Neural Stem Cells, TLX, and Neuroblastoma

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To my dear mom

Abstract

Neuroblastoma (NB) is one of the most commonly diagnosed extracranial tumors in children below five years of age. Moreover, NB accounts for almost 12-15% of all childhood cancer related fatalities. To date, the origin of NB has been linked to the neural crest derived sympathoadrenal progenitor cells. Progression and pathogenicity of the disease has been largely correlated with the ages of children and presence of undifferentiated neuroblasts and amplification of the *MYCN* oncogene. However from the clinical evidence gathered so far, *MYCN* amplification is related to poor prognosis but it occurs only in 20-25% of NB cases. Henceforth, there is a need for better diagnostic and predictive markers to stratify NB patients.

NB cells express a number of neural stem cell and progenitor markers such as Oct3/4, Sox2, CD133, ABCG2, and Nestin. Since self-renewal and differentiation of neural stem cells are predominantly regulated by a number of stem cell fate determinants such as Notch, Wnt, Hedgehog, PTEN, and TLX, it is speculated that deregulation of these genes may be responsible for the pathogenicity of the disease. TLX is an orphan nuclear receptor, which is predominantly expressed in the embryonic and adult forebrain, and is considered to be a crucial regulator of neurogenesis, because of its roles in neural stem cell self-renewal and maintenance. We have identified that upon hypoxia, TLX stimulates neural stem cell renewal by promoting Oct3/4 transcription in adult hippocampal progenitors. TLX is expressed at high levels in NB cell lines. In sphere forming cells generated from these cell lines, TLX was enriched and co-expressed along with the neural progenitor markers Nestin, Oct3/4, CD133, and HIF-2α. TLX was also co-expressed with the neural progenitor markers CD15 and MMP-2 in xenografts of primary NB-tumor initiating cells (TIC) derived from patients. Thus, TLX may be involved in the tumorigenesis of NB by promoting dedifferentiation of tumor cells.

NB develops through processes which may be defined as cellular “dedifferentiation”. The ability of tumors to form spheroids is one of the manifestations of dedifferentiation and transformation. To study the mechanisms of dedifferentiation, neuroblastoma cell lines will help us to identify new diagnostic markers. We generated spheroids of the NB cell line SK-N-BE2 and performed proteomics analysis to evaluate the differential expression pattern as compared with the wild-type cells. We identified 239 proteins which were affected by the dedifferentiation process. These proteins represented several regulatory processes, such as transcription, cell cycle regulation, apoptosis, cell adhesion, metabolism, intracellular transport, stress response, and angiogenesis. An extensive analysis using Cytoscape identified “DISC-1” and “DNA-PKcs”, both of which have been previously linked to dedifferentiation and cancer. The results contribute to better understanding of mechanisms involved in Neuroblastoma pathogenesis, along with identifying possible biomarkers for the disease that may be translated to the clinic.

Keywords: Neuroblastoma, TLX, neural stem cells, dedifferentiation, DISC-1, DNA-PKcs.

PAPERS IN THIS THESIS

This thesis is based on the following papers, which are referred in the text by roman numbers (I-III).

I. Chavali PL, **Saini RK**, Matsumoto Y, Ågren H, and Funa K (2011). Nuclear Orphan Receptor TLX induces Oct-3/4 for the Survival and Maintenance of Adult Hippocampal Progenitors upon Hypoxia. *J Biol Chem*, 286: 9393–9404.

II. Chavali PL, **Saini RK**, Zhai Q, Vizlin-Hodzic D, Ventakatabalasubramanian S, Hayashi A, Johansson E, Zeng Z-J, Mohlin S, Påhlman S, Hansford L, Kaplan D, and Funa K. TLX activates MMP-2, promotes self-renewal of tumor spheres in neuroblastoma, and correlates with poor patient survival. *Cell Death & Dis, In press.*

III. **Saini RK**, Attarha S, Santos C, Kolakowska J, Funa K, and Souchelnytskyi S. Proteomics of dedifferentiation of SK-N-BE2 neuroblastoma cells. *Cancer Science*, *Under revision.*

Papers not included in this thesis

I. Attarha S, **Saini RK**, Andersson S, Mints M, Souchelnytskyi S. PKN1 modulates TGFβ and EGF signaling in HEC-1-A endometrial cancer cell line. *OncoTargets and Therapy* 2014:7 1397–1408.

II. Santos C, Attarha S, **Saini RK**, Boaventura V, Costa J, Khouri R, Barral-Netto M, Brodskyn C, Souchelnytskyi S. Proteome profiling of Human Cutaneous Leishmaniasis lesion. *Journal of Investigatvie Dermatology, In Press*.

III. **Saini RK***, Woksepp H*, Zakharchenko O*, Gautier A*, Souchelnytskyi N, Hellman U, Souchelnytskyi S. Proteomics-based network signaling by TGFβ1 in 184A1 non-tumorigenic human breast epithelial cells, and its role in phosphorylation of p53 at Ser392 and regulation of cell proliferation, (*manuscript*).

Table of contents

Abbreviations

AHP	Adult Hippocampal Progenitors
aNSC	Adult Neural Stem Cells
ASCL1	Achaete-Scute homolog Complex1
bHLH	basic Helix-Loop-Helix
bFGF	basic Fibroblast Growth Factor
BMPs	Bone Morphogenetic Proteins
BrdU	5-Bromo-2-DeoxyUridine
CHIP	Chromatin Immunoprecipitation
CNS	Central Nervous System
CSCs	Cancer Stem Cells
DBH	Dopamine-Beta-hydroxylase
2-DE	two-Dimensional gel Electrophoresis
DISC-1	Disrupted In Schizophrenia
DNA-PKcs(PRKDC)	Protein Kinase, DNA-activated, catalytic subunit
EGF	Epidermal Growth Factor
EMSA	Electrophoretic Mobility Shift Assay
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GO	Gene Ontology
GFAP	Glial Fibrillary Acidic Protein
HIF	Hypoxia Inducible Factor
^{3}H-thymidine	Tritiated thymidine
H&E	Hematoxylin and Eosin
IGF-1	Insulin-like growth factor
IHC	Immunohistochemistry
LSD1	Lysine-Specific Demethylase (LSD1)
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time of Flight

MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide
miRNAs	microRNAs
MMPs	Matrix Metalloproteinases
NR2E1	Nuclear receptor 2 family E member1
NB	Neuroblastoma
NSCs	Neural Stem Cells
NGF	Nerve Growth Factor
PBS	Phosphate-Buffered Saline
pI	Isoelectric point
shRNA	small hairpin RNA
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SNS	Sympathetic Nervous System
Sox2	Sex-determining region Y (SRY)-Box2
TLX	Tailless homologue of drosophila
TH	Tyrosine Hydroxylase
TMA	Tissue Micro Array
VEGF	Vascular Endothelial Growth Factor

Neural Stem Cells, TLX, and Neuroblastoma

Neurogenesis

Neural crest stem cells and the sympathetic nervous system

Nervous system formation starts in the beginning of the third week of embryogenesis. At this stage, the ectoderm undergoes inwards turning of neural folds, which gives rise to the neural tube. Transient neural crest formation occurs at the edge of the ectoderm. The neural crest cells have the property to migrate throughout the body and reach almost all organs. During this migration, neural crest cells undergo differentiation and form Schwann or glial cells, melanocytes, and sympathoadrenal progenitor cells. From these sympathoadrenal progenitor cells, the sympathetic nervous system (SNS) is derived. SNS's special feature relates to the organism's "fight or flight" response. There are three types of cells present in the SNS: chromaffin cells (adrenal medullary cells), small intensely fluorescent cells, and sympathetic neurons (known as neuroblasts during embryogenesis) (1).

Bone morphogenetic proteins (BMPs) are the early-secreted initiating factors, which direct neural crest cells commitment towards the sympathoadrenal lineage by triggering migration through the neural tube towards the dorsal aorta (2,3). Activated neural crest cells by BMPs migrate to the destined locations including adrenal medulla, sympathetic ganglia, and para-ganglia. Some key transcription factors also promote the neural crest cells into neuronal differentiation (4). Eventually, neuronal proteins like NF, STMN2, and GAP43 are activated by these transcription factors (5). In addition, enzymes like dopamine-beta-hydroxylase (DBH) and tyrosine hydroxylase (TH) that take part in the synthesis of catecholamines to become active, which is a significant characteristic feature of neuronal cells in the SNS (6,7).

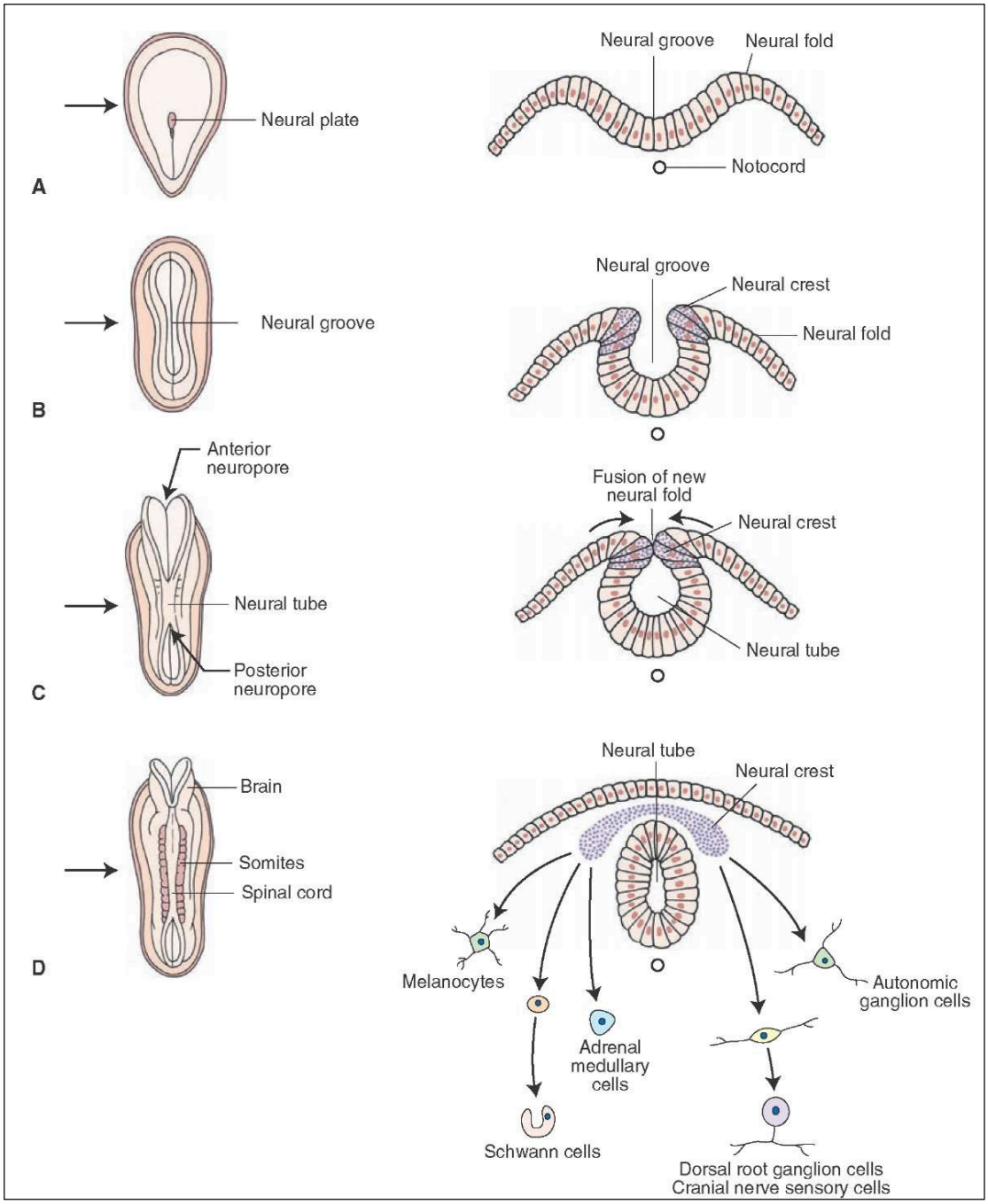

Figure 1: The illustration depicts neural tube development, neural groove formation, neural crest cells migration and differentiation into melanocytes, Schwann cells, adrenal medullary cells, dorsal root ganglion cells, cranial nerve sensory cells, and autonomic ganglion cells. Figure adapted from the following link: http://what-when-how.com/ neuroscience/development-of-the-nervous-system-gross-anatomy-of-the-brain-part-1 (8).

The transcription factor Achaete-Scute homolog Complex1 (ASCL1), also called Mash1 with a basic helix-loop-helix structure (bHLH), is activated by BMPs (6). Mutant ASCL1 mice have been shown to lack sympathetic neurons (9). In addition, other studies support the important role of ASCL1 in neuronal differentiation and also in activation of the transcription factor Phox2a. Another study reported that BMP, ASCL1, and Phox2a transcription factors together or alone are not sufficient to induce differentiation of sympathoadrenal progenitors (10). Furthermore, overexpression of BMP4 regulates expression of *dHAND* gene, which is yet another key regulator of sympathetic neuronal development, both *in vivo* and *in vitro*. Thus, BMPs regulate sympathetic neuron differentiation via Phox2a through its downstream activation of dHAND (11).

Growth factors such as basic Fibroblast Growth Factor (bFGF) and IGF influence sympathoadrenal progenitor cells for the expression of TrkC for the differentiation of sympathetic neurons (12). Depending on the availability of NGF, the neuroblasts are either stimulated, resulting in proliferation, differentiation, or undergo apoptosis (12,13). Disruption of the differentiation, survival, or apoptotic signaling in these embryonic neuroblasts is thought to be involved in the etiology of neuroblastomas (13-15).

Adult neural stem cells

For many years, the lack of substantial tissue repair or recovery of function after brain injury led to the assumption that new neuronal tissue was never produced in adult mammals. In the 1990s, different studies showed the presence of adult neural stem cells in both humans and rats, which could differentiate into neurons and glial cells (16-21). Repair mechanisms were thought to be limited to post-mitotic processes such as outgrowth of new axon terminals or synaptic reorganization. Since the development of methods using ^{3}H-thymidine to label dividing cells in the 1950s, evidence to the contrary has been mounting. More recently it has been shown that while neurogenesis is probably not possible in every area of brain tissue, there are a few specific sites containing stem cell-like cells that are capable of proliferation, generating a variety of mature central nervous system (CNS) cell types (22).

A variety of techniques can be used to investigate the capacity for proliferation and neurogenesis in adult organisms. An important *in vivo* method is the labeling of dividing and newly generated cells with exogenous nucleotides such as ^{3}H-thymidine or the more commonly used 5-Bromo-2-DeoxyUridine (BrdU) (23). Since BrdU is detectable by immunofluorescence it can be used in conjunction with antibody staining to confirm co-localization with specific cell type markers. Marker expression can itself be very useful, as there is a wide range of markers known to be fairly specific to different stages of neuronal differentiation (24,25). To exemplify, Sox transcription factors, GFAP, and Nestin may be expressed in neural stem or progenitor cells. Tuj1 and polysialylated neural cell adhesion molecule (PSA-NCAM) are found in immature neurons, and MAP-2 and NeuN are found in fully mature neurons. The main drawback to this approach is in the specificity of some of the markers, for example GFAP is expressed in mature astrocytes as well as stem cells, and PSA-NCAM can be re-expressed in some mature neurons under certain conditions. For this reason it is necessary to combine several markers together to confirm the identity of a cell (26,27).

Symmetric and asymmetric division

Characteristic feature of a stem cell (SC) is the capability to divide asymmetrically into two daughter cells, one stem cell and one non-stem cell. However, recent studies have shown that adult stem cells also divide asymmetrically (28,29).

Two types of stem cell divisions have been discussed in the past: 1) asymmetric division and 2) symmetric division. “Asymmetric division” suggests that each stem cell divides into one stem cell and one differentiated cell by the homeostatic regulation of the stem cell pool (28-30). Mechanisms regulating asymmetric divisions, involvement of cellular polarity, and orientation from the stem cell niche have been studied in *Drosophila* (30). However, the advantage of this model is to keep the SC population steady. The disadvantage of this is replenishment of stem cells during injuries. The other theory is “symmetric division” taking over to maintain the cell population steadies at the population level rather than at the single-cell level. Symmetric division results in two identical cells, but can result in two stem cells or two differentiated cells (31-34). However, maintenance of the stem cell niche is

regulated through numerous signals for proliferation and differentiation and has been studied by various research groups (35-53).

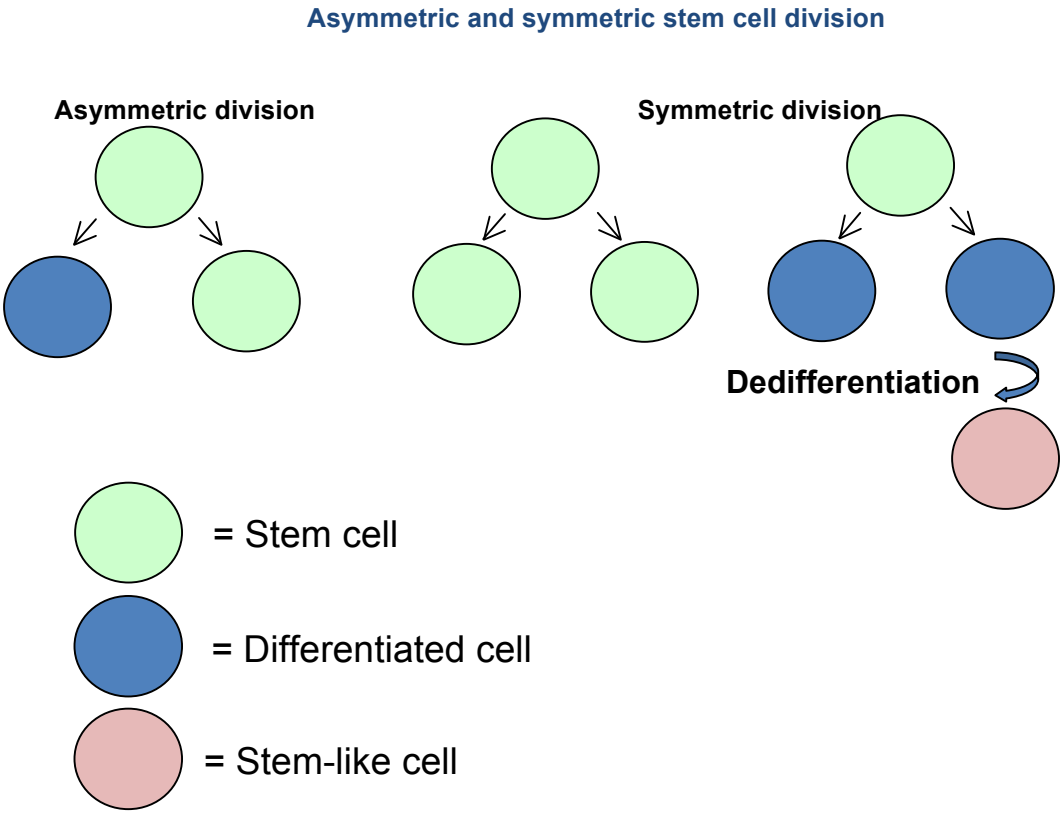

Figure 2: Illustration of asymmetric and symmetric division of stem cells. Asymmetric division gives rise to one stem cell and one differentiated cells, whereas symmetrical division gives rise to either two stem cells or two differentiated cells. Differentiated cell can revert and form stem-like cells through "dedifferentiation".

This thesis focuses on the regulation of stem cell-like characteristics in childhood cancer, namely neuroblastoma, which is derived from neural crest cells. There are different theories on how cancer stem cells arise, one is a stochastic, and another is a hierarchical model.

Stochastic and hierarchical models

The stochastic model and heirarchical model are contrary to each other. According to the stochastic model, each and every cell in cancer has the capability to become a cancer stem cell when needed. Various functions are partly controlled by some stochastically differing intrinsic factors. This means that activities are partially determined by the environmental conditions and the location of the cells. Experimentally, the stochastic model mimics the hierarchical model because of the low number of cancer stem cells (CSCs) present, differentiating with low proliferative capability. The hierarchical model posits that stem cells exist in tumors within the malignant population of a tumor, and are biologically very distinct. According to this model, CSCs only have the capability to maintain the self-renewing population. This model is designed to propagate malignant tumor cells from CSCs, although they are not genetically and functionally homogeneous. The clinical application of this model is to enable targeting of all CSCs to terminate tumor growth, which in turn will fail to relapse.

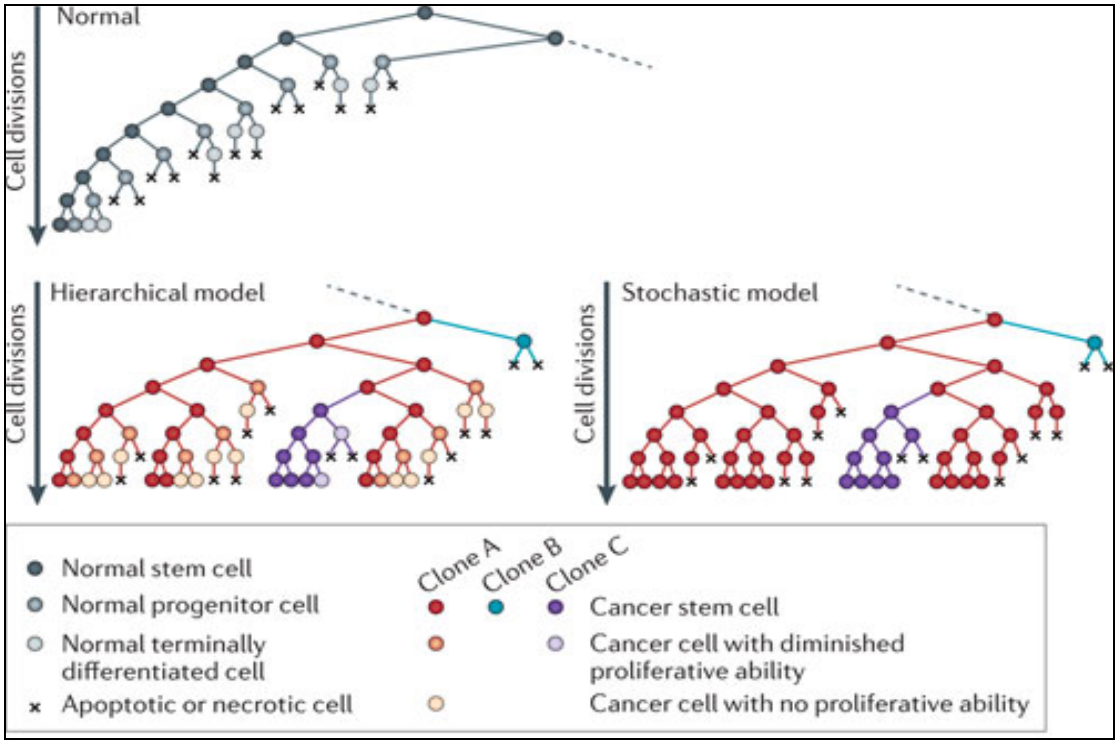

Figure 3: Schematic illustration showing cell division in normal and cancer cells. Normal cells have a flow to become progenitors and differentiate into specific lineage. In cancer two different theories are presented: Hierarchical and stochastic models. Hierarchical model says stem cells are important for keeping the cancer cell population, but in the stochastic model, any cell can become a cancer stem cell. Adapted from "Cancer stem cells: an evolving concept" *J Cell Phsyiol*. 2009 (54).

TLX (NR2E1)

Like any other stem cells, adult neural stem cells are regulated by various different transcription factors and epigenetic factors for maintenance, self-renewing, and differentiation of "neurogenic zone" throughout the life. TLX is an orphan nuclear receptor, as well as a transcription factor recognized for the first time in 2004 for its crucial role in maintenance of adult neural stem cells in self-renewing, undifferentiated, and proliferative states (55). Initially, TLX was discovered as a homolog of *Drosophila tailless* in vertebrates and is expressed in the neuroepithelial region of the embryonic brain (56). Later on, TLX has been reported for its varied expression and important roles in vertebrate brain (55, 57-63).

TLX expression in the mouse brain starts at embryonic day 8 (E8) and reaches its highest levels at E13.5, and after E16 it declines through neonate. Post-natal, TLX expression starts again and increases in the adult brain (58). TLX is expressed in vertebrate forebrains and in neural stem cells in the hippocampal dentate gyrus and in the subventricular zone (59). TLX is also expressed in the retina where it is important for retinal development and essential for vision (64,65). TLX knockout mice are normal at birth, but adult mice have reduced cerebral hemispheres, aggressive behavior, and show reduced learning abilities compared to wild-type mice. In addition, adult $TLX^{-/-}$ mutant mice also show severe retinopathy, as well as limbic and memory defects (58). TLX maintains neural stem cells in an undifferentiated and self-renewable state by co-operating with histone de-acetylases to repress downstream target genes, such as *p21* and *Pten* (66). Furthermore, TLX activates the Wnt/beta catenin pathway for maintaining adult neural stem cells in the self-renewing state (67). TLX also binds to its proximal promoter of the pluripotent stem cell marker Oct-3/4 during hypoxia in the presence of FGF for self-renewal of the adult neural stem cells (63).

In addition, TLX and Sox2 (SRY-box-containing gene2) proteins bind upstream of the *TLX* gene and physically interact with each other to form a complex on DNA that contains the consensus binding site for TLX, thereby regulating adult neural stem cells (68). MiR-137 is expressed in embryonic NSCs, where it controls cell fate determination. It negatively regulates embryonic NSCs for differentiating neural cells. Lysine-specific demethylase1 (LSD1) is a transcriptional co-repressor of TLX and

downstream target of miR-137. Whereas, miR-137 functions as a feedback regulatory loop of TLX and LSD1 to control neural stem cell proliferation and differentiation (69). MicroRNAs, miR-9 and let7b suppress TLX expression, leading to decreased proliferation and increased differentiation of NSCs. At the same time, TLX represses miR-9 pri-miRNA expression, forming a negative feedback loop, which may be important for keeping the balance between proliferation and differentiation of NSCs (70). In contrast, TLX is required for the activation of post-natal neural stem cells and also silencing of TLX does not give rise to glial differentiation spontaneously in mice (71).

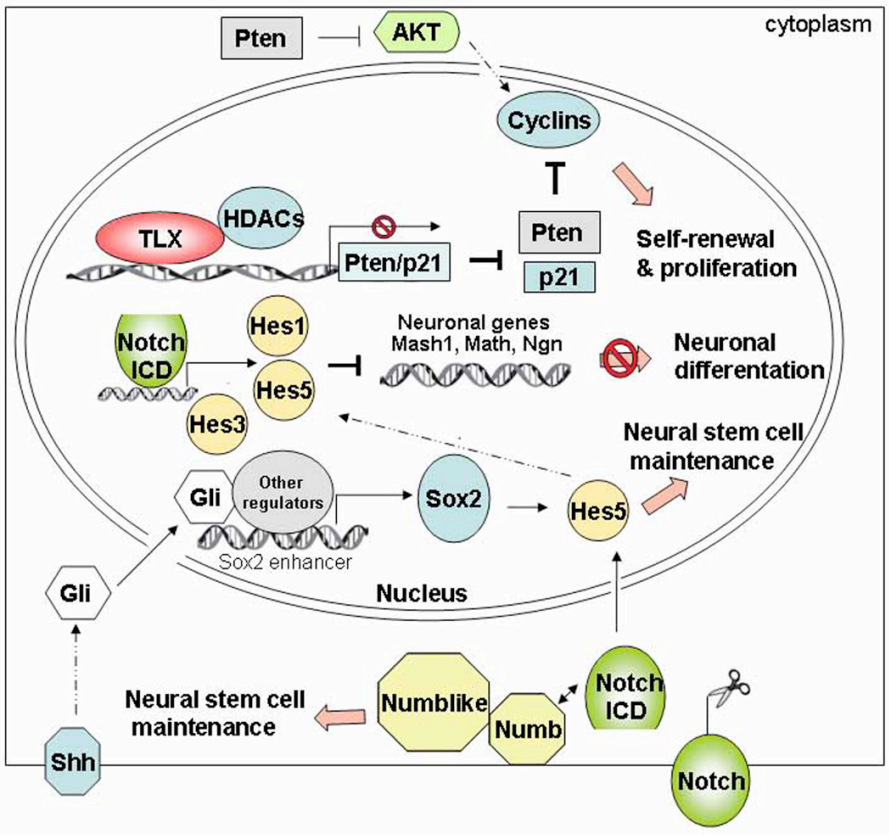

Figure 4: Schematic illustration showing TLX interacting with different molecules, directly or indirectly, to maintain neural stem cell self-renewal. TLX binds to the Pten/p21 and thereby maintains neural stem cells. Adapted from "Neural stem cells in the developing and adult brains" *J Cell Physiol*. 2009 (72).

Oxygen levels control TLX expression in the postnatal retina in pro-angiogenic astrocytes of postnatal retina. It is essential for the pro-angiogenic switch in response to hypoxic conditions, leading to assembly of fibronectin matrices and development of retinal vasculature. When astrocyte contact with blood vessels, oxygenation rapidly downregulates TLX expression and switches off proangiogenic activities, thus

maintaining vascular homeostasis (73). In concurrence with the important role of TLX in NSC self-renewal, TLX has been recognized to be involved in brain tumor initiation and progression. The *TLX* gene is amplified in certain primary glioblastoma samples and presents a poor survival (74). TLX expression can induce migration of NSCs from their natural niche and can initiate glioma development. Ectopic expression of TLX in the U87MG glioma cell line induced cell proliferation, colony formation and tumor formation *in vivo* (74,75).

So far, very little is known about the TLX interactome, it has been reported that it binds to the repressor atrophin-1, and the zinc finger transcription factor BCL11A (CTIP1/ Evi9) that is involved in many blood-related malignancies by playing a major role in the hematopoietic system. BCL11A is a novel co-regulator that might be involved in TLX-dependent gene regulation in the brain (76).

In neuroblastoma cell lines, TLX regulates angiogenesis by interacting with von Hippel-Lindau protein (pVHL) by direct binding to the pVHL to stabilize TLX. In normoxic condition, TLX binds and sequesters pVHL to stabilize HIF1-α. However, in hypoxia TLX increases its expression, and binds to the HIF-response element on the VEGF-promoter chromatin, and together with HIF2-α, recruiting RNA polymerase II to induce VEGF (77). TLX appears to be a component in regulating normal developmental and pathological processes. Further detailed studies are required to understand mechanisms used by *Tlx*.

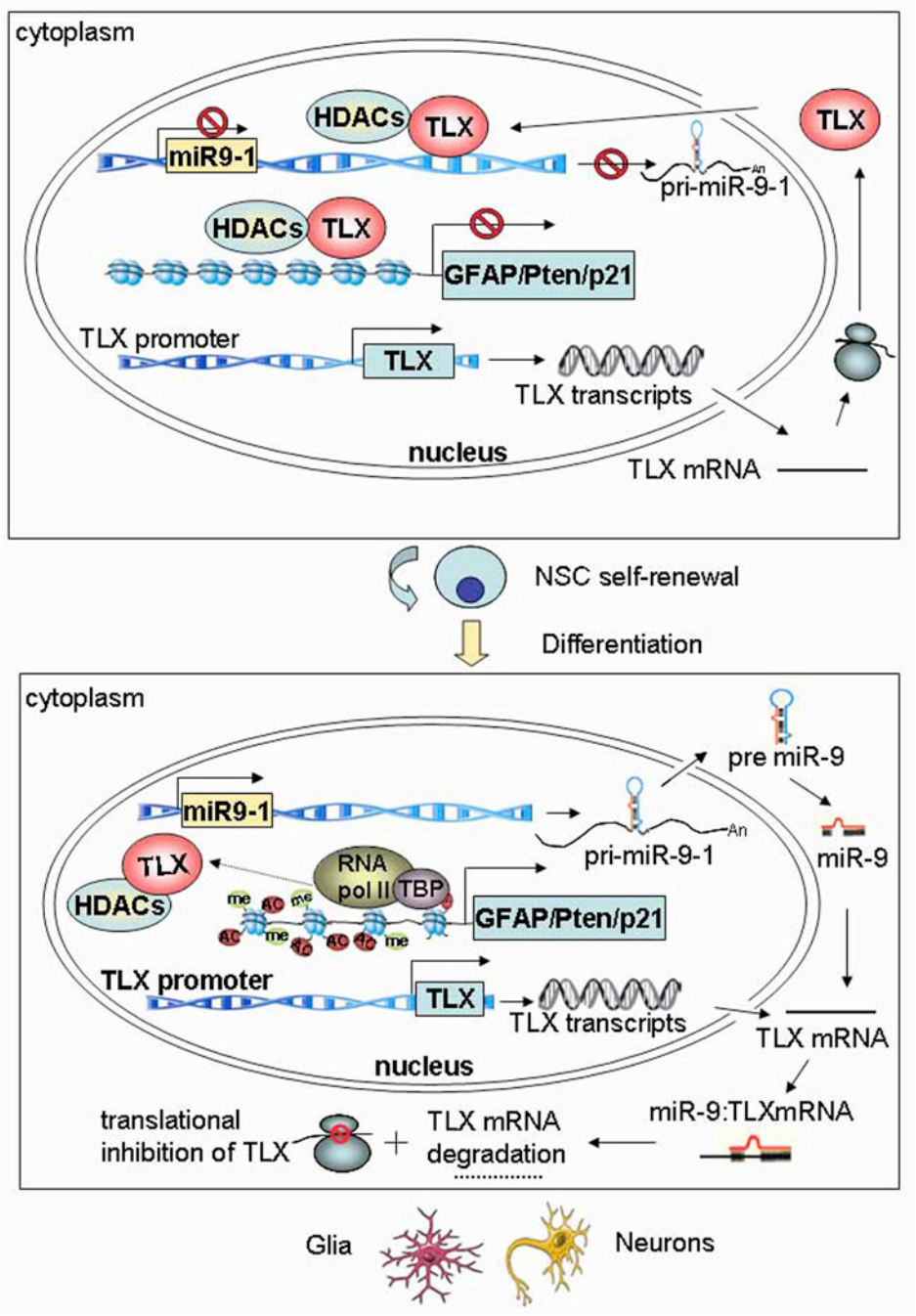

Figure 5: Illustration showing TLX interacting with miR9 promoter for self-renewing and differentiation of neural stem cells. Adapted from "Neural stem cells in the developing and adult brains" *J Cell Physiol*. 2009 (72).

Brief Introduction to Cancer

Cancer initiation and progression depends on various factors. Certain common features characterize cancers, essentially accumulation of genetic alterations. Causative mechanisms for the genetic abnormalities may vary, including mutations in DNA sequence, deletions, translocations, and amplifications, as well as epigenetic alterations. However, the result is often uncontrolled proliferation and immortality. Hanahan and Weinberg described these common features as “hallmarks” of cancer, including sustained proliferative signaling, inactivation of tumor suppressors, promotion of replicative immortality, resisting cell death, inducing angiogenesis, and activation of invasion and metastasis (79). When it comes to patient outcome the most important criteria is the type of cancer, the stage in which cancer is diagnosed, and if treatment is available (78).

Neural crest and disease development

Neural crest cells migrate to a large extent through the embryo, which is their most important feature. These cells gain various signals from their surrounding tissue microenvironments, which vary by developmental site and stage. The migrating neural crest cells are influenced by the molecular guidance given by various external factors and ligands acting on the receptors on the surface of the neural crest cells. The morphology, differentiation, and derivatives of the neural crest cells are also mediated by local tissue interactions. Genetically regulated cell-autonomous factors or exposure to environmental factors result in disturbances of differentiation, which then give rise to uncontrolled cell cycle or ectopic tissue formation. Disturbances in neural crest cell regulation are involved in different serious diseases such as neuroblastoma. Thus, neural crest cells have a major contribution in normal embryogenesis and in certain severe developmental diseases (80,81).

Neuroblastoma

Neuroblastoma is an embryonic tumor arising from the sympathetic nervous system during the fetal or early postnatal stage. It is believed to develop from incomplete precursor cells of the neural crest. It is a very heterogeneous disease and a common extracranial solid tumor. The median average age at diagnosis of the patients is 17

months and it is most commonly diagnosed during the first year after birth. Primary tumors frequently arise from the adrenal gland or sympathetic ganglia along the spinal cord, further metastasizing to the neck, chest, abdomen, or pelvis. A patient at low-risk have an excellent outcome with surgery, and sometimes the tumor regresses spontaneously. However for high-risk cases, tumors often relapse even after radiation and multimodal therapies. Neuroblastoma accounts for 12–15% of all childhood cancer deaths (83,84).

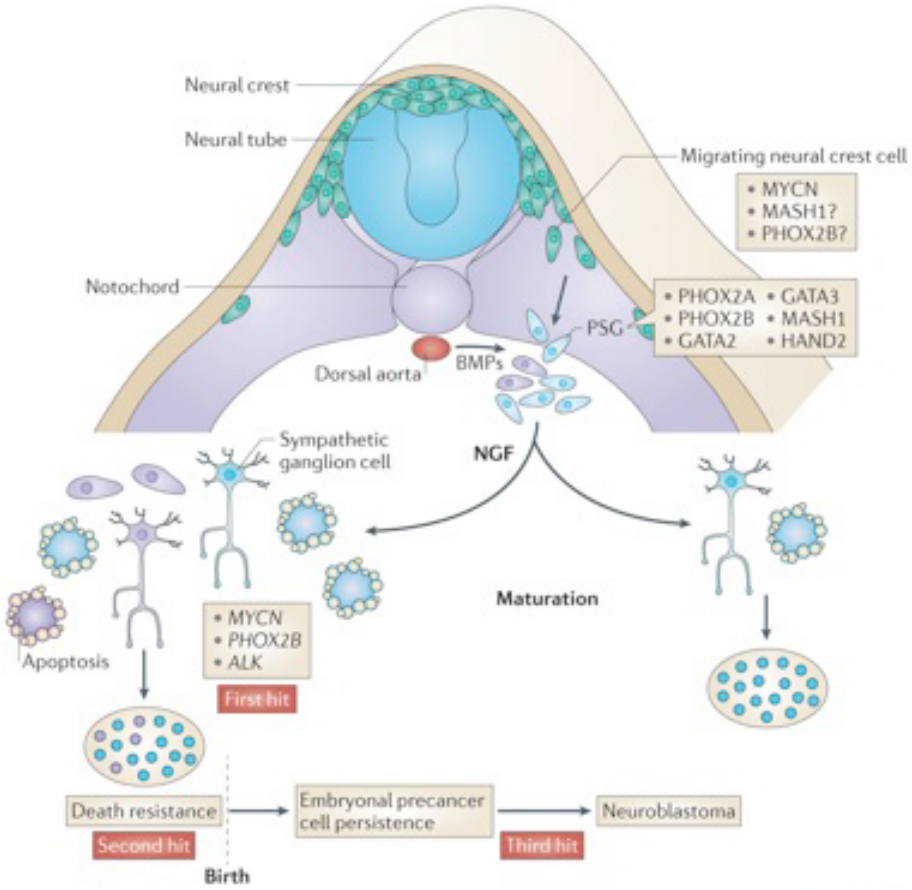

Figure 6: Illustration showing how neural crest cells migrate and neural tube formation. Proteins such as MYCN, MASH1, and PHOX2B help neural crest cells to differentiate. Mutations in various genes such as *MYCN, PHOX2B,* and *ALK* may cause neuroblastoma. Adapted from "The prenatal origins of cancer" from Nature Reviews Cancer, 2014 (82).

Histopathology, molecular characteristics, and risk factors of neuroblastoma

Neuroblastoma consists of cancerous cells that appear morphologically as small round and blue with hyperchromatic nuclei and a scant amount of cytoplasm (85). Tumors have been divided into four different histologic subtypes based on the degree of sur-

rounding tissues.

1. Schwannian stroma–neuroblastoma (stroma-poor)
2. Ganglioneuroblastoma–intermixed (stroma-rich)
3. Ganglioneuroblastoma–nodular (composite, stroma-rich/dominant, and stroma-poor)
4. Ganglioneuroma (stroma-dominant)

Furthermore, tumors are categorized into two prognostic subgroups based on the histology, *i.e.*, favorable or unfavorable. This includes the degree of neuroblast differentiation and also neuroblast nuclear morphology, as well as age of the patient (86). Molecular abnormalities include *MYCN* amplification, ploidy (DNA content), and allelic deletions of chromosome 1p and 11q. *MYCN* amplification occurs at the distal short arm of the chromosome 2 (2p24). In general, approximately 25% of the neuroblastoma patients have *MYCN* amplification. It is found in 5-10% of low-stage and 40% of advanced stages of the disease. In addition, it is seen with more than 10 gene copies per cell; is associated with advanced stages, tumor progression, and is a good prognostic indicator of aggressive behavior, being used in diagnosis since its discovery in 1983 (87,88). Normal human cells contain 23 pairs of chromosomes, whereas 55% of neuroblastoma is triploid or near triploid (also known as hyper-diploid), containing 58-80 chromosomes. The remaining 45% of neuroblastoma are either near diploid and contain 35-57 chromosomes, or near tetraploid and contain 81-103 chromosomes (89). There are allelic deletions at 1p and 11q. In tumors, deletions of genetic material indicate the loss of tumor suppressor genes. FISH experiments revealed that 30% of neuroblastoma have deletions at the short arm region 1p36, correlating with *MYCN* amplification and high-risk factors (90). The remaining 70% of advanced stage neuroblastoma have deletions at 1p (91).

Currently, the Children's Oncology Group risk stratification distinguished three risk groups of relapse, with prediction based on available patient biological prognostic variables from infants and children. The prolonged disease-free survival of a low risk are 95-100%, intermediate 85-90%, and the high risk are less than 30% (92). The low risk group includes infants at stage 4S with a favorable histology, no *MYCN* amplification, and with a DNA index of more than 1 (DI>1). The intermediate-risk group includes 0-12 year olds at stage 2A/2B with a favorable histology, no *MYCN*

amplification, with DI>1. This group also includes infants at 4S stage with unfavorable histology and DI=1. The high-risk group includes all patients older than 1½ years of age with stage 4, unfavorable histology, and DI=1 (92).

In a cohort of 194 high-risk neuroblastoma patients, 12.4% were found to carry a somatic mutation in the tyrosine kinase domain. Moreover, aberrant expression of anaplastic lymphoma kinase (*ALK*) is observed in hereditary neuroblastoma and the same has been attributed with mutations in chromosome 2, p23-p24 region. This finding suggests the role of *ALK* as one of the co-founding factors in the pathogenicity of hereditary neuroblastoma (93). Another gene, paired-like homeobox 2B (*PHOX2B)* has also been reported previously as a candidate gene for pre-disposition of neuroblastoma (94).

The identification of significant prognostic/diagnostic markers in clinical practice is a very complicated process. Sample selection, tumor heterogeneity, and differences in methods of data analysis of neuroblastoma markers make it a challenging task to identify diagnostic markers for NB. A systematic review of the literature between 1966 and 2000 concerning various cancer markers, revealed a total of 195 different neuroblastoma markers in 428 publications. The most frequent markers were studied to see how well they correlate with prognosis. It was found that these markers show variability both clinically and statistically. Thus, they have limited use in clinical applications (95).

Stem cells in neuroblastoma

Recent reports suggest that both neuroblastoma cell lines and tumors contain cancer stem/stem-like cells (96, 97), which is still a subject of debate. These cells were cultured under neural stem cell condition in the presence of bFGF, EGF, N2-supplement, and B-27 without serum. Under these conditions cells are able to self-renew, remain multipotent, highly malignant, and therapeutic resistant, and in tumors their frequency correlates with worse prognosis (96-98). Tumor-initiating cells (TICs) have been obtained from bone marrow metastases with high-risk neuroblastoma patients. These cells have been cultured under conditions promoting neurospheres and they recovered tumor-forming properties by xenograft (99). The identity of TICs in neuroblastoma *in vivo* is not yet fully identified because of their cellular

heterogeneity, which is a significant feature of neuroblastoma. Seventeen cell lines have been studied and existence of three different common features was observed among the cell lines and has been named N-type (neuroblastic), I-type (intermediate type), and S-type (substrate-adherent) cell lines. I-type cells have biochemical, morphological, tumorigenic, and differentiative properties. N-type cells have neuritic processes, scant cytoplasm, neurofilaments, granin, pseudoganglia, and expression of neurotransmitter enzymes. S-type cells have extensive cytoplasm, vimentin, and CD44 expression. I-type cells have bidirectional differentiation ability towards both N-type and S-type when induced by agents FSK, RA, and BUdR (forskolin, retinoic acid, and 5-bromouracil 2´deoxyribose). I-type cells are more malignant than N-type and S-type cells with significant 4-5 fold greater efficiency on soft agar colony formation assay and also 6-fold greater tumorigenicity in athymic nude mice (96).

Eight different cell lines of neuroblastoma including LA-N-5, SK-N-BE(2), IMR-32, CHIP-134, SHSY5Y, SK-N-SH, CHLA-20, and CHLA-79, from which the first four were found to be more malignant and have a high capacity for sphere formation. Three of these cell lines are capable of differentiating into multiple lineages, and LAN-5 cells have been showed to display verapamil sensitivity (96). Introducing genetically engineered oncolytic virus is the next generation anticancer therapy. Mahller et al (2009) has used nestin-targeted oncolytic herpes simplex virus (HSV), which killed TICs in neuroblastoma and also prevented the formation of the tumors in athymic nude mice (100).

Treatment regimen

Neuroblastoma is difficult to treat with medication in general, due to its heterogeneity. Towards testing new approaches in children, a low number of cases, heterogeneity, and ethical issues are complexities to overcome (101). Furthermore, drug kinetics, toxicity, and potential future side effects have to be considered when it comes to children. However, using the prognostic markers it is possible to diagnose and classify patients into different groups depending on the treatments required as in the table below.

Table 1. Phenotypic and Genetic Features of Neuroblastoma, Treatment and Survival According to Prognostic Category

	Prognostic Category			
Variable	***Low Risk***	***Intermediate Risk***	***High Risk***	***Stage 4S***
Stage*	1,2,3	1,2,3	3,4	4S
Pattern of Disease	Localized tumour; no *MYCN*-amplification	Localized tumour with locoregional lymph-node extension; metastases to bone marrow and bone; age at diagnosis <18 months; no *MYCN*-amplification	Metastases to bone marrow and bone; age at diagnosis > 18 months; *MYCN*-amplification may be present	Metastases to liveer and skin (with minimal bone marrow involvement); age at diagnosis<18 months; no *MYCN*-amplification
Tumour Genomics	Numerical aberrations	Numerical aberrations	Segmental aberrations	Numerical aberations
Treatment	Surgery	Surgery; Moderate-intensity chemotherapy	Dose-intensive chemotherapy, surgery, radiotherapy to primary tumour and resistant metastatic sitesM myeloablative chemotherapy with autologous hematopoietic stem-cell rescue; retinoic acid based therapy	Supportive care
Survival Rate (%)	>98	90 to 95	40 to 50	>90

Table 1: Stage categorization and risk classification with the treatment regimen is given in this table. Adapted from "Recent advances in neuroblastoma". *N Engl J Med (2010)* (102) Reproduced with permission from Maris JM.

Dedifferentiation

Dedifferentiation is defined as a cellular process in which differentiated cells go back to a less differentiated state, and possible to become stem-like/stem cells. Dedifferentiation is one of the important key features in progression of different cancers. However, little has been reported about mechanisms of dedifferentiation in

neuroblastoma. In a recent study by Jögi *et al.*, hypoxia-driven dedifferentiation was evaluated, and found that transcription factors Hif-1α and Hif-2α activate VEGF to induce dedifferentiation and contribute to tumorigenesis in neuroblastoma. Furthermore, hypoxia driven dedifferentiation seems to be a general phenomenon in solid tumors (105). Phox2b and its variants promote dedifferentiation of sympatho-adrenergic cells in neuroblastoma by increasing the expression of TLX3 (HOX11) and p75 (106). SOX2 is a pluripotent stem cell marker important for embryonic development and it has also been shown to be involved in cancer stem cells in which it promotes cell proliferation and tumorigenesis. In pancreatic cancer, SOX2 promotes cell proliferation and stemness/dedifferentiation through the regulation of a set of genes that controls cell cycle G1/S transition and epithelial-to-mesenchymal transition (EMT) phenotype (107). In colon cancer, elevated NF-κB expression promotes Wnt activation to induce dedifferentiation of cells and enhances tumor initiating capacity (108). TLX (NR2E1) is important for neuroblastoma dedifferentiation, thereby promoting tumorigenesis by targeting MMP2 and MMP9. Knowledge of mechanisms regulating dedifferentiation may lead to a better understanding of neuroblastoma and identification of novel tumor biomarkers and treatments (109).

Aim

The aim of the thesis was to investigate the role of TLX in adult neural stem cells and neuroblastoma, as well as to identify novel and potential diagnostic markers in neuroblastoma.

Materials and Methods

Cell Culture

Adult Hippocampal Cells (AHPs): Cells were cultured in Dulbecco's modified Eagle's medium/Ham's F-12 (1:1) containing N2 supplement (Invitrogen), L-glutamine (Cambrex), and 20 ng/ml of recombinant human bFGF (PeproTech EC). When used for experiments, cells were plated at different densities on polyornithine/laminin-coated plates in either proliferating condition, medium with 20 ng/ml bFGF, or in differentiating condition, medium without bFGF.

Neuroblastoma Cell lines: Cells were cultured in RPMI and DMEM medium containing 10% FBS, 1% ultraglutamine, and 1% penicillin streptomycin.

Sphere formation: neurospheres and tumor spheres

One of the most commonly used *in vitro* method for testing the proliferative and neurogenic capacity of neural progenitors is the neurosphere assay. Both non-renewing proliferative progenitors and self-renewing NSCs can be cultured as neurospheres, however only stem cell containing cultures can be repeatedly passaged. The frequency of colony forming cells can be determined by a limiting dilution neurosphere assay, in which cells are plated at reducing densities and the number of neurosphere negative wells used to calculate the proportion of proliferative cells. Cells cultured as neurospheres requires growth factors to maintain them, the most commonly used being EGF and bFGF, although Sonin Hedgehog (Shh) may be effective (16). Removal of these factors can be used to induce differentiation for analysis of the potential cell types generated, including neurons (112).

Immunoblotting and Immunofluorescence

Cells were harvested, proteins was separated on SDS gel, immunoblotted onto a PVDF membrane, and incubated with 5% bovine serum albumin in TBS with 0.1% Tween 20, and the membranes were probed with the monoclonal antibodies. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin-G was used as secondary antibody. Signals were detected by enhanced chemiluminescence.

Cells were fixed for 20 min with 4% paraformaldehyde in phosphate buffered saline

(PBS) for fixation. For intracellular protein staining, cells were permeabilized in 0.5% Triton X-100 for 3 min prior to staining. The cells were incubated in 10% FBS containing PBS for 1 hour at the room temperature for blocking. The cells were rinsed with 1× TBST and incubated with the primary antibodies diluted in the same blocking buffer overnight at 4°C. After three washes with PBST, the cells were incubated with Alexa Fluor 488/594 secondary antibodies (Molecular Probes) at a 1:2000 dilution. For nuclear counterstaining, the cells were incubated in 1 mg/ml Hoechst 33258 (Molecular Probes) for 30 min before being mounted in Dako fluorescent medium (Dakopatts AB). Cell counting was performed using Image 2000 analysis software (Zeiss) and confocal applying appropriate masks.

Migration Assay

Migration assays were performed using CytoSelectTM 24-well plate (Cell Biolabs, Inc., CBA-100-C), according to manufacturer's instructions. Briefly, an equal number of cells in suspension (1×10^5) in DMEM complemented with 2% fetal calf serum (FCS) were added to the upper compartment of the chamber, the bottom of which is a polycarbonate membrane insert with 8 mm pore size in a 24-well plate for migration assay to use serum as chemo-attractant, and incubated at 37°C for 16 h. The lower compartment contained DMEM with 10% FCS. At the end of the incubation period, non-migratory cells from the upper surface of the filter were wiped off with a swab. The lower surface of the filter was stained with a dye and extracted. A volume of 100 µl of each sample was transferred to a 96-well plate and the optical density (OD) at 560 nm wavelength was measured.

Enzyme linked immune sorbent assay (ELISA)

ELISA is a useful method for detection of the antigens that can be targeted by the antibody. Assay was performed using ELISA Kit (Invitrogen), according to manufacturer's instructions. Briefly, ELISA plates are coated with the specific antibody required from the company. Samples and controls were pipetted onto the coated wells. Plates were washed gently with ddH_2O once and 1× PBST twice. Blocking was done using blocking buffer for 60 min at room temperature. Wells were washed with 1×PBST and incubated with the biotin-conjugated primary antibody for an hour at room temperature then the wells were washed four times. Streptavidin-HRP solution

was added and incubated for 30 min at room temperature, after which the wells were washed four times. Stabilized chromogen was added and incubated in the dark for 30 min at room temperature. The OD was measured at 560 nm wavelength.

Animal xenograft

Tumors were established using a heterotopic model of tumorigenesis. Briefly, primary NB cells were re-suspended in PBS, mixed 1:3 with basement membrane extract (Trevigen) immediately prior to injection and injected in a 100µl volume into the inguinal fat pads of 4- to 5-week-old NOD/SCID (non-obese diabetic/severe combined immune-deficient) mice as approved by University Health Network's Animal Ethics Committee (protocols 09-004 and 2178.5). Animals were monitored weekly for evidence of tumor formation and were sacrificed when tumors reached 1.0-1.5 cm^3. Tumors were recovered and fixed in 10% formalin for 24 h prior to paraffin embedding. They were characterized as NB tumors by H&E staining and immunohistochemistry for the NB markers and determined to be free of Epstein-Barr Virus contamination by EBER1 *in situ* hybridization.

Electrophoretic mobility shift assay (EMSA)

The electrophoretic mobility shift assay is also called the gel retardation assay. It is used for detecting protein-nucleic acid interactions. It was initially developed for quantifying the interaction between DNA and proteins. However, since then it has evolved and can be used for different purposes, including the detection and also quantification of RNA-protein interactions. It is most commonly used for qualitative assays of DNA-binding proteins and of the respective consensus DNA or RNA sequences. In addition, under certain circumstances, it is also applied in quantitative studies including identification of binding affinities, stoichiometry, and kinetics (111, 112).

Briefly from the experiment, DNA probes were labeled with [^{32}P]ATP (ICN) using Klenow polymerase. The amplicons from Oct-3/4 promoter primers were gel-purified, and 500 ng was used for random labeling. The consensus TLX-binding site was synthesized as custom made oligos and annealed in the presence of salt. After further purification, they were used for isotope labeling. Nuclear extract was incubated in DNA binding buffer (10 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 1 mM

dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol) for 30 min at room temperature with ^{32}P-labeled DNA probe and 50 μg/ml poly(dI-dC) (Amersham Biosciences), with or without a 100-fold molar excess of cold competitors. Samples were separated on a 6–8% native polyacrylamide gel, and radiolabeled bands were visualized with BAS-2000.

Mass Spectrometry (MS)

MS is based on measuring molecular masses of biological molecules. This method can analyze a large number and a variety of chemical and biological compounds. The products of chemical synthesis, degradation of amino acids, proteins, lipids, nucleic acids, and glycans are analyzed by MS. Special names being based on the biological or chemical entities that can be analyzed include proteomics, glycomics, and lipidomics. Proteomics is a commonly used technique, which analyzes peptides, proteins, protein interactions, aglycans, or posttranslational modifications of proteins (PTMs). “Proteomics” is a study of the proteome, which means a whole protein complement from the cells or tissues. Proteomics is challenging due to the complexity of the proteomes. Recent advances concern improved identification and separation methods. MS became the core for one of the advanced methods in proteomic studies. Further, integration of MS with various other analytical methods made it easier to study many types of different samples derived from cells, tissues, organs, and organisms (113).

Proteome profiling

For proteome profiling, two-dimensional gel electrophoresis (2-DE), gel image analysis and MALDI-TOF mass spectrometry were used. In summary, cells were solubilized in urea-containing buffer for isoelectrofocusing. 2-DE was performed in IPG dry strips. The second dimension SDS-PAGE was performed in Dalt Six (GE Healthcare). This generated 2D gels which were stained with silver to detect proteins. Protein spots were analyzed using dedicated software (Image Master Platinum Version 6.0, GE Healthcare). All cellular proteins in 2D gels were considered for analysis. Protein spots which showed difference in expression between two tested conditions (dedifferentiated and differetiated cells) for more than 3-fold of the spot volume, or were unique, were taken for identification by MALDI-TOF mass

spectrometry. We used replicas of the gels to confirm identification of some of the proteins, i.e., a protein was identified independently in separate MS analysis of the same spots, excised from different replicas of 2D gels. Statistical significance of reproducibility of spot expression in 2D gels was evaluated by using the Image Master 2D Platinum Version 6.0 software. Student's t-tests were used to control significance of differences in expression of the selected spots. 2-DE allowed us to separate and detect proteins and their isoforms in native conditions, i.e. as full-length proteins as they were in cells and not as peptides generated by protease treatment. We could regularly detect 1000-1600 proteins were detected from speroid cultures in an average 2D gel.

Protein identification

Protein spots were excised from the gels, destained, and subjected to in-gel digestion with trypsin (modified, sequence grade porcine; Promega, USA), and tryptic peptides were concentrated and desalted using C18 ZipTip. Peptides were eluted with 65% acetonitrile, containing the matrix 4α-cyano acid CHCA, and applied directly onto the metal target and analyzed by MALDI-TOF MS on a M@LDI-R-TOF (Micromass/ Waters). Embedded software was used to process the mass spectra. Peptide spectra were internally calibrated using autolytic peptides from the trypsin (842.51, 1045.56, and 2211.10 Da). To identify proteins, we performed searches in the NCBInr sequence database using the ProFound search engine (http://65.219.84.5/service/prowl/profound.html). One missed cleavage, alkylation with iodoacetamide and partial oxidation of methionine, was allowed. Search parameters were set on mass tolerance less than 0.1 Da, no limitations of pI, limits of Mr of +20 and –20 kDa as compared to the migration position of a spot in 2D gel, and "mammalian" was selected for species search. Significance of the identification was evaluated according to the probability value, "Z" value, mass precision and number of the matched peptides, and sequence coverage (115).

Systemic analysis

Protein names were translated into gene ontology (GO) terms (http://biodbnet.abcc.ncifcrf.gov/). The GoMiner tool was used to identify functional domains affected by identified proteins from the systemic analysis and Cytoscape tools (http://discover.nci.

nih.gov/gominer/). GoMiner anlyses classification of identified proteins from proteomics into biologically coherent categories, and then assessing them. Interactions between the identified proteins from proteomics were analysed with Cytoscape, and a network of identified proteins was generated for analysis. MiMI plug-in was used to analyse related proteins and RNAs from the public databases. The network was viewed in Cytoscape; betweenness was analysed by network analysis, and network modules were extracted by AllegroMCODE tool. Fisher's exact test was used for calculating the P value to determine the network connectivity, and connections with $P<0.05$ were considered for the network. The FunCoup tool (www.funcoup.sbc.su.se) was used to build the small scale networks. The confidence score threshold was set at 0.50, to ensure significance of the network.

Results & Discussion

Paper I: Role of TLX in adult neural stem cells (aNSC)

Transcription factors are important for regulation of neural stem cell self-renewal and differentiation. However, regulation of these transcription factors is not fully understood. Recently, TLX has been implicated in maintaining aNSC in the stem cell self-renewing and proliferative state. We were interested in exploring the mechanism of TLX in maintaining aNSC. It is known that bFGF activates resting aNSC to initiate proliferation, and hypoxia further promotes it. Thus, we hypothesized that bFGF and hypoxia might synergize to enhance the expression of TLX in aNSC. Rat AHP cells were used in our study. We found that TLX was expressed in the presence of bFGF but not in the absence. While in hypoxia condition, TLX expression increased with the time course up to 24 hours, which further increased in the presence of bFGF (Figure 7).

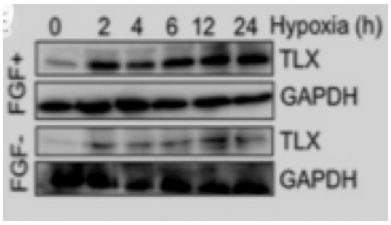

Figure 7: Immunoblot showing increased TLX expression upon hypoxia in the presence of FGF with the time course up to 24 hours. GAPDH was used as a loading control.

To elucidate the role of TLX upon hypoxia, differentiation of AHP cells was induced by withdrawing bFGF and addition of 0.2% FCS. After 7 days of culturing in these conditions, cells had formed extensive neurite growth and lost their round sphere morphology. TLX expression decreased by approximately 4-fold compared with undifferentiated cells. To see the TLX induction upon hypoxia, immunoblot was performed time-lapse in 0, 2, 4, 6, 12, and 24 hours and found a 2.5-3-fold increase in 2 hours, and 3-4-fold at 12 and 24 hours. After 24 hours, the level of TLX had stabilized. Since TLX is a transcription factor, we wondered whether TLX induces the reprogramming factor Oct3/4, which in turn, activates renewal of aNSC.

To assess whether there is a relationship between TLX induces Oct3/4, AHP cells were transiently transfected with TLX cDNA, and Oct3/4 mRNA was quantified by Q-PCR. Results showed TLX overexpression increased the transcript level of *Oct-3/4* by 2.1-4 fold. These results were also further validated by protein expression analysis by immunoblotting and immunofluorescence analysis.

To investigate TLX regulation of Oct3/4, we performed EMSA to assess its direct binding. EMSA results showed that TLX forms binding distinctly with the proximal promoter of Oct3/4. Results from overexpression and silencing of TLX experiments evidenced to support our results of TLX binding with the Oct3/4 promoter. In conclusion, our findings presented a novel role for TLX in regulating neuronal progenitor proliferation upon hypoxia by regulating the expression of the *Oct-3/4* gene

Paper II: Roles of TLX in neuroblastoma

Although, the mechanism of tumorigenesis in the nervous system is poorly understood, but recent studies show that cancer stem cells may initiate glioma (74,75). It has been known that the childhood nervous system malignancies arise either pre- or post-natal from existing embryonic cells. These cells originate at embryonic stages and initiate tumorigenesis. This indeed, may help researchers in identifying better strategies for early diagnostics and selection of therapeutic targets to prevent childhood cancers (116). As mentioned above, TLX is expressed highly at E13.5 in mice corresponding to the period of active neurogenesis, decreasing at birth. Later the expression increases in the aNSC again for their maintenance. The neural crest cells initiate migration at E10.5 from the dorsal aspect of the neural tube via dorsal aorta to further migrate to colonize the adrenal glands at E13.5 (82). It might be possible that these cells also express TLX, which remains to be examined.

We examined TLX expression in the neuroblastoma cell lines LAN-5, SK-N-BE2, SHSY-5Y, SK-N-SH, and IMR-32 and found expression in all of these cell lines. However, LAN-5, SK-N-BE2, and IMR-32 expressed higher TLX levels than SHSY-5Y and SK-N-SH. To find out the importance of TLX, we stably silenced TLX in IMR-32 cells and confirmed the efficiency of stable silencing with western blotting. Further analyses were performed using these stable clones.

To assess whether TLX is important for the sphere formation/dedifferentiation of neuroblastoma cells, both wild type and stably silenced TLX IMR-32 cells were cultured as neurospheres in serum-free media containing N2-supplement, B27-supplement, bFGF, and EGF. Cells were cultured for a period of three weeks by changing the medium every third day. After one week of culturing, cells formed distinct spheres in both wild type and siRNA control cells but stably silenced cells formed spheres very poorly even after three weeks, suggesting the importance of TLX for sphere formation. We tested these spheres for the expression of neural stem cell markers with co-expression of TLX and CD133, Nestin, Ki67, Oct3/4, and HIF-2α.

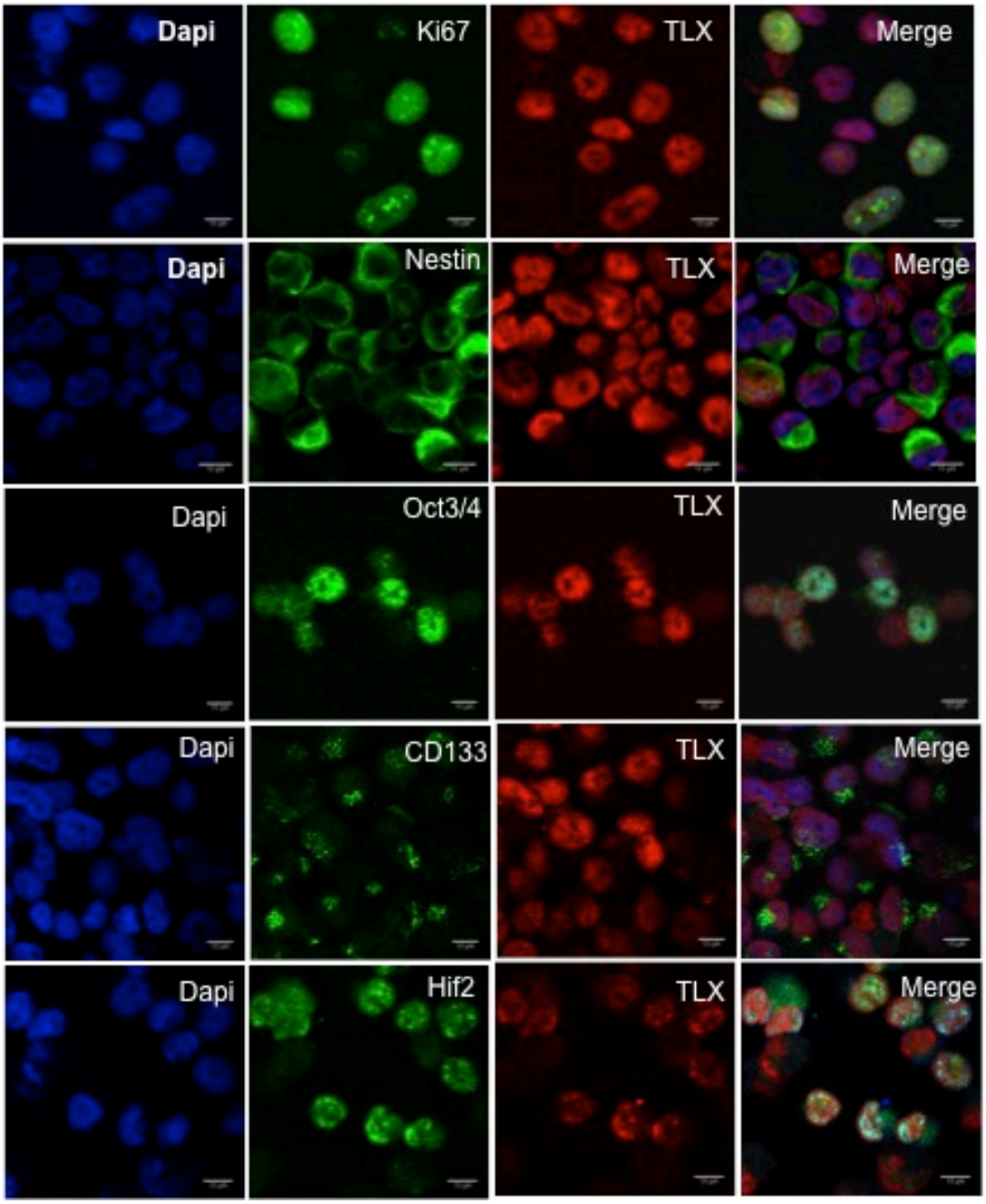

Figure 8: Immunofluorescence analysis shows that neuroblastoma SK-N-BE2c cell spheres were dispersed and stained for TLX (red) and co-stained for the indicated proteins (green). Scale bars, 10 μm.

Further, we wanted to see if these NB cell line-derived spheres form xenografts in nude mice. Thus, our collaborators in Canada performed the mice xenograft study with patient derived NB tumor initiating cells (TICs). We probed the tumor sections for the expression of TLX together with neuroprogenitor and migration markers. In the xenograft experiments, we found that TLX was co-expressed with MMP2 and CD15 in some areas of tumors. Therefore, we were also interested to see if TLX may promote metastasis in neuroblastoma cells since it is a property of neural stem cells to

migrate. Migration and invasion by colorimetric analysis of both IMR-32 wild type and TLX stably silenced cells showed a 2-3-fold reduction in migratory properties in TLX-silenced cells, suggesting that TLX promotes migration and invasion of neuroblastoma cells. These results were further supported by ELISA and RT-PCR analysis.

To identify interactions between TLX and MMP2, if any, we performed ChIP in both normoxic and hypoxic conditions. TLX-specific binding on the MMP-2 promoter consensus element by performing TLX capture using a biotinylated oligonucleotide encompassing the consensus element of TLX-binding site in the MMP-2 promoter. Our results show a 4.5-fold enrichment of TLX binding on the MMP-2 promoter site. Thereby, TLX promotes migration under hypoxic conditions by transcriptionally inducing MMP2.

We further wanted to confirm if the expression of TLX is also enhanced in neuroblastoma tissues obtained from patients. Two neuroblastoma tumor tissue microarrays including 10 cases of malignant neuroblastoma also showed increased expression of TLX when compared with 2 control tissues from normal peripheral nervous system normal sections. Furthermore, we have applied publicly available neuroblastoma-Versteeg-88 patients data of mRNA levels and clinical parameters, which showed that elevated expression of TLX correlated with the shorter survival in neuroblastoma patients by using Kaplan-Meier analysis. In summary, our results propose that TLX may be involved in tumorigenesis in neuroblastoma by activating different pathways. This means a synergistic amplification for “dedifferentiating” heterogeneous neuroblastoma cells maintained their stem like population surrounded by hypoxic environment, promoting malignancy. However, detailed studies are needed to confirm if TLX is a true driver of neuroblastoma malignancy by using a large number of clinical samples and more animal studies. In summary, the second paper demonstrates that TLX employs several pathways, amplifying each other to dedifferentiate NB cells and maintaining their progenitor population in a hypoxic environment.

Paper III: Novel diagnostic markers in neuroblastoma

Dedifferentiation is an obvious possibility that could promote tumorigenesis, malignancy, and that can confer resistance to treatment. Therefore, we furthered our efforts to study dedifferentiation of neuroblastoma cells by proteomics analysis to identify differentially regulated proteins between wild type and dedifferentiated tumor cells. This would give us an insight about the proteins that can promote stemness and tumorigenesis in neuroblastoma. The proteomics data would be further explored by a systemic analysis and validated with functional studies. Our previous study of TLX in neuroblastoma using a dedifferentiation model suggested that dedifferentiation is a process, which may promote neuroblastoma. The ability of neuroblastoma cells and tumors to form spheres is one of the manifestations of transformation and dedifferentiation.

To understand the mechanisms of dedifferentiation in neuroblastoma cells, we cultured spheres and performed a proteomics study to compare the spheres with wild type cells of the same cell lines. Our results showed that dedifferentiation induced extensive transformations in proteome profiles of the cells. We have identified 239 proteins that are altered due to dedifferentiation. These proteins are also involved in the hallmarks of cancer i.e. apoptosis, metabolism, angiogenesis, transcription, cell cycle, metabolism, adhesion, intracellular transport, and stress response. Further, we used Cytoscape analysis, which is a systems biology tool to establish the functional role of the identified proteins by evaluating the relationship between these proteins and publicly available data. We identified two proteins, DISC-1 and DNA-PKcs, that we find significantly centered and involved in the dedifferentiation and malignancy process. We have further validated our findings with the immunoblot analysis and Tissue Micro Array. Hence, we may propose that these two proteins may be good prognostic markers for aggressive neuroblastoma.

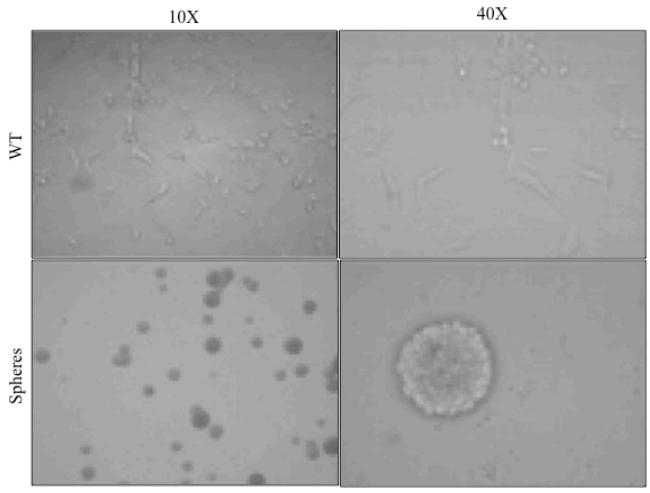

Figure 9: Neuroblastoma cell line SK-N-BE2 spheres cultured for two weeks in dedifferentiation medium, forming spheroids as compared with the wild-type (wt) cells. Phase contrast microscopic pictures taken by10× and 40× magnification, as indicated.

For dedifferentiation, we first cultured SK-N-BE2, IMR-32, and SHSY-5Y neuroblastoma cells in the sphere-forming medium. After two weeks of culturing, cells formed spheres. We used these spheres and wild type cells to perform western blot analysis to assess stemness markers such as CD133, Oct3/4, Sox2, Nestin, TLX, and Nanog. We further used these spheres and wild type cells to map protein expression with 2D gels. Though there was a similarity between wild type and spheres in total distribution of proteins in 2D gels, there was a significant variation among some proteins. We found similar patterns in all three cell lines, since it will be technically difficult to continue working with three cell lines, we continued our study with SK-N-BE2 cells.

We detected 533 common proteins expressed in spheres of SK-N-BE2 cells. A total of 330 proteins changed their expression and were unique and 3-fold more expression as identified by mass spectrometry. We unambiguously identified 239 proteins.

Among these, 120 showed elevated expression levels whereas 80 showed lower expression. To elucidate further biological functions of the identified proteins and its relationship between those proteins, we used GoMiner analysis. GoMiner analysis was used to identify biological processes and functions represented by the proteins and genes of interest. This tool helped us to identify functional clusters from the identified proteins. From this analysis, proteins were categorized based on their function, i.e. transcription, neurogenesis, development, and transport.

Cytoscape analysis gives the networks of interactions between the proteins and genes, which they may affect. We have used the identified proteins to generate networks between proteins and genes that are affected by functional categories “cancer”, “differentiated”, “stem cell”, and “secondary”. This analysis showed us the importance of regulators that were not previously associated with dedifferentiation and cancer, such as DISC1 and DNA-PKcs. PubMed searches helped us in supporting the information related to dedifferentiation, which involved 27 proteins identified in functional categories such as “cancer”, “dedifferentiation” and “stem cell”.

Further, we validated DISC1 and DNA-PKcs expression by immunohistochemistry tissue microarray. DISC1 and DNA-PKcs were selected also due to their positioning in the Cytoscape network and from the previous reports, showing that these two proteins may be important regulators of tumorigenesis. In addition, we have also searched publicly available data from the weblink http://hgserver1.amc.nl/cgi-bin/r2/main.cgi and found the neuroblastoma microarray data from 88 patients Versteeg MAS5.0 u133p2, which we used for DISC1 and DNA-PKcs. Kaplan-Meier analysis of the Versteeg R data indicated that increased expression of DNA-PKcs and DISC-1 indicates shorter survival rates for patients.

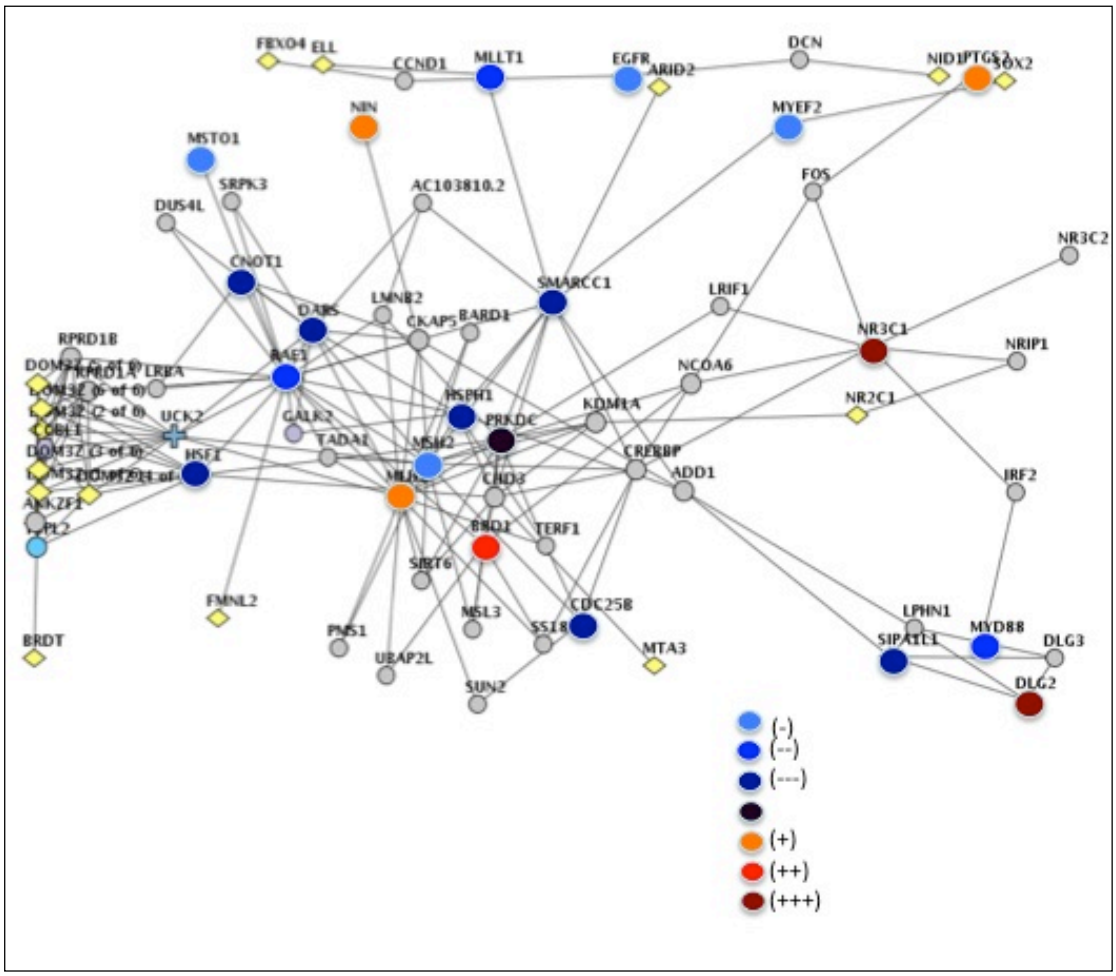

Figure 10: Image shows the network analysis from FunCoup of correlations between expression of the network nodes and survival of cancer patients. The network represents proteins involved in regulation of differentiation, stemness, and cancer, with color coding for their impact on survival of neuroblastoma patients. Color coding is indicated in the figure.

To conclude, our results show that proteins and genes which form a cluster in a network may have similar clinical value as prognostic markers. This demonstrates a new value of systemic analysis of omics-generated data for elucidation of functional roles of proteins and genes which have also shown similar clinical prognostic values.

Conclusions

This doctoral thesis focused on understanding of mechanisms behind tumorigenesis of the childhood cancer neuroblastoma. The goal was to study the key regulator TLX and to identify novel diagnostic markers for neuroblastoma.

- The mechanism of TLX in maintaining adult neural stem cells has been studied. TLX is a nuclear receptor and important regulator for maintenance of adult neural stem cells. TLX binds to the Oct-3/4 proximal promoter and thereby regulates NSCs proliferation, synergistically co-operating with hypoxia and bFGF.

- For the first time, TLX has been identified to be over-expressed in neuroblastoma and has been shown to be involved in progression of tumorigenesis. TLX promotes neuroblastoma cells to dedifferentiate and increase the expression of neural stem cell markers. TLX promotes invasion and migration by regulating MMP-2. *TLX* mRNA expression levels correlates with shorter survival rates analyzed by Kaplan-Meier analysis using neuroblastoma Versteeg-88 patient data.

- The proteomics study was focused on "transformation" and "dedifferentiation" as a hallmark of tumorigenesis in neuroblastoma. In this study, we used proteomics and systems biology to unveil the complexity of regulatory processes associated with dedifferentiation. We identified two novel diagnostic markers, i.e. DISC-1 and DNA-PKcs. This finding was further supported by results of our tissue micro array study and Kaplan-Meier analysis as described above with correlation to overall survival.

Future directions

The primary aim of this thesis was to elucidate the role of TLX in neuroblastoma. In addition, we have also studied novel diagnostic markers in this type of childhood cancer. We have used cell lines as a model system in a basic biology approach, which gave us a profound understanding of the mechanisms behind the development of this childhood cancer. However, for the future, some aspects such as early diagnosis and better treatment can be explored to make clinical translation possible.

We have mostly used neuroblastoma cell lines in our study, although we have also used some patient derived tumor initiating cells, tissues, and tissue microarrays. It will be important to clinically validate our study by using large-scale patient tissue samples, even if neuroblastoma is a rare disease presenting difficulties to collect patient tissues.

To be able to further validate our findings related to TLX, it is important to evaluate TLX expression and its role in a larger number of patient samples and in mice models. This also holds true for the novel diagnostic markers DISC-1 and DNA-PKcs.

Finally, dedifferentiation may have to be further evaluated because it may be a common phenomenon in many cancers. It is important to discern mechanisms behind this phenomenon in order to develop novel therapeutic strategies and targets.

These limitations are calling for further explorations and expansion of the studies reported in this thesis. Required experimental work is fully feasible, and I hope that the results presented in this thesis will be a good reference point for development of clinical applications, helping neuroblastoma patients.

Popular Science

Treasuring of "novel prognostic markers" in Neuroblastoma

Cancer has become a serious health problem worldwide, and scientists are focusing their attention on understanding how cancer develops. As scientists learn more about how cancer affects the body and hope to diagnose and treat cancer better.

Neuroblastoma is the most deadly common pediatric solid malignant tumor and causes death in 12-15% childhood cancers. Neuroblastoma starts in certain types of nerve cells (neural crest) found in the fetus. It usually occurs in infants and young children. Certain types of neuroblastoma may become very aggressive and therapy resistant. Primary tumors arise most commonly from the adrenal gland or sympathetic ganglia along the spinal cord, further metastasizing to the neck, chest, abdomen, or pelvis. Neuroblastoma varies between patients (heterogeneous) and some tumors can range from stage 4S (advanced stage) to spontaneous regression or differentiate into benign ganglioneuroblastoma or ganglioneuroma. The median age of diagnosis is less than two years of age and 80% of the cases are diagnosed before five years of age. In addition, tumors and cell lines revealed to have cancer stem cells. Presence of undifferentiated neuroblasts and amplification of the *MYCN* oncogene (gene that regulates cancer) predict the presence of the tumor in only 20% of the tumors. Hence, better screening and diagnostic markers are very much needed.

Neuroblastoma cell lines formed spheres (like small tumors) when cultured under neural stem cell media. These spheres expressed neural stem cell markers resembling cancer stem like cells. In the first paper of this thesis, we showed the mechanisms involved in maintenance of adult neural stem cells by a TLX protein. In the second paper, we identified how this protein regulates neuroblastoma and its prognostic value for the first time. Furthermore, in the third paper, we found two different proteins DISC1 and DNA-PKcs that can be very useful for better and early diagnosis in neuroblastoma. DNA-PKcs may be good diagnostic markers in human neuroblastoma. However, further validation studies by using a larger number of patient samples will help us to validate the clinical relevance of these proteins under diseased conditions.

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