TGF-β signaling in cancer

Erik Johansson

UNIVERSITY OF GOTHENBURG

Institute of Biomedicine

Department of Medical Chemistry and Cell Biology

The Sahlgrenska Academy

2010

ISBN 978-91-628-8208-2

Printed by Intellecta Infolog AB
Gothenburg, Sweden, 2010

ABSTRACT

Transforming Growth Factor Beta (TGF-β) is a cytokine regulating a wide range of cellular processes such as proliferation, differentiation, and migration. At the early stages of cancer development TGF-β functions as a tumor suppressor, mainly due to its inhibitory effect on cellular growth, but during cancer progression, mutations in TGF-β signal components switches TGF-β into a promoter of cancer cell proliferation, survival and metastasis.

The aim of this thesis was to study the role of TGF-β signaling in the progression of two different cancer types - diffuse-type gastric carcinoma and neuroblastoma. Furthermore, we wanted to investigate the function in neuroblastoma of the nuclear receptor TLX, a protein involved in neuronal stem cell maintenance, and if TLX interacts with TGF-β signaling.

We found that disruption of TGF-β signaling in diffuse-type gastric carcinoma cells led to accelerated tumor growth *in vivo* through the induction of angiogenesis, possibly due to the repression of anti-angiogenic proteins including TIMP2. In addition, TGF-β repressed expression of the cancer stem cell marker ABCG2 and diminished a subpopulation of cancer-initiating cells within the main cancer cell population, leading to reduced tumor formation.

TLX expression in neuroblastoma cells inhibited VHL-dependent degradation of HIF2α, which then could bind and activate the VEGF promoter. Silencing of TLX induced VHL protein levels, reduced hypoxia-dependent induction of HIF2α and inhibited cell proliferation. Furthermore, we found that TLX knockdown induced TGF-β response in neuroblastoma cells as seen by increased TGF-β dependent expression of its target genes p21 and Smad7. TLX physically interacts with Smad3 and knockdown of TLX led to increased TGF-β dependent nuclear translocation of Smad2/3.

In conclusion, the results from this thesis suggest that TGF-β signaling has an important tumor suppressive role in diffuse-type gastric carcinoma, due to both inhibition of tumor angiogenesis and repression of a cancer-initiating subpopulation of cancer cells. Furthermore, TLX was shown to be important for neuroblastoma cell proliferation, with a possible role in hypoxia-induced angiogenesis as well as in the regulation of TGF-β signaling in neuroblastoma.

List of publications

This thesis is based on the following articles which are referred to by their Roman numerals in the text:

I. **Johansson E**, Komuro A, Iwata C, Hagiwara A, Fuse Y, Watanabe A, Morishita Y, Aburatani H, Funa K, Kano MR, Miyazono K. Exogenous introduction of tissue inhibitor of metalloproteinase 2 reduces accelerated growth of TGF-β-disrupted diffuse-type gastric carcinoma.
Journal of Cancer Science 2010 Nov; 101(11):2398-2403

II. Ehata S, **Johansson E**, Katayama R, Koike S, Watanabe A, Hoshino Y, Katsuno Y, Komuro A, Koinuma D, Kano MR, Yashiro M, Hirakawa K, Aburatani H, Fujita N, Miyazono K. Transforming growth factor-β decreases the cancer-initiating cell population within diffuse-type gastric carcinoma cells.
Accepted Oncogene 2010

III. Zeng Z, Yoshida T, **Johansson E**, Lakshminarasimhan Chavali P, Hayashi A, Funa K. TLX controls angiogenesis through interaction with the Von Hippel-Lindau protein.
In manuscript

IV. **Johansson E**, Zeng Z, Yoshida T, Funa K. Nuclear receptor TLX inhibits TGF-β signaling in neuroblastoma.
In manuscript

Table of contents

List of abbreviations

ABCG2	ATP-binding cassette G2
ALK	anaplastic lymphoma kinase
Ang	angiopoietin
BMP	bone morphogenetic protein
CAF	cancer-associated fibroblast
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
Co-Smad	common-mediator Smad
CTGF	connective tissue growth factor
DAPK	death-associated protein kinase
DEC1	differentially expressed in chondrocytes 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
dnTβRII	dominant-negative TGF-β type II receptor
EB	epothilone B
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal transition
FBS	fetal bovine serum
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GIC	glioma-initiating cell
GSK3β	glycogen synthase kinase 3β
HBSS	Hank's balanced salt solution
HDAC	histone deacetylase
HGF	hepatocyte growth factor
HIF	hypoxia-inducible factor
HPRT	hypoxanthine phosphoribosyltransferase
HRE	hypoxia-responsive element
hTERT	human telomerase reverse transcriptase
IFN	interferon
IL	interleukin
I-Smad	Inhibitory Smad
LAP	latency-associated protein
LIF	leukaemia inhibitory factor
LTBP	latent TGF-β binding protein
MAPK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSC	neural stem cell

NG2	neuro-glial proteoglycan 2
OCT4	octamer-binding protein 4
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDGFRβ	PDGF receptor β
PECAM1	platelet/endothelial cell adhesion molecule 1
PHD	prolyl hydroxylase
RPMI	Roswell Park Memorial Institute medium
R-Smad	receptor-regulated Smad
SBE	Smad binding element
SDS	sodium dodecyl sulfate
SP	side population
TF	transcription factor
TRAF6	TNF receptor-associated factor 6
TAK1	TGF-β activated kinase 1
TβRI	TGF-β type I receptor
TβRII	TGF-β type II receptor
TBS	tris buffered saline
TGF-β	transforming growth factor-β
TIMP2	tissue inhibitor of metalloproteinase 2
TLX	tailless
TSP1	thrombospondin 1
VEGF	vascular endothelial growth factor
VEGFR	VEGF receptor
VHL	von Hippel-Lindau

1. Introduction

1.1 Transforming Growth Factor Beta (TGF-β)

1.1.1 TGF-β protein family

Transforming Growth Factor Beta is the prototypic member of a large family of secreted proteins that are involved in a wide range of cellular processes such as proliferation, differentiation, migration and apoptosis, and have important roles in embryonal development, tissue repair and immune system modulation. These proteins can be further divided into two subfamilies, depending on the signal pathways they activate - the TGF-β/Activin/Nodal subfamily and the bone morphogenetic protein (BMP) subfamily (Heldin et al., 1997).

1.1.2 TGF-β signaling

The TGF-β ligand, which exists in three isoforms (TGF-β1, TGF-β2, TGF-β3), is secreted into the extracellular matrix (ECM) in a heterotrimeric complex with latency-associated protein (LAP) and the latent TGF-β binding protein (LTBP). TGF-β remains inactive until it is activated and released from the latent complex in a mechanism involving thrombospondin 1 (TSP1), matrix metalloproteinases (MMPs) and integrins (Rifkin, 2005; Hyytiainen et al., 2004). In the canonical TGF-β signal pathway TGF-β initiates signaling by binding to and bringing together heteromeric complexes composed of TGF-β type I (TβRI; also called ALK-5) and type II (TβRII) receptor serine/threonine kinases on the cell surface. This allows TβRII to phosphorylate and activate the kinase domain of TβRI, which in turn transduces the signal through phosphorylation of the receptor-regulated Smads (R-Smads), Smad2 and Smad3, that form complexes with common-mediator Smad (Co-Smad), Smad4. The complexes then enter into the nucleus where they cooperate with other transcription factors and co-activators to regulate transcription of various target genes (Shi & Massagué, 2003).

1.1.3 Smad proteins

The Smad proteins are the vertebrate orthologs to *Drosophila* protein MAD, the first identified TGF-β signal mediator (Sekelsky et al., 1995). There are eight Smad proteins divided into three functional classes: five R-Smads, of which Smad 2 and 3 transduce TGF-β/Activin signaling while Smad 1, 5 and 8 transduce BMP signaling, the Co-Smad Smad4, and two inhibitory Smads (I-Smads), Smad6 and Smad7, that inhibit TGF-β signaling through competition with R-Smads for Co-Smad or receptor interaction as well as targeting receptors for degradation (Shi & Massagué, 2003).

R-Smads and Co-Smads have two conserved structural domains that are connected by a proline-rich linker region. The N-terminal MH1 domain interacts with the Smad Binding Element (SBE), which is defined as the sequence AGAC or the reverse complement GTCT, in the promoters of TGF-β target genes. The C-terminal MH2 domain is responsible for receptor interaction. The MH2 domain is highly conserved in all Smads. However, only the MH2 domains of R-Smads, but not Co- or I-Smads, contain Ser-Ser-X-Ser motifs which are phosphorylated by the type I TGF-β receptors (Souchelnytskyi et al., 1997).

R-Smad translocation and DNA-binding is also modified by several serine residues in the linker region that are phosphorylated by MAP kinases, cyclin dependent kinases and Ca^{2+}/Calmodulin kinase II (CaMKII), which indicates possible cross-talk with other signal pathways (Kretzschmar et al., 1997; Liu et al., 2005; Wicks et al., 2000). The linker region also contains a PY motif, which interacts with Smurf proteins that are E3 ubiquitin ligases that target Smads and TβRs for proteosomal degradation (Ebisawa et al., 2001). Smurfs also inhibit TGF-β signaling by binding to and facilitating Smad7 nuclear export (Kavsak et al., 2000).

Individual Smad proteins have relatively low specificity of DNA binding, so in order to produce specific transcriptional responses they must cooperate among each other and with other transcription factors and cofactors that may be cell-type restricted, thus enabling different gene responses in different cell types (Shi & Masague, 2003). Smads interact with both co-activators and co-repressors, which enables both activation and suppression of gene expression depending on the co-factors. Smads cooperate with proteins from various transcription factors families, such as the forkhead, homeobox, zinc-finger, AP1, Ets and basic helix-loop-helix families (Koinuma et al., 2009).

1.1.4 Non-Smad signal pathways

TGF-β also activates Smad-independent signal pathways, such as the Erk, JNK or p38 MAPK pathways, which may or may not regulate Smad activation. For instance, in cells with mutated TβRI that is unable to activate Smad, TGF-β was still able to activate p38 MAPK signaling (Yu et al., 2002). In response to TGF-β, TβRI interacts with TNF receptor-associated factor 6 (TRAF6) and promotes its binding to and ubiquitination of the MAPKKK TGF-β activated kinase 1 (TAK1) which activates the JNK and p38 pathways (Sorrentino et al., 2008; Yamaguchi et al., 2005). Furthermore, TβRII phosphorylates Par6, which recruits ubiquitin ligase Smurf1 that ubiquitinates and degrades the small GTPase RhoA, a protein regulating the assembly of tight junctions (Ozdamar et al., 2005).

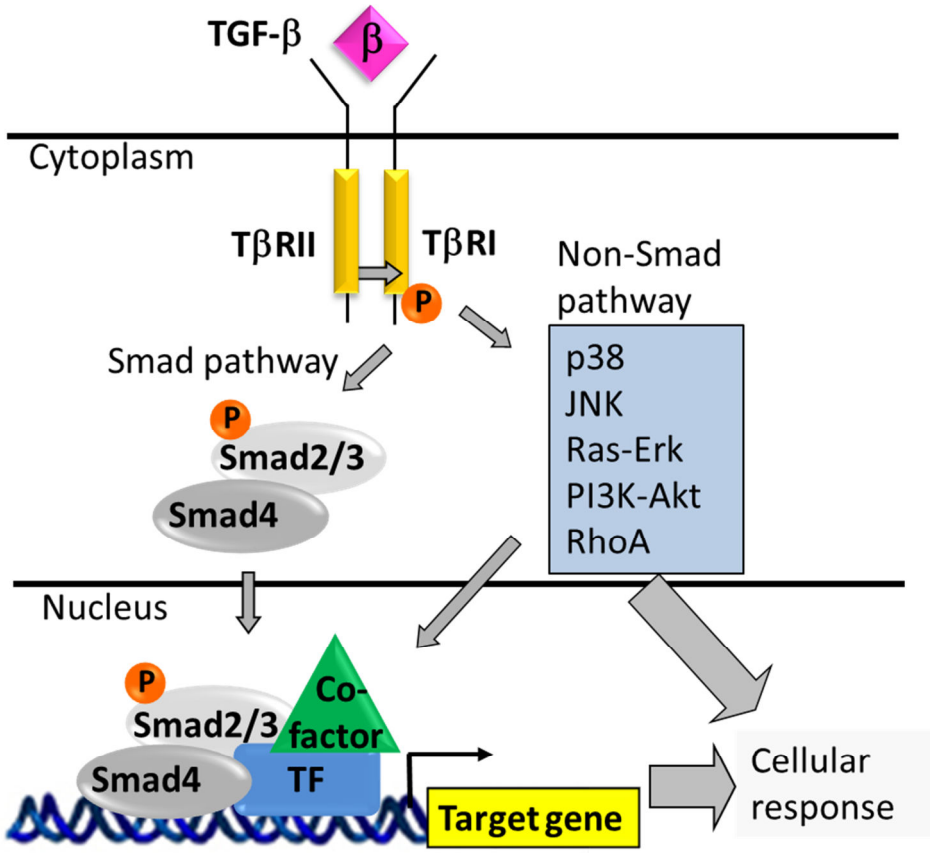

Figure 1: Schematic illustration of the TGF-β signaling pathway. (TF: transcription factor)

1.2 TGF-β in tumor development

The multifunctional nature of TGF-β signaling, affecting a wide range of cellular processes such as proliferation, differentiation, and migration, makes it one of the most important factors in the control of tumorigenesis. It is a potent antitumor agent mainly due to its inhibitory effect on cell proliferation and components of TGF-β signaling are therefore often mutated in malignancies. These mutations allow cancer cells to escape TGF-β dependent growth arrest, but may also lead to the conversion of TGF-β to an oncogene, inducing cancer cell proliferation, survival and metastasis at later stages of tumor development. The major functions of TGF-β signaling that affect tumorigenesis are summarized in figure 2.

1.2.1 Tumor suppressive functions of TGF-β

That TGF-β is an important tumor suppressor is evident by the frequent mutational defects of Smad4, TβRI or TβRII that are found in many malignancies such as cancers of the stomach, colon, prostate, breast, lung, liver and pancreas (Elliott & Blobe, 2005; Levy & Hill, 2006; Bornstein et al., 2009). Most malig-

nancies originate from epithelial cells, whose growth is inhibited by TGF-β that induces cell cycle arrest through the upregulation of cyclin-dependent protein kinase (CDK) inhibitors such as p15 and p21, as well as the repression of the growth-promoting transcription factors c-myc, inhibitor of DNA-binding-1 (Id1) and Id2 (Hannon & Beach, 1994; Datto et al., 1995; Yagi et al., 2002, Siegel & Massagué, 2003). Several pro-oncogenic factors such as SKI, MEL1 and BCL6 have been shown to assert its tumor promoting effects through the inhibition of TGF-β signaling (Suzuki et al., 2004; Heider et al., 2007; Takahata et al., 2009; Wang et al., 2008).

Apart from its ability to induce cell cycle arrest, TGF-β also inhibits cell growth by activating apoptosis through induction of proapoptotic proteins like Death-associated protein kinase (DAPK) and BIM (Yang et al., 2002; Ohgushi et al., 2005). In addition, recent evidence suggests that TGF-β has an inhibitory effect on cancer cell immortality by silencing the expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of telomerase, a protein that extend and stabilize chromosomal telomeres (Li & Liu, 2007). In the absence of hTERT, whose expression is undetectable in all normal cells except germ cells, telomeres are shortened in each cell division, ultimately leading to cell senescence and apoptosis, thus preventing dysregulated growth and survival (Li & Liu, 2007). However, in a majority of cancers, hTERT is expressed and telomerase is reactivated, leading to unlimited cell division and immortality of cancer cells (Shay & Bacchetti, 1997). TGF-β was found to inversely correlate with hTERT levels in colon and breast cancer cells and repress hTERT expression via direct binding of Smad3 to the hTERT promoter (Yang et al., 2001; Li, et al., 2006).

1.2.2 Oncogenic functions of TGF-β

Although TGF-β growth inhibition suppresses tumor progression in many cases, the expression of TGF-β1 in cancer cells was found to strongly correlate with progression and metastasis of prostate and colorectal cancers (Tsushima et al., 1996; Wikström et al., 1998; Friedman et al., 1995). TGF-β promotes proliferation of some mesenchymal cells, such as smooth muscle cells, as well as glioma and osteosarcoma tumor cells through the induction of platelet-derived growth factor (PDGF) A or B (Battegay et al., 1990; Bruna et al., 2007; Matsuyama et al., 2003). Hypomethylation of the *PDGFB* promoter led to increased PDGFB induction by TGF-β and associated with poor prognosis in glioma (Bruna et al., 2007).

In contrast to its proapoptotic effects, TGF-β was shown to inhibit apoptosis of mouse mammary carcinoma cells by inducing the expression of the transcription factor differentially expressed in chondrocytes 1 (DEC1) (Ehata et al., 2007).

DEC1 expression is high in breast cancer and correlates with tumor grade (Chakrabarti et al., 2004). Expression of a dominant-negative DEC1 mutant prevented metastasis of breast cancer cells *in vivo* (Ehata et al., 2007).

Furthermore, TGF-β also induces invasion and metastasis in many cancer types. TGF-β induces epithelial-mesenchymal transition (EMT), a process that occurs during embryonic development but also in pathological processes such as fibrosis and tumor metastasis. During EMT epithelial cells lose their cell-cell contacts and acquire a mesenchymal phenotype with increased motility and ability of invasion and vascular intravasalization, which are crucial properties for metastasis of cancer cells (Moustakas & Heldin, 2007). Apart from local invasion, TGF-β also contributes to the promotion of distal metastasis. TGF-β primes breast cancer cells for metastasis to the lungs through inducing expression of angiopoietin-like 4, a protein that facilitates the transendothelial passage of cancer cells (Padua et al., 2008).

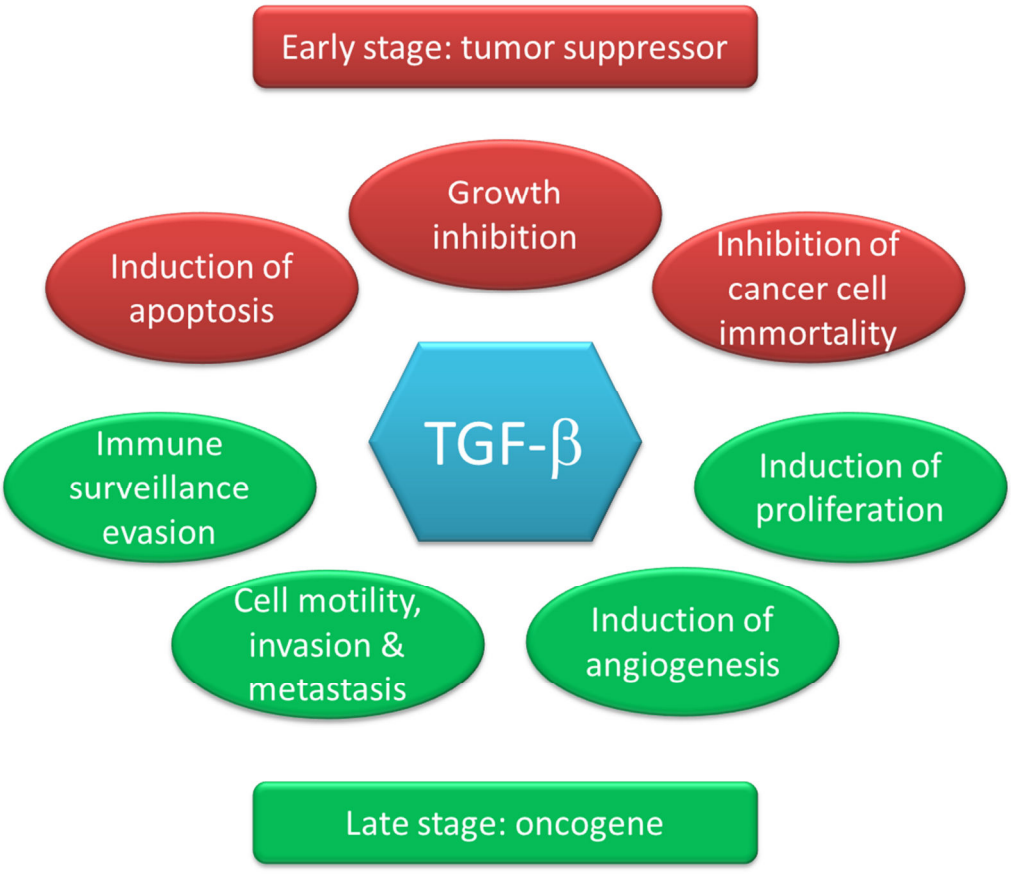

Figure 2: The major areas where TGF-β affects tumorigenesis (tumor suppressive functions in red and tumor promoting functions in green).

1.2.3 TGF-β effects on tumor microenvironment

Aside from its direct effect on cancer cells TGF-β also affects tumor growth through its regulation of the tumor microenvironment. Tumor cell production of TGF-β leads to tumor evasion from the host immune surveillance through several mechanisms, for instance by inhibiting the T-cell production of cytotoxic factors, such as the proapoptotic cytokines Fas-ligand and IFN-γ (Thomas & Massagué, 2005). However, loss of TGF-β signal in T-cells through the cell-specific knockout of Smad4 led to increased levels of cytokines including IL-5, IL-6, and IL-13 inducing expansion of stroma and spontaneous epithelial cancers in the gastrointestinal tract of mice (Kim et al., 2006)

TGF-β can activate fibroblasts into so-called cancer-associated fibroblasts (CAFs) that interact with and induce growth and cause malignant transformation of normal epithelial cells, which contributes to cancer progression (Stover et al., 2007). However, disruption of TGF-β signaling through loss of TβRII in fibroblasts induced proliferation of fibroblasts and promoted mammary carcinoma growth and invasion through upregulation of factors such as TGF-α, macrophage-stimulating protein (MSP) and hepatocyte growth factor (HGF) that were secreted by fibroblasts and activated their respective receptors on the adjacent carcinoma cells (Cheng et al., 2005).

TGF-β stimulates tumor angiogenesis and concurrent tumor growth through the induction of angiogenic factors such as connective tissue growth factor (CTGF) and vascular endothelial growth factor (VEGF) in epithelial cells and fibroblasts (Kang et al., 2003; Sánchez-Elsner et al., 2001), as well as the secretion and activation of matrix metalloproteinase (MMP)-2 and -9 in endothelial and cancer cells (Derynck et al., 2001). Mouse models using cells overexpressing TGF-β1 was shown to induce tumor angiogenesis and growth (Stearns et al., 1999).

1.3 Gastric carcinoma

Gastric cancer is the fourth most common cancer and the second leading cause of cancer death in the world. The incidence is especially high in East Asia, Eastern Europe, and parts of Central and South America, and is about twice as high in men than in women. The 5-year survival rate is less than 30% in most countries (Brenner et al., 2009; Hohenberger & Gretschel, 2003).

There are two types of gastric cancer, diffuse type and intestinal type, according to the Laurén classification (Laurén, 1985). Of the two histological subtypes of gastric carcinomas, there has been a marked decline in the incidence of distal, intestinal type gastric carcinoma, while the incidence of proximal, diffuse type gastric adenocarcinoma has been increasing, particularly in Western countries

(Miyahara et al., 2007; Henson et al., 2004). The diffuse type is more common in young patients and clinically characterized by rapid progression of disease and poor prognosis (Yashiro et al., 2005). Patients with this type of tumor often show thick stromal fibrosis with undifferentiated carcinoma cells scattered in the interstitium, which results in a stiff and thick gastric wall with reduced motility, but the tumors do not usually form ulcers or apparent mass lesions.

TGF-β signaling has been suggested to be an important factor in diffuse type gastric carcinoma. The thick stromal fibrosis observed in this cancer type is caused by interstitial collagen deposition induced by TGF-β produced by cancer cells and by cancer-associated fibroblasts (Tahara, 1990; Mizoi et al., 1993). Production of TGF-β1 was reported to associate with cancer progression and decreased survival (Kinugasa et al., 1998). However, disruption of TGF-β signaling due to loss of Smad4 expression has also been observed in diffuse-type gastric carcinoma (Kim et al., 2005).

1.4 Neuroblastoma

Neuroblastoma is the most common and deadly extracranial solid childhood malignancy, accounting for about 15% of all childhood tumor-related deaths (Brodeur & Maris, 2006). One hallmark of neuroblastoma is its considerable heterogeneity, both phenotypically and clinically, ranging from the relatively favorable highly differentiated tumors, which often spontaneously regress or differentiate into benign ganglioneuroblastoma, to the aggressive and highly metastatic undifferentiated tumors (Modak & Cheung, 2010). The median age of diagnosis is less than 2 years and 90% of the cases are diagnosed before 5 years of age (Brodeur & Castleberry, 2007). The 5-year survival rate, which was 74% for the period from 1999 through 2005, has improved in recent years, but this improvement is mainly due to increased cure rates in patients with benign tumor forms, while only slight improvement has been seen for children with high-risk neuroblastoma (Maris et al., 2007).

Neuroblastoma originates from immature or precursor cells of sympathetic neuronal lineage (Hoehner et al., 2006), and primary tumors occur where there is sympathetic nervous tissue, most commonly in the adrenal glands or along the spinal cord, giving rise to tumors in the neck, chest, abdomen or pelvis (Esiashvili et al., 2009). In a majority of neuroblastoma cases, metastasis have occurred at the time of diagnosis, with bone marrow, bone, liver and lymph nodes as the most common sites of spread (DuBois et al., 1999)

Few molecular markers have been found to associate with neuroblastoma outcome, with the most important being amplification of the *MYCN* oncogene,

which is strongly related to poor outcome when the copy number per cell is more than 10 (Schwab et al., 1983; Shimada et al., 1995). Neuroblastoma is most commonly a sporadic disease with only 1-2% of diagnosed cases displaying autosomal dominant inheritance (Knudson & Strong, 1972). Most cases of hereditary neuroblastoma are caused by activating mutations in the tyrosine kinase domain of the anaplastic lymphoma kinase (*ALK*) oncogene (Mossé et al., 2008).

1.5 Angiogenesis

Blood vessels are formed by two distinct processes – vasculogenesis and angiogenesis. Vasculogenesis, which mainly occurs during embryonal development, is a process in which angioblasts, endothelial cell precursors, differentiate into endothelial cells that proliferate and form a primitive tubular network (Papetti & Herman, 2002). During angiogenesis, this network of vessels is remodeled through sprouting and branching of new vessels from preexisting ones to form the interconnecting branching pattern characteristic of mature vasculature. Stabilization and maturation of vessel walls occur through the interaction of endothelial cells with supporting cells, such as smooth muscle cells and pericytes, and the surrounding extracellular matrix (ECM).

The ECM is a network of proteins and carbohydrates deposited in the extracellular space that bind together cells and give structural support to tissues, but also bind to and interact with cells to activate intracellular signal transduction that regulates processes such as adhesion, migration, proliferation and differentiation (Daley et al., 2008). The ECM is made up of three major components – the fiber-forming molecules such as the collagens, which provide tensile strength to tissues, the non-fiber-forming proteoglycans and glycoproteins, that give volume to the ECM and permits diffusion of small molecules between cells, and the matricellular proteins, that modulate cell-matrix interactions (Bornstein & Sage, 2002). The basement membrane is a specialized form of ECM that separate epithelium from stroma in all tissues, and also form a layer on the outside of blood vessels, supporting them and allowing molecules to pass between endothelial cells and the stroma (Kalluri, 2003).

Angiogenic sprouting is responsible for vascularisation of certain structures during normal development such as the neural tube, and also for most new vessel formation in the adult, such as during wound healing and the female reproductive cycle (Yancopoulus et al., 2000; Papetti & Herman, 2002). The sprouting process begins with destabilization of preexisting vessels through the removal of pericytes, leading to degradation of the vessel basement membrane and ECM, a process aided by MMPs (Moses et al., 1997). Endothelial cells can then, in re-

sponse to growth factors like VEGF and fibroblast growth factor (FGF), proliferate and migrate to form a new vessel branch, which then mature through the recruitment of pericytes, a process stimulated by the release of PDGFB from endothelial cells (Hellstrom et al., 1999), followed by formation of new ECM and basement membrane.

1.5.1 Vascular endothelial growth factor

Vascular endothelial growth factors (VEGFs) are important molecules for blood vessel formation, especially VEGFA, which is required for initiation of immature vessels by vasculogenesis or angiogenic sprouting both in development and in the adult. VEGF stimulates proliferation and migration and inhibits apoptosis of endothelial cells, inducing angiogenic sprouting and capillary formation (Nussenbaum & Herman, 2010).

VEGFA is a member of a family of growth factors also including VEGFB, -C and -D, that bind to a set of cell-surface receptor tyrosine kinases (mainly VEGFR-1, -2 and -3), triggering intracellular responses/signal transduction. The receptor mediating the major growth inducing properties of VEGFA is VEGFR-2 (also known as KDR or Flk-1), which is expressed on the surface of endothelial and hematopoietic precursors (Eichmann et al., 1997). VEGFR-2 knockout mice fail to develop vasculature (Shalaby et al., 1995). The same is seen in mice with VEGFA knockout (Carmeliet et al., 1996; Ferrara et al., 1996). The importance of regulation of VEGFA levels is evident in pathological conditions with induced VEGF expression, such as diabetic retinopathy, where an abnormal angiogenic response leads to leaky and haemorrhagic vessels (Aiello et al., 1994; Adamis et al., 1994).

1.5.2 Angiopoietins

Angiopoietins are growth factors that bind to Tie receptors on endothelial cells (Klagsbrun & Moses, 1999). Interaction between Angiopoietin 1 (Ang1) and the Tie2 receptor does not seem to be needed for the initial proliferation and tube formation of endothelial cells, but is important for pericyte recruitment, sprout formation and stability of vessels. Mice with knockout of either Ang1 or Tie2 develop normal primary vasculature but later die due to incomplete vascular remodeling (Suri et al., 1996). Overexpression of Ang1 in transgenic mice led to increased vessel number, size and branching, and also caused resistance to vessel leakage induced by VEGF or inflammatory agents (Suri et al., 1998; Thurston et al., 1999). Angiopoietin 2 (Ang2) binds to Tie2 and partly antagonizes Tie2 activation by Ang1, leading to pericyte loss and vessel destabilization which may prevent excess angiogenesis but also making vessels more plastic

and responsive to sprouting and tube formation induced by VEGF (Ferrara & Kerbel, 2005; van Kempen & Leenders, 2006).

1.5.3 Tumor angiogenesis

During the initial stages of tumor development the tumors are not vascularized, but survive on vasculature available in the surrounding host environment (van Kempen & Leenders, 2006). However, to grow past a size limit of about 1-3 mm^3 the tumor needs increased blood supply for nutrient delivery and waste removal. In order to continue growth the tumor induces a so-called angiogenic switch, induced by intra-tumor hypoxia, where the tumor changes to an angiogenic phenotype, inducing the formation of new blood vessels from the preexisting vasculature in a process similar to normal angiogenesis. However, these tumor-induced vessels are abnormal, usually made up of both endothelial and tumor cells (Chang et al., 2000) lacking functional pericyte coverage (Benjamin et al., 1999) and a complete basement membrane, which makes them leaky and dilated (Carmeliet et al., 1996).

1.5.4 Activators of tumor angiogenesis

Similar to normal angiogenesis, VEGFA expression, which is stimulated by hypoxic conditions in the central necrotic part of solid tumors (Graeber et al., 1996), has a central role in tumor angiogenesis. VEGFA mRNA is upregulated in most human cancers (Ferrara, 1999) and many tumor cell lines secrete VEGFA *in vitro* (Senger, et al., 1986). In metastatic human colon carcinoma, increased levels of VEGFA and VEGFR2 directly induced tumor vascularization (Takahashi et al., 1995). Apart from producing VEGFA themselves, tumors can also induce VEGFA production in surrounding stromal cells (Fukumura et al., 1998). Besides stimulation of vessel growth, VEGFA also increase the vessel permeability, enabling tumor cells to disseminate from the primary tumor site (Saaristo et al., 2000).

Although VEGFA seems to have the major role in tumor angiogenesis, there are many other factors that, often through cooperation with VEGFA, induce tumor neovascularization. The role of Ang2 in angiogenesis seems to be more important in tumor vasculature, where its expression is much higher than that in endothelial cells of normal blood vessels (Zagzag et al., 1999). Ang2 seems to have a specifically important role in a process called co-option, where tumors grow along existing vessels without inducing an angiogenic response (Yancopoulos et al., 1998). In the early, avascular stage of tumor growth, when VEGFA levels are low, Ang2 expression in tumor cells is induced, resulting in blood vessel destabilization and regression, which in the later stages, when VEGFA is highly expressed, make the vessels more responsive to VEGFA-

mediated growth (Maisonpierre et al., 1997). Furthermore, Ang2 contributes to the leakiness of tumor vessels through antagonizing Ang1 vessel stabilization (Carmeliet et al., 1996)

Fibroblast growth factor (FGF) was the first tumor-derived factor found to stimulate endothelial cell proliferation and neovascularization *in vivo* (Folkman et al., 1983). Inhibition of the FGF receptor led to decreased tumor growth and vessel density in mice implanted with tumor xenografts (Folkman et al., 1971; Compagni et al., 2000). The effect of FGF seems to be at a later stage in tumor development than that of VEGFA, which suggests that FGF is more important for maintenance than initiation of tumor angiogenesis (Compagni et al., 2000). FGF and VEGFA were shown to induce proliferation and formation of cordlike structures by bovine capillary endothelial cells in a synergistic manner (Goto et al., 1993). FGF also upregulates VEGFA mRNA and protein production (Deroanne et al., 1997; Stavri et al., 1995).

Interleukin 8 (IL8) is a growth factor produced by macrophages that appears to have a major role in tumor angiogenesis. Increased IL8 expression correlates with increased neovascularization and tumor growth (Luca et al., 1997, Yuan et al., 2000). IL8 stimulates production of MMP2 which degrades the basement membrane and remodels the ECM, thus initiating tumor angiogenesis and tumor cell invasion and metastasis (Klagsbrun & Moses, 1999).

1.5.5 Inhibitors of tumor angiogenesis

There are many endogenous inhibitors of angiogenesis whose expression is important for quiescence of vasculature in normal tissues. Loss of expression of these anti-angiogenic factors is associated with increased tumor angiogenesis and growth.

Thrombospondins are multidomain glycoproteins that are highly expressed during tissue remodeling associated with wound healing and tumor progression (Agah et al., 2002). Thrombospondin 1 (TSP1) was the first identified natural inhibitor of angiogenesis (Good et al., 1990). TSP1 contains three Type 1 Repeats (TSRs) that interact with endothelial cell membrane protein CD36 to inhibit migration and induce apoptosis of endothelial cells (Dawson et al., 1997; Jimenez et al., 2000). The TSR of TSP1 can also bind and activate latent TGF-β (Schultz-Cherry et al., 1994), which may lead to inhibition of tumor growth due to growth inhibitory effects of TGF-β (Miao et al., 2001). In addition, TSP1 inhibits MMP9, thus suppressing the release of VEGF from the ECM (Rodriguez-Manzaneque et al., 2001), and binds to VEGF directly and mediates its uptake and clearance (Greenaway et al., 2007).

Tissue inhibitor of metalloproteinase 2 (TIMP2) is a member of a family of endogenous inhibitors of MMPs that bind to the catalytic site of activated MMPs and inhibit their protease activity, indirectly inhibiting angiogenesis (Umenishi et al., 1991; Baker et al., 2002). TIMP2 also has direct effects on endothelial cell proliferation. TIMP2 interacts with integrin α3β1 on endothelial cells, leading to dissociation of the tyrosine phosphatase Shp1 from the integrin complex, and the subsequent association with and inactivation of FGFR and VEGFR2 and reduced FGF2- or VEGFA-dependent cell proliferation *in vitro* and angiogenesis *in vivo* (Seo et al., 2003). TIMP2 also induces Shp1-mediated induction of $p27^{Kip1}$ expression and growth arrest of endothelial cells (Seo et al., 2006). TIMP2 was reported to function as an inhibitor of tumor progression and invasion in several cancer models including breast and liver cancer (Mendes et al., 2007; Lee et al., 2005; Brand et al., 2000).

Other important inhibitors of tumor angiogenesis include the interferons (IFN-α, -β, -γ). IFN-α inhibits endothelial cell migration and IFN-α and -β both downregulate FGF expression in many cancer cells (Sato et al., 1995; Singh et al., 1995). Interleukin 4 (IL4) suppresses tumor growth through inhibition of tumor cell proliferation and induction of immune response against tumors (Topp et al., 1995, Tepper et al., 1992), but also negatively regulates tumor neoangiogenesis through inhibition of endothelial cell migration towards FGF *in vitro* and FGF-dependent vascularization of rat corneas *in vivo* (Volpert et al., 1998).

1.6 Hypoxia

Tissue hypoxia is a condition where there is a decrease in physiological oxygen levels in a tissue, and is observed in vascular disease or in cancer. Hypoxia activates a number of cellular processes with the aim of reinstating oxygen homeostasis.

Tumor hypoxia, a common feature of solid tumors, occurs when tumors grow to a size where the surrounding blood vessels no longer can deliver sufficient nutrients to the tumor tissue. Tumor cells located more than 0,1-0,2 mm from the nearest vessels will undergo apoptosis or necrosis (O'Reilly et al., 1996). Tumors might remain dormant in this avascular state for years, with an equal rate of proliferation and cell death. In order for tumor growth to progress beyond this state, the tumor cells adapt to the hypoxic conditions by inducing a switch from aerobic to anaerobic metabolism with upregulation of glucose uptake and induction of enzymes involved in glycolysis (Kim et al., 2006; Papandreou et al., 2006), as well as inducing an angiogenic phenotype by upregulating proangiogenic factors such as VEGF and PDGF (Semenza, 2001). The main regulators of this hypoxic response are the Hypoxia-inducible factor (HIF) proteins, that are

stabilized in hypoxia and induce expression of a number of genes involved in the hypoxic switch.

1.6.1 HIF proteins

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor consisting of an α-subunit and a β-subunit. While the β-subunit is constitutively expressed, independent of cellular oxygen concentration, the α-subunit is rapidly degraded in normoxia but stabilized in hypoxic conditions (Wang et al., 1995). The stabilized HIF heterodimer is translocated to the nucleus and binds to the hypoxia response element (HRE), ACGTG, in the promoters of its target genes, activating expression of a large number of hypoxia response genes such as VEGFA (Forsythe et al., 1996) and GLUT1 (Ebert et al., 1995).

There are three HIFα-subunits (HIF1α, HIF2α, HIF3α), of which HIF1α and 2α are the most studied, and many target genes such as VEGF, Tie2, Ang2 and VEGFR2 are induced by both subunits (Lau et al 2007). However, some genes are specifically regulated. Oct4 and transforming growth factor alpha (TGF-α) are, for instance, induced by HIF2α but not by HIF1α (Covello et al., 2006). Moreover, expression of HIF1α is ubiquitous while HIF2α is specifically expressed in vascular structures (Jain et al., 1998) as well as in distinct cell populations in organs such as brain, heart, lung, kidney and liver (Wiesener et al., 2003). HIF3α lacks the C-terminal transactivation domain present in HIF1α and HIF2α, and is thought to act as an inhibitor of the other α-subunits (Bárdos & Ashcroft, 2005).

1.6.2 VHL

Von Hippel-Lindau (VHL) disease is a hereditary cancer syndrome caused by mutation in the VHL tumor suppressor gene, leading to development of highly vascularized cancers such as clear cell renal carcinoma and hemangioblastomas of the retina and central nervous system (Lonser et al., 2003). The VHL gene product, pVHL, forms a protein complex with elongin B, elongin C, cullin2 and Rbx1 (Lonergan et al., 1998; Kamura et al., 1999). This complex has ubiquitin ligase activity which enables it to target proteins for proteasomal degradation (Iwai et al., 1999). The most well-known targets of the pVHL-complex are the isoforms of the alpha subunit of HIF (HIF1α, HIF2α and HIF3α) (Ohh, 2006). pVHL is crucial for binding to and subsequent degradation of HIFα. The interaction between pVHL and HIFα only occurs in the presence of oxygen, since HIFα needs to be prolyl hydroxylated by prolyl hydroxylases (PHDs), proteins that may work as a kind of cellular oxygen sensors, in order to bind to pVHL (Ivan et al., 2001; Jaakkola et al., 2001). *VHL* mutations in hemangioblastoma and clear cell renal carcinoma leads to deficient VHL inhibition of HIFα and

increased expression of HIF target genes such as VEGF and TGF-α (Reifenberger et al., 1995; Wizigmann-Voos et al., 1995; Petrides et al., 1990; Brown et al., 1993).

There are also several functions of pVHL that are independent of its interaction with HIF. It was found to have a crucial role in proper ECM assembly through direct interaction with collagen IV (Kurban et al., 2008; Ohh et al., 1998). It also associates with and stabilizes microtubules and, together with glycogen synthase kinase 3β (GSK3β), has an important role in maintenance of the primary cilium (Hergovich et al., 2003; Thoma et al., 2007).

1.7 Cancer stem cells

Normal stem cells are characterized by the capacity of self-renewal, a special cell division in which the stem cell produce one or two daughter stem cells with the same potential for development and replication as the mother cell, and pluripotency, the ability to differentiate into tissue-specific specialized cells (Lobo et al., 2007). Stem cells are often found in a certain microenvironment, the so-called niche, within an organ or tissue. The stem cells and the niche interact via paracrine factors and adhesion molecules, maintaining the characteristics of the stem cells (Iwasaki & Suda, 2009).

There is growing evidence that tumors contain a small subset of cells with the ability of self-renewal and generation of the diverse cells that comprise the heterogenous tumor mass. These cells are called cancer stem cells, or cancer-initiating cells, because they share properties with normal stem cells, such as self-renewal, long lifespan and resistance to apoptosis, and has the ability to continually sustain tumor growth. Cancer stem cells are considered to be critical for tumor proliferation and reoccurrence due to their natural resistance to cytotoxic drugs and capacity for DNA repair, allowing a small fraction of tumor cells to survive chemotherapy and support tumor regrowth (Dean et al., 2005). Cancer stem cells have been identified in a number of malignancies including brain tumors, melanoma, breast cancer, and colon cancer (Lobo et al., 2007; Visvader & Lindeman, 2008; Iwasaki & Suda, 2009).

1.7.1 ABCG2 and side population cells

ATP-binding cassette G2 (ABCG2) is a membrane protein involved in efflux of cytotoxic molecules from the cell, thus conferring the cell with drug resistance (Gottesman et al., 2002). Both normal and cancer stem cells express high levels of ABCG2 and other ABC-transporters that usually are inactive in differentiated cells (Scharenberg et al., 2002).

ABCG2 drug efflux is used as a marker for isolation of haematopoietic stem cells. While most cells take up and accumulate the fluorescent dye Hoechst 33342, this dye is transported out from the cell by ABCG2 in stem cells (Zhou et al., 2001; Goodell et al., 1996). This makes it possible to sort stem cells from other cells due to the low levels of Hoechst 33342 fluorescence. These sorted cells are called side population (SP) cells, because in flow-cytometric analysis SP cells are visualized as a negatively stained "side population" to one side of the majority of the cells in a dot blot (Goodell et al., 1996). The SP fraction isolated from murine bone marrow cells were found to be highly enriched in haematopoietic stem cells, that could reconstitute bone marrow and differentiate into lymphoid and myeloid lineages, demonstrating the pluripotency of these cells (Goodell et al., 1996). SP cells has been isolated from many tissues including brain, breast, lung and heart (Kim & Morshead, 2003; Alvi et al., 2003; Summer et al., 2003; Martin et al., 2004).

SP cells have been identified in many different cancer cells including neuroblastoma, glioblastoma, lung cancer and breast cancer cell lines as well as in a majority of the neuroblastoma patient samples analysed in one study (Hirschmann-Jax et al., 2004). A study of the rat glioma C6 cell line showed that isolated SP cells, and not non-SP cells, gave rise to both neuronal and glial lineages that produced tumors in mice, providing evidence that the SP fraction had cancer stem cell properties with high self-renewal ability and an immature phenotype (Kondo et al., 2004).

1.7.2 HIF proteins in cancer stem cells

Hypoxia has been shown to be important for maintenance of stem cells in the stem cell niches, such as the bone marrow for hematopoietic stem cells, adipose tissue for mesenchymal stem cells and the subventricular zone for neural stem cells (Mohyeldin et al., 2010). There is also increasing evidence that hypoxia and HIF proteins are important for maintenance of cancer stem cells in tumors.

Culture in hypoxic condition and HIF1α activation expanded a subpopulation of glioma cells positive for the cancer stem cell marker CD133 while markers for glial and neuronal differentiation were decreased (Soeda et al., 2009). Studies in glioblastoma also showed that several hypoxic response genes such as VEGF, Glut1 and Serpin B9 had higher expression in the cancer stem cell population compared to the normal cancer cell population (Bao et al., 2006; Li et al., 2009). While the levels of HIF1α was similar in both stem and non-stem tumor cell population, the levels of HIF2α was almost undetectable in non-stem cells while highly expressed in the stem cell population (Li et al., 2009). HIF knockdown in

glioblastoma stem cells inhibited self-renewal and proliferation *in vitro* and reduced their tumor-initiating properties *in vivo* (Li et al 2009).

HIF2α appears to have a more important role than HIF1α in cancer stem cell regulation and tumor progression. While HIF1α is stabilized only in severe hypoxia, HIF2α is stabile in a wider range of oxygen levels, from severe hypoxia to levels that are common for tumors (2-5%) (Holmquist-Mengelbier et al., 2006). Inhibition of HIF2α but not HIF1α suppressed renal cell carcinoma tumor growth *in vivo* (Kondo et al., 2003). Unlike HIF1α, HIF2α induces the expression of *OCT4*, a transcription factor that is important for stem cell pluripotency (Covello et al., 2006). HIF2α also binds to the promoter of the *ABCG2* gene and activates its expression in murine cardiac SP cells (Martin et al., 2008). These results suggest an important role of HIF2α in cancer stem cell maintenance. HIF2α expression induced an immature phenotype in neuroblastoma and breast cancer cells, induced growth of neuroblastoma tumors xenografted to athymic mice and correlated with poor outcome for neuroblastoma patients (Holmquist-Mengelbier et al., 2006; Pietras et al., 2008).

1.8 Nuclear receptor TLX

TLX (also called NR2E1) is the vertebrate homologue of the Drosophila *tailless* gene, and encodes an orphan nuclear receptor that is important for vertebrate brain functions (Yu et al., 1994; Shi et al., 2004). TLX expression in the mouse starts at embryonic day 8 (E8), peaks at E13.5 after which it gradually decreases. After birth the expression again increases to high levels in the adult brain (Monaghan et al., 1995).

TLX knockout mice appear normal at birth but mature knockout mice have reduced cerebral hemisphere and their behavior is more aggressive and they have reduced learning abilities (Monaghan et al., 1997). Embryonic brains of TLX-null mice demonstrate prolonged cell cycles and increased cell cycle exit, coupled with increased expression of cdk inhibitor p21 and decreased expression of cyclin D1 (Li et al., 2010).

Expression of TLX in adult brains is localized to neural stem cells (NSCs) in the subventricular zone and the hippocampal dentate gyrus (Shi et al., 2004). TLX maintains adult NSCs in an undifferentiated and self-renewable state with the ability to differentiate into all neural cell types *in vitro* (Shi et al., 2004). TLX has been shown to maintain NSCs in an undifferentiated proliferative state both through the transcriptional repression of cell cycle inhibitors p21 and Pten genes in cooperation with corepressors such as LSD1 and histone deacetylases

(HDACs) (Sun et al., 2010; Sun et al., 2007), and through the activation of the Wnt/β-catenin pathway (Qu et al., 2010).

TLX is also expressed in the retina where it is a key factor in retinal development and essential for vision (Yu et al., 2000). In the postnatal retina, TLX expression in proangiogenic astrocytes is controlled by oxygen levels and essential for the proangiogenic switch in response to hypoxic conditions, leading to assembly of fibronectin matrices and development of retinal vasculature (Uemura et al., 2006). Upon astrocyte contact with blood vessels, oxygenation rapidly downregulates TLX expression and switches off the proangiogenic activities, thus maintaining vascular homeostasis.

The microRNAs miR-9 and let7b both suppress TLX expression, leading to decreased proliferation and increased differentiation of neural stem cells (Zhao et al., 2009; Zhao et al., 2010). At the same time, TLX represses miR-9 primiRNA expression, forming a negative feedback loop which may be important in keeping the balance between proliferation and differentiation of neural stem cells (Zhao et al., 2009).

In concurrence with the important role of TLX in NSC self-renewal, its deregulation has been found to lead to brain tumor initiation and progression. TLX is overexpressed in primary glioblastoma samples, correlating with poor survival (Liu et al., 2010; Park et al., 2010). TLX expression induced migration of NSCs from their natural niche and initiated glioma development (Liu et al., 2010). Ectopic expression of TLX in the U87MG glioma cell line induced cell proliferation, colony formation *in vitro* and tumor formation *in vivo* (Park et al., 2010).

2. Aims of the studies

2.1 General aim

The general aim of this thesis was to study the role of TGF-β and TLX in different types of cancer and their involvement in regulation of tumor angiogenesis and stem-like properties of cancer cells.

2.2 Specific aims

I. To investigate the function of TIMP2 in the induced angiogenesis and accelerated growth of diffuse-type gastric carcinoma cells with disrupted TGF-β signaling.

II. To elucidate the role of TGF-β in regulation of cancer stem cells in diffuse-type gastric carcinoma.

III. To investigate the function of TLX and its interaction with VHL in hypoxia and angiogenesis of neuroblastoma.

IV. To study the role of TGF-β signaling and its interaction with TLX in neuroblastoma.

3. Materials and methods

3.1 Culture and drugs (Papers I-IV)

In order to study the effect of TGF-β and TLX on angiogenesis and carcinogenesis, a number of different cell lines were used for culture *in vitro*. The cell lines, their origins and culture media are summarized in table 1. Culture media were supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 60 mg/ml streptomycin. All cells were cultured in a 5% CO_2 incubator at 37ºC. For hypoxic conditions, cells were grown in 1.7% O_2.

The OCUM-2MLN cell line was obtained from a lymph node metastasis caused by orthotopic transplantation into a mouse of the OCUM-2M cell line (Fujihara et al., 1998), originally established from a patient with diffuse-type gastric carcinoma (Yashiro et al., 1995). To generate OCUM-2MLN cells with stable expression of GFP, dnTβRII or TIMP2, a lentiviral infection system was used. Using gene-specific primers (listed in table 2), cDNA was amplified using PCR and inserted into the multicloning site of the lentiviral vector pCSII-EF-Rfa, which was used for recombination with pCAG-HIVgp and pCMV-VSV-G-RSV-Rev vectors and virus production in 293FT cells. Lentiviral particles from 293FT conditioned media was then used for infection of OCUM-2MLN cells. OCUM-2MLN stably expressing ABCG2 or control short hairpin RNA (shRNA) were generated in a similar fashion, using the CS-Rfa-EG vector containing a GFP expressing cassette and shRNA oligonucleotides directed against ABCG2 or control shRNA.

To generate IMR-32 and SH-SY5Y cell lines with stable knockdown of TLX, cells were transfected with TLX shRNA or control shRNA vectors, and the antibiotic G-418 (4-5 μl/ml, Roche) was used to select for and maintain clones with stable shRNA expression.

Human TGF-β_1 (1-2 ng/ml, R&D Systems) was used for TGF-β stimulation. Bone morphogenetic protein 4 (BMP-4) (30 ng/ml) was used for BMP stimulation. Cycloheximide (1 μg/ml) was used for inhibition of protein translation. Hoechst 33342 (2-9 μg/ml) was used for visualisation of the SP fraction in flow cytometry. 2,2´-bipyridyl (BP) (100 μM, Sigma Aldrich) was used to mimick hypoxic conditions. MG132 (10 μM, Sigma Aldrich) was used to inhibit proteasomal degradation of proteins. Epothilone B (EB) and Nocodazole (25 nM and 3-10 μg/ml respectively, Sigma Aldrich) were used for perturbation of microtubuli stability.

Cell line	Species	Origin	Media	Paper
OCUM-2MLN	Human	Diffuse-type gastric carcinoma	DMEM	I, II
A549	Human	Non-small cell lung carcinoma	DMEM	II
HeLa	Human	Cervical adenocarcinoma	DMEM	II
HSC-43	Human	Diffuse-type gastric carcinoma	DMEM	II
HuH7	Human	Hepatocellular carcinoma	DMEM	II
MDA-MB-231	Human	Breast carcinoma	DMEM	II
OCUM-2D	Human	Diffuse-type gastric carcinoma	DMEM	II
OCUM-2M	Human	Diffuse-type gastric carcinoma	DMEM	II
OCUM-2MD3	Human	Diffuse-type gastric carcinoma	DMEM	II
OCUM-8	Human	Diffuse-type gastric carcinoma	DMEM	II
OCUM-9	Human	Diffuse-type gastric carcinoma	DMEM	II
HacaT	Human	Keratinocyte	DMEM	II, III, IV
786-O	Human	Renal cell carcinoma	RPMI 1640	III
786-VHL	Human	Renal cell carcinoma	RPMI 1640	III
AG1518	Human	Fibroblast	DMEM	III
LAN-5	Human	Neuroblastoma	RPMI 1640	III
PAE	Pig	Endothelial	DMEM	III
SKN2 BE-2	Human	Neuroblastoma	DMEM	III
COS-1	Monkey	Fibroblast	DMEM	III, IV
IMR-32	Human	Neuroblastoma	RPMI 1640	III, IV
SH-SY5Y	Human	Neuroblastoma	DMEM	III, IV

Table 1: List of cell lines used in this thesis.

3.2 Transient transfection and small interfering RNA

Transient transfection is used for exogenous introduction of genetic material to cells by using expression vectors, reporter plasmids or siRNA. The transfection methods used in this thesis was the Fugene6/FugeneHD (Roche) and Lipofectamine 2000 (Invitrogen) lipid based transfection reagents and the microporation technique using a Microporator (Digital Bio). All methods were used according to the manufacturer's instructions. Small interfering RNA (siRNA) duplex oligoribonucleotides against SMAD4 or control siRNA were synthesized by Invitrogen and used for transfection of OCUM-2MLN cells with Lipofectamine 2000 (Paper II). Transient transfection of IMR-32 and SH-SY5Y cells with siRNA against TLX (Qiagen) was done using microporation (Paper III).

3.3 RNA expression: Quantitative reverse transcriptase PCR analysis (Paper I-IV)

Reverse transcriptase polymerase chain reaction (RT-PCR) is used to determine the amount of specific RNA transcripts using reverse transcription of RNA into cDNA and subsequent PCR amplification with gene-specific primers. In quantitative (or real-time) RT-PCR, non-specific fluorescent dye that intercalates with double-stranded DNA allows the detection of amplified cDNA as the PCR reaction progresses in real time. By selecting a threshold value for fluorescence detection when the reaction is in the exponential phase (and no reagents are limiting the reaction) and determining the C_T value, i.e. the number of PCR cycles it takes for each cDNA sample to reach the threshold, the relative amounts of cDNA in the different samples can be determined. To accurately quantify the relative expression the measured cDNA amount is normalized to the measured amount of a housekeeping gene in the same sample.

Total RNAs from cultured cells or excised subcutaneous tumors were extracted using the RNeasy Mini Kit (QIAGEN) (Paper I-II) or using the TRIzol reagent (Invitrogen) and subsequent ethanol precipitation (Paper III-IV). cDNA was synthesized, using 1-2 μg RNA as template, with the Quantitect Reverse Transcription kit (QIAGEN) with random hexamer primers (Paper I-II) or by annealing oligo-dT primers and elongating with M-MuLV reverse transcriptase (Fermentas) (paper III-IV) according to the manufacturers' protocols. Transcript levels were measured and C_T values were estimated using the 7500 Fast Real-Time PCR System (paper I-II) or the StepOne Real-Time PCR system (paper III-IV) (Applied Biosystems). Transcript levels of the genes of interest were normalized to the expression of housekeeping genes GAPDH or HPRT. Sequences of the primers used for quantitative RT-PCR are listed in table 2.

3.4 Promoter reporter assay (Paper III-IV)

Promoter reporter assays are used to study how the activity of a specific promoter or enhancer region is regulated by drugs and transcription factors. A promoter or enhancer sequence of interest is cloned upstream of a reporter gene, in our case the luciferase gene, in a vector that is transfected into cells. The cells are treated with drugs or cotransfected with vectors expressing transcription factors or other proteins, or with siRNAs before they are lysed with lysis buffer. If the promoter is active the luciferase enzyme will be expressed and the enzymatic reaction when the luciferase substrate luciferin is added to the cell lysates produces light that can be quantified and give an indirect measurement of the degree of promoter activity.

The luciferase constructs used in this thesis were the HRE-containing promoter construct (Stewart et al., 2009), the VHL-promoter (gift from Dr Maher; Zatyka et al., 2002) (both in Paper III), a construct containing the 4.7 kb upstream sequence of the TLX gene (Paper III and IV), and the Smad Binding Element-containing $(SBE)_4$-lux construct (Paper IV). Cells were transfected with 0.3 μg reporter plasmid and 0.3 μg expression plasmid or mock vector. Cells were lysed 48 hours after transfection, procesed using the Luciferase Reporter Assay System (Promega) according to the manufacturer's instruction. Luciferase activity was determined by quantification of luminescense using a luminometer, and normalized to activity of co-transfected β-galactosidase or to total protein amount.

Primer	Forward sequence	Reverse sequence	Application	Species	Paper
TIMP2	5′-AAA GAA TTC ATG GGC GCC GCG G-3′	5′-ATA CTC GAG TTA TGG GTC CTC GAT GTC -3′	cDNA cloning	H	I
TIMP2	5′-AGA TGT AGT GAT CAG GGC CAA AG-3′	5′-GCT TGA TCT CAT ACT GGA TCC TCT TG-3′	RT-PCR	H	I
GAPDH	5′-GAA GGT GAA GGT CGG AGT C-3′	5′-GAA GAT GGT GAT GGG ATT TC-3′	RT-PCR	H	I, IV
HPRT1	5′-TGT TTG GGC TAT TTA CTA GTT G-3′	5′-ATA AAA TGA CTT AAG CCC AGA G-3′	ChIP	H	II
PAI-1	5′-GCA GGA CAT CCG GGA GAG A-3′	5′-CCA ATA GCC TTG GCC TGA GA-3′	ChIP	H	II
ABCG2	5′-CCC TGC GAC CCG GCT GAA AG-3′	5′-GAT CCC GGA GGC GGG AGT GT-3′	ChIP	H	II
ABCG2	5′-AGA TGG GTT TCC AAG CGT TCA T-3′	5′-CCA GTC CCA GTA CGA CTG TGA CA-3′	RT-PCR	H	II
SMAD4	5′-AAA ACG GCC ATC TTC AGC AC-3′	5′-AGG CCA GTA ATG TCC GGG A-3′	RT-PCR	H	II
HPRT	5′-TTT GCT TTC CTT GGT CAG GC-3′	5′-GCT TGC GAC CTT GAC CAT CT-3′	RT-PCR	H	II, IV
TLX FL	5′-GTT GAA TTC ATG AGC AAG CCG CC-3′	5′--GCG CTC GAG TTA GAT GTC ACT GGA TTT G-3′	cDNA cloning	M	III
TLX ΔN	5′-TAT GAA TTC GAA TCA GCT GCC AGG CTT-3′	5′--GCG CTC GAG TTA GAT GTC ACT GGA TTT G-3′	cDNA cloning	M	III
TLX ΔC	5′-GTT GAA TTC ATG AGC AAG CCG CC-3′	5′-TTT CTC GAG TTA ACA CAC GGA CTC AGT-3′	cDNA cloning	M	III
TLX ΔLB	5′-GTT GAA TTC ATG AGC AAG CCG CC-3′	5′-TTT CTC GAG TTA GAA TCG GCA GGG TTG G-3′	cDNA cloning	M	III
VEGF	5′-TTT TCA GGC TGT GAA CCT TG-3′	5′-GAT CCT CCC CGC TAC CAG-3′	ChIP	H	III
TLX	5′-CTG GCT GTA TCT GGC ATG AA-3′	5′-TCT AAA TCG AGC CAC CAC CT-3′	RT-PCR	H	III
VHL	5′-ATT AGC ATG GCG GCA CAC AT-3′	5′-TGG AGT GCA GTG GCA TAC TCA T-3′	RT-PCR	H	III
SMAD7	5′-CCT TAG CCG ACT CTG CGA ACT A-3′	5′-CCA GAT AAT TCG TTC CCC CTG T-3′	RT-PCR	H	IV
p21	5′-ACA GCA GAG GAA GAC CAT GTG-3′	5′-GGG CTT CCT CTT GGA GAA GAT-3′	RT-PCR	H	IV
PDGFRB	5′-GCA ATG TGA CGG AGA GTG TGA A-3′	5′-CCG ATG CAG CTC AGC AAA TT-3′	RT-PCR	H	IV

Table 2: List of primers used in this thesis.

3.5 Protein quantification: Immunoblotting (Paper I-IV)

Immunoblotting (also called Western blotting) is used to determine, by specific antibody detection, the presence and relative amounts of various proteins of interest in cells. Cultured cells were washed in phosphate buffered saline (PBS) and lysed on ice using lysis buffer with either Triton X-100 or Nonidet-P40 as detergents and containing phosphatase and protease inhibitors. Cellular debris was removed by centrifugation and equal amounts of protein were loaded to and separated on 8-15% SDS-PAGE gels. Proteins were then transferred to PVDF membranes that were blocked in 5% skim milk or 5% BSA and probed by primary antibodies (listed in table 3), followed by incubation with HRP-conjugated secondary antibody against mouse-, rabbit- or goat-IgG, depending on the species of origin for the primary antibody. Membranes were developed using the enhanced chemiluminescence (ECL) advance system (GE Healthcare) according to the manufacturer's protocol and scanned using LAS-1000 Plus (Fujifilm).

Antibody	Company	Source	Application	Paper
TIMP2	Abcam	Mouse	WB	I
PECAM1	BD Pharmingen	Rat	IF	I
NG2	Chemicon	Rabbit	IF	I
Hypoxyprobe	HPI, Inc	Mouse	IF	I
HA (3F10)	Roche	Mouse	WB	II
phospho-Smad2	Cell Signaling	Rabbit	WB	II
phospho-pRb	BD Pharmingen	Rabbit	WB	II
ABCG2/BCRP	Millipore	Mouse	WB	II
Smad2/3	BD Biosciences	Mouse	IF,ChIP	II, IV
VHL	BD Biosciences	Mouse	WB, IP, ChIP	III
β-tubulin	Sigma Aldrich	Mouse	WB	III
Actin (AC-40)	Sigma Aldrich	Mouse	WB	III
TLX	Abnova	Mouse	ChIP	III
GAPDH	Millipore	Mouse	WB	III, IV
β-Actin	Santa Cruz	Mouse	WB	III, IV
TLX	Lifespan Biosciences	Rabbit	WB, IP, IF	III, IV
Flag (M2)	Sigma Aldrich	Mouse	WB, IP	III,IV
c-Myc (9E10)	Santa Cruz	Mouse	WB, IP	IV
phospho-Smad2	Gift from CH Heldin	Rabbit	WB	IV
Smad2	Gift from CH Heldin	Rabbit	WB	IV
PDGFR-β	Santa Cruz	Rabbit	WB	IV

Table 3: List of antibodies used in this thesis.

3.6 Protein binding assay: Coimmunoprecipitation (Paper III-IV)

Immunoprecipitation is used to enrich a protein of interest by binding it with specific antibodies, and pull down the protein-antibody complex with agarose beads. Co-immunoprecipitation enables the study of protein-protein interaction, since other proteins that interact with the immunoprecipitated protein will also be pulled down and can then be detected and identified by immunoblotting. Co-immunoblotting was performed either on endogenously expressed proteins or on Flag-, Myc-, or HA-tagged proteins that were transfected into cells and exogenously expressed. Cells were lysed in lysis buffer (48 h after transfection if exogenous proteins were used) and the lysate was incubated with antibody. Then protein A/G agarose beads were added to the lysate and incubated for 4 h or overnight in 4ºC. In the case of Flag-tagged proteins, anti-Flag M2 antibody conjugated to agarose (Sigma-Aldrich) was directly added to the lysate and incubated overnight at 4°C. The agarose beads were washed, boiled in SDS-sample buffer, and immunoblotting was performed as described above.

3.7 Immunofluorescense: Immunohistochemistry (Paper I) & Immunocytochemistry (Paper III-IV)

Immunofluorescense is a method used to fix tissues (immunohistochemistry) or cells (immunocytochemistry) and determine the expression and localization of an antigen of interest, by incubation with antigen-specific antibodies followed by addition of fluorescent labeled secondary antibodies that can be detected with a fluorescence microscope.

For immunohistochemistry, tumor tissue from mice were excised and fixed in 10% neutral buffered formalin followed by sequential washes in PBS containing sucrose at gradually increasing percentages (10-15-20 %). Tissues were then embedded in optimal cutting temperature compound and snapped frozen in dry-iced acetone, sectioned at 10 μm thickness in a cryostat, and incubated with first primary antibodies (listed in table 3) and then Alexa Fluor-488 or Alexa Fluor-594-conjugated secondary antibodies (Molecular Probes). Samples were observed with an LSM510 Meta confocal microscope (Zeiss) and images were imported into Adobe Photoshop and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

For immunocytochemistry, cells were cultured on chamber slides, fixed in ice-cold 50:50 Methanol:Aceton for 5 min, permeabilized with tris buffered saline (TBS) containing Triton-X, incubated in blocking buffer (TBS + 10% FBS) for

1 hour and incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Cells were then incubated with secondary antibodies as described above. For nuclear staining, 1 μg/ml of Hoechst 33258 (Molecular Probes) was added. Cells were observed and analyzed with an epi-illumination fluorescence microscope (Zeiss Axiovision or Olympus High-Content).

3.8 In vivo protein-DNA interaction: Chromatin immunoprecipitation (Paper II-III)

Chromatin immunoprecipitation (ChIP) is used to study *in vivo* binding of specific proteins to gene regulatory regions such as promoters and enhancers, by cross-linking DNA and proteins with formaldehyde fixation followed by sonication to shear DNA into fragments of 200-1000 base pairs. Protein-DNA complexes are then immunoprecipitated with antibodies specific to proteins that are assumed to bind the regulatory region of interest. When the DNA fragments bound by proteins have been collected, the protein-DNA cross-linking is reversed, proteins and RNA are degraded, and DNA is purified and amplified with PCR using primers specific to the regulatory region in question. If the protein bound to the specific DNA region this DNA sequence will be amplified.

Protein and DNA were cross-linked by incubating cells with formaldehyde at a final concentration of 1% for 10 min. After cell lysis, crosslinked DNA was sonicated and incubated at 4°C with antibodies against Smad2/3 (10 μg, paper II) or TLX, HIF2α, RNA Polymerase II, VHL (2 μg, paper III), along with mouse/rabbit IgG (negative control). The antibody-protein-DNA complex was collected using anti-mouse IgG Dynabeads (paper II) or Protein A/G-agarose (paper III). Immunoprecipitates were subjected to five washes in ChIP wash buffer (50 mM HEPES-KOH pH 7, 0.5 M LiCl, 1mM EDTA, 0.7% deoxycholate, 1% Igepal CA630) (paper II) or subsequent washes in low salt, high salt and LiCl wash buffers (paper III), and finally washed in TE buffer. Immunoprecipitates were then eluated and incubated with NaCl at 65ºC for 4 h or overnight to reverse the DNA-protein crosslinks. RNA and protein was degraded using RNaseA and ProteinaseK, respectively, and DNA was extracted with a PCR Purification Kit (Qiagen) (paper II) or using phenol:chloroform extraction and ethanol precipitation (paper III), and used for quantitative or semi-quantitative RT-PCR analysis, using promoter-specific primers as listed in table 2.

3.9 Cell proliferation assay

Cell proliferation assay is used to compare the growth rate of cells in different conditions. The growth rate was determined by two methods: cell count or the MTT colorimetric assay.

For cell count, cells were seeded at 2-2.5 × 10^4 cells per well in 12- or 24-well plates and counted manually in triplicates using a counter-chamber at the indicated time points after seeding (paper I, III) or counted in duplicates with a hemocytometer at day 4 after TGF-β treatment (paper II). The cell numbers were compared to that of the day of seeding or the day of TGF-β treatment.

In MTT assay (paper IV), cell survival is assessed indirectly, on the basis of the breakdown of the yellow tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into blue formazan salt, which can be spectrophotometrically quantified. This color change reflects the enzyme activity of mitochondrial dehydrogenases in living cells. Cells were seeded at 1500 cells per well in 96-well plates with at least 8 replicates for each time-point and treatment. At the respective time-points after seeding, cells were incubated with 20 µl of MTT dissolved in PBS. After 6 h incubation at 37°C, media was removed, formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm. The absorbance values were compared to the value of cells at day 1 after seeding.

For determining the effect of TGF-β treatment on growth rates, TGF-β1 (1-2 ng/ml) was added to cells the following day after seeding (paper II, IV).

3.10 Colony formation assay in soft agar (Paper II-III)

Colony formation in soft agar is used to study non-adhesive cellular growth in three dimensions and is an assay to determine tumorigenicity *in vitro*. Agar (Nacalai Tesque or Chemicon) was dissolved in culture medium to 0.5 or 0.8% and plated in culture plates (bottom layer). After the bottom layer had coagulated, cells were seeded in 0.3 or 0.4% agar and added on top of the bottom agar layer. Cells were covered with liquid growth media and cultured at 37°C in a 5% CO_2 incubator for approximately 3 weeks. Cell viability was determined by manual count of colonies or measured using Cell Count Reagent SF (Nacalai Tesque), where water-soluble tetrazolium salt is incubated with cells for 2 hours, followed by measurement of absorbance at 450 nm.

3.11 Side population (SP) analysis and sorting (Paper II)

Side population (SP) analysis is a method used to quantify and isolate cells that are positive for the stem cell marker ABCG2. The fluorescent dye Hoechst 33342 will be taken up by most of the cells, but the dye will only be effluxed by cells that express ABCG2. This makes it possible to sort and collect ABCG2+ cells because of their low levels of Hoechst 33342 fluorescence. These cells are called side population cells, and they are enriched in stem cells.

Cells were resuspended in ice-cold HBSS +2% FBS and treated with 2-9 μg/mL Hoechst 33342 dye (Invitrogen) for 60 min at 37°C. After wash with PBS, cells were analyzed and collected using a flow cytometer and histograms were generated by the FlowJo software (Tree Star).

3.12 In vivo xenograft models (Paper I-II)

Transplantation of human tumor tissue or cells into immunodeficient mice such as athymic (nude) or severe combined immune deficient (SCID) mice is a method used to study the biology and treatment of solid human cancers *in vivo*. For easy operation and visualisation of tumor formation subcutaneous transplantation of tumors or cells is commonly used. However, in order to study the tumor biology in an environment similar to the original primary site or to study tumor metastasis, orthotopic transplantation is used. In orthotopic transplantation models, cancer cells are transplanted to the organ or tissue of origin, such as mouse mammary pads in the case of breast cancer.

In this thesis male BALB/c *nu/nu* nude mice (4-5 weeks of age) purchased from Oriental Yeast, Co were used. All animal experimental protocols were performed in accordance with the policies of the Animal Ethics Committee of the University of Tokyo. In the subcutaneous xenograft model (paper I) a total of 5×10^6 cells resuspended in 100 μl of culture medium were injected into mice. Tumor size was measured from one week after injection. In the orthotopic model (paper I) a total of 5×10^6 OCUM-2MLN cells resuspended in 50 μl of culture medium were injected into the gastric walls of mice. About four weeks later the mice were sacrificed and tumors were measured and harvested. In the subcutaneous model using non-SP and SP-cells (paper II), cells were resuspended in 50% matrigel (BD Bioscience).

Subcutaneous xenografts were measured externally every other day until the end of evaluation periods, and orthotopic xenografts were measured after harvest. Tumor volume was approximated using the equation $\text{vol} = (\mathbf{a} \times \mathbf{b}^2)/2$, where vol is volume, **a** the length of the major axis, and **b** is the length of the minor axis.

Relative tumor volume was calculated by dividing tumor volume by that measured on the first day of evaluation.

To study the degree of hypoxia in tumor tissue (paper I), hypoxyprobe (pimonidazole hydrochloride; HPI, Inc.), which is a molecule that binds to hypoxic cells, was injected through the tail vein of nude mice at 60 mg/kg body weight 30 min before subcutaneous tumor harvest. Hypoxic area of the tissue was analyzed using immunohistochemistry with primary antibody against hypoxyprobe.

3.13 In vivo cell isolation and gene expression analysis (Paper I-II)

To study the difference in gene expression of xenografted tumors formed by different cancer cells, tumor tissue was digested with 1 mg/ml collagenase (Worthington) and 0,25% Trypsin-EDTA (Invitrogen). The resulting single cell suspensions were subjected to MACS cell separation using microbeads conjugated to CD326 antibody (Miltenyi Biotec) allowing the selection of CD326 expressing human cancer cells from CD326 negative mouse stromal cells. Total RNA from the isolated cancer cells were purified using the RNeasy Mini Kit (Qiagen) and used for gene expression analysis, which was performed using the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix) as described (Komuro et al., 2009).

3.14 Statistical analysis

For data storage and analysis Excel software (Microsoft) was used. Statistical differences were compared by Student's t test and expressed as mean values ± standard errors.

4. Results

4.1 Paper I – Exogenous introduction of tissue inhibitor of metalloproteinase 2 reduces accelerated growth of TGF-β-disrupted diffuse-type gastric carcinoma

Diffuse-type gastric cancer is characterized by rapid progression, poor prognosis and thick stromal fibrosis associated with high levels of TGF-β produced by cancer cells and cancer-associated fibroblasts (Yashiro et al., 1995; Mizoi et al., 1993). However, since the role of TGF-β in this cancer type had not been well characterized before, we previously investigated the function of TGF-β signaling using the diffuse-type gastric carcinoma cell line OCUM-2MLN, to which we introduced a dominant negative form of the TGF-β type II receptor (dnTβRII) which binds to TGF-β ligands but does not transduce intracellular signals (Komuro et al., 2009). Expression of dnTβRII caused accelerated tumor growth through the induction of tumor angiogenesis *in vivo*.

4.1.1 Regulation of TIMP2 expression by TGF-β signaling

Using oligonucleotide microarray analysis with RNA isolated from cancer cells from tumors with normal or dysfunctional TGF-β signaling we found that the expression of tissue inhibitor of metalloproteinase 2 (TIMP2) was downregulated in dnTβRII tumors *in vivo*, and we also found that TGF-β induced TIMP2 mRNA expression in OCUM-2MLN cells *in vitro*. TIMP2 is an endogenous inhibitor of matrix metalloproteinases (MMPs) and has been shown to inhibit tumor progression and invasion in several cancer models (Lee et al., 2005; Brand et al., 2000), but since its involvement in gastric cancer was unknown we decided to study TIMP2 function in the OCUM-2MLN diffuse-type gastric carcinoma model.

4.1.2 TIMP2 overexpression abolished the accelerated growth of dnTβRII cells in vivo

We used previously established OCUM-2MLN cells expressing green fluorescent protein (GFP) or dnTβRII and lentivirally reinfected these cells with GFP or TIMP2 genes to establish three new OCUM-2MLN cell lines: GFP+GFP, dnTβRII+GFP and dnTβRII+TIMP2. We then used these cells for subcutaneous transplantation to nude mice. Compared to the GFP+GFP controls, tumors expressing dnTβRII+GFP displayed accelerated growth, which was completely abolished in tumors where TIMP2 was coexpressed with dnTβRII. These results were confirmed in an orthotopic model where the same cell lines were transplanted to the gastric wall of nude mice.

4.1.3 The effect of TIMP2 overexpression on tumor angiogenesis

Since we previously showed that the accelerated tumor growth in dnTβRII expressing tumors is due to induced tumor angiogenesis, we wanted to know if the inhibitory effect of TIMP2 on tumor growth was due to inhibition of angiogenesis. We therefore studied tumor vascular density using immunohistochemical staining of PECAM1, a marker of vascular endothelium. PECAM1-positive area was significantly increased in dnTβRII+GFP tumors compared to GFP+GFP tumors. Coexpression of TIMP2 with dnTβRII only slightly decreased the total PECAM1 staining, but the vessel length appeared to be decreased in the TIMP2 expressing tumors.

4.1.4 TIMP2 overexpression reduced the function of tumor vessels

The decreased vessel length in dnTβRII+TIMP2 tumors suggested that blood vessel functionality may be reduced in these tumors. To evaluate the function of tumor vessels we monitored oxygen delivery by using Hypoxyprobe (pimonidazole hydrochloride), a molecule that binds to hypoxic cells. At day 15 after subcutaneous tumor transplantation, Hypoxyprobe was injected through the tail vein of mice 30 min before tumor harvest. Hypoxic area in tumor tissue was then analyzed with immunohistochemistry using antibody against Hypoxyprobe (figure 3A). Intratumor hypoxic area was significantly reduced in dnTβRII+GFP tumors compared to GFP+GFP control tumors, but in dnTβRII+TIMP2 tumors hypoxic area was significantly increased compared to dnTβRII+GFP tumors, with hypoxic staining even in areas with high vessel density, suggesting that the shorter vessels in TIMP2 expressing tumors were less functional and unable to efficiently deliver oxygen to the tumor tissue.

We next investigated pericyte-coverage of tumor vessels. Pericytes stabilise blood vessels and are important for vasculature maturation. Pericytes were identified as cells around the vessels staining positive for the pericyte marker neuroglial proteoglycan 2 (NG2). Double staining of PECAM1 and NG2 revealed a much more diffuse vascular structure in GFP+GFP and dnTβRII+TIMP2 tumors with less pericyte-coverage of vessels than in dnTβRII+GFP, correlating with the difference in vessel functionality (figure 3B).

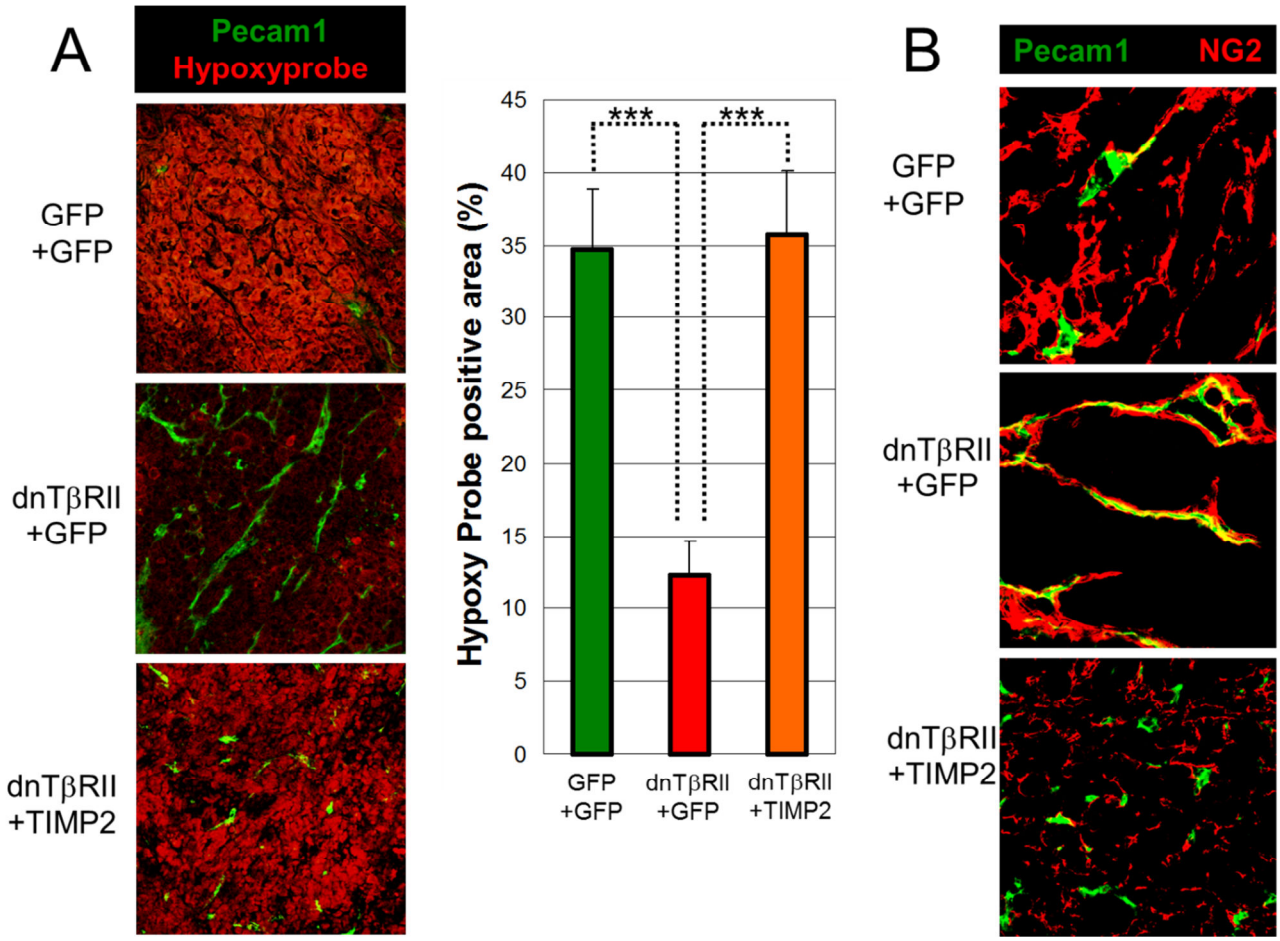

Figure 3: TIMP2 expression decreased oxygen delivery (A) and pericyte-coverage (B) of tumor vessels.

4.2 Paper II - Transforming growth factor-β decreases the cancer-initiating cell population within diffuse-type gastric carcinoma cells

Most studies of human cancers are based on analysis of the tumor mass as a whole, but recent findings suggest that the tumor cell population is heterogenous with respect to tumor-forming ability, with a small fraction of the cell population – so-called cancer stem cells or cancer initiating cells – being the driving force of tumor proliferation (Stingl & Caldas, 2007). We previously found that disruption of TGF-β signaling by introduction of dnTβRII to OCUM-2MLN diffuse-type gastric carcinoma cell lines led to accelerated tumor growth due to induced tumor angiogenesis (Komuro et al., 2009). However, our aim with this study was to see if TGF-β signaling also played a part in regulation of cancer stem cells in diffuse-type gastric carcinoma.

4.2.1 Cancer-initiating cells are enriched in 2MLN-dnTβRII cells

First we examined if 2MLN-dnTβRII cells contained a higher proportion of tumor initiating cells compared to 2MLN-GFP control cells. For this purpose, various numbers of cells were transplanted to mice, and were compared for tumor formation frequency. As few as $1x10^3$ dnTβRII cells were sufficient for tumor formation, while for GFP cells no tumors were formed when transplanting $3x10^3$ cells or less, suggesting that disruption of TGF-β signaling induced tumor formation through enrichment of a cancer-initiating cell population.

4.2.2 ATP-binding cassette G2 is upregulated in dnTβRII tumors

In order to find candidate genes that would be responsible for the increased stem cell like properties of 2MLN-dnTβRII cells we analyzed data from the oligonucleotide microarray that we performed previously (Komuro et al., 2009). We found that one of the genes upregulated in dnTβRII tumors was *ATP-binding cassette G2* (*ABCG2*), which codes for a membrane protein involved in efflux of cytotoxic molecules from the cell. *ABCG2* is highly expressed in both normal stem cells and cancer stem cells, making it a prime target of focus in our study.

4.2.3 TGF-β regulates ABCG2 expression through the Smad pathway

Upon treatment of OCUM-2MLN cells with TGF-β, ABCG2 mRNA and protein expression was decreased, but expression was unchanged in cells expressing dnTβRII. Knockdown of Smad4 expression with siRNA abolished the negative effect of TGF-β on ABCG2 expression, indicating that TGF-β activation of the Smad pathway was responsible for ABCG2 regulation. This was confirmed by ChIP analysis, which showed that Smad2/3 binds to the ABCG2 promoter in response to TGF-β treatment.

4.2.4 OCUM-2MLN cells contain an SP cell fraction with tumorigenic properties

To confirm the functionality of ABCG2 in OCUM-2MLN cells we used flow cytometric analysis with Hoechst 33342 staining to identify SP cells. We found a distinct fraction of SP cells making up 1.45% of the cell population, and this fraction was decreased by treatment with transporter inhibitors reserpine or fumitremorgin. We next sorted SP cells and non-SP cells from OCUM-2MLN and transplanted equal number of SP or non-SP cells into mice. Tumors formed by SP cells were significantly larger than that of non-SP cells, suggesting that OCUM-2MLN cells is a heterogenous cell population and that the SP cell fraction is more tumorigenic than the non-SP cell fraction.

4.2.5 TGF-β diminished the SP fraction of OCUM-2MLN cells

To test our hypothesis that TGF-β diminishes the OCUM-2MLN SP fraction through repression of ABCG2 we treated cells with TGF-β and performed flow cytometric analysis with Hoechst 33342 staining and found that the SP-fraction was significantly reduced by TGF-β (figure 4). TGF-β also inhibited colony formation of OCUM-2MLN cells in soft agar *in vitro* and tumor formation *in vivo*.

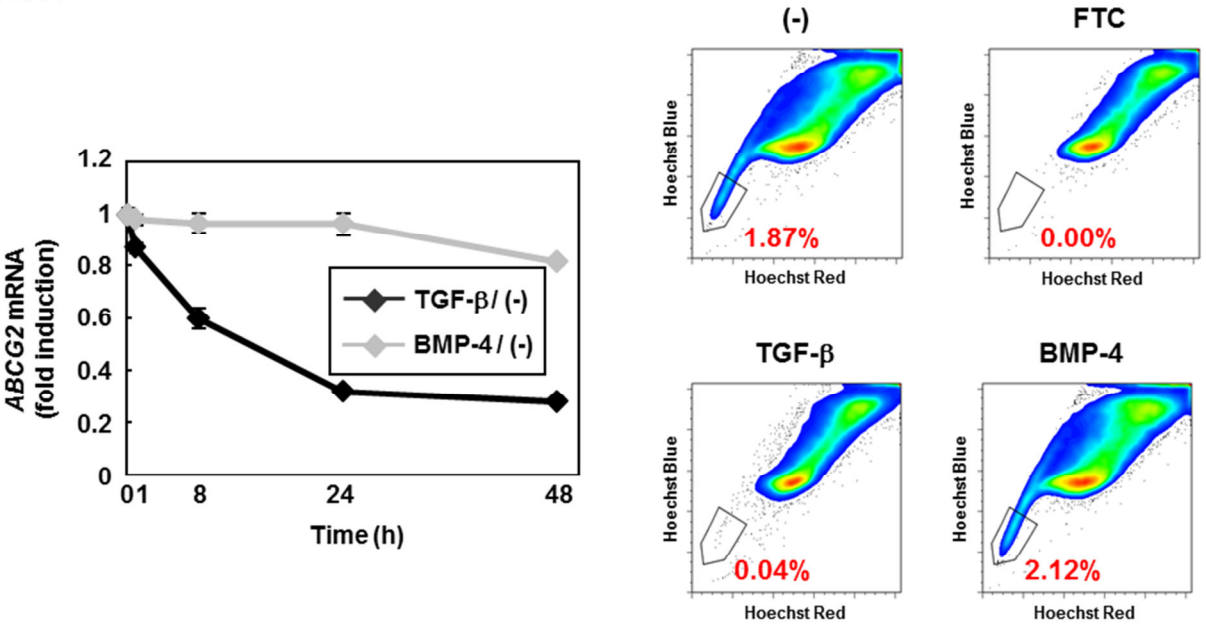

Figure 4: TGF-β downregulated ABCG2 expression and SP-fraction of OCUM-2MLN cells.

4.2.6 ABCG2 expression and SP-fractions in gastric carcinoma cell lines

Finally, we looked at ABCG2 expression and SP cell percentage in four different diffuse-type gastric cancer cell lines and found that both ABCG2 expression and SP fraction was significantly higher in OCUM-2MLN cells, and that only this cell line, and not the other three, formed tumors *in vivo*.

4.3 Paper III - TLX creates hypoxic microenvironment in neuroblastoma

There is growing evidence of the existence of a subpopulation of cancer-initiating cells, with properties similar to stem cells, in many solid tumors including neuroblastoma, a cancer of the peripheral (sympathetic) nervous system. Since the orphan nuclear receptor TLX has a central role in maintaining stemness of neural stem cells in the central nervous system, we wanted to study the role of TLX in neuroblastoma. Two neuroblastoma cell lines were the main fo-

cus of this study: IMR-32, which expresses high levels of TLX, and the more differentiated SH-SY5Y cell line which has lower TLX expression.

4.3.1 Hypoxic conditions induced TLX and suppressed VHL protein expression in IMR-32 and SH-SY5Y neuroblastoma cells

It is well-known that hypoxia has an important role in both normal stem cell and cancer stem cell maintenance. Hypoxic conditions induced TLX protein expression in IMR-32 and SH-SY5Y cells while expression of VHL was downregulated. VHL and TLX mRNA levels were not significantly altered by hypoxia, suggesting that TLX and VHL expression is mainly regulated on the protein level.

4.3.2 VHL binds to and stabilizes TLX

Overexpression of VHL in 786-0 cells, lacking functional endogenous VHL, led to reduction of HIF2α levels presumably due to VHL targeting of HIF2α for proteasomal degradation. However, VHL overexpression led to stabilization of TLX in both presence and absence of proteasomal inhibitor MG132. Using co-immunoprecipitation we found that TLX bound to VHL wild-type protein but not to the Δ95-123 mutant, suggesting that this region is indispensable for binding to TLX, just as it is for binding between VHL and HIFα or tubulin (Hergovich et al., 2003).

4.3.3 Hypoxia-responsive promoter activity was repressed by VHL but activated by TLX

It has been shown that hypoxia-dependent induction of TLX stimulates angiogenesis in the developing retina (Uemura et al., 2006). Since TLX enters the nucleus in hypoxia we examined if TLX cooperates with HIFα through binding to the HIF-responsible element (HRE) found in promoters of hypoxia-responsive genes. There is a TLX binding element next to the HRE in the VEGF-promoter. In VHL-deficient 786-0 cells but not in 786-VHL cells, we found induced luciferase activity of the HRE-reporter in response to TLX overexpression. Hypoxic conditions induced HRE-reporter activity in SH-SY5Y cells, and transfection of VHL repressed the activity, but this repression was counteracted by TLX overexpression.

4.3.4 TLX silencing in neuroblastoma led to growth retardation, reduced hypoxia-dependent HIF2α induction and increased VHL expression

In order to study the biological role of endogenous TLX in neuroblastoma we made IMR-32 and SH-SY5Y clones with stable knockdown of TLX. TLX-silenced cells displayed significant growth retardation compared to control cells,

and in IMR-32, TLX silencing led to reduced colony formation in soft agar and induced expression of the early apoptosis marker Caspase 3. Hypoxia-induced expression of HIF2α was reduced in silenced cells, but pVHL levels were increased both in normoxia and hypoxia (figure 5).

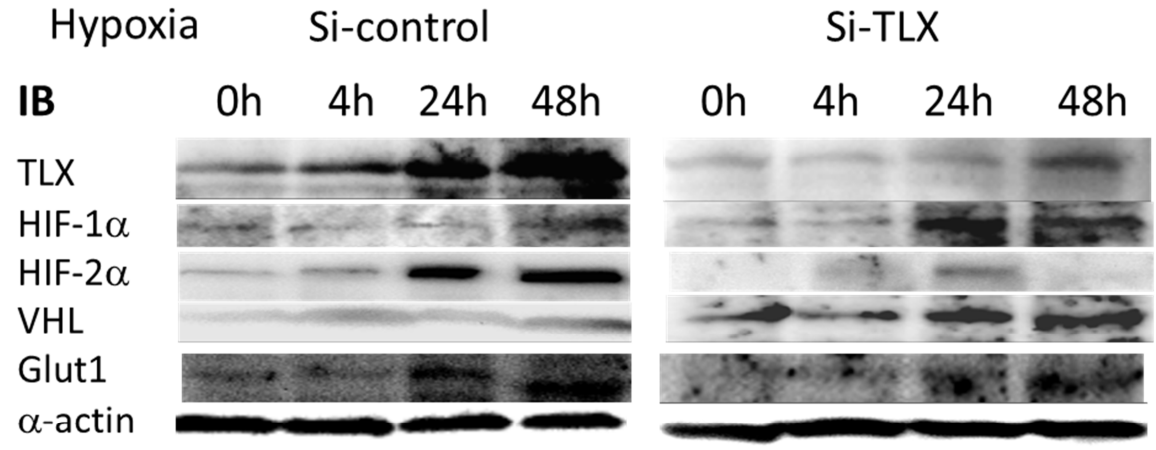

Figure 5: Knockdown of TLX increased pVHL protein levels and reduced hypoxia-induced HIF2α expression in IMR-32 cells.

4.3.5 TLX is recruited to the VEGF promoter in hypoxia and is needed for promoter binding of HIF2α

Using ChIP assay for the VEGF promoter, we found that although TLX did not bind in normoxia, silencing of TLX led to reduced promoter binding of HIF2α. In hypoxia, both HIF2α and TLX were bound to the promoter, but silencing not only reduced TLX binding but also reduced promoter binding of HIF2α.

4.3.6 TLX binding to VHL and microtubules is important for HIF stability

Since both HIF2α and TLX interacted in binding to the HRE site of the VEGF promoter, we next studied if TLX and HIF2α are part of a complex with pVHL. Using immunoprecipitation with antibody against pVHL in IMR-32 and SH-SY5Y cells we found that both TLX and HIF2α were pulled down with VHL and that the stability of all three proteins were increased at 4 hours of hypoxia.

Furthermore, both β-tubulin and acetylated α-tubulin were present in the VHL-TLX complex, and their binding increased in hypoxia, but knockdown of TLX or treatment with microtubule-depolymerisation interfering drug Epothilone B reduced binding of TLX, HIF2α, β-tubulin and acetylated α-tubulin to VHL. These data suggest that TLX expression and its binding to microtubules and VHL is important for complex stabilization.

4.4 Paper IV - Nuclear receptor TLX inhibits TGF-β signaling in neuroblastoma

TGF-β has an important role in many cancer types, where it can function either as a tumor suppressor or oncogene. The function of TGF-β in neuroblastoma, however, has not yet been thoroughly investigated. Our aim with this study was to study TGF-β signaling and its possible interaction with TLX in neuroblastoma.

4.4.1 TLX silencing increased growth response of IMR-32 cells to TGF-β

Stable knockdown of TLX in both IMR-32 and SH-SY5Y neuroblastoma cell lines led to significant growth retardation, and TGF-β dependent growth inhibition was found in both control and TLX knockdown cells for SH-SY5Y. However, growth of IMR-32 cells were unaffected by TGF-β treatment in control cells, while stable TLX knockdown led to increased proliferation in response to TGF-β. These results suggested that high TLX expression might be responsible for the lack of TGF-β response in IMR-32 cells.

4.4.2 TLX silencing increased TGF-β dependent promoter activity and induction of TGF-β target genes

To study the effect of TLX on TGF-β signaling we performed luciferase assay using a TGF-β responsive promoter construct and found that knockdown of TLX led to increased promoter activity in both IMR-32 and SH-SY5Y cells. Furthermore, overexpression of TLX in SH-SY5Y cells led to reduced promoter activity. These results were confirmed by quantitative PCR where knockdown of TLX in SH-SY5Y and IMR-32 cells led to increased TGF-β responsive induction of mRNA levels of well-known TGF-β targets p21 and Smad7. These results indicated that TLX inhibits TGF-β response in these neuroblastoma cells.

4.4.3 TLX interacts with Smad3 and prevents Smad2/3 nucelar translocation in IMR-32 cells

We next wanted to study the mechanism by which TLX inhibits TGF-β signaling. Using coimmunoprecipitation we found that TLX protein binds to Smad3, a central component of canonical TGF-β signaling. We then hypothesized that TLX might bind to and inhibit phosphorylation of the Smad2/3 complex by TGF-β receptors, but found no significant effect on phosphorylation when TLX was knocked down or overexpressed. Instead we studied if TLX binding to Smad2/3 might prevent TGF-β dependent translocation of Smad2/3 to the nucleus. We found that in IMR32 cells with stable knockdown of TLX there was

increased nuclear localization of Smad2/3 protein compared to control cells (figure 6). However, in SH-SY5Y, which exhibited TGF-β responsive nuclear translocation of Smad2/3 in almost all cells, TLX knockdown or overexpression had no significant effect.

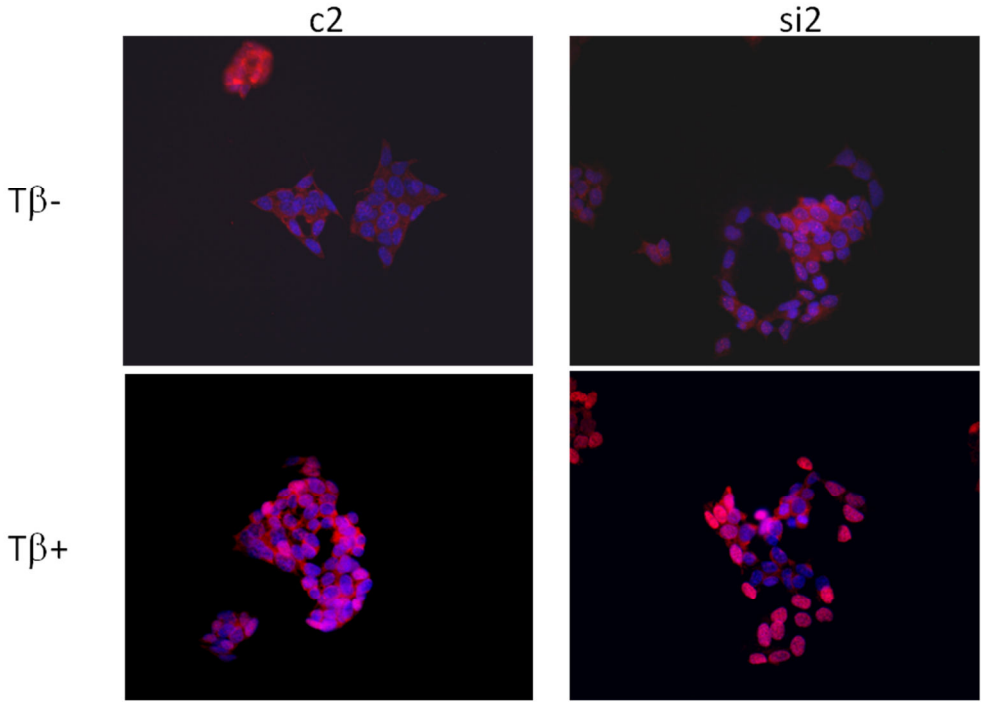

Figure 6: IMR-32 cells with silenced TLX (si2) displayed increased Smad2/3 nuclear translocation compared to control cells (c2) when treated with TGF-β (blue: nucleus; red: Smad2/3).

5. Discussion

5.1 Regulation of angiogenesis by TGF-β in diffuse-type gastric carcinoma

In paper I and II we studied the function of TGF-β in diffuse type gastric carcinoma, where TGF-β production was reported to be associated with progression and decreased survival, but since loss of Smad4 had also been reported in this cancer type, the role of TGF-β in diffuse-type gastric carcinoma was unclear.

We found that disruption of TGF-β signaling due to overexpression of a dominant-negative mutant form of the TGF-β type II receptor (dnTβRII) significantly induced tumorigenicity of OCUM-2MLN diffuse-type gastric carcinoma cells *in vivo*, and that this effect was due to induced angiogenesis. One of the major tumor-promoting properties of TGF-β is its stimulation of angiogenesis through the induction of angiogenesis-promoting factors such as PDGFB, VEGF as well as the secretion of MMP2 and -9. Concurrent with this, we found through microarray analysis of gene expression in dnTβRII and control tumors that disruption of TGF-β signaling led to repressed PDGFB, VEGF and MMP2 mRNA expression, but that it also led to repression of angiogenesis-inhibitors such as TSP1 and TIMP2 (Komuro et al., 2009; Paper I). It appeared that in this tumor model the repression of angiogenesis-inhibitors by dnTβRII expression had more impact on angiogenesis and tumor growth than the repression of angiogenesis-inducing genes.

When we previously coexpressed TSP1 with dnTβRII in OCUM-2MLN cells we found reduced vessel density as seen by decreased Pecam1 staining of tumor samples (Komuro et al., 2009). However, when we coexpressed TIMP2 with dnTβRII there was no significant reduction of vessel density but, similar to GFP control tumors, the vessels in TIMP2 tumors were shorter and had less pericyte coverage, resulting in leaky vessels that were unable to efficiently deliver oxygen to the tumor tissue. TIMP2 is a known inhibitor of endothelial cell proliferation, but its possible effect on pericyte proliferation or recruitment to vessels has not been reported previously. The mechanism behind this remains to be studied.

5.2 The role of TGF-β in cancer stem cell regulation

From our *in vivo* microarray data we found that two genes encoding ABC-transporters, proteins that are highly expressed in normal and cancer stem cells, were upregulated in diffuse-type gastric tumors with disrupted TGF-β signaling. In paper II, we studied the effect of TGF-β on the cancer stem cell population and found that TGF-β reduced the percentage of cancer-initiating cells in this gastric cancer model, which is contrary to the function of TGF-β in glioma-initiating cells (GICs). In GICs, TGF-β dependent induction of the leukemia inhibitory factor (LIF) and subsequent activation of the JAK-STAT pathway led to increased self-renewal and tumorigenesis (Peñuelas et al., 2009). By another mechanism, TGF-β was shown to induce *SOX4* and subsequently *SOX2* gene expression, which also led to increased stemness and tumor forming ability of GICs (Ikushima et al., 2009). Similar effects on maintenance of stemness in cancer-initiating cells have been reported in other cancer types such as chronic myeloid leukaemia (Naka et al., 2010).

However, similar to our results, TGF-β stimulation of transformed human breast epithelial cells led to loss of stem cell-like properties and mammosphere formation in one study (Tang et al., 2007), although another study on similar cells showed that TGF-β maintained stemness and tumorigenic activity of these cells through the induction of EMT (Mani et al., 2008). Just as in most aspects of TGF-β in cancer development, TGF-β thus appears to have a dual role in the control of cancer stem cells, and the mechanisms that regulate this balance needs to be addressed in the future.

In paper IV, we reported that not only does TLX inhibit TGF-β signaling in neuroblastoma cells, but TGF-β also seems to repress TLX promoter activity, suggesting a reciprocal interaction between these two molecules. TLX is a major regulator of stemness in neural stem cells, and unpublished data from our group strongly suggests that it also has an important role in maintaining stem like properties of cancer-initiating cells in neuroblastoma. TGF-β, on the other hand, seems to be a negative regulator of neural stem cells. Inhibition of signaling of the TGF-β family was shown to commit embryonic stem cells to form embryonic neural stem cells that can differentiate to neurons, astrocytes and oligodendrocytes of the central nervous system (Temple, 2001). TGF-β also negatively regulates Wnt-induced self-renewal of neural stem cells in the mid brain, where ablation of the *TBRII* gene led to midbrain enlargement and increased expression of components of the Wnt signal pathway (Falk et al., 2008).

It remains to be elucidated if TGF-β has a similar inhibitory function in the regulation of cancer-initiating cells in neuroblastoma, and if its interaction with TLX is important for this regulation.

5.3 Cancer stem cells and tumor metastasis

There have been reports indicating that cancer stem cells are more prone to metastasize than the main fraction of cells in a tumor. For instance, a distinct subpopulation of metastatic pancreatic cancer stem cells, profiled as $CD133^+$ $CXCR4^+$, exhibited stronger migratory properties than the regular $CD133^+$ stem cell fraction, and removal of this subpopulation from the cancer stem cell pool almost completely abrogated metastasis but not primary tumor formation *in vivo* (Hermann *et al*., 2007).

In agreement with these reports, we found in paper II that diffuse-type gastric carcinoma cells isolated from metastases (OCUM-2MLN and OCUM-2MLD3) from mice xenografted with the parent cell line OCUM-2M expressed higher levels of ABCG2 and had a larger SP fraction than the parent cell line. When these cells were implanted into mice, OCUM-2MLN and OCUM-2MD3 cells gave rise to lymph node and intraperitoneal metastases, respectively, while no metastases were formed by OCUM-2M (Fujihara et al., 1998; Yashiro et al., 1996). These data suggest that the 2MLN and 2MD3 cell lines, which have a relatively large SP cell fraction, have acquired a higher metastatic ability than the parent cell line, and that metastasis of diffuse-type gastric carcinoma might be due to a small subpopulation of metastatic tumor cells.

5.4 Induction of tumor angiogenesis by TLX

Many recent reports suggest that there is a strong association between high HIF2α expression and the maintenance of cancer-initiating cells in different types of tumors, including glioma and neuroblastoma, where HIF2α correlates with high VEGF expression, angiogenesis and poor prognosis (Li et al., 2009; Holmquist-Mengelbier et al., 2006). Furthermore, HIF2α, unlike HIF1α, activates the expression of genes associated with stemness, such as *OCT4* and *ABCG2* (Covello et al., 2006; Martin et al., 2008).

TLX was previously shown to be an important oxygen sensor for hypoxia-regulated angiogenesis in the postnatal retina (Uemura et al., 2006). In paper III, we showed that expression of TLX might promote angiogenesis through different mechanisms depending on the oxygen levels. In normoxia TLX binds to and sequesters pVHL, which leads to stabilization of HIF2α and enhanced binding of HIF2α to the VEGF promoter, while in hypoxia TLX directly binds and activates the VEGF promoter. It is possible that cancer progression in response to high levels of HIF2α in neuroblastoma and other malignancies are in part due to indirect HIF2α stabilization by TLX.

We found that both β-tubulin and acetylated α-tubulin were present in a complex with TLX-pVHL-HIF2α, and that their binding to the complex appeared to increase in hypoxia but decrease if TLX was silenced. Furthermore, treatment with a microtubule-interfering drug reduced the binding of β-tubulin, acetylated α-tubulin, TLX and HIF2α to pVHL. These findings suggest that TLX is important for the interaction between microtubules and the TLX-pVHL-HIF2α complex, and it is possible that binding to microtubule may be needed for the hypoxia-dependent translocation of TLX and HIF2α into the nucleus.

5.5 The interaction between TLX and TGF-β signaling in neuroblastoma cell proliferation

In paper IV, we show that in IMR-32 neuroblastoma cells that normally are refractory to TGF-β stimulation, knockdown of TLX expression make the cells more responsive to TGF-β stimulus and lead to TGF-β dependent induction of proliferation. We found that upregulation of the cdk inhibitor p21 by TGF-β, which is a well-known mechanism for TGF-β growth repression, is enhanced when TLX is knocked down, which is in agreement with the report that p21 is inhibited by TLX (Sun et al., 2007). However, since TGF-β treatment led to growth induction and not repression, it is likely that p21 is not an important regulator of proliferation in these cells.

Our group previously reported high expression levels of PDGF receptor β (PDGFRβ) in IMR-32 cells (Wetterskog et al., 2009). We now found that PDGFRβ mRNA and protein levels are significantly reduced in cells with TLX-knockdown, but that expression is induced by TGF-β treatment. TGF-β dependent induction of PDGFA and B, the ligands for PDGFRβ, has been found to promote proliferation in glioma and osteosarcoma cells (Bruna et al., 2007; Matsuyama et al., 2003), and PDGF signaling could also be important for proliferation of neuroblastoma cells. The constitutively high levels of PDGFRβ, perhaps partly due to high TLX expression, in wild-type IMR-32 cells might explain the high proliferation and lack of growth response to TGF-β in these cells, while cells where TLX is silenced have retarded growth due to low basal levels of PDGFRβ.

The proximal promoter of the *PDGFRB* gene contains putative binding sites both for TLX and Smad, suggesting competitive or synergistic effects of TLX and Smad on *PDGFRB* expression. We are presently studying the interaction between TLX and Smad in regard to promoter binding and their effect on regulation of potential common target genes such as *PDGFRB*.

5.6 The dual role of TGF-β in cancer progression

As a general model, it is thought that TGF-β functions as a tumor suppressor in the early phases of tumorigenesis, but during cancer progression it can be switched into a promoter of tumor growth and metastasis (Bierie & Moses, 2006). Many cancers such as glioma, prostate cancer and breast cancer have mutations that disrupt only the tumor suppressive signal pathways induced by TGF-β while the mitogenic effect remains intact, leading to increased malignancy (Massagué, 2008).

The genetic and epigenetic mechanism behind the switch that turns TGF-β from a tumor suppressor into a tumor promoter is still poorly understood, though the mutation and activation of several oncogenes might be involved. Aberrant expression of c-Myc in tumor cells repress the expression of cell cycle regulators p15 and p21, thus abolishing the growth suppressive response to TGF-β (Pardali & Moustakas, 2007). Activation of the oncogenic Wnt/β-catenin pathway in cancers of the colon, breast and liver leads to binding of β-catenin and LEF-1 to the c-Myc promoter and induced c-Myc expression, and also prevents TGF-β from suppressing c-Myc (Sasaki et al., 2003).

Another major oncogene affecting TGF-β signaling is Ras and its downstream signaling through the Raf/MAPK pathway. Ras-transformed mammary epithelial cells became resistant to TGF-β dependent growth inhibition but induced TGF-β secretion leading to increased TGF-β dependent invasive properties of these cells (Oft et al., 1996). In Panc-1pancreatic carcinoma cells, constitutively active Ras cooperated with TGF-β to specifically induce the expression of Snail, a transcription factor that promotes EMT, while other known TGF-β target genes were unaffected (Horiguchi et al., 2009).

In the inhibition of early stage tumor cell proliferation the tumor suppressor p53 cooperates with TGF-β (Adorno et al., 2009). However, when p53 is mutated, Smad proteins cooperate with mutant p53 to antagonize the ability of p63 to suppress metastasis through the downregulation of sharp-1 and cyclin G2. Mutation of p53 in non-invasive tumor cells enhances pro-invasive and migratory effects of TGF-β (Adorno et al., 2009).

In a recent paper, downregulation of disabled homolog 2 (DAB2) in squamous cell carcinoma cells blocked TGF-β-dependent growth inhibition, while it increased TGF-β dependent induction of motility, anchorage-independent growth and *in vivo* tumor growth, although the mechanism for the dual action of DAB2 is yet unclear (Hannigan et al., 2010).

6. Conclusions

I. Disruption of TGF-β signaling due to introduction of dnTβRII to OCUM-2MLN cells increases tumor angiogenesis and growth. Expression of TIMP2 is induced by TGF-β *in vitro* and repressed in dnTβRII tumors *in vivo*. Exogenous expression of TIMP2 abolishes the accelerated growth of dnTβRII tumors. TIMP2 expressing tumors display increased hypoxia and shorter vessels with less pericyte coverage compared to dnTβRII tumors.

II. OCUM-2MLN cells contain an SP cell fraction with increased tumorigenic properties. TGF-β represses expression of ABCG2 through binding of Smad2/3 to the *ABCG2* promoter and diminishes the SP-cell population in OCUM-2MLN, leading to reduced tumor formation *in vivo*.

III. TLX expression in neuroblastoma inhibits VHL-dependent degradation of HIF2α, leading to its stabilization and enhancement of binding of HIF2α to the VEGF promoter. Knockdown of TLX increases VHL protein levels and reduces hypoxia-dependent stabilization of HIF2α, leading to cell growth inhibition and apoptosis.

IV. Knockdown of TLX increases growth in response to TGF-β in IMR-32 cells and induces TGF-β-dependent promoter activity and mRNA expression of TGF-β target genes, p21 and Smad7, in IMR-32 and SH-SY5Y cells. TLX physically interacts with Smad3 and prevents TGF-β induced nuclear translocation of Smad2/3 in IMR-32.

7. Acknowledgements

This work was carried out at the Institute of Biomedicine at the University of Gothenburg and at the Department of Molecular Pathology at the Graduate School of Medicine, University of Tokyo, Japan. I would like to thank the following people that have enabled me to complete this thesis.

First of all, my supervisor, **Keiko Funa**, for accepting me as a PhD student in her group, for supporting me through all the years, and for enabling me to do part of my PhD in Japan.

My co-supervisor in Tokyo, **Kohei Miyazono**, for allowing me to work in his lab and for making it possible to extend my period in Tokyo.

Everyone at the Department of Molecular Pathology in Tokyo, especially the following:
Mitsunobu R Kano, my group leader in Tokyo, for all his advice and help.
Akiyoshi Komuro for allowing me to be part of his project, for his kindness, and for teaching me a lot of techniques.
Caname Iwata, for his patience and kindness, and for invaluable help with my animal experiments.
Shogo Ehata, for including me in his project and for experimental advice.
Yasuyuki Morishita, for help with tissue preparation and for the opera singing.
Masako Oka for spreading a positive atmosphere in the lab, and for "taking care" of me at times when the Japanese conversation became too difficult.
Kunihiko Kiyono, for being down-to-earth and always easy to talk to, for his technical advice and for introducing me to the lab when I first came to Tokyo.
Hironori Matsuyama, for the almost daily lunch company, for izakaya visits, walks and talks (Nanika ii koto arimasu youni!).
Kaori Harada, for her friendliness, and for izakaya visits and Shinto festivals.
Hiroshi Suzuki for advice, lunches and help with the microarray analysis.
Hajime Mihira for treating me to yakiniku and ostrich liver, for the lunch company and positive spirit.
Yo-taro Shirai, **Kimihito Kawabata**, **Yoshiko Matsumoto**, for good company in the lab.

Akira Watanabe, for good advice and for arranging and analysing the microarray.

Present members of the lab in Gothenburg:
Amiko Hayashi, for the coffee breaks and creating a happy mood in the lab.
Ravikanth Rao, for high spirits and for being a good roommate.
V. Sivaramakrishnan, for his positivity and for being one of the kindest persons I have met.

Past lab members and colleagues:
Pavithra Chavali for the cakes, coffee and technical advice and for being an inspiration in the lab.
Zhaojun Zeng, for experimental advice, especially regarding Western blots, for keeping me company in the evenings at the lab, for the singing and the music.
Bodil Nordlander, for the discussions, and for good lab and lunch company
Muna Elmi, for making me feel welcome when I first joined the group, and for being such a kind and helpful person.
Rie Tajima, for teaching me a lot of methods during the first year in the group.
Yoshiki Matsumoto, for the lunch company and advice before going to Japan.
Weiwen Yang, for being a friendly and helpful person.
Takeshi Yoshida, for introducing me to his project, technical advice and lunch company.
Zackary Chiragwandi, for his positive thinking and for introducing me to the world of physics.
Abtin and **Alicia Moshiri**, for being nice and friendly people.

Erica, **Anna**, **Nico** and **Gisela**, for the lunches and Friday coffees.
Daniel Andersson, for teaming up with me and for putting up with my ignorance during the Statistics course.

All the administrators at the Institute of Biomedicine, for the invaluable help.

Daniel Wetterskog, for being a great friend, for the lunches, many Japanese movie nights, and for all your advice about Swedish PhD life when I came back from Tokyo.

Claes Molin, soon-to-be "Double Doctor", for the friendship, music talks, football-nights and Cinemateket-movies.

Johan Sejnelid, for friendship, beer drinking at Pronto and Tokyo walks, for the discussions about everything Japanese and non-science.

My closest family, for all their support through the years:
my mother **Margareta**, my father **Bo** and his wife **Marianne**, my brother **Lars**, his wife **Jorun**, my niece **Hilma** and nephews **Henric** and **Linus**.

My two grandmothers, mormor **Karin** and farmor **Irma**, for always believing in me/worrying about me. I miss you.

Noriko Kokubo, for her love and support, and for a happy future together.

8. References

Adamis, A.P., Miller, J.W., Bernal, M.T., D'Amico, D.J., Folkman, J., Yeo, T.K., and Yeo, K.T. (1994). Increased vascular endothelial growth factor levels in the vitreous of eyes with proliferative diabetic retinopathy. Am J Ophthalmol *118*, 445-450.

Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V.*, et al.* (2009). A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell *137*, 87-98.

Agah, A., Kyriakides, T.R., Lawler, J., and Bornstein, P. (2002). The lack of thrombospondin-1 (TSP1) dictates the course of wound healing in double-TSP1/TSP2-null mice. Am J Pathol *161*, 831-839.

Aiello, L.P., Avery, R.L., Arrigg, P.G., Keyt, B.A., Jampel, H.D., Shah, S.T., Pasquale, L.R., Thieme, H., Iwamoto, M.A., Park, J.E.*, et al.* (1994). Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med *331*, 1480-1487.

Alvi, A.J., Clayton, H., Joshi, C., Enver, T., Ashworth, A., Vivanco, M.M., Dale, T.C., and Smalley, M.J. (2003). Functional and molecular characterisation of mammary side population cells. Breast Cancer Res *5*, R1-8.

Baker, A.H., Edwards, D.R., and Murphy, G. (2002). Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci *115*, 3719-3727.

Bao, S., Wu, Q., Sathornsumetee, S., Hao, Y., Li, Z., Hjelmeland, A.B., Shi, Q., McLendon, R.E., Bigner, D.D., and Rich, J.N. (2006). Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor. Cancer Res *66*, 7843-7848.

Bardos, J.I., and Ashcroft, M. (2005). Negative and positive regulation of HIF-1: a complex network. Biochim Biophys Acta *1755*, 107-120.

Battegay, E.J., Raines, E.W., Seifert, R.A., Bowen-Pope, D.F., and Ross, R. (1990). TGF-beta induces bimodal proliferation of connective tissue cells via complex control of an autocrine PDGF loop. Cell *63*, 515-524.

Benjamin, L.E., Golijanin, D., Itin, A., Pode, D., and Keshet, E. (1999). Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest *103*, 159-165.

Bierie, B., and Moses, H.L. (2006). Tumor microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer *6*, 506-520.

Bornstein, P., and Sage, E.H. (2002). Matricellular proteins: extracellular modulators of cell function. Curr Opin Cell Biol *14*, 608-616.

Bornstein, S., White, R., Malkoski, S., Oka, M., Han, G., Cleaver, T., Reh, D., Andersen, P., Gross, N., Olson, S.*, et al.* (2009). Smad4 loss in mice causes spontaneous head and neck cancer with increased genomic instability and inflammation. J Clin Invest *119*, 3408-3419.

Brand, K., Baker, A.H., Perez-Canto, A., Possling, A., Sacharjat, M., Geheeb, M., and Arnold, W. (2000). Treatment of colorectal liver metastases by adenoviral transfer of tissue inhibitor of metalloproteinases-2 into the liver tissue. Cancer Res *60*, 5723-5730.

Brenner, H., Rothenbacher, D., and Arndt, V. (2009). Epidemiology of stomach cancer. Methods Mol Biol *472*, 467-477.

Brodeur, G.M., and Maris, J.M. (2006). in Principles and Practice of Pediatric Oncology, eds Pizzo, P.A., and Poplack, D.G. (J.B. Lippincott, Philadelphia), pp. 933–970.

Brown, L.F., Berse, B., Jackman, R.W., Tognazzi, K., Manseau, E.J., Dvorak, H.F., and Senger, D.R. (1993). Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol *143*, 1255-1262.

Bruna, A., Darken, R.S., Rojo, F., Ocana, A., Penuelas, S., Arias, A., Paris, R., Tortosa, A., Mora, J., Baselga, J.*, et al.* (2007). High TGFbeta-Smad activity confers poor prognosis in glioma patients and promotes cell proliferation depending on the methylation of the PDGF-B gene. Cancer Cell *11*, 147-160.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C.*, et al.* (1996a). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature *380*, 435-439.

Carmeliet, P., Mackman, N., Moons, L., Luther, T., Gressens, P., Van Vlaenderen, I., Demunck, H., Kasper, M., Breier, G., Evrard, P.*, et al.* (1996b). Role of tissue factor in embryonic blood vessel development. Nature *383*, 73-75.

Chakrabarti, J., Turley, H., Campo, L., Han, C., Harris, A.L., Gatter, K.C., and Fox, S.B. (2004). The transcription factor DEC1 (stra13, SHARP2) is associated with the hypoxic response and high tumor grade in human breast cancers. Br J Cancer *91*, 954-958.

Chang, Y.S., di Tomaso, E., McDonald, D.M., Jones, R., Jain, R.K., and Munn, L.L. (2000). Mosaic blood vessels in tumors: frequency of cancer cells in contact with flowing blood. Proc Natl Acad Sci U S A *97*, 14608-14613.

Cheng, N., Bhowmick, N.A., Chytil, A., Gorksa, A.E., Brown, K.A., Muraoka, R., Arteaga, C.L., Neilson, E.G., Hayward, S.W., and Moses, H.L. (2005). Loss of TGF-beta type II receptor in fibroblasts promotes mammary carcinoma growth and invasion through upregulation of TGF-alpha-, MSP- and HGF-mediated signaling networks. Oncogene *24*, 5053-5068.

Compagni, A., Wilgenbus, P., Impagnatiello, M.A., Cotten, M., and Christofori, G. (2000). Fibroblast growth factors are required for efficient tumor angiogenesis. Cancer Res *60*, 7163-7169.

Covello, K.L., Kehler, J., Yu, H., Gordan, J.D., Arsham, A.M., Hu, C.J., Labosky, P.A., Simon, M.C., and Keith, B. (2006). HIF-2alpha regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. Genes Dev *20*, 557-570.

Daley, W.P., Peters, S.B., and Larsen, M. (2008). Extracellular matrix dynamics in development and regenerative medicine. J Cell Sci *121*, 255-264.

Datto, M.B., Li, Y., Panus, J.F., Howe, D.J., Xiong, Y., and Wang, X.F. (1995). Transforming growth factor beta induces the cyclin-dependent kinase inhibitor p21 through a p53-independent mechanism. Proc Natl Acad Sci U S A *92*, 5545-5549.

Dawson, D.W., Pearce, S.F., Zhong, R., Silverstein, R.L., Frazier, W.A., and Bouck, N.P. (1997). CD36 mediates the In vitro inhibitory effects of thrombospondin-1 on endothelial cells. J Cell Biol *138*, 707-717.

Dean, M., Fojo, T., and Bates, S. (2005). Tumor stem cells and drug resistance. Nat Rev Cancer *5*, 275-284.

Deroanne, C.F., Hajitou, A., Calberg-Bacq, C.M., Nusgens, B.V., and Lapiere, C.M. (1997). Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. Cancer Res *57*, 5590-5597.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001). TGF-beta signaling in tumor suppression and cancer progression. Nat Genet *29*, 117-129.

DuBois, S.G., Kalika, Y., Lukens, J.N., Brodeur, G.M., Seeger, R.C., Atkinson, J.B., Haase, G.M., Black, C.T., Perez, C., Shimada, H.*, et al.* (1999). Metastatic sites in stage IV and IVS neuroblastoma correlate with age, tumor biology, and survival. J Pediatr Hematol Oncol *21*, 181-189.

Ebert, B.L., Firth, J.D., and Ratcliffe, P.J. (1995). Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J Biol Chem *270*, 29083-29089.

Ebisawa, T., Fukuchi, M., Murakami, G., Chiba, T., Tanaka, K., Imamura, T., and Miyazono, K. (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J Biol Chem *276*, 12477-12480.

Ehata, S., Hanyu, A., Hayashi, M., Aburatani, H., Kato, Y., Fujime, M., Saitoh, M., Miyazawa, K., Imamura, T., and Miyazono, K. (2007). Transforming growth factor-beta promotes survival of mammary carcinoma cells through induction of antiapoptotic transcription factor DEC1. Cancer Res *67*, 9694-9703.

Eichmann, A., Corbel, C., Nataf, V., Vaigot, P., Breant, C., and Le Douarin, N.M. (1997). Ligand-dependent development of the endothelial and hemopoietic lineages from embryonic mesodermal cells expressing vascular endothelial growth factor receptor 2. Proc Natl Acad Sci U S A *94*, 5141-5146.

Elliott, R.L., and Blobe, G.C. (2005). Role of transforming growth factor Beta in human cancer. J Clin Oncol *23*, 2078-2093.

Esiashvili, N., Anderson, C., and Katzenstein, H.M. (2009). Neuroblastoma. Curr Probl Cancer *33*, 333-360.

Falk, S., Wurdak, H., Ittner, L.M., Ille, F., Sumara, G., Schmid, M.T., Draganova, K., Lang, K.S., Paratore, C., Leveen, P.*, et al.* (2008). Brain area-specific effect of TGF-beta signaling on Wnt-dependent neural stem cell expansion. Cell Stem Cell *2*, 472-483.

Ferrara, N. (1999). Molecular and biological properties of vascular endothelial growth factor. J Mol Med *77*, 527-543.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-Braxton, L., Hillan, K.J., and Moore, M.W. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature *380*, 439-442.

Ferrara, N., and Kerbel, R.S. (2005). Angiogenesis as a therapeutic target. Nature *438*, 967-974.

Folkman, J., Langer, R., Linhardt, R.J., Haudenschild, C., and Taylor, S. (1983). Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. Science *221*, 719-725.

Folkman, J., Merler, E., Abernathy, C., and Williams, G. (1971). Isolation of a tumor factor responsible for angiogenesis. J Exp Med *133*, 275-288.

Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. (1996). Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol *16*, 4604-4613.

Friedman, E., Gold, L.I., Klimstra, D., Zeng, Z.S., Winawer, S., and Cohen, A. (1995). High levels of transforming growth factor beta 1 correlate with disease progression in human colon cancer. Cancer Epidemiol Biomarkers Prev *4*, 549-554.

Fujihara, T., Sawada, T., Hirakawa, K., Chung, Y.S., Yashiro, M., Inoue, T., and Sowa, M. (1998). Establishment of lymph node metastatic model for human gastric cancer in nude mice and analysis of factors associated with metastasis. Clin Exp Metastasis *16*, 389-398.

Fukumura, D., Xavier, R., Sugiura, T., Chen, Y., Park, E.C., Lu, N., Selig, M., Nielsen, G., Taksir, T., Jain, R.K.*, et al.* (1998). Tumor induction of VEGF promoter activity in stromal cells. Cell *94*, 715-725.

Good, D.J., Polverini, P.J., Rastinejad, F., Le Beau, M.M., Lemons, R.S., Frazier, W.A., and Bouck, N.P. (1990). A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. Proc Natl Acad Sci U S A *87*, 6624-6628.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med *183*, 1797-1806.

Goto, F., Goto, K., Weindel, K., and Folkman, J. (1993). Synergistic effects of vascular endothelial growth factor and basic fibroblast growth factor on the proliferation and cord formation of bovine capillary endothelial cells within collagen gels. Lab Invest *69*, 508-517.

Gottesman, M.M., Fojo, T., and Bates, S.E. (2002). Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer *2*, 48-58.

Graeber, T.G., Osmanian, C., Jacks, T., Housman, D.E., Koch, C.J., Lowe, S.W., and Giaccia, A.J. (1996). Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumors. Nature *379*, 88-91.

Greenaway, J., Lawler, J., Moorehead, R., Bornstein, P., Lamarre, J., and Petrik, J. (2007). Thrombospondin-1 inhibits VEGF levels in the ovary directly by binding and internalization via the low density lipoprotein receptor-related protein-1 (LRP-1). J Cell Physiol *210*, 807-818.

Hannigan, A., Smith, P., Kalna, G., Lo Nigro, C., Orange, C., O'Brien, D.I., Shah, R., Syed, N., Spender, L.C., Herrera, B.*, et al.* (2010). Epigenetic downregulation of human disabled homolog 2 switches TGF-beta from a tumor suppressor to a tumor promoter. J Clin Invest *120*, 2842-2857.

Hannon, G.J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. Nature *371*, 257-261.

Heider, T.R., Lyman, S., Schoonhoven, R., and Behrns, K.E. (2007). Ski promotes tumor growth through abrogation of transforming growth factor-beta signaling in pancreatic cancer. Ann Surg *246*, 61-68.

Heldin, C.H., Miyazono, K., and ten Dijke, P. (1997). TGF-beta signaling from cell membrane to nucleus through SMAD proteins. Nature *390*, 465-471.

Hellstrom, M., Kalen, M., Lindahl, P., Abramsson, A., and Betsholtz, C. (1999). Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development *126*, 3047-3055.

Henson, D.E., Dittus, C., Younes, M., Nguyen, H., and Albores-Saavedra, J. (2004). Differential trends in the intestinal and diffuse types of gastric carcinoma in the United States, 1973-2000: increase in the signet ring cell type. Arch Pathol Lab Med *128*, 765-770.

Hergovich, A., Lisztwan, J., Barry, R., Ballschmieter, P., and Krek, W. (2003). Regulation of microtubule stability by the von Hippel-Lindau tumor suppressor protein pVHL. Nat Cell Biol *5*, 64-70.

Hermann, P.C., Huber, S.L., Herrler, T., Aicher, A., Ellwart, J.W., Guba, M., Bruns, C.J., and Heeschen, C. (2007). Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell *1*, 313-323.

Hirschmann-Jax, C., Foster, A.E., Wulf, G.G., Nuchtern, J.G., Jax, T.W., Gobel, U., Goodell, M.A., and Brenner, M.K. (2004). A distinct "side population" of cells with high drug efflux capacity in human tumor cells. Proc Natl Acad Sci U S A *101*, 14228-14233.

Hoehner, J.C., Gestblom, C., Hedborg, F., Sandstedt, B., Olsen, L., and Pahlman, S. (1996). A developmental model of neuroblastoma: differentiating stroma-poor tumors' progress along an extra-adrenal chromaffin lineage. Lab Invest *75*, 659-675.

Hohenberger, P., and Gretschel, S. (2003). Gastric cancer. Lancet *362*, 305-315.

Holmquist-Mengelbier, L., Fredlund, E., Lofstedt, T., Noguera, R., Navarro, S., Nilsson, H., Pietras, A., Vallon-Christersson, J., Borg, A., Gradin, K.*, et al.* (2006). Recruitment of HIF-1alpha and HIF-2alpha to common target genes is differentially regulated in neuroblastoma: HIF-2alpha promotes an aggressive phenotype. Cancer Cell *10*, 413-423.

Horiguchi, K., Shirakihara, T., Nakano, A., Imamura, T., Miyazono, K., and Saitoh, M. (2009). Role of Ras signaling in the induction of snail by transforming growth factor-beta. J Biol Chem *284*, 245-253.

Hyytiainen, M., Penttinen, C., and Keski-Oja, J. (2004). Latent TGF-beta binding proteins: extracellular matrix association and roles in TGF-beta activation. Crit Rev Clin Lab Sci *41*, 233-264.

Ikushima, H., Todo, T., Ino, Y., Takahashi, M., Miyazawa, K., and Miyazono, K. (2009). Autocrine TGF-beta signaling maintains tumorigenicity of glioma-initiating cells through Sry-related HMG-box factors. Cell Stem Cell *5*, 504-514.

Iwai, K., Yamanaka, K., Kamura, T., Minato, N., Conaway, R.C., Conaway, J.W., Klausner, R.D., and Pause, A. (1999). Identification of the von Hippel-lindau tumor-suppressor protein as part of an active E3 ubiquitin ligase complex. Proc Natl Acad Sci U S A *96*, 12436-12441.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science *292*, 464-468.

Iwasaki, H., and Suda, T. (2009). Cancer stem cells and their niche. Cancer Sci *100*, 1166-1172.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J.*, et al.* (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science *292*, 468-472.

Jain, S., Maltepe, E., Lu, M.M., Simon, C., and Bradfield, C.A. (1998). Expression of ARNT, ARNT2, HIF1 alpha, HIF2 alpha and Ah receptor mRNAs in the developing mouse. Mech Dev *73*, 117-123.

Jang, C.W., Chen, C.H., Chen, C.C., Chen, J.Y., Su, Y.H., and Chen, R.H. (2002). TGF-beta induces apoptosis through Smad-mediated expression of DAP-kinase. Nat Cell Biol *4*, 51-58.

Jimenez, B., Volpert, O.V., Crawford, S.E., Febbraio, M., Silverstein, R.L., and Bouck, N. (2000). Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat Med *6*, 41-48.

Kalluri, R. (2003). Basement membranes: structure, assembly and role in tumor angiogenesis. Nat Rev Cancer *3*, 422-433.

Kamura, T., Koepp, D.M., Conrad, M.N., Skowyra, D., Moreland, R.J., Iliopoulos, O., Lane, W.S., Kaelin, W.G., Jr., Elledge, S.J., Conaway, R.C.*, et al.* (1999). Rbx1, a component of the VHL tumor suppressor complex and SCF ubiquitin ligase. Science *284*, 657-661.

Kang, Y., Siegel, P.M., Shu, W., Drobnjak, M., Kakonen, S.M., Cordon-Cardo, C., Guise, T.A., and Massague, J. (2003). A multigenic program mediating breast cancer metastasis to bone. Cancer Cell *3*, 537-549.

Katayama, R., Koike, S., Sato, S., Sugimoto, Y., Tsuruo, T., and Fujita, N. (2009). Dofequidar fumarate sensitizes cancer stem-like side population cells to chemotherapeutic drugs by inhibiting ABCG2/BCRP-mediated drug export. Cancer Sci *100*, 2060-2068.

Kavsak, P., Rasmussen, R.K., Causing, C.G., Bonni, S., Zhu, H., Thomsen, G.H., and Wrana, J.L. (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell *6*, 1365-1375.

Kim, B.G., Li, C., Qiao, W., Mamura, M., Kasprzak, B., Anver, M., Wolfraim, L., Hong, S., Mushinski, E., Potter, M.*, et al.* (2006a). Smad4 signaling in T cells is required for suppression of gastrointestinal cancer. Nature *441*, 1015-1019.

Kim, J.W., Tchernyshyov, I., Semenza, G.L., and Dang, C.V. (2006b). HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab *3*, 177-185.

Kim, J.Y., Park, D.Y., Kim, G.H., Choi, K.U., Lee, C.H., Huh, G.Y., Sol, M.Y., Song, G.A., Jeon, T.Y., Kim, D.H.*, et al.* (2005). Smad4 expression in gastric adenoma and adenocarcinoma: frequent loss of expression in diffuse type of gastric adenocarcinoma. Histol Histopathol *20*, 543-549.

Kim, M., and Morshead, C.M. (2003). Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. J Neurosci *23*, 10703-10709.

Kinugasa, S., Abe, S., Tachibana, M., Hishikawa, Y., Yoshimura, H., Monden, N., Dhar, D.K., Nagasue, N., and Nagaoka, S. (1998). Overexpression of transforming growth factor-beta1 in scirrhous carcinoma of the stomach correlates with decreased survival. Oncology *55*, 582-587.

Klagsbrun, M., and Moses, M.A. (1999). Molecular angiogenesis. Chem Biol *6*, R217-224.

Knudson, A.G., Jr., and Strong, L.C. (1972). Mutation and cancer: neuroblastoma and pheochromocytoma. Am J Hum Genet *24*, 514-532.

Koinuma, D., Tsutsumi, S., Kamimura, N., Imamura, T., Aburatani, H., and Miyazono, K. (2009a). Promoter-wide analysis of Smad4 binding sites in human epithelial cells. Cancer Sci *100*, 2133-2142.

Koinuma, D., Tsutsumi, S., Kamimura, N., Taniguchi, H., Miyazawa, K., Sunamura, M., Imamura, T., Miyazono, K., and Aburatani, H. (2009b). Chromatin immunoprecipitation on microarray analysis of Smad2/3 binding sites reveals roles of ETS1 and TFAP2A in transforming growth factor beta signaling. Mol Cell Biol *29*, 172-186.

Komuro, A., Yashiro, M., Iwata, C., Morishita, Y., Johansson, E., Matsumoto, Y., Watanabe, A., Aburatani, H., Miyoshi, H., Kiyono, K.*, et al.* (2009). Diffuse-type gastric carcinoma: progression, angiogenesis, and transforming growth factor beta signaling. J Natl Cancer Inst *101*, 592-604.

Kondo, K., Kim, W.Y., Lechpammer, M., and Kaelin, W.G., Jr. (2003). Inhibition of HIF2alpha is sufficient to suppress pVHL-defective tumor growth. PLoS Biol *1*, E83.

Kondo, T., Setoguchi, T., and Taga, T. (2004). Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. Proc Natl Acad Sci U S A *101*, 781-786.

Kretzschmar, M., Doody, J., and Massague, J. (1997). Opposing BMP and EGF signaling pathways converge on the TGF-beta family mediator Smad1. Nature *389*, 618-622.

Kurban, G., Duplan, E., Ramlal, N., Hudon, V., Sado, Y., Ninomiya, Y., and Pause, A. (2008). Collagen matrix assembly is driven by the interaction of von Hippel-Lindau tumor suppressor protein with hydroxylated collagen IV alpha 2. Oncogene *27*, 1004-1012.

Lau, K.W., Tian, Y.M., Raval, R.R., Ratcliffe, P.J., and Pugh, C.W. (2007). Target gene selectivity of hypoxia-inducible factor-alpha in renal cancer cells is conveyed by post-DNA-binding mechanisms. Br J Cancer *96*, 1284-1292.

Lauren, P. (1965). THE TWO HISTOLOGICAL MAIN TYPES OF GASTRIC CARCINOMA: DIFFUSE AND SO-CALLED INTESTINAL-TYPE CARCINOMA. AN ATTEMPT AT A HISTO-CLINICAL CLASSIFICATION. Acta Pathol Microbiol Scand *64*, 31-49.

Lee, Y.K., So, I.S., Lee, S.C., Lee, J.H., Lee, C.W., Kim, W.M., Park, M.K., Lee, S.T., Park, D.Y., Shin, D.Y.*, et al.* (2005). Suppression of distant pulmonary metastasis of MDA-MB 435 human breast carcinoma established in mammary fat pads of nude mice by retroviral-mediated TIMP-2 gene transfer. J Gene Med *7*, 145-157.

Levy, L., and Hill, C.S. (2006). Alterations in components of the TGF-beta superfamily signaling pathways in human cancer. Cytokine Growth Factor Rev *17*, 41-58.

Li, H., and Liu, J.P. (2007). Mechanisms of action of TGF-beta in cancer: evidence for Smad3 as a repressor of the hTERT gene. Ann N Y Acad Sci *1114*, 56-68.

Li, H., Xu, D., Toh, B.H., and Liu, J.P. (2006). TGF-beta and cancer: is Smad3 a repressor of hTERT gene? Cell Res *16*, 169-173.

Li, W., Sun, G., Yang, S., Qu, Q., Nakashima, K., and Shi, Y. (2008). Nuclear receptor TLX regulates cell cycle progression in neural stem cells of the developing brain. Mol Endocrinol *22*, 56-64.

Li, Z., Bao, S., Wu, Q., Wang, H., Eyler, C., Sathornsumetee, S., Shi, Q., Cao, Y., Lathia, J., McLendon, R.E.*, et al.* (2009). Hypoxia-inducible factors regulate tumorigenic capacity of glioma stem cells. Cancer Cell *15*, 501-513.

Liu, F., and Matsuura, I. (2005). Inhibition of Smad antiproliferative function by CDK phosphorylation. Cell Cycle *4*, 63-66.

Liu, H.K., Wang, Y., Belz, T., Bock, D., Takacs, A., Radlwimmer, B., Barbus, S., Reifenberger, G., Lichter, P., and Schutz, G. (2010). The nuclear receptor tailless induces long-term neural stem cell expansion and brain tumor initiation. Genes Dev *24*, 683-695.

Lobo, N.A., Shimono, Y., Qian, D., and Clarke, M.F. (2007). The biology of cancer stem cells. Annu Rev Cell Dev Biol *23*, 675-699.

Lonergan, K.M., Iliopoulos, O., Ohh, M., Kamura, T., Conaway, R.C., Conaway, J.W., and Kaelin, W.G., Jr. (1998). Regulation of hypoxia-inducible mRNAs by the von Hippel-Lindau tumor suppressor protein requires binding to complexes containing elongins B/C and Cul2. Mol Cell Biol *18*, 732-741.

Lonser, R.R., Glenn, G.M., Walther, M., Chew, E.Y., Libutti, S.K., Linehan, W.M., and Oldfield, E.H. (2003). von Hippel-Lindau disease. Lancet *361*, 2059-2067.

Luca, M., Huang, S., Gershenwald, J.E., Singh, R.K., Reich, R., and Bar-Eli, M. (1997). Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. Am J Pathol *151*, 1105-1113.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N.*, et al.* (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science *277*, 55-60.

Mani, S.A., Guo, W., Liao, M.J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M.*, et al.* (2008). The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell *133*, 704-715.

Maris, J.M., Hogarty, M.D., Bagatell, R., and Cohn, S.L. (2007). Neuroblastoma. Lancet *369*, 2106-2120.

Martin, C.M., Ferdous, A., Gallardo, T., Humphries, C., Sadek, H., Caprioli, A., Garcia, J.A., Szweda, L.I., Garry, M.G., and Garry, D.J. (2008). Hypoxia-inducible factor-2alpha transactivates Abcg2 and promotes cytoprotection in cardiac side population cells. Circ Res *102*, 1075-1081.

Martin, C.M., Meeson, A.P., Robertson, S.M., Hawke, T.J., Richardson, J.A., Bates, S., Goetsch, S.C., Gallardo, T.D., and Garry, D.J. (2004). Persistent expression of the ATP-binding cassette transporter, Abcg2, identifies cardiac SP cells in the developing and adult heart. Dev Biol *265*, 262-275.

Massague, J. (2008). TGF beta in Cancer. Cell *134*, 215-230.

Matsuyama, S., Iwadate, M., Kondo, M., Saitoh, M., Hanyu, A., Shimizu, K., Aburatani, H., Mishima, H.K., Imamura, T., Miyazono, K.*, et al.* (2003). SB-431542 and Gleevec inhibit transforming growth factor-beta-induced proliferation of human osteosarcoma cells. Cancer Res *63*, 7791-7798.

Mendes, O., Kim, H.T., Lungu, G., and Stoica, G. (2007). MMP2 role in breast cancer brain metastasis development and its regulation by TIMP2 and ERK1/2. Clin Exp Metastasis *24*, 341-351.

Miao, W.M., Seng, W.L., Duquette, M., Lawler, P., Laus, C., and Lawler, J. (2001). Thrombospondin-1 type 1 repeat recombinant proteins inhibit tumor growth through transforming growth factor-beta-dependent and -independent mechanisms. Cancer Res *61*, 7830-7839.

Miyahara, R., Niwa, Y., Matsuura, T., Maeda, O., Ando, T., Ohmiya, N., Itoh, A., Hirooka, Y., and Goto, H. (2007). Prevalence and prognosis of gastric cancer detected by screening in a large Japanese population: data from a single institute over 30 years. J Gastroenterol Hepatol *22*, 1435-1442.

Mizoi, T., Ohtani, H., Miyazono, K., Miyazawa, M., Matsuno, S., and Nagura, H. (1993). Immunoelectron microscopic localization of transforming growth factor beta 1 and latent transforming growth factor beta 1 binding protein in human gastrointestinal carcinomas: qualitative difference between cancer cells and stromal cells. Cancer Res *53*, 183-190.

Modak, S., and Cheung, N.K. (2010). Neuroblastoma: Therapeutic strategies for a clinical enigma. Cancer Treat Rev *36*, 307-317.

Mohyeldin, A., Garzon-Muvdi, T., and Quinones-Hinojosa, A. (2010). Oxygen in stem cell biology: a critical component of the stem cell niche. Cell Stem Cell *7*, 150-161.

Monaghan, A.P., Bock, D., Gass, P., Schwager, A., Wolfer, D.P., Lipp, H.P., and Schutz, G. (1997). Defective limbic system in mice lacking the tailless gene. Nature *390*, 515-517.

Monaghan, A.P., Grau, E., Bock, D., and Schutz, G. (1995). The mouse homolog of the orphan nuclear receptor tailless is expressed in the developing forebrain. Development *121*, 839-853.

Moses, M.A. (1997). The regulation of neovascularization of matrix metalloproteinases and their inhibitors. Stem Cells *15*, 180-189.

Mosse, Y.P., Laudenslager, M., Longo, L., Cole, K.A., Wood, A., Attiyeh, E.F., Laquaglia, M.J., Sennett, R., Lynch, J.E., Perri, P.*, et al.* (2008). Identification of ALK as a major familial neuroblastoma predisposition gene. Nature *455*, 930-935.

Moustakas, A., and Heldin, C.H. (2007). Signaling networks guiding epithelial-mesenchymal transitions during embryogenesis and cancer progression. Cancer Sci *98*, 1512-1520.

Naka, K., Hoshii, T., Muraguchi, T., Tadokoro, Y., Ooshio, T., Kondo, Y., Nakao, S., Motoyama, N., and Hirao, A. (2010). TGF-beta-FOXO signaling maintains leukaemia-initiating cells in chronic myeloid leukaemia. Nature *463*, 676-680.

Nussenbaum, F., and Herman, I.M. (2010). Tumor angiogenesis: insights and innovations. J Oncol *2010*, 132641.

O'Reilly, M.S., Holmgren, L., Chen, C., and Folkman, J. (1996). Angiostatin induces and sustains dormancy of human primary tumors in mice. Nat Med *2*, 689-692.

Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. (1996). TGF-beta1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. Genes Dev *10*, 2462-2477.

Ohh, M. (2006). Ubiquitin pathway in VHL cancer syndrome. Neoplasia *8*, 623-629.

Ohh, M., Yauch, R.L., Lonergan, K.M., Whaley, J.M., Stemmer-Rachamimov, A.O., Louis, D.N., Gavin, B.J., Kley, N., Kaelin, W.G., Jr., and Iliopoulos, O. (1998). The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol Cell *1*, 959-968.

Ozdamar, B., Bose, R., Barrios-Rodiles, M., Wang, H.R., Zhang, Y., and Wrana, J.L. (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science *307*, 1603-1609.

Padua, D., Zhang, X.H., Wang, Q., Nadal, C., Gerald, W.L., Gomis, R.R., and Massague, J. (2008). TGFbeta primes breast tumors for lung metastasis seeding through angiopoietin-like 4. Cell *133*, 66-77.

Papandreou, I., Cairns, R.A., Fontana, L., Lim, A.L., and Denko, N.C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. Cell Metab *3*, 187-197.

Papetti, M., and Herman, I.M. (2002). Mechanisms of normal and tumor-derived angiogenesis. Am J Physiol Cell Physiol *282*, C947-970.

Pardali, K., and Moustakas, A. (2007). Actions of TGF-beta as tumor suppressor and pro-metastatic factor in human cancer. Biochim Biophys Acta *1775*, 21-62.

Park, H.J., Kim, J.K., Jeon, H.M., Oh, S.Y., Kim, S.H., Park, M.J., Soeda, A., Nam, D.H., and Kim, H. (2010). The neural stem cell fate determinant TLX promotes tumorigenesis and genesis of cells resembling glioma stem cells. Mol Cells.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A *104*, 5431-5436.

Penuelas, S., Anido, J., Prieto-Sanchez, R.M., Folch, G., Barba, I., Cuartas, I., Garcia-Dorado, D., Poca, M.A., Sahuquillo, J., Baselga, J*., et al.* (2009). TGF-beta increases glioma-initiating cell self-renewal through the induction of LIF in human glioblastoma. Cancer Cell *15*, 315-327.

Petrides, P.E., Bock, S., Bovens, J., Hofmann, R., and Jakse, G. (1990). Modulation of pro-epidermal growth factor, pro-transforming growth factor alpha and epidermal growth factor receptor gene expression in human renal carcinomas. Cancer Res *50*, 3934-3939.

Pietras, A., Gisselsson, D., Ora, I., Noguera, R., Beckman, S., Navarro, S., and Pahlman, S. (2008). High levels of HIF-2alpha highlight an immature neural crest-like neuroblastoma cell cohort located in a perivascular niche. J Pathol *214*, 482-488.

Qu, Q., Sun, G., Li, W., Yang, S., Ye, P., Zhao, C., Yu, R.T., Gage, F.H., Evans, R.M., and Shi, Y. (2010). Orphan nuclear receptor TLX activates Wnt/beta-catenin signaling to stimulate neural stem cell proliferation and self-renewal. Nat Cell Biol *12*, 31-40; sup pp 31-39.

Reifenberger, G., Reifenberger, J., Bilzer, T., Wechsler, W., and Collins, V.P. (1995). Coexpression of transforming growth factor-alpha and epidermal growth factor receptor in capillary hemangioblastomas of the central nervous system. Am J Pathol *147*, 245-250.

Rifkin, D.B. (2005). Latent transforming growth factor-beta (TGF-beta) binding proteins: orchestrators of TGF-beta availability. J Biol Chem *280*, 7409-7412.

Rodriguez-Manzaneque, J.C., Lane, T.F., Ortega, M.A., Hynes, R.O., Lawler, J., and Iruela-Arispe, M.L. (2001). Thrombospondin-1 suppresses spontaneous tumor growth and inhibits activation of matrix metalloproteinase-9 and mobilization of vascular endothelial growth factor. Proc Natl Acad Sci U S A *98*, 12485-12490.

Saaristo, A., Karpanen, T., and Alitalo, K. (2000). Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. Oncogene *19*, 6122-6129.

Sanchez-Elsner, T., Botella, L.M., Velasco, B., Corbi, A., Attisano, L., and Bernabeu, C. (2001). Synergistic cooperation between hypoxia and transforming growth factor-beta pathways on human vascular endothelial growth factor gene expression. J Biol Chem *276*, 38527-38535.

Sasaki, T., Suzuki, H., Yagi, K., Furuhashi, M., Yao, R., Susa, S., Noda, T., Arai, Y., Miyazono, K., and Kato, M. (2003). Lymphoid enhancer factor 1 makes cells resistant to transforming growth factor beta-induced repression of c-myc. Cancer Res *63*, 801-806.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron-Maguire, M., Gridley, T., Wolburg, H., Risau, W., and Qin, Y. (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. Nature *376*, 70-74.

Scharenberg, C.W., Harkey, M.A., and Torok-Storb, B. (2002). The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. Blood *99*, 507-512.

Schultz-Cherry, S., Lawler, J., and Murphy-Ullrich, J.E. (1994). The type 1 repeats of thrombospondin 1 activate latent transforming growth factor-beta. J Biol Chem *269*, 26783-26788.

Schwab, M., Alitalo, K., Klempnauer, K.H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M., and Trent, J. (1983). Amplified DNA with limited homology to myc cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumor. Nature *305*, 245-248.

Schwarte-Waldhoff, I., Volpert, O.V., Bouck, N.P., Sipos, B., Hahn, S.A., Klein-Scory, S., Luttges, J., Kloppel, G., Graeven, U., Eilert-Micus, C.*, et al.* (2000). Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. Proc Natl Acad Sci U S A *97*, 9624-9629.

Sekelsky, J.J., Newfeld, S.J., Raftery, L.A., Chartoff, E.H., and Gelbart, W.M. (1995). Genetic characterization and cloning of mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics *139*, 1347-1358.

Semenza, G.L. (2001). Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol Med *7*, 345-350.

Senger, D.R., Perruzzi, C.A., Feder, J., and Dvorak, H.F. (1986). A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. Cancer Res *46*, 5629-5632.

Seo, D.W., Li, H., Guedez, L., Wingfield, P.T., Diaz, T., Salloum, R., Wei, B.Y., and Stetler-Stevenson, W.G. (2003). TIMP-2 mediated inhibition of angiogenesis: an MMP-independent mechanism. Cell *114*, 171-180.

Seo, D.W., Li, H., Qu, C.K., Oh, J., Kim, Y.S., Diaz, T., Wei, B., Han, J.W., and Stetler-Stevenson, W.G. (2006). Shp-1 mediates the antiproliferative activity of tissue inhibitor of metalloproteinase-2 in human microvascular endothelial cells. J Biol Chem *281*, 3711-3721.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L., and Schuh, A.C. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature *376*, 62-66.

Shay, J.W., and Bacchetti, S. (1997). A survey of telomerase activity in human cancer. Eur J Cancer *33*, 787-791.

Shi, Y., Chichung Lie, D., Taupin, P., Nakashima, K., Ray, J., Yu, R.T., Gage, F.H., and Evans, R.M. (2004). Expression and function of orphan nuclear receptor TLX in adult neural stem cells. Nature *427*, 78-83.

Shi, Y., and Massague, J. (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell *113*, 685-700.

Shimada, H., Stram, D.O., Chatten, J., Joshi, V.V., Hachitanda, Y., Brodeur, G.M., Lukens, J.N., Matthay, K.K., and Seeger, R.C. (1995). Identification of subsets of neuroblastomas by combined histopathologic and N-myc analysis. J Natl Cancer Inst *87*, 1470-1476.

Siegel, P.M., and Massague, J. (2003). Cytostatic and apoptotic actions of TGF-beta in homeostasis and cancer. Nat Rev Cancer *3*, 807-821.

Singh, R.K., Gutman, M., Bucana, C.D., Sanchez, R., Llansa, N., and Fidler, I.J. (1995). Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. Proc Natl Acad Sci U S A *92*, 4562-4566.

Soeda, A., Park, M., Lee, D., Mintz, A., Androutsellis-Theotokis, A., McKay, R.D., Engh, J., Iwama, T., Kunisada, T., Kassam, A.B.*, et al.* (2009). Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha. Oncogene *28*, 3949-3959.

Sorrentino, A., Thakur, N., Grimsby, S., Marcusson, A., von Bulow, V., Schuster, N., Zhang, S., Heldin, C.H., and Landstrom, M. (2008). The type I TGF-beta receptor engages TRAF6 to activate TAK1 in a receptor kinase-independent manner. Nat Cell Biol *10*, 1199-1207.

Souchelnytskyi, S., Tamaki, K., Engstrom, U., Wernstedt, C., ten Dijke, P., and Heldin, C.H. (1997). Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-beta signaling. J Biol Chem *272*, 28107-28115.

Stavri, G.T., Zachary, I.C., Baskerville, P.A., Martin, J.F., and Erusalimsky, J.D. (1995). Basic fibroblast growth factor upregulates the expression of vascular endothelial growth factor in vascular smooth muscle cells. Synergistic interaction with hypoxia. Circulation *92*, 11-14.

Stearns, M.E., Garcia, F.U., Fudge, K., Rhim, J., and Wang, M. (1999). Role of interleukin 10 and transforming growth factor beta1 in the angiogenesis and metastasis of human prostate primary tumor lines from orthotopic implants in severe combined immunodeficiency mice. Clin Cancer Res *5*, 711-720.

Stewart, G.D., Nanda, J., Brown, D.J., Riddick, A.C., Ross, J.A., and Habib, F.K. (2009). NO-sulindac inhibits the hypoxia response of PC-3 prostate cancer cells via the Akt signaling pathway. Int J Cancer *124*, 223-232.

Stingl, J., and Caldas, C. (2007). Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. Nat Rev Cancer *7*, 791-799.

Stover, D.G., Bierie, B., and Moses, H.L. (2007). A delicate balance: TGF-beta and the tumor microenvironment. J Cell Biochem *101*, 851-861.

Summer, R., Kotton, D.N., Sun, X., Ma, B., Fitzsimmons, K., and Fine, A. (2003). Side population cells and Bcrp1 expression in lung. Am J Physiol Lung Cell Mol Physiol *285*, L97-104.

Sun, G., Alzayady, K., Stewart, R., Ye, P., Yang, S., Li, W., and Shi, Y. (2010). Histone demethylase LSD1 regulates neural stem cell proliferation. Mol Cell Biol *30*, 1997-2005.

Sun, G., Yu, R.T., Evans, R.M., and Shi, Y. (2007). Orphan nuclear receptor TLX recruits histone deacetylases to repress transcription and regulate neural stem cell proliferation. Proc Natl Acad Sci U S A *104*, 15282-15287.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N., and Yancopoulos, G.D. (1996). Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell *87*, 1171-1180.

Suri, C., McClain, J., Thurston, G., McDonald, D.M., Zhou, H., Oldmixon, E.H., Sato, T.N., and Yancopoulos, G.D. (1998). Increased vascularization in mice overexpressing angiopoietin-1. Science *282*, 468-471.

Suzuki, H., Yagi, K., Kondo, M., Kato, M., Miyazono, K., and Miyazawa, K. (2004). c-Ski inhibits the TGF-beta signaling pathway through stabilization of inactive Smad complexes on Smad-binding elements. Oncogene *23*, 5068-5076.

Tahara, E. (1990). Growth factors and oncogenes in human gastrointestinal carcinomas. J Cancer Res Clin Oncol *116*, 121-131.

Takahashi, Y., Kitadai, Y., Bucana, C.D., Cleary, K.R., and Ellis, L.M. (1995). Expression of vascular endothelial growth factor and its receptor, KDR, correlates with vascularity, metastasis, and proliferation of human colon cancer. Cancer Res *55*, 3964-3968.

Takahata, M., Inoue, Y., Tsuda, H., Imoto, I., Koinuma, D., Hayashi, M., Ichikura, T., Yamori, T., Nagasaki, K., Yoshida, M.*, et al.* (2009). SKI and MEL1 cooperate to inhibit transforming growth factor-beta signal in gastric cancer cells. J Biol Chem *284*, 3334-3344.

Tang, B., Yoo, N., Vu, M., Mamura, M., Nam, J.S., Ooshima, A., Du, Z., Desprez, P.Y., Anver, M.R., Michalowska, A.M.*, et al.* (2007). Transforming growth factor-beta can suppress tumorigenesis through effects on the putative cancer stem or early progenitor cell and committed progeny in a breast cancer xenograft model. Cancer Res *67*, 8643-8652.

Temple, S. (2001). The development of neural stem cells. Nature *414*, 112-117.

Tepper, R.I., Coffman, R.L., and Leder, P. (1992). An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. Science *257*, 548-551.

Thoma, C.R., Frew, I.J., Hoerner, C.R., Montani, M., Moch, H., and Krek, W. (2007). pVHL and GSK3beta are components of a primary cilium-maintenance signaling network. Nat Cell Biol *9*, 588-595.

Thomas, D.A., and Massague, J. (2005). TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell *8*, 369-380.

Thurston, G., Suri, C., Smith, K., McClain, J., Sato, T.N., Yancopoulos, G.D., and McDonald, D.M. (1999). Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. Science *286*, 2511-2514.

Topp, M.S., Papadimitriou, C.A., Eitelbach, F., Koenigsmann, M., Oelmann, E., Koehler, B., Oberberg, D., Reufi, B., Stein, H., Thiel, E.*, et al.* (1995). Recombinant human interleukin 4 has antiproliferative activity on human tumor cell lines derived from epithelial and nonepithelial histologies. Cancer Res *55*, 2173-2176.

Tsushima, H., Kawata, S., Tamura, S., Ito, N., Shirai, Y., Kiso, S., Imai, Y., Shimomukai, H., Nomura, Y., Matsuda, Y.*, et al.* (1996). High levels of transforming growth factor beta 1 in patients with colorectal cancer: association with disease progression. Gastroenterology *110*, 375-382.

Uemura, A., Kusuhara, S., Wiegand, S.J., Yu, R.T., and Nishikawa, S. (2006). Tlx acts as a proangiogenic switch by regulating extracellular assembly of fibronectin matrices in retinal astrocytes. J Clin Invest *116*, 369-377.

Umenishi, F., Umeda, M., and Miyazaki, K. (1991). Efficient purification of TIMP-2 from culture medium conditioned by human hepatoma cell line, and its inhibitory effects on metalloproteinases and in vitro tumor invasion. J Biochem *110*, 189-195.

van Kempen, L.C., and Leenders, W.P. (2006). Tumors can adapt to anti-angiogenic therapy depending on the stromal context: lessons from endothelial cell biology. Eur J Cell Biol *85*, 61-68.

Wang, D., Long, J., Dai, F., Liang, M., Feng, X.H., and Lin, X. (2008). BCL6 represses Smad signaling in transforming growth factor-beta resistance. Cancer Res *68*, 783-789.

Wang, G.L., Jiang, B.H., Rue, E.A., and Semenza, G.L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A *92*, 5510-5514.

Wetterskog, D., Moshiri, A., Ozaki, T., Uramoto, H., Nakagawara, A., and Funa, K. (2009). Dysregulation of platelet-derived growth factor beta-receptor expression by DeltaNp73 in neuroblastoma. Mol Cancer Res *7*, 2031-2039.

Wicks, S.J., Lui, S., Abdel-Wahab, N., Mason, R.M., and Chantry, A. (2000). Inactivation of smad-transforming growth factor beta signaling by Ca(2+)-calmodulin-dependent protein kinase II. Mol Cell Biol *20*, 8103-8111.

Wiesener, M.S., Jurgensen, J.S., Rosenberger, C., Scholze, C.K., Horstrup, J.H., Warnecke, C., Mandriota, S., Bechmann, I., Frei, U.A., Pugh, C.W.*, et al.* (2003). Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. FASEB J *17*, 271-273.

Wikstrom, P., Stattin, P., Franck-Lissbrant, I., Damber, J.E., and Bergh, A. (1998). Transforming growth factor beta1 is associated with angiogenesis, metastasis, and poor clinical outcome in prostate cancer. Prostate *37*, 19-29.

Visvader, J.E., and Lindeman, G.J. (2008). Cancer stem cells in solid tumors: accumulating evidence and unresolved questions. Nat Rev Cancer *8*, 755-768.

Wizigmann-Voos, S., Breier, G., Risau, W., and Plate, K.H. (1995). Up-regulation of vascular endothelial growth factor and its receptors in von Hippel-Lindau disease-associated and sporadic hemangioblastomas. Cancer Res *55*, 1358-1364.

Volpert, O.V., Fong, T., Koch, A.E., Peterson, J.D., Waltenbaugh, C., Tepper, R.I., and Bouck, N.P. (1998). Inhibition of angiogenesis by interleukin 4. J Exp Med *188*, 1039-1046.

Yagi, K., Furuhashi, M., Aoki, H., Goto, D., Kuwano, H., Sugamura, K., Miyazono, K., and Kato, M. (2002). c-myc is a downstream target of the Smad pathway. J Biol Chem *277*, 854-861.

Yamaguchi, K., Shirakabe, K., Shibuya, H., Irie, K., Oishi, I., Ueno, N., Taniguchi, T., Nishida, E., and Matsumoto, K. (1995). Identification of a member of the MAPKKK family as a potential mediator of TGF-beta signal transduction. Science *270*, 2008-2011.

Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J., and Holash, J. (2000). Vascular-specific growth factors and blood vessel formation. Nature *407*, 242-248.

Yancopoulos, G.D., Klagsbrun, M., and Folkman, J. (1998). Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border. Cell *93*, 661-664.

Yang, H., Kyo, S., Takatura, M., and Sun, L. (2001). Autocrine transforming growth factor beta suppresses telomerase activity and transcription of human telomerase reverse transcriptase in human cancer cells. Cell Growth Differ *12*, 119-127.

Yashiro, M., Chung, Y.S., Nishimura, S., Inoue, T., and Sowa, M. (1995). Establishment of two new scirrhous gastric cancer cell lines: analysis of factors associated with disseminated metastasis. Br J Cancer *72*, 1200-1210.

Yashiro, M., Chung, Y.S., Nishimura, S., Inoue, T., and Sowa, M. (1996). Peritoneal metastatic model for human scirrhous gastric carcinoma in nude mice. Clin Exp Metastasis *14*, 43-54.

Yu, L., Hebert, M.C., and Zhang, Y.E. (2002). TGF-beta receptor-activated p38 MAP kinase mediates Smad-independent TGF-beta responses. EMBO J *21*, 3749-3759.

Yu, R.T., Chiang, M.Y., Tanabe, T., Kobayashi, M., Yasuda, K., Evans, R.M., and Umesono, K. (2000). The orphan nuclear receptor Tlx regulates Pax2 and is essential for vision. Proc Natl Acad Sci U S A *97*, 2621-2625.

Yu, R.T., McKeown, M., Evans, R.M., and Umesono, K. (1994). Relationship between Drosophila gap gene tailless and a vertebrate nuclear receptor Tlx. Nature *370*, 375-379.

Yuan, A., Yang, P.C., Yu, C.J., Chen, W.J., Lin, F.Y., Kuo, S.H., and Luh, K.T. (2000). Interleukin-8 messenger ribonucleic acid expression correlates with tumor progression, tumor angiogenesis, patient survival, and timing of relapse in non-small-cell lung cancer. Am J Respir Crit Care Med *162*, 1957-1963.

Zagzag, D., Hooper, A., Friedlander, D.R., Chan, W., Holash, J., Wiegand, S.J., Yancopoulos, G.D., and Grumet, M. (1999). In situ expression of angiopoietins in astrocytomas identifies angiopoietin-2 as an early marker of tumor angiogenesis. Exp Neurol *159*, 391-400.

Zatyka, M., Morrissey, C., Kuzmin, I., Lerman, M.I., Latif, F., Richards, F.M., and Maher, E.R. (2002). Genetic and functional analysis of the von Hippel-Lindau (VHL) tumor suppressor gene promoter. J Med Genet *39*, 463-472.

Zhao, C., Sun, G., Li, S., Lang, M.F., Yang, S., Li, W., and Shi, Y. (2010). MicroRNA let-7b regulates neural stem cell proliferation and differentiation by targeting nuclear receptor TLX signaling. Proc Natl Acad Sci U S A *107*, 1876-1881.

Zhou, S., Schuetz, J.D., Bunting, K.D., Colapietro, A.M., Sampath, J., Morris, J.J., Lagutina, I., Grosveld, G.C., Osawa, M., Nakauchi, H*., et al.* (2001). The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med *7*, 1028-1034.