (penicillin/streptomycin) from Invitrogen. For paper III, the HS181 culture plates were instead pre-coated with truncated recombinant human vitronectin and cultured in Essential 8 media from Invitrogen according to manufacturer's instructions (Invitrogen). Immunohistochemistry and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) were used for routine analysis of markers of pluripotency as described (Gertow et al. 2004).

3.5 Animals

Male mice at the age 6-12 weeks, from severely immunodeficient strains were used for the experiments.

The mice were kept at the MTC animal facility with ad libitum food and water, at 20-24°C temperature, 50% relative humidity and a 14/10 hour light-dark cycle. The mice were monitored following all procedures, according to the ethical and mouse experimental regulations of Karolinska Institutet and to protocols in the ethical permissions.

SCID beige mice (C.B.-17/GbmsTac-scid-bgDF N7). (**Papers I and II**)

The genetic profile of severe combined immunodeficiency (SCID)-beige mice includes the Beige mutation leading to defect macrophages and impaired NK-cell. The SCID mutation causes lack of both T and B lymphocytes. (Croy and Macdougall 1990). These mice were obtained from M&B, Denmark via the MTC animal facility, Karolinska Institutet.

NSG mice (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ JAX® strain). (**Paper III**)

The NSG strain represents today the most highly immunodeficient mouse model; exhibiting absence of mature B, T cells and NK cells as well as having defective macrophages and dendritic cells and lacking complement (Shultz et al. 2005). The NSG mice from Jackson laboratories were obtained via the MTC animal facility, Karolinska Institutet.

3.6 In Vivo model – PSCT



Figure 11. The PSCT *in vivo* model

3.6.1 Generation of experimental teratoma

Experimental teratomas were generated by injection of 1×10^4 - 1×10^5 HS181 cells (in 50 μ l culture medium) under the testicular capsule with anaesthesia (3.0 % isofluran). HS181 cells were immediately prior to injection mechanically collected from the culture plate, re-suspended in medium and injected using a 1-ml syringe with 27G $\frac{3}{4}$ '' Nr. 20 0.4-19mm grey needle (both from BD Falcon). For injection, the abdomen was opened with a small cut allowing access to the testis. For details of the surgical procedure, see Gertow et al 2007. The abdomen was closed with re-absorbable suture (Vicryl) and post-operative administration of Tamgesic was applied. Teratomas were

allowed to develop for 40-45 days resulting in mature experimental teratoma (Gertow et al 2004).

3.6.2 Growth of human tumours to the PSCT model

3.6.2.1 Transplantation of cell lines (*Paper I, III*)

Tumour cell lines in logarithmic growth phase were harvested with enzymatic detachment (Trypsin, Invitrogen) and kept on +4° ice until injected. Cells were resuspended in cold PBS and transported to the surgery on wet ice (+4°C). 2x10⁶ million cells were injected in a volume of 50 µl centrally in the teratoma. For this a second operation was performed to access the testis/teratoma via the abdomen, similar to the surgery for the first implantation of HS181 cells. Two weeks later chemotherapy was started.

3.6.2.2 Transplantation of clinical tumours (*Paper II*)

After receiving fresh patient material the tumour was aliquoted into small pieces of 1-4 mm, and when possible saved in liquid N₂ for later injection, or alternatively injected fresh.

Material for injection was transported to the animal facility on wet ice in medium. Small pieces of 1mm³ were inserted intact, or injected as cell suspension. The cell suspension was prepared by plunger of a 5-ml syringe (BD Falcon), and by filtering the cell suspension through a 70µm Nylon cell strainer (BD Falcon). The suspension This was injected with a 1ml-syringe (BD Falcon) and 27G^{3/4} Nr. 20 0.4-19mm grey needle (BD Falcon), similar to the procedure used for tumour cell lines. Table 1 illustrates an overview of the patient tumour material with diagnosis and clinical history for the patients in Paper II.

	Diagnosis	Stage	History	Chr. Aberration
Patient 1	Supratentorial peripheral neuroectodermal tumour (sPNET)	Very aggressive irradiated and chemo	2:nd relapse	p53 mutation
Patient 2	Pilocytic astrocytoma	Low malignancy	Primary tumour Hydrocephalous	N/A
Patient 3	Classic medulloblastoma	Highly malignant	Primary tumour	p53 mutation
Patient 4	Peripheral primitive neuroectodermal tumour (pPNET)	Non-- irradiated	Primary tumour	EwS-Fli1 translocation
Patient 5	Neuroblastoma	M (INRG) 4 (INSS)	First relapse. Metastasis in skull.	11q- del

Table 2: Details of surgical tumours included in Paper II.

3.7 Chemotherapy treatment (Paper III)

Doxorubicin treatment: The animals were randomised into treatment groups and therapy started 14 days after tumour transplantation.

Doxorubicin (Sandoz®; Ebewe) was diluted in PBS (DPBS GIBCO™ Life technology) and administered by intraperitoneal injection at the indicated doses, 4mg/kg 8mg/kg and 12mg/kg.

Control animals were MOCK treated with an injection of PBS of equal volume.

3.8 Euthinization

Animals were sacrificed at the endpoint of the experiment, by cervical dislocation or using CO₂ gas. Selected material was harvested for analysis.

3.9 Tissue sampling

Testis/teratoma/tumour was extracted and fixed in 4% PFA over night and transferred to 70% EtOH and then paraffin embedded (using the CCK core facility service). Additional organs (liver, gut and kidney) were preserved in some cases.

The tissue was dehydrated through a series of alcohol to xylene in a standardized manner before paraffin embedding. Samples were sectioned on Super frost plus glass (menzels or thermo scientific) into 5µm thick sections (= FFPE) for analysis.

For further analysis some material was snap frozen in liquid nitrogen and saved in -80°C. Suggested analysis for these frozen material was to extract RNA.

3.10 Analysis

3.10.1 Histology and morphology

Hematoxylin & Eosin (HE) staining was performed on 5µm thick FFPE sections to provide a general overview of the tissue morphology. The Hematoxylin stains the nucleus blue and the Eosin binds eosinophilic structures providing a pink colour. The stained FFPE sections were scanned and the location of tumour growth, together with histological structures, were mapped using FISH and IHC, this way indicating areas of interest. Immunohistochemistry was extensively used in all papers. A panel of antibodies have been applied to identify different selected targets (listed below in Tables 2-5)

Antibody	Dilution	Source
Oct4	1:50	Chemicon, MAB4401
Nanog	1:50	R&D, AF1997
TRA1-81	1:50	Chemicon, ab90233
TRA1-60	1:50	Chemicon, ab90232
SSEA-4	1:50	Chemicon, ab90230

Table 3. Antibodies used for indication of pluripotency in hESC.

Marker	Source	Dilution	Secondary antibody detection
B-catenin	Invitrogen 18-0226	1:100	Bond Kit nr: DS9800 Lieca
CD31	Dako M0823	1:200	Bond Kit nr: DS9800 Lieca
CD99	NCL-CD99	1:50	Bond Kit nr: DS9800 Lieca
CGA	Neomarker MS324	1:800	Bond Kit nr: DS9800 Lieca
E-cadherin	NCL-E-CAD	1:40	Bond Kit nr: DS9800 Lieca
Ki67	Ventana 790-4286	Ready to use	UltraView Universal DAB
NeuN	Chemicon MAB377	1:50	Bond Kit nr: DS9800 Lieca
NSE	NCL-NSE-435	1:200	Bond Kit nr: DS9800 Lieca
Synaptophysin	NCL-L-SYNAP-299	1:400	Bond Kit nr: DS9800 Lieca
Vimentin	Dako M7020	1:400	Bond Kit nr: DS9800 Lieca
NFP	NCI-NF200-N52	1:50	Bond Kit nr: DS9800 Lieca

Table 4. Antibodies used in Paper I

Antigen	Source	Dilution	2:nd detection
αSMA	Novocastra/leica, NCL - SMA	1:50	DakoCytomation EnVision+System-HRP
CD31	DAKO, M 0823	1:20	DakoCytomation EnVision+System-HRP
CD44	DAKO, M 7082	1:50	DakoCytomation EnVision+System-HRP
Chromogranin A	DAKO, A 0430	1:2000	Bond Polymer Refine detection kit, Leica
Cripto	Rockland, 600-401-99337	1:400	4+ Biotinylated Goat Anti-Rabbit-IgG, Biocase Medical
E-cadherin	Abcam, ab1416	1:50	Vectastain Universal Elite ABC
HIF2α	Novus Biologicals, NB100-132	1:150	DakoCytomation EnVision+System-HRP
Ki67	Abcam, ab833	1:50	Vectastain Universal Elite ABC
Lefty	Santa Cruz, SC-7408	1:25	4+ Biotinylated Mouse Anti-Goat-IgG, Biocase Medical
Nodal	Life Span BioScience, LS-B3955	1:100	4+ Biotinylated Mouse Anti-Goat-IgG, Biocase Medical
NSE	Novocastra/leica, NCL-NSE-435	1:200	DakoCytomation EnVision+System-HRP
Synaptophysin	Novocastra/leica, NCL-L-Synap-299	1:100	DakoCytomation EnVision+System-HRP
Tyrosinase	Leica, PA 0322	1:100	DakoCytomation EnVision+System-HRP
Vimetin	DAKO, M 7020	1:100	DakoCytomation EnVision+System-HRP

Table 5. Antibodies used in Paper II

Antigen	Source	Dilution	2:nd detection
Ki67	Abcam AB:833-500.Rabbit pk ab. Lot 713408	1:50	Vectastain Universal Elite DAB- ABC
Caspase-3	BD-PharMingen Purified rabbit anti-active caspase-3 Cat: 559565	1:1000	Vectastain Universal Elite ABC
HuCD34	DAKO mouse Anti-Human CD34 ClassII. m.ab; M 7165.	1:500 1:50	Jackson Immunoresearch laboratory IF: Cy3 goat anti mouse IHC : DAB, Vectastain Universal elite, ABC

Table 6. Antibodies used in Paper III

Paraffin-embedded pre-baked material sectioned in 4µm on superfrosted glass slides were used for these experiments. The antibodies included generally had different optimization protocols including different pre-treating steps and solutions. Antigen retrieval was performed with either boiling in high pH buffer (like Tris-EDTA buffer or DAKO pH9 buffer) or low pH buffer (for example Citrate buffer) enzymatic treatment with weak HCl and pepsin was common, however in some cases no pre-treatment was needed. (Details are defined in the respective papers) regarding specific conditions and secondary detection system applied, (Papers I-III).

Analysis using Fluorescent in situ hybridization (FISH) was a key tool in the performed studies. This was used to separately identify human PSCT and engrafted tumour cells in the model. FFPE sections from tumour xenografts were used as positive control in FISH experiments.

The following FISH probes were used (all from Abbott Molecular/Vysis);

- A. FISH probe - Spectrum Red labelled total human genomic DN
- B. FISH probe - CEP X SpectrumOrange CEP Y SpectrumGreen
- C. FISH probe - LSI n-myc, 2p24 Spectrum orange
- D. FISH probe - LSI n-myc, 2p24 Spectrum Green/ CEP2 Spectrum orange

Probe A. was used to human confirm origin of the formed embryonic tissues in PSCT.
Probe B. was used to detect the X (PSCT cell) and Y-chromosome (engrafted male tumour cell).

Probe C. was used to detect tumours amplified MYCN, but not for LSC analysis .

Probe D. was primary used for LSC analysis of MYCN in SKNBE2.

The protocol for FISH analysis was carried out strictly according to the manufacturers instructions (Vysis protocol). In brief, the procedure included deparaffinized using xylene, rehydration using a series of Ethanol and water. Antigen retrieval with boiling in Citrate buffer (pH 6.0) and 0.01% HCL+pepsin at 37°C for 5 min. Denaturation of

double-stranded DNA at 75°C for 5 min and probe hybridization at recommended temperature (either 38°C for NMYC or 42°C for X/Y) overnight. SSC solution was provided by the vysis kit. Final fixing of sections with Vectasheild HardSet Antifade mountingmedia with DAPI.

3.10.2 Image analysis

Image analysis was performed using a Zeiss Axiovert 200M microscope and software from QCapture and Openlab.

FFPE slides were scanned using a Hamamatsu scanning system NDP SCAN 2.0.

High throughput screening of single cells of PSCT FFPE tissue specimens was performed using a CompuCyte LSC 2 Laser Scanning Cytometer equipped with 2 lasers (488nm and 633nm) and using the WinCyte 3.7 software (CompuCyte).

3.10.3 Statistical analysis

Statistical analysis: This was performed using one-way analysis of variance (ANOVA). The Bonferroni correction was applied to adjust for multiple testing. The GraphPad Prism 6.0 software was used for testing normal distribution and for the generation of graphs.

4 ETHICAL PERMISSIONS

The work was conducted with approved ethical applications:

Permissions number 514/00 and 2008/307/31 obtained from the Local Ethics Committee at Karolinska Institute.

Permissions number N105/07, N112/09, N173/10, N101-13 and N118/14, from the Stockholm Northern Animal Review Board.

5 RESULTS and DISCUSSION

5.1 Paper I

Neuroblastoma Cells Injected Into Experimental Mature Teratoma Reveal A Tropism For Embryonic Loose Mesenchyme.

Seema Jamil, Jessica Cedervall, **ISABELL HULTMAN**, Rouknuddin Ali, Naira V. Margaryan, Agnes Rasmuson, John Inge Johnsen, Baldur Sveinbjörnsson, Tina Dalianis, Lena Kanter, Abiel Orrego, Luigi Strizzi, Mary.J.C. Hendrix, Bengt Sandstedt, Per Kogner, Lars Ährlund-Richter

Published in the Int J Oncology. 43: 831-838. 2013.

BACKGROUND and AIM: Earlier studies in our research group have raised the possibility that the embryonic microenvironment in PSCT could be particularly well suited for studies on neural childhood tumours. This study aim to answer the question; Can the human non-malignant embryonic process in stem-cell induced mature experimental teratoma function as an *in vivo* microenvironment for studies of the growth of childhood neuroblastoma (NB) cell lines?

APPROACH: Three human NB cell lines, IMR-32, Kelly and SK-N-BE(2), were compared for growth patterns in the PSCT model and when injected as xenografts.

RESULTS and DISCUSSION: Proliferative index for each NB cell line was similar in both models, and growth was readily detected from the three cell lines showing histology of a poorly differentiated NB tumour with variable amount of fibrovascular stroma. NB cells were in the PSCT model found integrated in embryonic loose mesenchymal stroma and never observed in areas with well-differentiated somatic tissue i.e. bone, muscle, gut or areas of other easily identifiable tissue types. Histology recapitulated NB native presentation in patients. As expected, a perivascular preference was observed for the instigation of NB colonies.

The tissue selectivity of the microcolonisation of NB with strong preference to loose mesenchymal stroma is intriguing. This tropism may be due to e.g. i) the open contextual nature of mesenchyme providing space, ii) the rigidity of present ECM providing physical cues, or iii) presence of potential developmental cues that could attract and promote integration. The latter option is particularly intriguing since embryonic mesenchyme is rich in neurotrophic factors and their receptors, with potential to support cancer / stem cell like growth.

Aggressive IMR-32 tumour growth showed expression of the TGF β superfamily member Nodal and its co-receptor Cripto-1, but not the Nodal antagonist Lefty, concurring with Nodal pathway regulation in early embryonic patterning.

SIGNIFICANCE. We conclude that mature PSCT provide an embryonic niche well suited for further *in vivo* studies of NB. The observed tissue tropism provide a clear advantage towards conventional xenograft studies with possibilities to explore cues that attracted or promoted the microcolonisation of NB in human stroma.

5.2 Paper II

Tropism Of The *In Situ* Growth From Biopsies Of Childhood Neuroectodermal Tumours Following Transplantation Into Experimental Teratoma

Seema Jamil, ISABELL HULTMAN, Jessica Cedervall, Rouknuddin Q. Ali, Gabriel Fuchs, Bengt Gustavsson, Jurate Asmundsson, Bengt Sandstedt, Per Kogner, Lars Ährlund-Richter.

Published in the Int. J. Cancer. 134:1630–1637. 2014.

BACKGROUND and AIM: In Paper I we demonstrated how the PSCT model could provide an experimental human *in vivo* model for neuroblastoma using NB cell lines. Here we explore whether the model also provides a relevant neoplastic niche suited for transplantation studies, of surgery-material from patients with primitive neuroectodermal tumours

APPROACH: Five tumour specimens were analysed for growth after transplantation to the PSCT model and after injection as xenografts. Tumours included; Supratentorial primitive neuroectodermal tumour (sPNET); Pilocytic astrocytoma of the brainstem; Classic medulloblastoma; peripheral primitive neuroectodermal tumour (pPNET) or neuroblastoma (NB), respectively, was transplanted to the PSCT model. Each patient specimen was transplanted into 2-6 separate PSCT.

RESULTS and SIGNIFICANCE:

This study demonstrated that biopsies from childhood neuroectodermal tumours could instigate microcolonies in specific embryonic components of human experimental teratoma.

Engraftment was detected in PSCT for three of the five transplanted tumours (pPNET, sPNET, and NB) after analysis of up to 120 FFPE sections of each PSCT. Protruding growth from the latter two tumours were detected, and selected for detailed examination. The failure to find engraftment could be due to lack of growth from the tumour, but it is important to note that for technical and resource reasons only small portions of the PSCTs were analysed. Likely, additional growth can be detected in further analysis of the material. Small biopsies (1mm) were allowed to grow for limited time (one month). When transplanted as xenografts no palpable growth was observed after four months. Similar to results from NB cell lines, a strict tissue tropism with a non-random integration into embryonic structural compartments was observed confirming for a clinical neuroblastoma engraftment into loose mesenchyme. For the sPNET tumour, instead we found exclusive intergration into areas of neural condensation. Also, tumour colonisation showed a perivascular preference resulting in histology closely recuperating observations for the original patient tumour. Although the original tumour histology was kept, when tested for a panel of markers the NB lost IHC expression of neuroendocrine markers, Synaptophysin, NeuN, NFP, NSE. In contrast, an sPNET tumour showed preserved histology and marker profile.

5.3 Paper III

Chemotherapy Resistance In Microcolonies Of Neuroblastoma Analysed In Homologous Embryonic Tissue Environment In Vivo.

Hultman I, Rognmo I, Jansson Edqvist J, Blomberg E, Pernicová Z, Björk L, Ali R, Phillips, L, Sandstedt B, Kogner P, Åhrlund-Richter L.

Manuscript

AIM: Chemotherapy is widely accepted as part of first-line therapy for high-risk paediatric neuroblastoma (NB). The goal of this study was to examine *in situ* tumour chemotherapy responsiveness/resistance, using a previously developed human embryonic model for NB.

BACKGROUND: Childhood cancers show fundamental differences to most common adult solid tumours in their *cancer-causing genetics*, cell biology and their local tissue microenvironment. Effective treatments will be attainable when the molecular events that are specific to childhood tumourigenesis are better understood. However, it is in this context critical to consider both species and developmental aspects when looking for the relevant signalling.

APPROACH: We report for the first time *in situ* studies of neuroblastoma chemotherapy responsiveness/ resistance performed in a homologous embryonic *in vivo* microenvironment.

Two well characterised paediatric neuroblastoma cell lines; IMR-32 and SK-N-BE(2) were transplanted to an *in vivo* model comprising a benign experimental teratoma, generated from human diploid bona fide pluripotent stem cells (the PSCT model) in NSG mice. Fourteen days after inoculation of two million tumour cells, emerging neuroblastoma micro-colonies were subjected to chemotherapy intraperitoneally with the topoisomerase inhibiting anthracycline doxorubicin. Twelve and 96 hours later the total PSCT with neuroblastoma growth and its surrounding human tissue environment were removed and assayed in formalin-fixed paraffin-embedded sections. We used a combination of conventional immunohistochemistry (IHC) and cytology to measure proliferation, apoptosis and cell death. A high throughput single cell screening system using laser scanning cytometry (LSC) to measure chemotherapy-induced changes of nuclear DNA index (sub G1 fraction) was also applied.

RESULTS and DISCUSSION: The efficacy of microcolonisation and expansion of NB colonies in homologous embryonic stroma were similar to previous studies using cell lines. The results demonstrated a dose dependent response of NB cells to a single-dose regimen of doxorubicin (chemotherapy drug belonging to the anthracycline group).

The strongest anti-NB effects were observed from a recurrent treatment of 4+4 mg/kg doxorubicin given with a 48 hours interval. Administration of the same total dose, 8mg/kg, as a single injection appeared less effective for both NB lines.

Following a recurrent doxorubicin regimen (repeated administration with 48h interval), we observed the presence of an asynchronous cellular response within individual tumour colonies, with cells exhibiting cytotoxic effects or enhanced proliferative index.

The toxicity from the administered doses of doxorubicin on non-malignant PSCT tissues was assessed on embryonic tissues with high mitotic activity, i.e. embryonic neural epithelium, cartilage and muscle. Selectivity for transplanted NB cells over non-malignant PSCT cells was observed for all tested doxorubicin regimens.

SIGNIFICANCE:

This study illustrates how chemotherapy outcome may be monitored in a pre-clinical assay in a homologous *in vivo* setting.

These findings illustrate the feasibility of the approach and are a encouraging for clinically relevant further studies of patient material, regarding intra tumour heterogeneity and asynchronous response to therapy, of neuroblastoma and other primitive neuroectodermal tumours originating early in life.

The illustration of a heterologous and asynchronous response after recurrent doxorubicin treatment is intriguing and may be of importance for the understanding of tumour resistance.

6 CONCLUSIONS

We conclude that the homologous complex microenvironment with compartmentalised embryonic tissues in human experimental teratoma (the PSCT model) can provide:

1. **PSCT presents a novel human *in vivo* model for neuroblastoma**, (Paper 1)
 - Structural components of the embryonic process in stem-cell induced teratoma supply an experimental *in vivo* microenvironment for the instigation of microcolonies of injected childhood neuroblastoma (NB) cell lines.
 - Microcolonisation showed a tissue tropism, with specificity to embryonic loose mesenchyme.
 - Individual variations in molecular profiles for the three neuroblastoma cell lines were observed, providing additional information on the neuroblastoma tumour biology.
2. **PSCT presents relevant *in vivo* neoplastic niches for studies on invasion and microcolonisation of primitive neuroectodermal tumours originating early in life** (Paper II)
 - Transplantation of biopsies from childhood neuroectodermal tumours showed microinvasion into specific embryonic components of human experimental teratoma. The tumour progression in the model showed striking similarities to the tumour conditions in the young patient, maintaining most of their original characteristics.
 - Histological examination revealed a tropism with nonrandom integration into morphologically identifiable tissues.
3. **PSCT presents a model for *in situ* evaluation of early outcome parameters and death of tumour cells following chemotherapy of solid tumours** (Paper III).
 - This study illustrates how chemotherapy outcome may be monitored in a pre-clinical assay. It was possible to measure dose dependent and diversified responses to doxorubicin chemotherapy.
 - The illustration of a heterologous and asynchronous response after recurrent doxorubicin treatment is intriguing and may be of important for understanding of tumour resistance.

7 FUTURE PROSPECTS

Building on the proof of concept presented in this thesis, it is now important to ensue detailed studies on the interplay of tumour and human microenvironment. This would address if, or how, signalling pathways differ between tumour xeno-interactions and homologous interactions with surrounding tumour microenvironment. Such studies could reveal important information in the quest for new targets in therapeutics.

The PSCT model provides theoretically not only an opportunity to study homologous interactions but also to generate an autologous system. We have in a collaborative study with the KI Biobank and Dr Anna Falk, KI, derived iPSC from two young patients with MYCN amplified NB, using skin fibroblasts. These will next be used to perform similar experiments as presented in this thesis, but in an autologous setting.

Moving from cell lines to patient tumour material is also a goal. Here, it is important to broaden the repertoire of tested tumour types and to increase the numbers of tested. Partly, this is already on going. A total of 5 additional patient tumour materials have been collected, including material from 3 male patients diagnosed with high risk NB. These have been grafted to the PSCT (fresh and frozen/thawed) and subjected to chemotherapy (Table 7).

Patient number	pat. 1	pat. 2	pat. 3
Diagnosis	Highrisk neuroblastoma	Highrisk neuroblastoma	Ganglioneuroblastoma, intermixed subtype
Age at biopsy	23*	45*	10y 8m*
Survival	21*+	12*+	11*+
Outcome	NED 2015	NED 2015	In remission
Treatment	High-risk protokoll	Rapid-Cojec + TVD	High-risk protokoll
Chromosomal aberrations	11-q deletion, 17q +	MYCN amplified	11-q del, MYCN unknown
Total nr of injected PSCT	11	8	6
Fresh tumour NT	1	2	-
Fresh tumour +DOX treatment	4 (4mg/kg DOX)	2 (4+4mg/kg DOX)	-
Thawed tumour NT	3	2	3
Thawed tumour +DOX treatment	3 (8mg/kg DOX)	2 (4+4mg/kg DOX)	3 (4+4mg/kg DOX)
Findings	Analysis ongoing Growth identified in one PSCT	Analysis ongoing	Analysis ongoing

All patients included were males, diagnosed tumor at stage 4 and metastasis present at sampling
*M=months. Y=years

INSS: International Neuroblastoma Staging System Committee system.

INRGSS: The International Neuroblastoma Risk Group Staging System.

NED: No evidence of disease; DOX: doxorubicin; NT: not treated; TVD: topotecan, Vincristine, doxorubicine

Table 7. Overview of additional un-published studies on patient material.

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