Transcriptional Regulation of the Platelet-Derived Growth Factor β-receptor by p53 Family Members

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2008

ISBN 978-91-633-3331-6
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Printed by Intellecta Docusys AB
Gothenburg, Sweden

ABSTRACT

Transcriptional Regulation of the Platelet-Derived Growth Factor β-receptor by p53 Family Members

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Aims: The platelet-derived growth factor β-receptor (PDGFRB) is critically involved in embryonic development and has a role in many diseases. In cells, signaling through PDGFRB affects growth, migration and death. The role of PDGFRB in these crucial processes necessitates strict regulation and therefore it is regulated in many ways, including transcription, dephosphorylation, internalization, and degradation. Of these, transcriptional regulation is the least studied. Previously, PDGFRB transcription has been shown to be under the control of the transcription factors nuclear factor y (NF-Y), specificity protein 1 (Sp1) and the p53 family member p73. In the present thesis we investigated the role of p53 family members and their mechanisms for transcriptional regulation of PDGFRB.

Results: In search for the mechanism behind p73α-mediated repression of the PDGFRB, we found that p73α competed with histone acetyltransferases for binding to NF-Y. The recruitment of p73α and ΔNp73 to the PDGFRB promoter corresponded with PDGFRB expression. In repression of the PDGFRB promoter, p73 was recruited with the co-repressor HDAC1. ΔNp73 and the co-activator p300, on the other hand, was recruited in induction of the PDGFRB promoter.

Overexpression of the p53-interacting viral large T antigen (LT) in NIH3T3 fibroblasts resulted in repressed PDGFRB promoter activity and decreased expression of PDGFRB protein and mRNA. The same type of overexpression in c-Myc$^{-/-}$ HO15.19 fibroblasts, Rb$^{-/-}$NIH3T3, and pRb- and p53-lacking osteosarcoma, Saos2, did not repress PDGFRB promoter activity, showing the importance of these molecules for LT-mediated repression of PDGFRB.

In order to identify the role of p53, we overexpressed p53 in mouse embryonic fibroblasts (MEF), p53$^{-/-}$ MEF, and Saos2, which induced repression of PDGFRB promoter activity and decreased mRNA and protein expression. Endogenous p53, activated by MMC treatment, also downregulated PDGFRB expression. Experiments showed that p53 could bind the PDGFRB promoter region surrounding the CCAAT-motif. Upon p53 induction, when PDGFRB expression was repressed, p53 and HDAC1 bound the PDGFRB promoter and the co-activator p300 was dismissed.

The role of ΔNp73 in PDGFRB expression was investigated using the neuroblastoma cell line IMR-32 which had dysregulated PDGFRB expression and SH-SY5Y which had regulated expression. Silencing of ΔNp73 repressed PDGFRB promoter activity and protein expression in IMR-32, but not in SH-SY5Y, and ΔNp73 was constitutively bound to the PDGFRB promoter only in IMR-32. Treatment with the anticancer drug cisplatin decreased PDGFRB protein, mRNA and promoter activity in both cell lines. In IMR-32, cisplatin was found to dismiss ΔNp73 and p300 from the PDGFRB promoter and recruit HDAC4.

Conclusions: Results presented in this thesis suggest a role for p53 family members in downregulation of PDGFRB expression upon growth stimulation or in response to DNA damage. In addition, we demonstrated that ΔNp73 have a role in dysregulated PDGFRB expression. Also, we propose a potential for tyrosine kinase inhibitors in the treatment of neuroblastoma with high ΔNp73 and PDGFR expression.

List of Publications

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

I. Uramoto, H., **Wetterskog, D**., Hackzell, A., Matsumoto, Y., Funa, K. p73 competes with co-activators and recruits histone deacetylase to NF-Y in the repression of PDGF β-receptor.
Journal of Cell Science 117 (2004) 5323-5331.

II. Uramoto, H., Hackzell, A*****., **Wetterskog, D***., Ballagi, A., Izumi, H., Funa, K. pRb, Myc and p53 are critically involved in SV40 large T antigen repression of PDGF β-receptor transcription.
Journal of Cell Science 117 (2004) 3855-3865. ***joint second authors**

III. Yang, W*****., **Wetterskog, D***., Matsumoto, Y., Funa, K. Kinetics of repression by modified p53 on the PDGF β-receptor promoter.
International Journal of Cancer 123 (2008) 2020-2030. ***joint first authors**

IV. **Wetterskog, D.**, Ozaki, T., Nakagawara, A., Funa, K. Dysregulation of PDGF β-receptor expression by ΔNp73 in neuroblastoma.
Manuscript

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List of Abbreviations

ATM	ataxia-telangiectasia mutated
ATR	ataxia-telangiectasia Rad3-related
CDDP	cisplatin
CDK	cyclin dependent kinase
ChIP	chromatin immunoprecipitation
CIP	CDK inhibitory proteins
CML	chronic myeloid leukemia
CNS	central nervous system
COL1A1	collagen type 1α1 chain
CSF1R	colony stimulating factor 1 receptor
CUB	C1r/C1s, Uegf and Bmp1
DFSP	dermatofibrosarcoma protuberans
DRG	delayed response genes
ECM	extracellular matrix
EMSA	electrophoretic mobility shift assay
ERG	early response genes
ETS	E26 transformation-specific
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
GFP	green fluorescent protein
GST	glutathione S transferase
HA	hemagglutinin
HAT	histone acetyltransferase
HDAC	histone deacetylase
HFM	histone fold motif
IGF1	insulin like growth factor 1
INK	inhibitors of kinase 4
INR	initiator element
JNK	c-Jun N-terminal kinase
LOH	loss of heterozygosity
LRP1	low density lipoprotein-related protein 1
LT	large T antigen
Mdm2	murine double minute 2
MEF	mouse embryonic fibroblast
MMC	mitomycin C
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
NES	nuclear export signal
NF-Y	nuclear factor y
NLS	nuclear localization signal
P/CAF	p300/CBP-associated factor
PBS	phosphate buffered saline
PC	proprotein convertase
PDGF	platelet-derived growth factor
PDGFRA	platelet-derived growth factor receptor α
PDGFRB	platelet-derived growth factor receptor β
PIN1	peptidylprolyl cis/trans isomerase
PML	promyelocytic leukemia

pRb	retinoblastoma protein
Pol II	RNA polymerase II
RTK	receptor tyrosine kinase
SAM	sterile alpha motif
SDS	sodium dodecyl sulfate
SH2	Src homology 2
SHP	SH2 tyrosine phosphatase 1
SMC	smooth muscle cell
Sp1	specificity protein 1
SV40	simian virus 40
TAF	TBP associated factors
TBP	TATA binding protein
TBS	tris buffered saline
TPA	tissue plasminogen activator
UPA	urokinase plasminogen activator
VEGF	vascular endothelial growth factor
vSMC	vascular smooth muscle cell
YHD	yeast homology domain

Quod te non occidit, te fortiorem faciet

Introduction

Background

In the late 1960's, studies on culturing of cells raised the question about the identity of the serum-derived factors that promoted DNA and RNA synthesis, cell division, and reduction of sensitivity to contact inhibition (Todaro et al., 1965; Todaro et al., 1967; Holley and Kiernan, 1968, 1974). From the studies of Samuel Balk in the early 1970's on chicken fibroblast cells, it was determined that these factors derived from blood serum rather than plasma (Balk, 1971). Additional insight was gained with the findings that addition of platelets to platelet-poor plasma restored the proliferative effect of plasma on smooth muscle cells (SMCs), fibroblasts, and glial cells (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976). The purification and characterization of these platelet-derived growth factors (PDGFs) took until the late 1970's and early 1980's when, from independent studies, two factors were found; PDGF-A and PDGF-B (Antoniades et al., 1979; Heldin et al., 1979; Deuel et al., 1981; Raines and Ross, 1982).

Through the use of radio-iodinated PDGF (^{125}I-PDGF), PDGF receptors were identified on glial cells, fibroblasts, and smooth muscle cells (Heldin et al., 1981; Bowen-Pope and Ross, 1982; Huang et al., 1982; Williams et al., 1982). These receptors were found to be large membrane-bound proteins that, upon binding of PDGF, became phosphorylated on tyrosine residues (Ek and Heldin, 1982; Ek et al., 1982; Nishimura et al., 1982; Frackelton et al., 1984). It was also discovered that these receptors carried intrinsic tyrosine-kinase activity (Bishayee et al., 1986). The cloning and sequencing of both the mouse and human PDGF receptor genes came in the late 1980's and revealed two different PDGF receptors, PDGF receptor α (PDGFRA) and receptor β (PDGFRB) (Yarden et al., 1986; Claesson-Welsh et al., 1988; Gronwald et al., 1988; Matsui et al., 1989). The promoters for the PDGF and PDGF receptor genes were cloned in the early and mid 1990's, thereby providing templates for analysis of the importance of transcriptional regulation on PDGF signaling (Takimoto et al., 1991; Jin et al., 1993; Wang and Stiles, 1994; Ballagi et al., 1995).

After the discoveries of the PDGF ligands and receptors, PDGF signaling was characterized. Immediate downstream signal transduction molecules were identified as those containing the Src homology 2 (SH2) domain and initiated signaling cascades mainly through MAP kinases and serine/threonine kinases. This PDGF signaling regulated various cellular processes such as chemotaxis, cell growth, and apoptosis (as reviewed in Heldin et al., 1998). In mouse knockout models of PDGF and PDGF receptors, their importance for development was determined by the fact that all knockout mice died either during embryogenesis or shortly after birth (Leveen et al., 1994; Soriano, 1994; Bostrom et al., 1996; Soriano, 1997).

Since over-activity of PDGF was shown to be involved in atherosclerosis, fibrotic diseases, and tumor progression, the regulation and inhibition of PDGF-signaling was investigated (as reviewed in Heldin and Westermark, 1999). Cells were found to utilize different feedback mechanisms in order to regulate PDGF signaling where, after activation, the PDGF receptor was found to be dephosphorylated by phosphatases, internalized and degraded in endosomes and transcriptionally downregulated. In tumors, expression of PDGF and PDGF receptors was suggested to activate autocrine or paracrine stimulation of growth. In order to be able to inhibit PDGF signaling, many tyrosine kinase inhibitors have been tested as anti-cancer drugs, with varying success and specificity for PDGF receptors (Board and Jayson, 2005).

Twenty years after the discovery of the PDGF-A and -B, two new ligands were found, in each case by three different research groups (Hamada et al., 2000; Li et al., 2000; Tsai et al., 2000; Bergsten et al., 2001; Hamada et al., 2001; LaRochelle et al., 2001). Although initially differently designated, today the established names for the two new ligands are PDGF-C and PDGF-D.

Studies of the transcriptional regulation of the PDGFRB from our group have previously identified nuclear factor y (NF-Y), specificity protein 1 (Sp1), c-Myc and p73α as important regulators (Ishisaki et al., 1997; Izumi et al., 2001; Molander et al., 2001; Hackzell et al., 2002). Investigations presented in this thesis have addressed the role of p53 family members in transcriptional regulation of PDGFRB.

Structure and function of PDGF

The PDGF-family consists of PDGF-A, -B, -C, and -D. Common for all PDGFs is the growth factor domain, also called the PDGF/VEGF homology domain. Extensions to the N- and C-terminal of the VEGF/PDGF domain modify the biological activity of the ligands. In the endoplasmic reticulum the monomers dimerize as PDGF-AA, -BB, -AB, -CC or -DD (Ostman et al., 1992). These are then proteolytically cleaved to generate the active forms that can bind the PDGF receptors (figure 1).

The common VEGF/PDGF domain in PDGF is about 100 amino acids in length and has eight conserved cysteine residues (as reviewed in Fredriksson et al., 2004a). Two of these cysteine residues are involved in dimer formation and the other six stabilizes the internal structure by making a tight cysteine knot by forming three disulphide bridges. This causes exposure of hydrophobic residues to the aqueous surroundings and further promotes dimerization of the monomers in order to make a hydrophobic core. It should be noted that PDGF-C has two more cysteines and PDGF-D has four more but the function of these cysteines are unknown.

For activation, the N-terminal extensions of PDGF-A and -B are cleaved. C-terminal extensions on the other hand are involved in interactions with extracellular matrix and retention inside the cell (LaRochelle et al., 1991). For PDGF-A and -B the proteolytic cleavage occurs intracellularly in the Golgi complex (Ostman et al., 1992). PDGF-A is cleaved by the proprotein convertase furin (Siegfried et al., 2003). PDGF-B is also cleaved by proprotein convertases but by which one(s) has not yet been determined (Siegfried et al., 2005). In contrast, PDGF-C and D differ in their N-terminal extensions where a sequence, called hinge, separates the growth factor domain from the CUB (C1r/C1s, Uegf and Bmp1) domain. For PDGF-C and D, proteolytic cleavage of the CUB domain occurs extracellularly and enables binding to the PDGF receptors (Li et al., 2000; Bergsten et al., 2001; LaRochelle et al., 2001). *In vivo*, the tissue plasminogen activator (tPA) cleaves PDGF-C while the urokinase plasminogen activator (uPA) cleaves PDGF-D (Fredriksson et al., 2004b; Ustach and Kim, 2005).

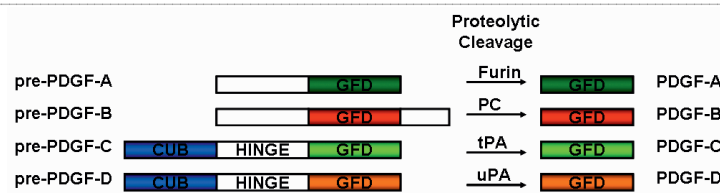

Figure 1. Proteolytic cleavage of PDGF ligands to their active form.

Structure and function of PDGF receptors

PDGFRA and PDGFRB are the membrane-bound receptors for the platelet-derived growth factors. Their primary structure shows that they belong to the family of receptor tyrosine kinases (RTKs) in the class III subgroup (Ullrich and Schlessinger, 1990). Other members of this family include the macrophage colony stimulating factor 1 receptor (CSF1R), KIT, and the VEGF receptor FLT3. This subgroup of RTKs is characterized by having five to seven extracellular immunoglobulin domains, a single pass membrane spanning region and a split internal tyrosine kinase domain (figure 2).

In addition to the extracellular domain, the transmembrane sequence, and the intracellular split tyrosine kinase domain, the PDGF receptors also consist of a juxtamembrane domain and a C-terminal domain (Williams, 1989). For both receptors, two tyrosine residues in the C-terminal domain have been shown to be targets for autophosphorylation. Tyrosine residues in the split tyrosine kinase domains are also autophosphorylated, as are residues in the sequence between the tyrosine kinase domains where phosphorylation of Tyr-751 in PDGFRB is involved in substrate specificity (Kazlauskas and Cooper, 1989). The juxtamembrane domain of PDGFRB contains Tyr-579 which in addition to being an autophosphorylation site is important for receptor internalization upon ligand induced signaling (Mori et al., 1994). This domain also binds members of the Src family. The transmembrane sequence is the only hydrophobic sequence long enough to traverse the cell membrane and is arranged as a rigid alpha helix (Escobedo et al., 1988). Moreover, the extracellular part of the PDGF receptors contains five immunoglobulin domains. These domains are responsible for the binding of PDGF ligands and dimerization of the receptors. It is mainly the domains which are furthest away from the cell membrane that are involved in ligand binding. For example, in PDGFRB, domains number 2 and 3 are sufficient to initiate PDGF signaling (Lokker et al., 1997). Domain 4, on the other hand, is important for receptor dimerization and subsequent signaling (Omura et al., 1997).

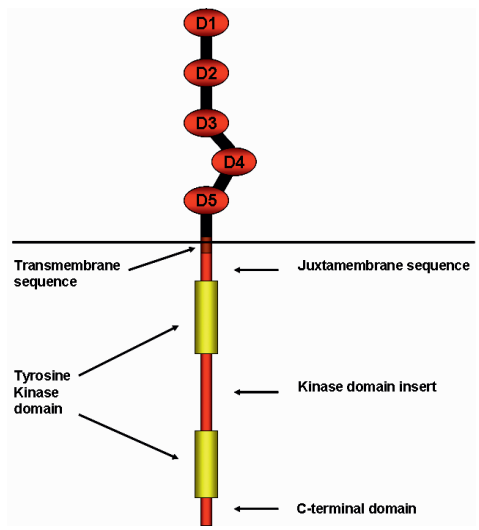

Figure 2. Structure of the PDGF receptor.

PDGF signaling

PDGF signaling is initiated by the binding of dimerized PDGFs to PDGF receptors. Since both PDGFs in the dimer can bind a receptor, this will bring two receptors in close proximity to each other. Which receptor complex is formed will depend on the PDGF dimer, since the different PDGFs bind with different affinity to the receptors. AA, AB, BB, CC and DD will create an α-α, α-β or β-β receptor complex, where the α-α receptor complex can bind all PDGF dimers except DD, the α-β receptor complex can bind all PDGF ligands except AA and the β-β receptor complex only binds BB and DD (figure 3). Although the receptor complexes interact with similar downstream signaling molecules (figure 4), findings from knockout mice indicate that there is no complete redundancy in PDGF signaling, since knockout mice die around birth.

When PDGF receptors form a complex, tyrosine residues in the internal kinase domains will become autophosphorylated (Kelly et al., 1991). Autophosphorylation of tyrosine 849 and 857 located inside the kinase domain of PDGFRA and PDGFRB, respectively, will increase the catalytic effect of the receptor (Kazlauskas and Cooper, 1989). Furthermore, autophosphorylation of tyrosine residues outside and in between the kinase domains will serve as docking sites for a wide range of signaling molecules. Common for all these signaling molecules is that they contain the SH2-domain (Kypta et al., 1990; Fantl et al., 1992). These interacting molecules will be activated either through phosphorylation or conformational changes and initiate signaling through interactions with downstream mediators (as reviewed in Heldin et al., 1998). The Src family of kinases is activated through interaction with phosphorylated Tyr-589 on PDGFRB and Tyr-572 on PDGFRA. Activated Src phosphorylates and activates the membrane-associated c-Abl kinase (Plattner et al., 1999). Moreover, in response to DNA damage, nuclear c-Abl phosphorylates and activates the p53 family members p53 and p73 (Goga et al., 1995; Agami et al., 1999). c-Abl also induces the expression of c-Myc (Furstoss et al., 2002). Activation of the phosphoinositide 3-kinase (PI3K) through interaction with Tyr-758 and Tyr-763 on PDGFRB activates Akt by phosphorylation. Akt then activates the mammalian target of rapamycin (mTOR) which downregulates PDGFRB expression (Zhang et al., 2007). Also, Akt activation results in downregulation of the proapoptotic genes BAD and caspase 9 (del Peso et al., 1997; Cardone et al., 1998). In conclusion, the effect PDGF signaling elicits on a cell depends on which receptor complex is formed and what signaling molecules that bind the complex.

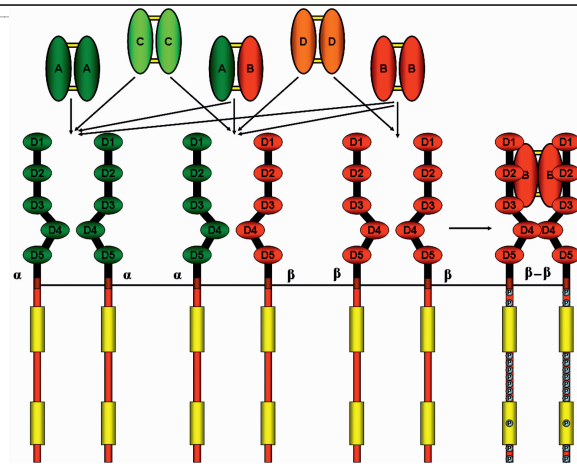

Figure 3. PDGF-induced phosphorylation of PDGF receptors.

Expression of PDGF ligands and receptors

PDGF ligands and receptors are expressed by a wide variety of cells. Expression patterns are generally distinct, with specific cells expressing specific ligands or receptors. Most cell types do not simultaneously express a PDGF ligand and its corresponding receptor, indicating that the mode of action for PDGF is predominantly paracrine. However, fibroblasts express PDGF-A, -B, -D as well as both receptors and thus autocrine or juxtacrine signaling loops may exist. Moreover, in neurons, where both PDGF-A and -B as well as both of the receptors can be expressed, the receptors seem to signal differently, *i.e.*, PDGFRA for progenitor proliferation and PDGFRB for differentiation and cell survival (Smits et al., 1991). The mesangial cells of the kidney express both PDGF-B and PDGFRB, thus being likely to activate an autocrine stimulation of growth (Alpers et al., 1992).

Neighboring cells with distinct expression of PDGF and a cognate receptor include the Oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells which express PDGFRA (Hart et al., 1989). These progenitors respond to the release of PDGF-A from type-1 astrocytes by dividing and postponing their differentiation into oligodendrocytes (Noble et al., 1988; Raff et al., 1988). Also, in the kidney, PDGF-D is expressed in visceral epithelial cells and binds to PDGFRB presented on mesangial cells and fibroblasts (Bergsten et al., 2001; Changsirikulchai et al., 2002). Culturing of cells will also affect the expression of PDGF ligands and receptors. For example, PDGF receptor expression in SMCs and fibroblasts increases upon tissue culturing (Terracio et al., 1988). Also, the presence of growth factors will affect PDGF expression.

The pattern of expression of PDGF ligands and receptors is especially of importance for organ development and wound healing. Furthermore, overexpression of PDGF receptors and ligands are involved in various diseases.

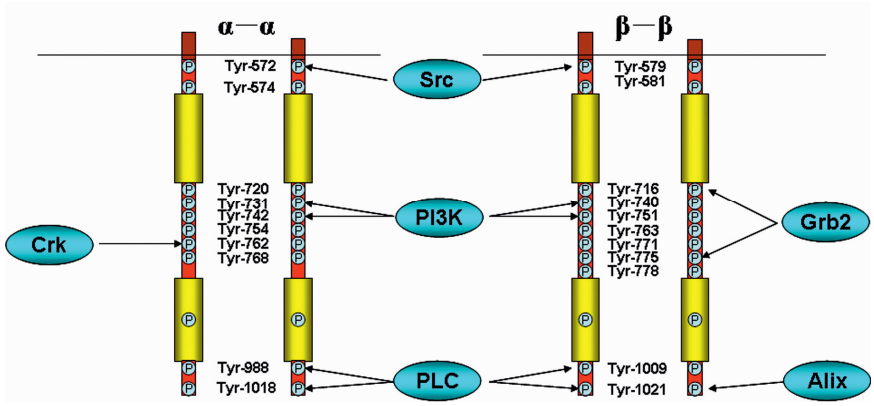

Figure 4. Examples of PDGFRA and PDGFRB interacting molecules.

Physiological role of PDGF signaling

The PDGFs and PDGFRs all have a role in embryonic development as seen from knockout mice. In the adult, PDGF signaling is one of the main actors in wound healing and is involved in all phases of wound healing.

Role in development

Through the generation of knockout mice for PDGF ligands and receptors a greater understanding of the role of PDGF signaling in development has been gained. These knockout studies demonstrate that many of the well-characterized cellular responses to PDGFs such as proliferation, survival, and migration are essential during development with the PDGF ligand and receptor knockouts all being prenatally or perinatally lethal.

Knockout of PDGF-A affects, among other things, the development of the lung, CNS and hair follicles. PDGF-A null mice that survive to postnatal age develop lung emphysema due to loss of alveolar septation (Bostrom et al., 1996). In PDGF-A null mice, the alveolar myofibroblasts are absent in the developing lung. Usually, PDGF-A is found expressed in the lung epithelium, and cells positive for PDGFRA are found in the mesenchyme and putative alveolar myofibroblast progenitors. These progenitors are committed to alveolar myofibroblast differentiation and require PDGF-A for their growth and migration (Lindahl et al., 1997b). In the development of the CNS, PDGF-A knockout leads to a reduced number of PDGFRA expressing O-2A cells. This is due to a lack of PDGF-A driven proliferation of an initially normal population of PDGFRA-expressing progenitor cells. Also, and possibly as a result of the reduced O-2A population, the brain and especially the optic nerve becomes hypomyelinated (Fruttiger et al., 1999). In hair follicle development, PDGF-A control the proliferation of PDGFRA-positive dermal mesenchymal progenitor cells (Karlsson et al., 1999), and as a result the PDGF-$A^{-/-}$ mice have thinner dermis and malformed hair follicles.

Patch mutant mice carrying a deletion that covers the PDGFRA gene initially provided insight into the role of PDGFRA in development. However, better understanding was brought with the generation of PDGFR$A^{-/-}$ mice (Soriano, 1997). These mice have defects in multiple tissues and have skeletal defects which are lethal to the embryo. This indicates a role of PDGFRA in early development. The cleft face phenotype, seen in PDGFRA null mice, is associated with a defect of migration of neural crest cells (Morrison-Graham et al., 1992). Loss of PDGFRA in the neural crest cells disables their participation in the cranial mesenchyme (Tallquist and Soriano, 2003). Skeletal defects are attributed to defective myotome formation rather than the sclerotome. However, the myotome expresses PDGF-A and -C but not PDGFRA. Instead the sclerotome expresses the PDGFRA. This indicates a form of PDGFRA dependent feedback mechanism from the sclerotome in the myotome formation and somatic patterning.

Interestingly, in knockout studies of PDGF-C, a double knockout for both PDGF-A and -C phenocopied the loss of PDGFRA (Ding et al., 2004). This shows that PDGF-A and -C are the principal *in vivo* ligands for the PDGFRA. Moreover, PDGF-C null mice die perinatally due to difficulties in feeding and respiration because of a deformed palate. Both PDGF-A and C are expressed in the epithelium of the palate but only PDGF-C null mice show a deformed palate. This shows a unique role for PDGF-C / PDGFRA signaling in palatogenesis.

PDGF-B null mice and PDGFRB null mice have highly similar phenotypes. Both knockouts show hematological defects, have dilated blood vessels and “spotted kidneys” and die perinatally from hemorrhaging (Leveen et al., 1994; Soriano, 1994). The blood of PDGF-B and PDGFRB

null mice contains fewer platelets (thrombocytopenia) and has lower hemoglobin levels (anemia) than wild-type mice. However, the reason for the hematological defects is unknown, but the cause for hemorrhage is deficiency in pericyte recruitment (Lindahl et al., 1997a). The pericyte is a relatively undifferentiated cell that serves to support blood vessels. Developing pericytes express PDGFRB while endothelial cells of the capillaries express PDGF-B. Creation of vSMCs and pericytes are PDGF-B/PDGFRB independent while migration and proliferation of the existing pool of vSMC and pericytes are PDGF-B/PDGFRB dependent (Hellstrom et al., 1999). Mice lacking PDGF-B are unable to attract PDGFRB-expressing pericytes and as a result the associated capillaries are weakened and rupture upon increased blood flow. The spotted kidney seen in the null mice is due to abnormally developed glomeruli with blood filled structures therein. In wild-type but not in knockout mice, mesangial cells are found around blood vessels in the kidney. Moreover, mesangial cells in the glomerulus proliferate and migrate in response to PDGF-B (Shultz et al., 1988; Barnes and Hevey, 1990). In accordance, during glomerulogenesis, PDGF-B is expressed in vascular endothelial cells and recruits and stimulates proliferation of PDGFRB-positive mesangial cells (Lindahl et al., 1998).

Since no knockout mouse for PDGF-D has been generated, studies so far have been focused on PDGF-D tissue expression, overexpression and the use of inhibitory peptides in order to gain insight into its role in development. Tissue expression is widespread for PDGF-D with the highest expression in the pancreas, heart, ovary, and the adrenal gland (Bergsten et al., 2001; LaRochelle et al., 2001). In transgenic mice, overexpression of PDGF-D in keratinocytes increases the recruitment of macrophages in skin (Uutela et al., 2004). Furthermore, PDGF-D, as PDGF-B, increases interstitial fluid pressure.

Role in the adult

Most knowledge of the role of PDGF signaling comes from its role in development. The role in adult life is much less known. However, the best characterized role for PDGF signaling in the adult is its involvement in wound healing.

Wound healing consists of separate but overlapping phases; hemostasis, inflammation, proliferation and remodeling (as reviewed in Diegelmann and Evans, 2004). In hemostasis, platelets release clotting factors, cytokines and growth factors in response to injury. Thereafter, leukocytes migrate into the wounded area, as part of the inflammatory phase, to remove bacteria, foreign materials and damaged tissue. In the subsequent proliferative phase, extracellular matrix (ECM) molecules are produced, mainly by fibroblasts, to form new ECM, create epithelia and stimulate blood vessel formation. In the last phase, the wound undergoes constant alterations. This remodeling phase is enabled through the constant degradation of previously produced collagen by proteases produced by fibroblast and macrophages.

PDGF signaling is implied in all phases of wound healing. Upon injury, as part of hemostasis, platelets degranulate and release their contents including PDGFs. In addition, PDGF is produced by fibroblasts (Paulsson et al., 1987), endothelial cells (Harlan et al., 1986), SMCs (Walker et al., 1986) and keratinocytes (Ansel et al., 1993), all of which are present at the site of injury. The produced PDGFs stimulate recruitment of neutrophils and macrophages to initiate the inflammatory phase. Also, PDGFR-expressing fibroblasts and SMCs are recruited and stimulated to proliferate. At the site of injury, macrophages will start producing PDGF to further recruit and stimulate proliferation of fibroblasts and SMCs. In the following proliferative phase, PDGF signaling stimulates the production of collagen (Canalis, 1981), fibronectin (Blatti et al., 1988) and proteoglycans (Schonherr et al., 1991). Since PDGF can induce the production of collagenase it might play a role in the remodeling phase as well (Bauer et al., 1985).

PDGF in disease

PDGF signaling has a causative or functional role in various diseases. Fibrotic diseases, vascular diseases and cancer are categories that fit most, if not all, of the PDGF-driven diseases. For all these diseases there is a potential to use drugs affecting PDGF signaling as therapy.

Fibrotic diseases

In fibrotic diseases, generally, release of PDGF from macrophages stimulates proliferation and recruitment of PDGFR-expressing myofibroblasts, while release of inflammatory cytokines from macrophages stimulates PDGFR expression in the myofibroblasts. Furthermore, PDGF and transforming growth factor β (TGF-β) will stimulate myofibroblasts to produce ECM such as collagen. Deposition of these ECM molecules will lead to scarring of the affected tissue and in the end loss of function of the organ in question. Fibrotic diseases driven by the mechanism described above include pulmonary fibrosis, renal fibrosis, hepatic fibrosis and dermal fibrosis.

Pulmonary fibrosis is caused by injury to the lung, which leads to a chronic inflammatory response where activated alveolar macrophages are the main source for PDGF (Martinet et al., 1987). In renal fibrosis, PDGFRB is upregulated in mesangial cells in response to inflammation in the kidney (Fellstrom et al., 1989). Upon release of PDGFs from invading macrophages the mesangial cells will be recruited and stimulated to proliferate and produce extracellular matrix. Moreover, in hepatic fibrosis, hepatic stellate cells of the liver are activated in response to injury to the liver. TGF-β, which is produced by stellate cells and macrophages, will increase PDGFRB expression in the stellate cells making them responsive to PDGFs (Pinzani et al., 1995). In the autoimmune disorder scleroderma, there is a progressive dermal fibrosis in skin and other organs. In scleroderma, the source of PDGF-B is activated and infiltrating macrophages (Gay et al., 1989). Tyrosine kinase inhibitors show promising results for treatment of these fibrotic diseases (Gilbert et al., 2001; Distler et al., 2007).

Vascular diseases

In the healthy adult, PDGF expression remains low in the arteries. However, in response to a pathological stimulus, all cell types that make up the arterial wall, as well as inflammatory cells that can infiltrate the artery can be induced to produce PDGF. Depending on which disease, production of PDGF will recruit vascular SMCs (vSMCs) or pericytes, stimulate their proliferation and induce the production of ECM.

Atherosclerosis is an inflammation of medium and large arteries (for a review see Ross, 1999). A role of PDGF in atherosclerosis is supported by numerous studies. There are numerous sources available for PDGF in atherosclerosis such as SMCs and platelets, but the main source is considered to be the macrophages (Ross et al., 1990). Production of PDGF-B from the macrophages will recruit SMCs (Rubin et al., 1988), which will proliferate and produce ECM. If the cause of the inflammation is not resolved a situation of chronic inflammation can arise. Moreover studies on the low-density lipoprotein receptor-related protein (LRP1) show that SMC migration induced by PDGF-B is reduced by LRP1 (Boucher et al., 2003). LRP1 forms a complex with PDGFRB leading to endocytosis of PDGFR. Inactivation of LRP1 leads to increased PDGFR presence and increased susceptibility for formation of atherosclerosis. This susceptibility can be prevented and counteracted with imatinib.

Cancer

PDGF signaling is involved in the proliferation, metastasis, drug resistance, stromal cell recruitment, and vascularization of many tumors. This signaling is either paracrine or autocrine and

where paracrine signaling mostly is involved in the recruitment of stromal cells, vascularization and regulation of interstitial pressure affecting drug uptake. Autocrine PDGF signaling on the other hand gives rise to a stimulation of growth by the cell itself and can also affect metastasis. The first indication of autocrine PDGF signaling in cancer came 25 years ago with the discovery that the simian sarcoma virus expresses a product *v-sis* that is identical to PDGF-B (Doolittle et al., 1983). This product causes transformation of PDGFR-expressing cells and tumors in animals by an autocrine PDGF signaling of growth (Uhrbom et al., 1998). Since then, involvement of all members in the PDGF family and their receptors have been shown in autocrine signaling in cancer (Ostman, 2004). For many of the PDGF-driven cancer forms, clinical trials with tyrosine kinase inhibitors such as imatinib are underway.

Paracrine PDGF signaling
The recruitment of stromal cells, mostly consisting of pericytes and fibroblasts, is dependent on tumor-derived PDGF (Forsberg et al., 1993; Skobe and Fusenig, 1998). Pericyte recruitment is crucial for the development of functional capillaries. For example, in melanoma, PDGF-B and -D expression recruit PDGFRB-expressing pericytes (Furuhashi et al., 2004). These pericytes stabilize the tumor vessels, promote angiogenesis and increase tumor growth. Interestingly, the combination of a VEGFR inhibitor and imatinib is very efficient in regressing well-vascularized tumors (Bergers et al., 2003). Treatment with a VEGFR inhibitor alone is only efficient on tumors not yet well vascularized, while the opposite is seen with imatinib, which reduces tumor pericyte recruitment and vascularity even in well-vascularized tumors.

Stromal fibroblasts are often positive for PDGFRA and PDGFRB. As seen in immortalized non-tumorigenic keratinocytes (HaCaT), expressing PDGF-B, one effect of the recruited fibroblasts can be to convert nontumorogenic cells to become tumorigenic (Skobe and Fusenig, 1998). This might be a result of paracrine signaling of the keratinocyte growth factor from the PDGF-activated fibroblasts. Other clues to the effect of PDGFR signaling come from studies on fibrosarcomas and human lung tumors where tumor-derived PDGF-A and -C recruits PDGFRA-positive fibroblasts. In the case of fibrosarcoma, recruited fibroblasts produce VEGF which activates endothelial cells thereby stimulating angiogenesis (Dong et al., 2004). Findings in human lung tumors confirm the *in vivo* role of this signaling in the promotion of tumor growth (Tejada et al., 2006). Imatinib treatment or FGF-inhibition of cervical cancer-associated fibroblasts expressing FGF2 and FGF7 are both effective in reducing tumor growth (Pietras et al., 2008).

A clinically important aspect of cancer treatment is the uptake of anti-cancer drugs in tumors. This uptake is greatly affected by the interstitial pressure inside the solid tumor. Studies on phosphorylation mutants of PDGFRB show that PDGFRB signaling through PI3K is important for proper regulation of interstitial pressure (Heuchel et al., 1999). In colonic carcinomas inhibition of PDGF-B or treatment with imatinib reduces PDGFRB signaling in stromal cells and results in lower interstitial pressure (Pietras et al., 2001). Moreover, decrease of PDGFRB signaling with imatinib increase the uptake of anticancer drugs (Pietras et al., 2002).

PDGF driven tumors
Autocrine PDGF signaling plays a role in the establishment, growth, maintenance, and malignancy in glioma brain tumors, where expression of PDGFs and cognate PDGF receptors is frequent (Nister et al., 1988). For PDGF-A and -B, an increase from very low expression to high expression is seen with increasing grades of gliomas. PDGFRA, on the other hand, is found highly expressed in all grades of glioma (Hermanson et al., 1992). New possibilities for autocrine signaling in glioma was opened up with the discovery of high expression of PDGF-C and -D in

glioma cell lines and glioblastoma tissues (Lokker et al., 2002). Since expression of PDGFRB is also found in surrounding endothelial cells indicates that PDGFRB signaling might be involved in tumor angiogenesis. Cell-cycle regulatory pathways are often disturbed in gliomas with 60% of gliomas having an INK4a-ARF deletion or inactivation (Costello et al., 1996) while the other 40% often have a deletion of p53 (Louis, 1994). Overexpression of PDGF-B causes formation of highly malignant PDGF-B and PDGFRA positive brain tumors in newborn mice (Uhrbom et al., 1998; Dai et al., 2001) where $p53^{-/-}$ mice and $Ink4\text{-}Arf^{-/-}$ mice respond more strongly to PDGF-B retroviral overexpression with increased tumor frequency (Hesselager et al., 2003). Imatinib has been shown to slow down glioma cell growth in experimental cell models (Servidei et al., 2006) and gives increased survival rate for patients with high grade glioma (Dresemann, 2005).

Dermatofibrosarcoma protuberans (DFSP) is a slow-growing, but recurrent, malignant skin tumor. More than 95% of DFSP is associated with a specific chromosome 17/22 translocation (Pedeutour et al., 1996). This translocation fuses the collagen type 1α1 chain (COL1A1) in frame with exon 2 of the PDGF-B gene and will change its transcriptional regulation so that it comes under the control of the Col1a1 promoter which is highly expressed in dermal fibroblasts (Simon et al., 1997). In addition, DFSP fibroblasts express both PDGFRA and PDGFRB, creating a powerful autocrine stimulation of growth upon PDGF-B expression (Kikuchi et al., 1993). Imatinib induced blockage of autocrine PDGFRB signaling in DFSP, show reduced growth both *in vitro* and *in vivo* (Sjoblom et al., 2001).

Gastrointestinal stromal tumors (GIST) are a rare disease of the gastrointestinal system. This form of tumor is often characterized by a mutation of KIT (CD117) (Taniguchi et al., 1999). However, tumors lacking a mutation of KIT often contain a mutation in PDGFRA (Heinrich et al., 2003) leading to ligand-independent activation of PDGFRA and its downstream signaling molecules PI3K and Signal Transducers and Activators of Transcription (STAT). Imatinib is currently being used successfully in treatment of patients with GISTs benefiting from the fact that it targets both KIT and PDGFRA.

Chronic myeloid leukemia (CML) is a form of leukemia characterized by the unregulated growth of myeloid cells in the bone marrow. It was the first disorder to be reported to derive from a chromosomal translocation. This translocation produces the Bcr/Abl product which is a constitutively active tyrosine kinase that promotes growth and impairs DNA repair (Shtivelman et al., 1985). Another common translocation involves the PDGFRB gene that is fused to ETS variant gene 6 (ETV6 or TEL oncogene) (Golub et al., 1994). The ETV6–PDGFRB fusion results in a protein containing the transmembrane and cytoplasmic part of the receptor fused to the helix-loop-helix domain of TEL (Carroll et al., 1996). This protein cannot bind to PDGF, but is constitutively active through TEL-mediated dimer formation. In treatment of CML, imatinib has proven extremely successful, and although the patients are not cured *per se* and needs continuous treatment, the 5-year survival rate is almost 90% (Soverini et al., 2008).

Neuroblastoma is the most common solid tumor found in children. It is an embryonic malignancy of the sympathetic nervous system arising from neuroblasts, but the cause is unknown. It is grouped into four stages (1–4) depending on location. Further divisions are made depending on age, presence of N-Myc amplifications, and loss of heterozygosity (LOH) of 1p36 (for a review see Maris et al., 2007). Expression of PDGFRA, PDGFRB, PDGF-A, and PDGF-B have been detected in neuroblastoma (Matsui et al., 1993), indicating the possibility for an autocrine stimulation of growth in this type of cancer. This is supported by the fact that treatment with imatinib reduces growth of neuroblastoma cell lines and diminishes tumor sizes in mice (Beppu et al., 2004).

Cell cycle

Addition of growth factors to growth-arrested cells will within minutes induce transcription of early response genes (ERGs). c-Myc, c-Fos and c-Jun are three examples of hundreds of immediate early genes, many of which encode for transcription factors that activate transcription of delayed response genes (DRGs). One such DRG product, E2F, is converted from a transcriptional activator to a repressor when bound to an unphosphorylated form of the tumor suppressor pRb which in turn interacts with histone deacetylases to repress transcription. Phosphorylation of pRb at mid-G1 by Cdk4/6 and cyclin D inhibits its repressive function on E2F and allows E2F to activate gene transcription. This initial phosphorylation of pRb leads to production of cyclin E and Cdk2, which further stimulates phosphorylation of pRb in late-G1. At this point passage through cell cycle is independent of Cdk4/6 and cyclin D so progression occurs even if cyclin D expression drops *i.e.* the restriction point has been passed, permitting entry into S phase. After completion of mitosis, CDK and cyclin levels fall and pRb becomes unphosphorylated and can again inhibit E2F in early G1 phase of the cell cycle.

Another way for the cell to regulate cell cycle progression is through cyclin kinase inhibitors. These are divided into two families, the CDK inhibitory proteins (CIP) and inhibitors of kinase 4 (INK4). CIPs bind to G1 cyclin-CDK complexes and inhibit them. The most well-known CIP is $p21^{CIP}$ which inhibits Cdk4/6-cyclin D complexes. INK4 proteins like $p16^{INK4}$ can bind both monomeric Cdk4 and 6 as well as CDK-cyclin complex, forming an inactive ternary complex.

Stimulation of growth-arrested cells with PDGF enables entry into the G1-phase of the cell cycle. However, PDGF is unable to promote further progress through G1 in order to enter the S-phase. Therefore PDGF signaling is said to act as a competence factor for the cell. This competent state is achieved within 30 min of PDGF treatment. To progress in the cell cycle, the competent cells require additional components usually present in serum. These additional factors, termed progression factors, are mimicked by the epidermal growth factor (EGF) and insulin-like growth factor 1 (IGF-1).

Regulation of PDGF signaling

PDGF signaling is extensively regulated by a variety of feedback mechanisms. These feedback mechanisms act on many levels in PDGF signaling and include ligand-mediated endocytosis, receptor dephosphorylation by protein tyrosine phosphatases, ubiquitin-mediated degradation, and transcriptional regulation.

Ligand-mediated endocytosis

PDGF receptors are located in distinct membrane invaginations on the cell membrane, caveolae (Liu et al., 1996), where many PDGF receptor interacting proteins also are located (Chang et al., 1994). The caveolae are involved in endocytosis, and upon binding of PDGF, PDGF receptors are internalized into the cell in endosomes (Rosenfeld et al., 1984). There, in the endosome, the ligand-receptor complex dissociates and the receptor is either cycled back to the cell surface (Karlsson et al., 2006), or degraded through fusion of the endosomes with lysosomes (Sorkin et al., 1991). The rate of internalization is dependent on the kinase activity of the receptor, as supported by findings that mutations of the Src binding site Tyr-579 (Mori et al., 1994) and the PI-3 kinase binding sites Tyr-740 and Tyr-751 (Joly et al., 1994) of the PDGFRB impair internalization.

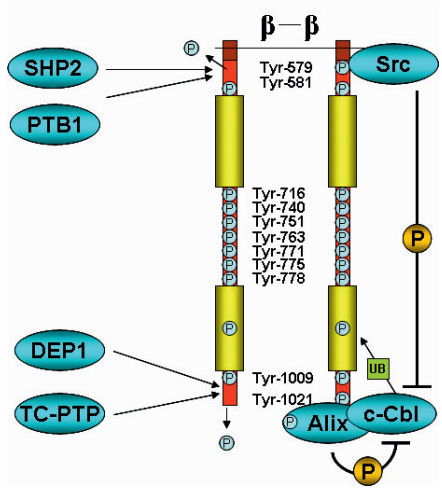

Figure 5. Dephosphorylation and ubiquitination of PDGFRB.

Receptor dephosphorylation by protein tyrosine phosphatases

Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. PDGF receptor activity is usually negatively controlled by protein tyrosine phosphatases (figure 5). This control can either be general or site-specific and allows the cell to stop or fine-tune the signaling (Ostman and Bohmer, 2001). At least six protein tyrosine phosphatases, PTP-PEST, SHP1, SHP2, PTP-1B, DEP1, and TC-PTP can form a complex with PDGFRB (Kovalenko et al., 2000; Markova et al., 2003). Of these, PTPs, DEP1, and TC-PTP show site-specific dephosphorylation of Tyr-1021, while PTB-1 and SHP2 dephosphorylate Tyr-579 and Tyr-771, respectively. Dephosphorylation of Tyr-1021 and Tyr-579 decreases signaling via PLC and Src, respectively.

Ubiquitin-mediated degradation

Ubiquitination is the process where one or more ubiquitin molecules are attached to lysine residues on a protein. This modification will target proteins for degradation either in the proteasome or lysosome (Hershko, 1991). Upon ligand binding, both PDGF receptors are ubiquitinated and targeted for degradation (Mori et al., 1992). In this process, the E3 ubiquitin ligase c-Cbl (Casitas B-lineage lymphoma) plays a crucial role (Miyake et al., 1999). For PDGFRB, c-Cbl increases the PDGF-induced ubiquitination and subsequent lysosomal degradation through binding to PDGFRB pTyr-1021 (figure 5). In addition, this inhibits binding of PLC to pTyr1021, which in turn inhibits cell migration (Reddi et al., 2007).

Moreover, the adapter protein Alix constitutively interacts with PDGFRB and becomes tyrosine phosphorylated in response to ligand stimulation. When phosphorylated, Alix phosphorylates c-Cbl and targets c-Cbl for proteasomal degradation (Lennartsson et al., 2006). The same scenario is seen for the PDGF receptor interacting protein Src which phosphorylates c-Cbl, causing it to self-ubiquitinate and become degraded (Bao et al., 2003).

Transcriptional regulation

Gene expression is controlled by transcriptional regulation. In this process, binding of transcription factors to the regulatory region called promoter, upstream of the transcription initiation site, affect the recruitment of the basal transcription machinery. This recruitment is further affected by the acetylation status of histones close to the promoter region. Acetylated histones create a more loosely packed DNA and thus provide better access for the basal transcription machinery to DNA.

Basal transcription

Initiation of transcription requires the binding of RNA polymerase II (Pol II). This binding is achieved by the formation of a preinitiation complex on proximal promoters that usually contain core recognition motifs, such as TATA and CAAT. GC-rich sequences, to which the transcription factor Sp1 binds, are also often found in promoters. For a TATA-box containing promoter, TFIIB and the TATA-binding protein (TBP) binds to start forming the preinitiation complex. Subsequent binding of TFIIA stabilizes the existing interactions and recruits TBP associated factors (TAFs), to form TFIID. Pol II binds to the formed complex together with TFIIF, which will help to speed up the polymerization process. Association of TFIIE to Pol II enables it to move down the strands. TFIIH is a large protein complex that contains both helicase and kinase activities. TFIIH binds specifically to the template strand to ensure that the correct strand of DNA is transcribed, and separate the two strands using its helicase activity. Through the kinase activity of TFIIH the Pol II becomes phosphorylated and activated to start producing mRNA.

Transcription factors

Transcription factors are sequence-specific DNA-binding factors that bind promoter regions and enhancers to regulate Pol II-controlled gene expression. Transcription factors are often members of large protein families and in these families, members display similar DNA binding but are differently activated. Their activity is also affected by posttranslational modifications such as acetylation, phosphorylation, and methylation. The majority of transcription factors are either activators or repressors. However, some can function as both, depending on cofactors. Transcription factors have a DNA-binding domain and an activation or repression domain, but can also include an oligomerization domain and regulatory domains.

DNA-binding motifs are necessary for specific binding of the transcription factors to their target sequences on the promoters. There are many DNA binding motifs, which include helix-turn-helix, helix-loop-helix, zinc-finger and leucine-zipper motifs. Less is known about the structure of activation domains but they include glutamine rich motifs, proline-rich motifs, and hydrophobic β-sheets. However, the main function of the transcription factors is the recruitment of coactivators and corepressors through direct protein-protein interactions. These recruited coregulators will affect the activity of Pol II both directly and indirectly. For direct regulation, the recruited proteins serve as a bridge between the transcription factors and members of the preinitiation complex in order to adjust the rate of transcription. Indirect regulation, on the other hand, includes recruitment of histone modifying enzymes such as histone acetyltransferases (HATs) and histone deacetylases (HDACs) in order to change the availability for preinitiation complex to DNA.

Chromatin modifications

Chromatin is a structure consisting of DNA and histones, where 146 base pairs of DNA are wrapped around an assembled histone H3-H4 tetramer and two H2A-H2B dimers to form a nucleosome. This structure is repeated and densely packed throughout the chromosome. The

interaction between DNA and histones is achieved from the negatively charged DNA backbone with the positively charged histones. In this state, the chromatin is condensed and highly condensed chromatin (heterochromatin) is transcriptionally silent. For transcription factors and Pol II to gain access to DNA, the DNA needs to be unwrapped from the histones. Histone modifications affect the condensation of chromatin and the main modifications are acetylation, methylation, and phosphorylation. In histone acetylation, acetyl groups are added to lysine residues on the histones and thereby neutralize the positive charge. As an effect, the ionic interaction between DNA and histone is decreased, thus reducing chromatin condensation. Hence, recruitment of HATs and HDACs by transcriptional activators and repressors, respectively, can change the condensation of the chromatin.

Transcriptional regulation of the PDGFRB

In addition to receptor downregulation from internalization and degradation of the ligand-activated receptor, stimulation by ligands can induce decreases in the mRNAs encoding their cognate receptors as seen for PDGFRB (Vaziri and Faller, 1995). These negative feedback mechanisms provide ways to desensitize cells to subsequent stimulation by the ligand and result in reduced or abolished response in cells when chronically exposed to a ligand.

The basal transcriptional regulators of PDGFRB expression are NF-Y and Sp1. These transcription factors interact with the basal transcription machinery and associate with HATs or HDACs either directly or indirectly through coregulatory proteins. Both c-Myc and p73 are two coregulators involved in repression of PDGFRB expression, in response to mitogenic stimulation. However, oncogenic factors can also affect PDGFRB expression as seen for ΔNp73 and the large t antigen (LT) of SV40 (Wang et al., 1996; Hackzell et al., 2002).

PDGFRB promoter

The PDGFRB promoter does not contain a TATA box or TATA-like sequence. Instead it has a CCAAT sequence and a GC-rich area (figure 6). In the mouse PDGFRB promoter, the CCAAT sequence is located 60 base pairs upstream of the transcriptional start site and is crucial for transcription, as CCAAT-mutations decrease promoter activity (Ballagi et al., 1995). The main binding partner to the CCAAT sequence in the PDGFRB promoter is NF-Y (Ishisaki et al., 1997) but C/EBP might play a role as well (unpublished observations). Moreover, deletion of the GC-rich region, located 100 base pairs upstream of the transcriptional start site, also causes a decrease in transcriptional activity of the PDGFRB promoter (Molander et al., 2001). In this GC-rich area, two GC-boxes are present to which Sp1 binds, thus likely to be the main interacting protein for this sequence.

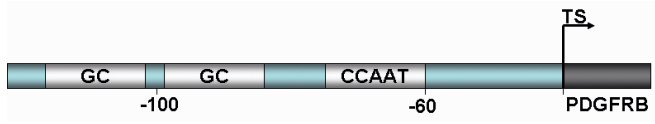

Figure 6. The PDGFRB promoter.

NF-Y

NF-Y is a CCAAT-specific transcription factor (Dorn et al., 1987) which consists of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA-binding and transcriptional activation (Maity et al., 1992; Sinha et al., 1995). NF-Y binds as a trimer to DNA. This trimer is created through the formation of a NF-YB-NF-YC dimer, providing a surface to which NF-YA can bind.

Conserved regions in the subunits of NF-Y, the so called yeast homology domain (YHD), correspond to sites involved in DNA-binding and subunit interaction sites necessary for DNA-binding (Li et al., 1992). The YHD lies in the C-terminal of NF-YA, in the central part of NF-YB, and in the N-terminal of NF-YC. In NF-YB and NF-YC, these domains contain the histone fold motif (HFM) (Baxevanis et al., 1995). In histones, this motif mediates histone dimerization and formation of non sequence-specific interactions with DNA (Arents and Moudrianakis, 1995). For NF-YB and NF-YC, HFMs are required for dimer formation, NF-YA association, and CCAAT-binding (Kim et al., 1996). In contrast to NF-YB and NF-YC, the conserved domain of NF-YA does not resemble any of the known DNA-binding motifs. Instead, this domain contains two distinct halves, each 20 amino acids long with one part required for NF-YB-NF-YC association and the other for DNA-binding (Xing et al., 1993). Both NF-YA and NF-YC have domains that are glutamine rich and contains hydrophobic residues (Coustry et al., 1995). For transcription factors like Sp1, glutamine-rich domains contain the activation function and this is true also for NF-Y (Courey and Tjian, 1988). In NF-YA, the activation domain is in the N-terminal, while in the C-terminal for NF-YC (Coustry et al., 1996). NF-YB, however, does not carry an apparent activation domain (figure 7).

In its activation of transcription, NF-YB and NF-YC recruit and interact with TBP and associated TAFs through the C-terminal end of their YHDs (Bellorini et al., 1997; Frontini et al., 2002). HAT and HDAC activities are also directly and indirectly associated with transcriptional regulation by NF-Y (Jin and Scotto, 1998). In activation of transcription, NF-YA and NF-YB interact with P/CAF and p300 both carrying HAT, respectively (Jin and Scotto, 1998; Li et al., 1998). Thus, the trimer could theoretically associate with both HATs simultaneously.

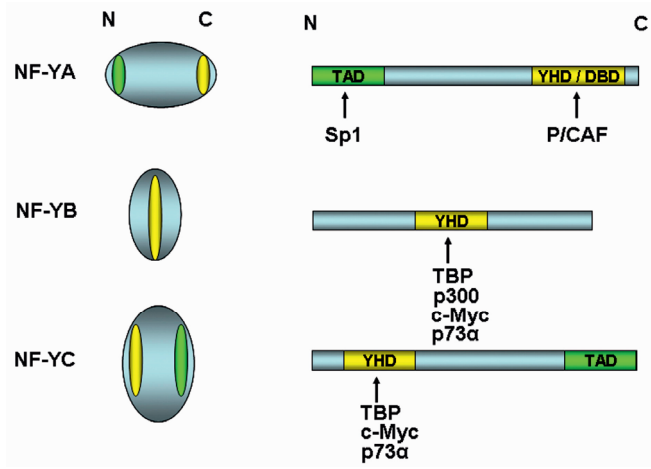

Figure 7. NF-Y domains and interacting proteins.

The CCAAT box is almost always flanked by at least one functionally important promoter element, and NF-Y affects neighboring transcription factors to synergistically increase transcriptional activation. This is achieved by either increasing the affinity of the neighboring factors for DNA or by interacting with parts of these factors other than the DNA-binding domain, so that their respective contacts with DNA are stabilized as reported for Sp1 (Wright et al., 1995).

NF-Y is a crucial activator of the PDGFRB promoter (Ishisaki et al., 1997). In regulation of PDGFRB promoter activity, NF-Y interacts with Sp1, c-Myc, and p53 family members. Sp1 seems necessary for the effect of NF-Y since deletion of Sp1-binding sites disables the function of NF-Y to increase promoter activity (Molander et al., 2001). c-Myc, on the other hand, binds directly to NF-YB and NF-YC not affecting their DNA binding, but repressing transactivation (Izumi et al., 2001). However, the exact mechanism of this repression is unknown. Also, p73α, a member of the p53-family, binds to NF-YB and NF-YC to repress PDGFRB promoter activity (Hackzell et al., 2002).

Sp1

Specificity protein 1 (Sp1) binds as a monomer or multimer to GC-rich consensus sequences, GC-boxes, in order to regulate transcription (Kadonaga et al., 1987). Sp1 is part of the large Sp/KLF transcription factor family with the Sp-subfamily consisting of Sp1–9. Sp1–4 have glutamine-rich transactivation domains (TAD) while Sp5–9 do not, and of these only Sp1 and Sp3 are ubiquitously expressed. Sp1 regulates expression of many different genes in response to oncogenes, growth stimulation and differentiation (as reviewed by Safe and Abdelrahim, 2005).

Sp1 possesses three C_2H_2-type zinc fingers as its DNA-binding domain (Kadonaga et al., 1987), and carries four TADs designated A, B, C, and D (Pascal and Tjian, 1991). Two of the activation domains, A and B, located in the N-terminus of Sp1 are rich in glutamine and each can stimulate transcription when bound to DNA (Courey and Tjian, 1988). Domain C has highly charged amino acids and carries a weak transactivation potential. Domain D, on the other hand, lacks any clear amino acid setup but is required for certain synergistic activation together with A and B (figure 8) (Pascal and Tjian, 1991).

Domains A and B bind directly to TBP in order to stimulate transcription initiation (Emili et al., 1994). In addition, both domains also interact with a TBP-associated factor such as TAF4 (Gill et al., 1994). Sp1 also recruits TFIIB, TFIIE and TFIIA into the preinitiation complex (Choy and Green, 1993). This recruitment and stabilization of many members of the initiation complex explains the ability of Sp1 to induce the transcription of genes with TATA-less promoters.

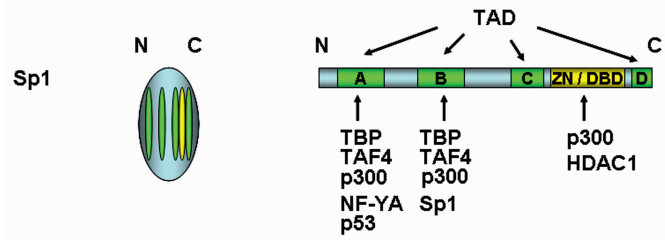

Figure 8. Sp1 domains and interacting proteins.

Moreover, Sp1 interacts directly or indirectly with HATs and HDACs to regulate transcription (Xiao et al., 2000). Through binding of p300, thus activating each of the two Sp1 A- and B-domains, Sp1 can be a transcriptional activator (Suzuki et al., 2000). By binding HDAC1 to its DNA-binding domain it can act as a repressor (Zhang and Dufau, 2002). It is plausible that a Sp1-multimer binding to a promoter can simultaneously recruit HATs and HDACs in order to create a more dynamic histone and transcription factor acetylation profile.

There are at least three ways for Sp1 to activate transcription: first by transactivation by a single Sp1-binding site recruiting the basal transcription machinery, second through synergistic transactivation between two or more Sp1 without cooperative DNA-binding, and third by superactivation of the Sp1-mediated transcription as described below.

Sp1 bound to a single site can, as mentioned, activate transcription, by recruiting the basal transcription machinery. When binding to two neighboring GC-boxes, Sp1 mostly bind as a multimer. As a multimer, Sp1 presents multiple docking sites for interacting proteins. Sp1 requires three of its activation domains, A, B, and D, to achieve synergistic activation on two adjacent binding sites. In superactivation, Sp1 bound to DNA interacts with the superactivator which does not bind DNA. Sp1 transactivates gene expression synergistically with a large variety of transcription factors (Wierstra, 2008). For example, Sp1 binds the members of the p53-family, p53, p63, and p73, acting synergistically with p53 and p73 in activation of the p53 target gene p21 (Koutsodontis et al., 2005). Also, Sp1 interacts with NF-YA through their respective TADs, and in some cases acts synergistically rather than additive for activation (Roder et al., 1999).

Sp1 plays an essential role in PDGFRB transcriptional regulation. Two out of three GC-boxes in the GC-rich area of the PDGFRB promoter binds Sp1 and deletion of these boxes lead to loss of response to Sp1 overexpression (Molander et al., 2001).

c-Myc

c-Myc belongs to the Myc-family of transcription factors and binds as a heterodimer with its partner, Max, to enhancer box (E-box) sequences with the consensus binding sequence CACGTG, to activate transcription (Blackwood and Eisenman, 1991). However, it can also act via other transcription factors and DNA-binding sites and then mostly acts as a transcriptional repressor. c-Myc regulates many key genes involved in cell proliferation, and overexpressed c-Myc contributes to many processes involved in tumor proliferation.

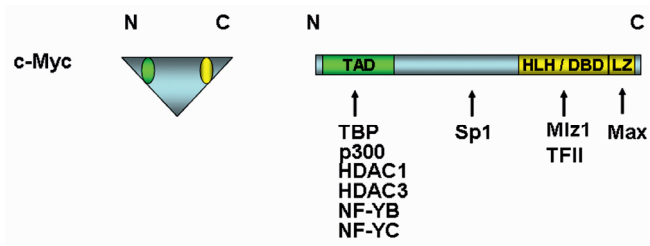

Figure 9. c-Myc domains and interacting proteins.

c-Myc contains a basic-helix-loop-helix (bHLH) leucine-zipper motif in its C-terminus and a TAD in its N-terminus (figure 9). The leucine-zipper motif mediates interaction with Max while the bHLH mediates contact with DNA (Blackwood and Eisenman, 1991). Also, the bHLH mediates interaction with the transcription factors Miz1 and TFII-I (Gartel and Shchors, 2003). The TAD contains three motifs, Myc box I and II, both of which are necessary for transactivation, and Myc box III involved in proteasomal destruction of c-Myc and recruitment of HDACs.

c-Myc interacts with TBP as well as a subunit of TFIIF, RAP74, via its TADs (Hateboer et al., 1993; McEwan et al., 1996). This suggests that c-Myc at least in part exerts its effect through the recruitment of TBP. Another action of c-Myc in transcriptional regulation is the recruitment of HATs and HDACs such as CBP, p300, P/CAF, and HDACs 1, 3, and 4. CBP stimulates transcriptional activity of c-Myc and acetylates c-Myc *in vitro,* resulting in increased stability of the normally short lived c-Myc (Vervoorts et al., 2003). GCN5 and P/CAF acetylate c-Myc *in vivo* and also increase its stability (Patel et al., 2004). The sites of acetylation are within a nuclear localization sequence and the leucine- zipper motif. Interestingly, interaction between p300 and the TAD domain of c-Myc results in acetylation, but instead of an increase in stability, turnover is increased. However, upon acetylation the transcriptional activity of c-Myc is also increased (Faiola et al., 2005). HATs might serve to activate c-Myc by acetylation, or act as a bridge to other transcription factors,or by acetylating histones. In transcriptional repression by c-Myc, the Myc box III motif binds to HDAC3 in repression of transcription of certain promoters (Kurland and Tansey, 2008). Also,in transcriptional repression of the HIV type 1 promoter, c-Myc recruits HDAC1 (Jiang et al., 2007).

c-Myc expression leads to downregulation of a number of genes. This transcriptional repression can be either direct or indirect. For genes under the control of a core promoter element, the initiator (INR), c-Myc can interfere with the INR-transcription factors TFII-I, YY-1, and Miz-1. Association of c-Myc with TFII-I (Roy et al., 1993) and YY-1 (Shrivastava et al., 1996), respectively, prevents their activation of transcription. However, the most studied and best described transcriptional repression of c-Myc is that on Miz-1 where c-Myc recruits DNA methyltransferase 3 (DNMT3) and possibly displaces p300 bound to Miz-1, to inhibit expression of Miz-1 target genes (Brenner et al., 2005). Binding to other transcription factors likely involves similar mechanisms to disturb the formation of the preinitiation complex or to compete with coactivators. A more direct role of c-Myc repression of transcription would be through recruitment of HDACs.

c-Myc plays an important role in control of the PDGFRB where it in response to growth stimuli downregulates PDGFRB (Oster et al., 2000). This control is exerted through NF-Y where c-Myc binds NF-YB and NF-YC but not NF-YA. Interaction is dependent on the HFMs of NF-Y and the homology boxes in c-Myc (Izumi et al., 2001). Binding of c-Myc to NF-Y does not affect the DNA-binding but affect the transactivation ability of NF-YC. It still remains to be determined whether c-Myc through the interaction with NF-Y can recruit HDACs to further repress transcription.

SV40 large T antigen

SV40 belongs to the polyoma virus family and is dependent on its oncogenic protein, LT, for replication. Following infection, LT affects gene expression by binding to transcription factors that are important for both replication and cell cycle regulation, such as p53 and pRb (Moens et

al., 1997). Inactivation of the Rb and p53 tumor suppressors by LT is one of the central features of transformation of the host cell by SV40.

The LT is a 90-kDa phosphoprotein where the N-terminus binds and dissociates the E2F-pRb complex, thereby preventing pRb repression of E2F (DeCaprio et al., 1988), and the C-terminus interacts with the DNA binding domain of p53 to inactivate it (Kierstead and Tevethia, 1993). In the N-terminal region of LT, the LXCXE motif and the J domain are the crucial regions for binding and inactivation of pRb (figure 10). The J domain is homologous to the molecular chaperone DnaJ from *E. coli*. Mutations in the J domain region inactivate the LT (Stubdal et al., 1997). Thus, it is possible that LT could inactivate pRb by behaving like a molecular chaperone.

LT interacts with TBP, $hTAF_{II}130$, $hTAF_{II}32$, TFIIB, and Pol II, but the effect of these interactions are unclear. The C-terminus of LT interacts with the N-terminus of TBP at the same place where p53 interacts with TBP (Gruda et al., 1993). Thus, LT might compete with p53 for binding to TBP. However, LT cannot activate promoters only containing a TATA-box and needs additional interactions for activation. This suggests a role for LT as a bridging molecule in transcriptional activation.

In regulating transcription, LT binds CBP/p300 (Eckner et al., 1996) which causes changes in the phosphorylation status of both CBP and p300, resulting in the repression of their transcriptional activities. The interaction depends on a region surrounding the pRb interaction motif LXCXE since LT mutants lacking this domain is unable to affect p300 in any way.

LT indirectly activates transcription of a wide range of genes involved in G1/S progression by its activation of E2F. However, a more direct role in transcription is indicated by the finding that LT can activate promoters containing an Sp1, ATF or AP1-binding sites, in conjunction with a TATA element (Gilinger and Alwine, 1993). Again, this could point to a role for LT as a bridging factor between other transcription factors within the basal transcription machinery. Also, LT represses mRNA expression of PDGFRA and PDGFRB, and for PDGFRA in fibroblasts, independently of p53 and Rb (Wang et al., 1996).

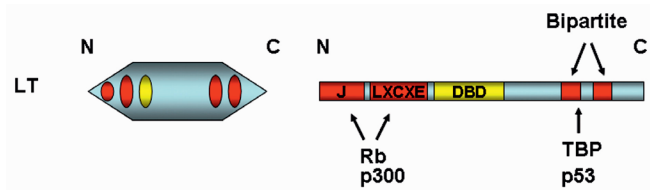

Figure 10. LT domains and interacting proteins.

p53 family members

The p53 family of tumor suppressors consists of three members, p53, p63, and p73, all of which are crucial transcription factors for cellular stress responses and development. p53 was identified in 1979 by its interaction with the LT of SV40 from studies by David Lane (Lane and Crawford, 1979). Some 18 years later, p73 was discovered as a protein sharing large homology with p53 and located at a chromosomal region frequently deleted in neuroblastoma (Kaghad et al., 1997). p63, the most recent family member, was isolated in 1998, and found to consist of many isoforms including N-terminal deleted variants (Yang et al., 1998). This finding sparked investigations into whether the same was true for the other p53 family members and indeed, p73 was found to consist of many isoforms (Kaghad et al., 1997; De Laurenzi et al., 1998; De Laurenzi et al., 1999; Ishimoto et al., 2002), and recently this was found for p53 as well (Bourdon et al., 2005).

From the discovery, the role of p53 family members in cancer and development has been intensely investigated. In human cancers, p53 is found mutated in 50% of the cases (Greenblatt et al., 1994), while mutated forms of p63 and p73 rarely are found. Instead, for p63 and p73, the ΔN-isoforms act as dominant inhibitors of the full-length forms of p53, p63, and p73 (for a review see Yang et al., 2002). This blocks activation of p53 target genes important for apoptosis and cell-cycle arrest, thus serving as oncogenic stimuli for cancer progression. In development, p63 and p73, rather than p53, have important roles as supported by knockout mice of p63 and p73, which unlike p53, show developmental abnormalities (Yang et al., 1999; Yang et al., 2000). Isoforms of p63 and p73 have different C-terminals from that of p53, and some contain the sterile alpha motif (SAM) domain which often is found in proteins involved in development.

The p53 family proteins are sequence-specific transcription factors where all members can bind to the canonical p53-binding sequence, with various efficiencies, in order to transactivate genes that mediate cell cycle arrest or apoptosis. Current research focuses on the importance of post-translational modifications of p53 members for association to target genes and their functional effects.

Structure and Function of p53 Family Members

Through alternative promoters and splicing, the p53 family members can express many mRNA variants encoding for proteins with distinct N- or C-terminal endings. p63 has six mRNA variants coding for six isoforms. Transcription is initiated from either of two distinct promoters, upstream of exon 1 or in intron 3, to generate full-length p63 (TAp63) or N-terminally deleted p63 (ΔNp63). C-terminal splicing gives rise to either α, β, γ isoforms (Yang et al., 1998). Similarly, p73 mRNA transcripts encode for as many as twenty-nine p73 isoforms. But so far, only twelve p73 isoforms have been described. Like p63, transcription is initiated from two promoters, one upstream of exon 1 and one in intron 3, yielding TAp73 or ΔNp73, respectively. Alternative splicing of the C-terminal produces α, β, γ, δ, ε, ζ or η isoforms (figure 11) whilst N-terminal splicing produces p73 lacking exon 2 or both exon 2 and 3. p53, p53β and p53γ C-terminal isoforms are generated by different splicing of intron 9. N-terminal deleted p53 is generated from an alternative promoter in intron 4, resulting in p53 lacking the first 132 amino acids, called Δ133p53. p53 lacking the first 40 amino acids is generated through alternative splicing of intron 2 or through alternative initiation of translation. The purpose of this many isoforms remains to be elucidated.

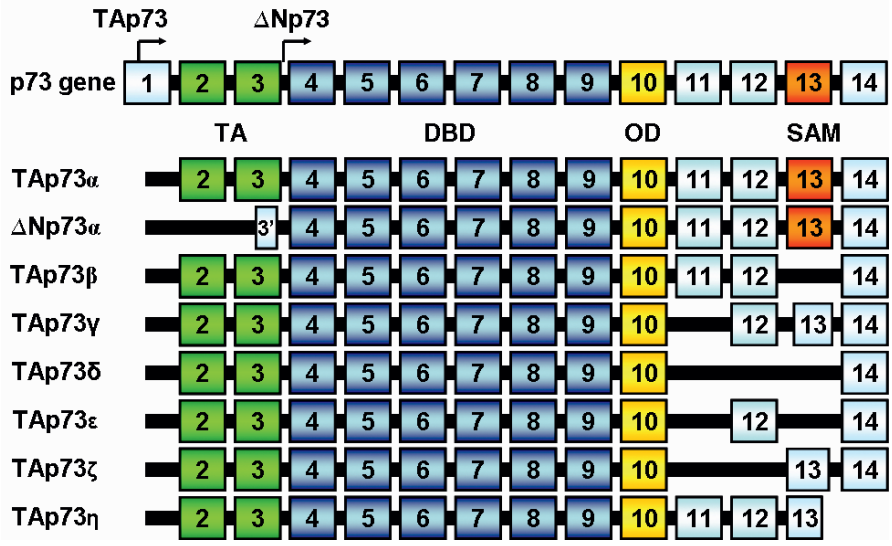

Figure 11. Exon structure of TAp73 isoforms and ΔNp73α.

The domains required for transcriptional activity, cell cycle arrest, and apoptosis are the N-terminal transcriptional activation domains (TADs), the DNA-binding domain (DBD), the oligomerization domain (OD), and the C-terminal basic domain (BD). The BD is present in p53 but not in p63 or p73, while the SAM domain is present in p63 and p73 alpha-isoforms but lacking in p53 (Harms and Chen, 2006). Overall a great deal of homology exists between the members especially in the DBD, but less in the OD, and the least in the TADs (figure 12).

p53 contains two N-terminal TADs with TA1 consisting of residues 1-42 and TA2 of residues 43-92 or 43-63 when excluding the proline-rich domain between residues 64-92. p63 and p73 contain one TAD. The activation domains are responsible for interactions with the basal transcription machinery and coregulators. While Δ40p53 lacks the first TAD, ΔNp63 and ΔNp73 lack the full-length activation domain, but instead have a unique activation domain consisting of 13 or 14 residues. Whether this indicates that N-terminal deleted p63 and p73 activate a distinct subset of genes from their full-length counterparts, or if they as originally believed, bind to the same DNA as their full-length counterparts to inhibit their function, remains to be elucidated. Similar to the C-terminal, many residues in the N-terminal are targets for posttranslational modifications in response to various kinds of stresses.

The DBD of the p53 family proteins carries the greatest homology in between family members. A fully functional DBD is essential, as pointed out by the fact that most mutations of p53 reside in this domain. The DBD of p53 family proteins all recognize the p53-responsive element consisting of the decamer RRRCWWGYYY, where R is a purine, Y a pyrimidine and W an adenine or thymine. However, p53 family members can bind to other sequences as well.

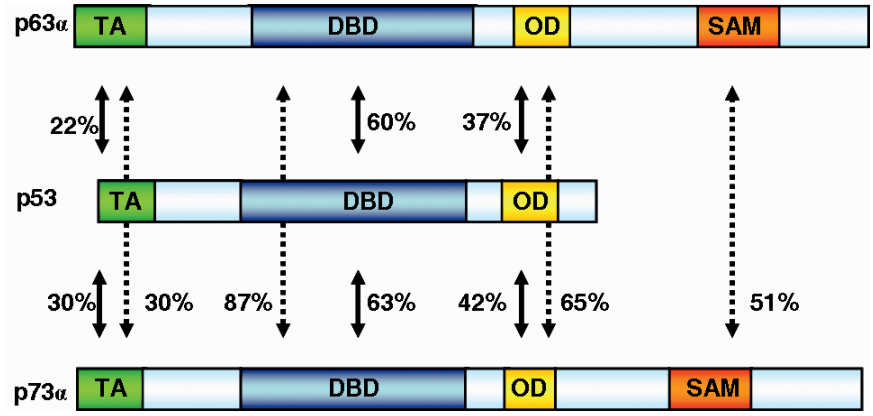

Figure 12. Homology of p53 family members.

High affinity binding and transcriptional activation require the formation of a tetramer through the OD. With the finding that p53 family members are expressed as so many isoforms, the role of heterodimerization of these isoforms needs to be considered.

The C-terminal BD found in p53 is a regulatory domain, where nearly all residues are targets for posttranslational modifications. p63α and p73α contain the SAM domain. The SAM domain is known to mediate protein-protein interactions and the fact that it is lacking in p53 could explain the different activities of the proteins. Interestingly, p73β lacking the SAM domain is more efficient than p73α to induce apoptosis. Mutation studies confirm that the SAM domain exerts some form of negative regulation of the TAD domain of p73 (Liu and Chen, 2005).

The p53 family proteins possess both a nuclear localization signal (NLS) and a nuclear export signal (NES), which are essential for nuclear-cytoplasmic shuttling. p53 contains a NLS located in between residues 305-322 that is active for importin A binding and subsequent nuclear import. In addition, p53 has two NESs, one in TAD1 (residues 11–27) and one in the OD (residues 340-351). The residues comprising the p53 NLS and the C-terminal NES are conserved in p63/p73 and are functional in p73. The effect of these signals is regulated by posttranslational modifications.

Posttranslational modifications of p53 family members

Posttranslational modifications of p53 family members, such as phosphorylation, acetylation and ubiquitination, alter their stability and DNA-binding affinities (as reviewed in Maisse et al., 2003; Lavin and Gueven, 2006). A variety of different stresses, such as UV radiation, DNA double-stranded breaks and chemical DNA-damaging agents, such as cisplatin induce posttranslational modifications of p53 family members. Posttranslational modifications and protein interactions include Mdm2-mediated ubiquitination, kinase-mediated phosphorylations, p300-mediated acetylation and prolyl isomerization. The principal locations of modifications on p53 family members in response to stress are located in the N-terminal TADs and C-terminal regions.

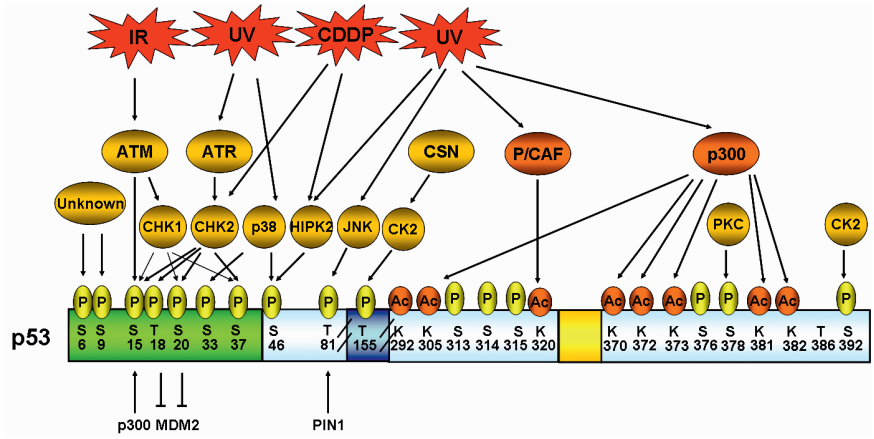

Figure 13. Posttranslational modifications of p53 and their effects on p53 interacting proteins.

In unstressed cells, p53 levels are kept low by the ubiquitin ligase Mdm2. Mdm2 does this by ubiquitinating p53 and targeting it for proteasomal degradation (Haupt et al., 1997). The binding of Mdm2 to the TAD of p53 also blocks the transcriptional activity. After a stress signal, Mdm2 polyubiquitinates itself and is degraded. p73 is also inhibited by binding to Mdm2. However, Mdm2 does not ubiquitinate p73 and target it for degradation, instead it interferes with the binding of p73 to p300 (Zeng et al., 1999). As p53, p73 induces Mdm2 expression as a negative feedback mechanism.

In response to stress, p53 family members become phosphorylated by various protein kinases, including ataxia-telangiectasia mutated (ATM), ataxia-telangiectasia and Rad3-related (ATR), Chk1, Chk2, JNK (Jun NH_2-terminal kinase), p38, and c-Abl (figure 13). The most studied p53 phosphorylation is that of Ser-15 which occurs in response to many stress signals. In response to DNA breaks caused by ionizing radiation, Ser-15 is phosphorylated by the serine/threonine kinase ATM. This phosphorylation decreases binding of Mdm2 to p53 and promotes phosphorylation of the neighboring residues Thr-18 and Ser-20. The phosphorylation at Ser-15, Thr-18, and Ser-20 promotes the recruitment of p300, CREB binding protein (CBP) and P/CAF to the TAD which then acetylates the C-terminal lysines to modify the interaction of p53 with target genes.

The tyrosine kinase c-Abl plays a central role in DNA damage-induced stabilization of p53 and p73. Upon DNA damage, c-Abl is phosphorylated and activated by ATM. Activated c-Abl stops Mdm2-mediated degradation of p53 (Sionov et al., 2001) and binds to the PXXP motif of p73 and phosphorylates it on Tyr-99 (Agami et al., 1999). In addition, c-Abl indirectly promotes p73 phosphorylation on Ser/Thr-Pro residues through the activation of the p38 MAP kinase pathway, which promotes transactivation of p73 (Sanchez-Prieto et al., 2002).

p300 and CBP enhance the transcriptional activity of p53 family members. For p53, p300 binds to the TAD and acetylates the C-terminal lysine residues (Gu and Roeder, 1997). This acetylation regulates the ability of p53 to recognize its DNA sequence and to a small degree its activation of transcription. Furthermore, acetylation of lysine residues might prevent ubiquitination of the

same residues thus preventing proteasomal degradation. p73 also interacts with p300 to increase transcriptional activation and apoptosis (Zeng et al., 2000).

Acetylation of p53 by CBP (Pearson et al., 2000), and p300-mediated acetylation of p73 (Bernassola et al., 2004) have been linked to their localization to promyelocytic leukemia (PML) bodies. The acetylation-induced protein stabilization is promoted by the PML protein, which, in turn, regulates p73 transcriptional activity. Interestingly, p38-mediated phosphorylation of p73 (Bernassola et al., 2004), and the HIPK2-mediated Ser-46 phosphorylation of p53 (Hofmann et al., 2002) favor their binding to PML and recruitment in the PML-NB, thus promoting their stabilization and activation.

Stress-induced phosphorylation of p73 on Ser/Thr-Pro residues and phosphorylation of p53 on Ser-33, Thr-81 and Ser-315 leads to interaction with the prolyl isomerase Pin1. This results in prolyl isomerization, conformational changes, acetylation, stabilization, and enhancement of activity (Zheng et al., 2002; Mantovani et al., 2004). Thus, only when a number of stress kinases alter p53 family members at specific sites does Pin1 bind to it efficiently. It is also of interest that Pin1 associates with p73 after genotoxic stress to promote its acetylation by p300 and increases the stability of p73.

Basal transcription machinery and p53 family members

Of the three family members, only p53 has so far been shown to directly interact with members of the basal transcription machinery. When p53 is bound to a p53 responsive element in the promoter it affects transcription by direct interaction, through its N-terminal domain, with TBP (Truant et al., 1993) and the TBP associated factors $TAF_{II}31$ and $TAF_{II}70$ (Thut et al., 1995; Farmer et al., 1996).

Chromatin remodeling and p53 family members

All p53 family members use HATs and HDACs to function. For example, p53, p63, and p73 utilize p300 as a co-activator where the binding of p300 can serve both as a bridge to the RNA II polymerase and to acetylate histones around target promoters (Gu et al., 1997; Lill et al., 1997; Zeng et al., 2000; MacPartlin et al., 2005). Moreover, the binding of p300 to p53 and p63γ leads to their acetylation and subsequent stabilization and activation. In contrast, acetylation of p73 by p300 is not needed for transcriptional activation. All p53 family members interact with p300 through their N-terminal TADs. Thus, all N-terminal deleted variants of the p53 family members are unable to interact with p300, allowing them to function as dominant negative against the full-length proteins. Less is known about the interaction between p53 family members and HDACs in transcriptional repression, but p53 can interact indirectly with HDAC1 through the corepressor mSin3a (Murphy et al., 1999). In this repression, HDAC1 deacetylates lysine residues on histones, tightening the chromatin, thereby repressing gene transcription.

Transcriptional regulation of p53 family members

In some genes, the binding of p53 family members to their responsive element results in direct repression of that gene. Apart from the possible recruitment of HDACs, p53 family members can repress transcription by interfering with DNA binding and through inactivation of transcription factors. Competition for binding to DNA involves an overlapping p53 binding site with that of another transcription factor, while inactivation of a transcription factors occurs through protein-protein interactions.

p53 family members and Sp1 bind and cooperate in activation of target genes, such as p21 and the human immunodeficiency virus 1 (HIV-1) long terminal repeat (Gualberto and Baldwin,

1995; Koutsodontis et al., 2001). However, association with Sp1 can also result in repression, as seen for cyclin B1 and vascular endothelial growth factor (VEGF) in which p73 decreases transcription through Sp1 binding sites (Salimath et al., 2000; Innocente and Lee, 2005), and for epidermal growth factor (EGFR) where p63γ interacts with Sp1 to inhibit its binding to DNA (Nishi et al., 2001). For the human telomerase reverse transcriptase (hTERT), p53 downregulates expression through Sp1-binding sites (Kanaya et al., 2000). Also, for the insulin-like growth factor receptor (IGFR), p53 negatively regulates gene transcription via protein-protein interaction with Sp1 (Ohlsson et al., 1998).

Another way of transcriptional repression by p53 family members is achieved through binding and inactivation of NF-Y. This is seen in the transcriptional repression of the heat shock protein 70 (hsp70) where p53 represses and interacts with NF-Y (Agoff et al., 1993). Studies on cell division cycle 2 (cdc2) also support the role for NF-Y in p53-mediated repression (Yun et al., 1999). However, a broader role of inactivation of NF-Y by p53 family members comes from studies of replicative senescence, where p53, p63γ and p73β downregulate Cdk1 and cyclin B expression and decrease binding of NF-Y to DNA (Jung et al., 2001). This suggests that protein-protein interactions between p53 family members and NF-Y may be a mechanism by which they regulate gene expression.

Interplay of p53 family members

In the p53, p63 and p73 subfamilies, respectively, the assembly of a tetramer including one or more N-terminal deleted variants leads to a reduction of transactivation ability. Tetramerization of different C-terminal isoforms is also likely to affect transactivation but the outcome of such tetramers is much more difficult to predict. The dominant negative effect of ΔNp73 over p53 and p73 is the best characterized heterooligomerization. ΔNp73 can either bind physically to p53 or p73 to inhibit their activity or compete for binding to p53-responsive elements in promoters (Zaika et al., 2002). Interestingly, p53 cannot induce apoptosis without the presence of either p63 or p73 (Flores et al., 2002). Moreover, p53 and p73 bind and activate the ΔNp73 promoter to induce its transcription as part of a negative feedback mechanism (Grob et al., 2001).

Transcriptional regulation of PDGFRB of p53 family members

Of the p53 family members, only p73α so far, has been found to repress PDGFRB transcription (Hackzell et al., 2002). This p73α-mediated repression is dependent on NF-Y since p73α is unable to downregulate PDGFRB expression in the presence of DN-NF-Y. p73α binds both NF-YB and NF-YC through their HFMs. The TAD of p73α is also necessary for PDGFRB repression, and ΔNp73, lacking the TAD domain, is incapable of repression.

Aims of the studies

Overall Aim

The overall aim of this thesis was to clarify the role of p53 family members in transcriptional regulation of the PDGFRB and to characterize the mechanisms of regulation.

Specific aims

I. To elucidate the mechanism of the p73 induced transcriptional repression of the PDGFRB.

II. To elucidate the mechanism of the LT induced transcriptional repression of the PDGFRB.

III. To clarify the role of p53 in the transcriptional regulation of PDGFRB.

IV. To investigate the cause of dysregulation of PDGFRB expression in neuroblastoma.

Materials and Methods

Cell culturing and drugs (Papers I-IV)

In order to elucidate the transcriptional regulation of the PDGFRB, a wide range of cell lines have been used. A summary of these cell lines, their origin, characteristics and growth conditions is found in Table 1.

A summary of the drugs used, their effects and concentrations used is found in Table 2. In order to increase acetylation status of cellular proteins we used the HDAC inhibitor Trichostatin A. To create the Saos2-LT stable cell lines the antibiotic G418 was used to select clones with stably integrated LT. Doxycyclin was used to activate the Tet-On promoter in the Tet-On-p53 Saos2. Cisplatin was chosen for its well known effect to stabilize and induce both p53 and p73, while mitomycin C (MMC) was chosen for its stabilizing and activating effect on p53 (Fang et al., 1999; Gong et al., 1999). In order to study the biological effect of PDGFRB signaling the protein tyrosine kinase receptor inhibitor STI-571 was used.

Table 1

Cell line	Species	Origin	Media	Culture conditions	Paper
COS-1	Monkey	Epithelial	DMEM	5% CO_2, 37°C	I
NIH3T3	Mouse	Fibroblast	DMEM	5% CO_2, 37°C	I, II
$Rb^{-/-}$ 3T3	Mouse	Fibroblast	DMEM	5% CO_2, 37°C	II
HO15.19 (c-$Myc^{-/-}$)	Rat	Fibroblast	DMEM	5% CO_2, 37°C	II
ST-15A	Rat	Cerebellum	DMEM	5% CO_2, 33°C	II
SL2 ($Sp1^{-/-}$, NF-$Y^{-/-}$)	Drosophila	Embryo	Schneider's Drosophila	Air, 25-28°C	II
Saos2 ($pRb^{-/-}$, $p53^{-/-}$)	Human	Osteosarcoma	DMEM	5% CO_2, 37°C	II, III
MEF	Mouse	Fibroblast	DMEM	5% CO_2, 37°C	III
$p53^{-/-}$ MEF	Mouse	Fibroblast	DMEM	5% CO_2, 37°C	III
IMR-32	Human	Neuroblastoma	RPMI	5% CO_2, 37°C	IV
SH-SY5Y	Human	Neuroblastoma	DMEM	5% CO_2, 37°C	IV

Table 2

Drug	Effect	Concentrations used	Paper
TSA	Inhibits histone deacetylases	0.4 µg/ml	I
G418	Disrupts proofreading	500 µg/ml	II
Doxycyclin	Induces Tet-On promoters	2 µg/ml	III
Mitomycin C	Stabilizes and activates p53	10 µg/ml	III
STI-571	Inhibits protein-tyrosine kinases	0.1-10 µM	III, IV
Cisplatin	Stabilizes and activates p53 and p73	5-40 µM	IV

Transfection methods (Papers I-IV)

Transfection is a technique to introduce exogenous gene material, such as plasmids and siRNA into cells. The transfection method used for each cell line was chosen depending on the transfection efficiency. This was determined by levels of protein expression, as examined by immunoblotting or percentage of cells positive for GFP following transfection by a GFP expression vector or fluorescent siRNA. All transfection methods were used according to the manufacturer's instructions. A summary of transfection reagents used for each cell line is found in Table 3.

Promoter Reporter Assay – Luciferase assay (Papers I-IV)

Promoter reporter assays are tools for studying promoters, enhancers and transcription factors. In these methods, the regulatory sequence of interest is introduced upstream of a reporter gene and the resulting vector is transfected into cells with or without vectors expressing transcription factors or siRNAs. For luciferase gene reporter assay the reporter expression is measured by adding luciferin to cell lysates and measuring the resulting luminescence.

Cells were transfected with 0.2-0.25 µg of constructs containing different lengths of the mouse PDGFRB promoter upstream of the luciferase gene with or without 0.25 to 0.5 µg of an expression plasmid. Standardization was usually made by co-transfecting a β-galactosidase reporter plasmid or by measuring protein concentration. Cells were lysed with 100-150 µl reporter lysis buffer (Promega) and luciferase activity was measured according to the vendor's instruction (Promega). All experiments were replicated at least three times and values represent means plus/minus standard deviation of one representative experiment, each done in triplicate. Overall significance was determined by submitting data to one-way analysis of variance. Significance of between-group differences was determined by Scheffé post-hoc comparisons. p values < 0.05 were considered to be statistically significant.

Table 3

Transfection Reagent	Company	Description	Cell Line	Paper
Fugene6	Roche	Lipid based	COS-1, NIH3T3, MEF, HO15.19, SH-SY5Y	I, II, III, IV
N-ter	Sigma	Nanoparticle	p53-/- MEF	III
Lipofectamin 2000	Invitrogen	Lipid based	p53-/- MEF, IMR-32	III, IV
HiPerfect	Qiagen	Lipid based	SH-SY5Y, IMR-32	IV
Combimag	OZ biosciences	Magnetic beads	SH-SY5Y, IMR-32	IV
Microporator	Digitalbio	Microporation	SH-SY5Y, IMR-32	IV

Flow Cytometry (Papers III and IV)

Flow cytometry is a method to stain cells against the antigen of interest using an antibody coupled to a flourophore or a fluorescent intercalating agents that stain nucleic acids to measure its contents in cells.

Cells were seeded in 10 cm-dishes and harvested at 50-80% confluency. Cells were trypsinized and centrifuged at 1200 rpm for 5 min and washed twice with PBS before dissolving the cell pellet in 200 µl PBS. Thereafter, cells were fixed for 30 min by addition of ice cold 70% ethanol. Next, cells were centrifuged and washed in PBS before staining with propidium iodine (40 µg/ml) in the presence of RNAseA (50 µg/ml) for 30 min at 37°C. Cells were washed and cell aggregates were removed using a mesh. Cell cycle analysis was performed on the FACSCalibur (Beckton Dickinsson) using the CellQuest software. FL2-H, FL2-A and FL2-W were detected. Using FL2-H against FL2-W dot plot, aggregates were gated out and at least 10000 events were gated and analyzed.

Immunocytochemistry (Papers I and III)

Immunocytochemistry is a method where you fix cells and incubate them with antibodies against antigen of your interest followed by addition of a fluorescent labeled secondary antibody. By using a fluorescence microscope the sub-cellular localization of the antigen is determined.

Cells were fixed in 4% paraformaldehyde for 20 min at room temperature. Then cells were incubated in TBS-based blocking buffer containing 0.1% Triton X-100, 3% bovine serum albumin (BSA), and 3% normal horse serum, for 1 h at room temperature. Thereafter, cells were incubated with primary antibodies diluted in blocking buffer overnight at 4°C. After three washes with TBS, cells were incubated with Alexa Fluor-488 or Alexa Fluor-594 (Molecular Probes), for 2-3 hours at room temperature. For nuclear staining, cells were incubated 30 min with 1 µg/ml Hoechst.

Table 4

Primer	Region	Species	Forward Sequence	Reverse Sequence	Application	Paper
PDGFRB	Promoter	M	5'-GGGAGGGAGCAGGAG GGAAAGGAG-3'	5'-GAATCAGGGGAATGGA GAGGGTGC-3'	ChIP	I, II, III
PDGFRB	Promoter	M	5'-CCTCAGGTAGTCATGG TCTC-3'	5'-TGCCAGACCACAGGAT AATG-3'	ChIP	I
PDGFRB	Coding	M	5'-AAACACACCTTCTTGCA GCGACACTCC-3'	5'-GTAGCTGAGCACTGGT GAGTCCTTGAT-3'	RT-PCR	II, III
Actin	Coding	H/M	5'-AAGATGACCCAGATCA TGTTTGAG-3'	5'-AGGAGGAGCAATGATC TTGATCTT-3'	RT-PCR	II, III, IV
p73	Coding	M	5'-CAGCAGCAGCTCCTAC AGAGG-3'	5'-CGGTACTGGTCAGGGA CCTTC-3'	RT-PCR	III
PDGFRB	Promoter	H	5'-AGCATCCCCTCACATC CTGAGCGA-3'	5'-CAGGAGCTCACACCAC TATGGGCT-3'	ChIP	III, IV
PDGFRB	Coding	H	5'-TCAACGTCTCTGTGAA CGCAGTGC-3'	5'-GCCCAGGGTGCGGTTG TCTTTGAA-3'	RT-PCR	III, IV
p73	Coding	H	5'-CCGGGAGAACTTTGAG ATCC-3'	5'-ATCTTCAGGGCCCCCA GGTC-3'	RT-PCR	IV
ΔNp73	Coding	H	5'-CGCCTACCATGCTGTA CGTCA-3'	5'-GTGCTGGACTGCTGGA AAGT-3'	RT-PCR	IV
p21	Coding	H	5'-ATGAAATTCACCCCCT TTCC-3'	5'-CCCTAGGCTGTGCTCA CTTC-3'	RT-PCR	IV
GAPDH	Coding	H	5'-ACCTGACCTGCCGT CT AGAA-3'	5'-TCCACCACCCTGTTGC TGTA-3'	RT-PCR	IV

Reverse transcriptase PCR (Papers II- IV)

Reverse transcriptase polymerase chain reaction (RT-PCR) is a method to amplify, detect and semi-quantitatively determine specific RNA-transcripts using reverse transcription of RNA to cDNA and subsequent PCR with primers to amplify the cDNA.

Total RNA was extracted either by RNeasy Mini kit (Qiagen) according to the manufacturer's instruction or using a protocol based on the RNA isolation by guanidinium thiocyanate-phenol-chloroform extraction of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Cultured cells were incubate on ice with a denaturing buffer containing 4 M guanidium thiocyanate, 0.1 M β-mercaptoethanol, 25 mM sodium citrate, and 0.5% sarcosyl. NaAc was added to samples and vortexed, followed by phenol and chloroform-isoamylalcohol (24:1) and subsequently vortexed between each addition. After centrifugation the upper phase containing total RNA was transferred to a new tube and isopropanol was added to precipitate the RNA. Following ethanol wash the RNA pellet was dissolved in RNase free water and spectrophotometrically quantified. An amount of 1-3 µg of RNA was then reverse transcribed using either Moloney murine leukaemia virus (MMLV) reverse transcriptase (Invitrogen) or SuperScriptIII reverse transcriptase (Invitrogen). PCR was performed by using Taq polymerase (Fermentas) with primers (Table 4) against specific RNA transcripts. Depending on the length of amplified transcript, samples were analyzed on a 1-2% TAE agarose gels containing ethidium bromide and scanned using a FLA-2000 plate reader (Fujifilm). β-Actin was used as a control housekeeping gene, allowing semi-quantitative evaluation of the RNA-transcripts.

Immunoblotting (Papers I-IV)

Immunoblotting, also known as Western Blotting is a technique to detect the presence, relative amount and molecular weight of specific antigens through separation of proteins on SDS polyacrylamid gel then transferring them to nitrocellulose membranes to be detected by specific antibodies.

Cultured cells were washed in PBS and incubated with lysis buffer (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100) or lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol), and protease inhibitors and reductants i.e., PMSF, aprotinin and DTT and shaken on ice for 15 minutes. Lysates were sonicated (VibraCell) 12 times with 0.5 second pulses at 80% power to ensure complete lysis of cells. To remove cell debris, lysates were centrifuged for 10 min at 4°C at 15000 rpm. The protein concentration of lysates was determined by the Bradford protein assay (Bio-Rad). Depending on the molecular weight of the protein, samples were separated either on an 8%, 10% or 12% SDS-PAGE and transferred onto a Hybond-C extra PVDF membrane (AmershamBiosciences). The membrane was blocked overnight with 5% skim milk in TBS-T at 4°C to reduce unspecific binding of the primary antibody. Primary antibodies (Table 5) were diluted in 5% skim milk and incubated with the membrane for 1 hour at room temperature. After 3 x 5 min of TBS-T washes, the membrane was incubated in anti-mouse, anti-rabbit or anti-goat IgG secondary antibody diluted in 5% skim milk for 1 h. After subsequent TBS-T washing, the membrane was developed using the enhanced chemiluminescence (ECL) Advance system (GE Healthcare) and scanned using LAS-1000 Plus (Fujifilm).

Immunoprecipitation (Papers I and III)

Immunoprecipitation is a method to separate and enrich proteins of interest by using specific antibodies against an antigen and pull down the antibody-antigen complex with agarose beads. Immunoprecipitated proteins are separated on an SDS-PAGE and blotted to a PVDF membrane and immunoblotted with antibodies against the antigen. Co-immunoprecipitation, studying proteins interactions, uses the same principles as immunoprecipitation but after blotting to a PVDF membrane it is immunobloted against antigens known or thought to interact with the immunoprecipitated antigen.

Cells were lysed either in a lysis buffer containing 25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, 1% Triton X-100 or a lysis buffer containing 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 120 mM NaCl, 0.5% Nonidet P-40, 10% glycerol. If cells were transfected with expression vectors, they were lysed 48 h after transfection and a part of the supernatant was used for expression control of transfected plasmids. The soluble supernatants were mixed with an antibody (Table 5), and incubated for 2 h at 4°C. Protein G-Sepharose beads were added and incubated for 1 h at 4°C. The samples were washed with the lysis buffer three times and the bound proteins were eluted by boiling in the SDS-sample buffer. Immunoblotting was performed as described above.

Table 5

Antibody	Company	Species	Application	Paper
p300 (585X)	Santa Cruz	Rabbit	ChIP	I
Anti-acetyl Lysine	Upstate	Rabbit	WB (1:1000)	I
HA (3F10)	Roche	Rat	WB (1:500)	I
HA (F7)	Santa Cruz	Rabbit	IP	I
Flag (M2)	Roche	Rabbit	WB (1:1000), IP	I
HDAC1 (H-3284)	Sigma	Rabbit	ChIP	I, III
NF-YB (FL-207X)	Santa Cruz	Rabbit	ChIP	I, II, III
p73 (H79)	Santa Cruz	Rabbit	WB (1:1000), ChIP	I, III, IV
SV40 T (PAb-108)	Santa Cruz	Rabbit	WB (1:1000), ChIP	II
Sp1 (1C6)	Santa Cruz	Mouse	WB (1:1000)	II
c-Myc (9E10)	Santa Cruz	Mouse	WB (1:1000), ChIP	II, III
PDGFRB (958)	Santa Cruz	Rabbit	WB (1:1000)	II, III, IV
p300 (N-15)	Santa Cruz	Rabbit	ChIP	III, IV
Actin (AC-40)	Sigma	Mouse	WB (1:1000)	II, III, IV
p53 (DO-1)	Santa Cruz	Mouse	WB, IF (1:1000), ChIP	II, III, IV
p53 (FL-393)	Santa Cruz	Rabbit	WB, IF (1:1000), ChIP	III
p53 (PAb-421)	Calbiochem	Rabbit	EMSA, IF (1:1000)	III
Anti-acetyl Lys-373-p53	Upstate	Rabbit	WB (1:1000), ChIP	III
Anti-methyl Lys-370-p53	Upstate	Rabbit	ChIP	III
p-PDGFRB (Tyr1021)	Santa Cruz	Rabbit	WB (1:1000)	III
Sp1 (PEP2)	Santa Cruz	Rabbit	ChIP	III
p73 (E4)	Santa Cruz	Mouse	WB (1:1000), ChIP	III, IV
GAPDH (6C5)	Chemicon	Mouse	WB (1:5000)	III, IV
HDAC4 (H-92)	Santa Cruz	Rabbit	ChIP	III, IV
ΔNp73	SigmaGenosis	Rabbit	WB (1:1000), ChIP	IV

In vitro translation (Paper I)

In vitro translation is a method where, in the test tube, one combines DNA template with a mixture of RNA polymerase and amino acids together with the translational machinery from a cell lysate to produce a desired protein.

Proteins were made by using a coupled transcription and translation (TNT) system (Promega) according to the manufacturer's instructions. An amount of 0.5 µg DNA was added directly to 20 µl of TNT rabbit reticulocyte lysate with 1 µl of [^{35}S] methionine and reactions were carried out at 30°C for 90 min.

In vitro binding assay (Paper I)

In vitro binding assay is used to detect protein-protein interactions. One of protein is *in vitro* translated and labeled with [^{35}S] methionine. The other interaction partner is GST-tagged, expressed in *E.coli* and pulled down with gluthathione-sepharose. The proteins are mixed and bound proteins are eluted and samples separated on a gel.

GST-HDAC were incubated with 30 µl gluthathione-sepharose (Pharmacia) in 800 µl of a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, 0.5% NP-40, 10% glycerol and 1 mM PMSF. After 1 h incubation, the beads were washed three times with binding buffer and incubated with *in vitro* translated HA-p73α. After incubation for 2 h at 4°C, beads were washed 3 times with binding buffer, and analyzed by SDS-PAGE. The gels were dried and analyzed by autoradiography on X-ray film using FLA-2000 (Fujifilm).

EMSA (Paper III)

EMSA is an *in vitro* method to study the interaction of proteins to DNA. A labeled DNA fragment is either incubated with an *in vitro* translated protein or a nuclear extract and run on a non-denaturing-PAGE. If the *in vitro* translated protein binds the DNA a slower migrating band can be detected as compared to the unbound DNA. When incubating with a nuclear extract, antibodies are added to the mixture. If the antigen binds DNA the antibody will make a complex that migrates slower and the band representing the DNA-protein complex will be reduced in strength as compared to controls.

EMSA were performed by using a double-stranded synthetic oligonucleotide labeled with [^{32}P]-ATP (DuPont NEN) using polynucleotide kinase (BoehringerMannheim), and separated by a G-25 column (AmershamBiosciences). Either *in vitro* translated proteins, using TNT System (Promega), or nuclear extracts were mixed with the labeled DNA. Supershifts were made by adding antibodies. Mixtures were loaded and analyzed by non-denaturing-PAGE. The gels were dried and analyzed by autoradiography on X-ray film using FLA-2000 (Fujifilm).

Chromatin immunoprecipitation (Papers I-IV)

Chromatin immunoprecipitation (ChIP) assay is a method to study more of an *in vivo* binding situation, compared to EMSA, of specific proteins to gene regulatory regions such as promoters and enhancers. This is achieved by crosslinking DNA and proteins by formaldehyde fixation. The crosslinked DNA is sonicated to sizes between 300-1000 base pairs and immunoprecipitated

using a specific antibody (Table 5) against the protein assumed to bind the regulatory region. The crosslinking is then reversed and proteins and RNA are degraded while the DNA is purified using phenol chloroform extraction. The DNA is amplified with PCR using primers (Table 4) against the regulatory region of interest and if the antigen binds the region a band will be detected when run on an agarose gel.

Cells were cultured in 10-cm dishes to almost confluence. Protein and DNA were cross-linked by incubating cells with 1% formaldehyde for 10 min at 37°C. Cells were lysed in Buffer X (50 mM Tris-HCl at pH 8, 1 mM EDTA, 120 mM NaCl, 0.5% NP-40, 10% glycerol and 1 mM PMSF) or RIPA buffer. The lysate was sonicated and soluble chromatin was pre-cleared by addition of Protein A-sepharose. An aliquot of the chromatin was frozen at -80°C and used in subsequent PCR analysis. The remainder of the chromatin was diluted with Buffer X or RIPA buffer and incubated with 2-3 µg of antibody or mouse IgG or normal rabbit serum. Immunocomplexes were collected by incubation with Protein G-agarose (Santa Cruz). In Re-ChIP experiments, following the first immunoprecipitation the precipitates were extensively washed with a low pH buffer, and immunoprecipitated with another antibody. Immunoprecipitates were washed once with Buffer X or RIPA buffer, high salt Buffer X (500 mM NaCl) or High Salt Ripa Buffer and LiCl buffer (10 mM Tris, 1 mM EDTA, 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, pH 8.1) and twice with TE (10 mM Tris, 1 mM EDTA, pH 8.0). Immunocomplexes were eluted twice with elution buffer (0.1 M $NaHCO_3$, 1% SDS). To reverse crosslinking, eluted samples were incubated with 0.2 M NaCl for 4 h at 65°C. Samples were digested with Proteinase K (0.04 mg/ml) for 2 h at 45°C and then with RNase A (0.02 mg/ml) for 30 min at 37°C. DNA was purified with phenol:chloroform extraction followed by ethanol precipitation and resuspended in H_2O. Aliquots of 2 µl serial dilutions were analyzed by PCR with the appropriate primer pairs. Amplification was performed for an optimal number of cycles. PCR products were separated by electrophoresis on 2% agarose gels and were stained with ethidium bromide.

Receptor Binding Assay (Paper II)

Receptor binding assays can be used to estimate the relative amounts of functional receptor in a cell line under different conditions. A cold ligand is used to see its ability to compete with [^{125}I]-labeled ligands for binding to the cells. Cells were grown on 24-well plates (Becton Dickinson). Cell cultures were washed once in binding buffer containing PBS, 1 mg/ml BSA, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$ followed by incubation at 0°C for 2 h in 200 ml binding buffer containing various dilutions of ligands. The cells were washed in binding buffer before addition of the labeled ligand (0.5-2 ng containing 15000-30000 cpm). After incubation at 0°C for 1 h, the cells were washed with binding buffer, and then lysed in 200 ml of 20 mM Tris-HCl, pH 7.5, 1% Triton X-100 and 10% glycerol at room temperature for 20 min. The amount of solubilised [^{125}I] radioactivity was measured in a gamma-counter.

Results

Paper I - p73 competes with co-activators and recruits histone deacetylase to NF-Y in the repression of PDGF β-receptor

p73 is a member of the p53 tumor suppressor family and similar to p53 in DNA-binding and transcriptional activation of p53-responsive genes. We have previously shown that serum-stimulation induces p73α which leads to downregulation of PDGFRB expression by transcriptional repression (Hackzell et al., 2002). In that report, the C-terminal SAM domain of p73α was shown to bind NF-YB and NF-YC, thereby interfering with the NF-Y-mediated transactivation of the PDGFRB promoter. The aim of this study was to elucidate the mechanism for p73α repression of NF-Y activity on the PDGFRB promoter.

Since several studies have demonstrated that NF-Y activation is dependent on the presence of p300 and P/CAF (Currie, 1998; Faniello et al., 1999), we first investigated the importance of acetylation for PDGFRB promoter activity. Treatment with the histone deacetylase inhibitor TSA enhanced the PDGFRB promoter activity even above control levels. This confirmed the presence of endogenous deacetylase activity on the PDGFRB promoter. Furthermore, transfection of p300 significantly increased the promoter activity which suggested the presence of endogenous acetylation targets on the PDGFRB promoter. Our finding that both TSA treatment and p300 transfection were dependent on an intact NF-Y binding site indicated that NF-Y was the target of acetylation.

In protein interaction studies, we confirmed the previous findings that NF-YB and NF-YC bound P/CAF and that NF-YB also bound p300 (Currie, 1998; Li et al., 1998). Additionally, we found that p300 acetylated NF-YC, an acetylation which in turn was reduced by overexpression of p73α. This was in accordance with our findings that overexpression of p300 and P/CAF reduced p73α-mediated repression of PDGFRB promoter activity, and that overexpression of p73α reduced p300 or P/CAF mediated activation of PDGFRB promoter activity. However, the exact mechanism of this antagonistic effect of p300 and p73α was unknown.

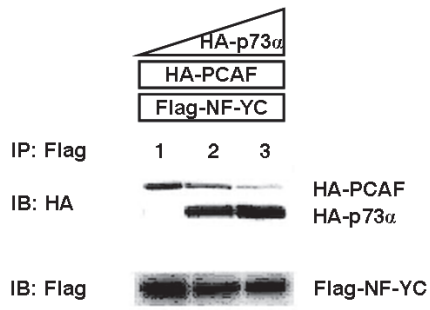

Figure 14. p73 interferes with PCAF binding to NF-Y.

Since both p300 and p73α can bind NF-Y, we investigated the role of protein interactions on the PDGFRB promoter. Overexpression of p73α was shown to compete out the binding of HATs to NF-YB and NF-YC thus providing an explanation into the mechanism of the observed antagonistic effect of p300 and p73α (figure 14). However, this did not explain the strong repression of p73 on PDGFRB promoter activity and the effect of TSA. Our *in vitro* binding assays showed that p73α bound HDAC1. This indicated that there were other mechanisms than competition with co-activators to repress the PDGFRB promoter.

ChIP assays confirmed the *in vivo* binding of p73α to the PDGFRB promoter, a binding that decreased upon TSA treatment. The recruitment of p73α and ΔNp73 to the PDGFRB promoter was shown to occur in accordance with PDGFRB expression. p73α and HDAC1 were bound when PDGFRB expression was repressed, and ΔNp73 and p300 were bound when expression was increased. Immunofluorescence staining supported this finding with the strongest nuclear co-localization of p73α and HDAC1 when PDGFRB expression was repressed. In conclusion, our results show that histone acetyltransferases and histone deacetylases are crucially involved in p73α-mediated repression on the PDGFRB promoter (figure 15).

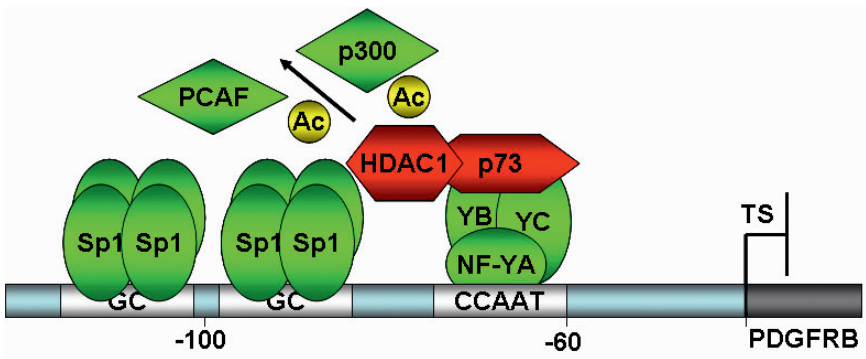

Figure 15. Illustration of p73 interference with HAT binding to NF-Y.

Paper II - pRb, Myc and p53 are critically involved in SV40 large T antigen repression of PDGF β-receptor transcription

LT is the only viral protein essential for replication of the polyoma virus SV40. Following infection, LT affects the host genome and growth control by binding to a wide variety of transcription factors that are important for both replication and cell cycle regulation including p53, retinoblastoma family proteins and other tumor suppressors (Moens et al., 1997). Previously it has been shown that the LT and small t antigen of SV40 downregulated the expression of PDGF receptors in fibroblasts (Wang et al., 1996). The aim of this study was to clarify the mechanism that LT uses to repress PDGFRB expression.

Overexpression of LT in NIH3T3 resulted in decreased expression of PDGFRB protein and mRNA, confirming previous findings (Wang et al., 1996). In further support, LT was found to induce repression of PDGFRB promoter activity. Since the LT-mediated downregulation of the PDGFRB was shown to arise from the effect on the transcriptional activity, we investigated the role of NF-Y for this regulation. Co-expression of LT with dominant negative NF-YA showed only half as effective repression of PDGFRB promoter activity as compared to transfection of LT alone. This demonstrated the importance of NF-Y, although it may not be indispensible for LT repression on PDGFRB promoter activity. Moreover, overexpression of LT in the c-Myc$^{-/-}$ HO15.19 fibroblast cell line did not alter the expression level of PDGFRB. Instead of repression there was an induction of the promoter activity, indicating that c-Myc is involved in LT-induced repression of PDGF β-receptor expression.

In order to clarify whether the major targets of LT, pRb and p53, were directly involved in the LT-induced repression of the PDGFRB, we studied PDGFRB promoter activity in Saos2 cell line lacking p53 and pRb. LT mutants that cannot bind p53 or pRb were also tested for their ability for the repression (figure 16). We found that the pRb-binding mutant LT, K1 or C105G, caused more than a two-fold increase of PDGFRB promoter activity while the LT p53 binding mutant, Δ434-444, and the pRb-binding mutant H42Q, did not affect the promoter activity. Accordingly, LT could not alter the PDGFRB promoter activity in p53$^{-/-}$ pRb$^{-/-}$ Saos-2 cells or Rb$^{-/-}$ 3T3 cells. These findings suggested that the binding of LT to both pRb and p53 plays an important role in the repression of PDGFRB promoter activity.

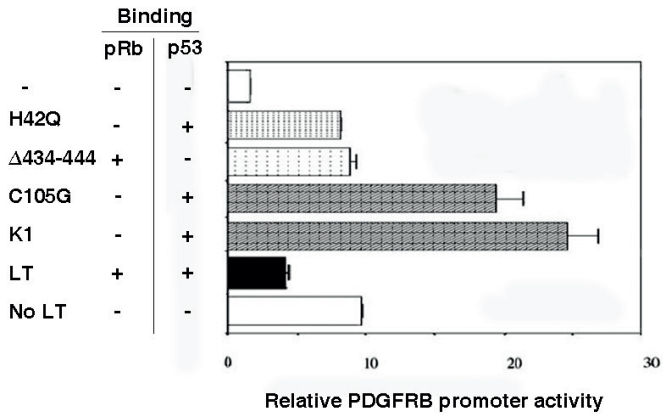

Figure 16. Binding of both p53 and pRb is necessary for LT repression on PDGFRB promoter activity.

Furthermore, $Rb^{-/-}$ 3T3 cells had high expression of the PDGFRB, and in contrast to NIH3T3 no decrease was seen in response to serum-stimulation of serum-starved cells. Also, c-Myc expression increased after serum-stimulation in both cell lines but was followed by a much slower decrease compared to the rapid decrease seen in normal 3T3 cells. This suggests that downregulation of the PDGFRB, as well as that of c-Myc, is impaired in the absence of pRb.

Overexpression of p53 was shown to increase the PDGFRB promoter activity through the NF-Y and Sp1 binding motifs. Addition of p53 yielded an additive effect with NF-Y but a synergistic effect with Sp1 on the promoter activation. All these factors together brought about a significant activation due to the synergistic effect between Sp1 and NF-Y and the synergistic effect between p53 and Sp1. It is thus likely that p53 activates the promoter, mainly through the Sp1-binding site.

In order to see the *in vivo* binding of LT and p53 to the PDGFRB promoter we performed ChIP assays and found that LT bound the PDGFRB promoter *in vivo* in ST15A cells at 33°C when LT was expressed in the cells. Binding of p53 to the promoter was stronger when LT was not expressed. Furthermore, LT was found to bind the proximal promoter in Saos-2-LT cells, suggesting that LT can bind the promoter even in the absence of pRb and p53. In conclusion, LT affects the PDGFRB promoter by interfering with the activation by NF-Y and Sp1, through a mechanism involving c-Myc, pRb and p53 (figure 17).

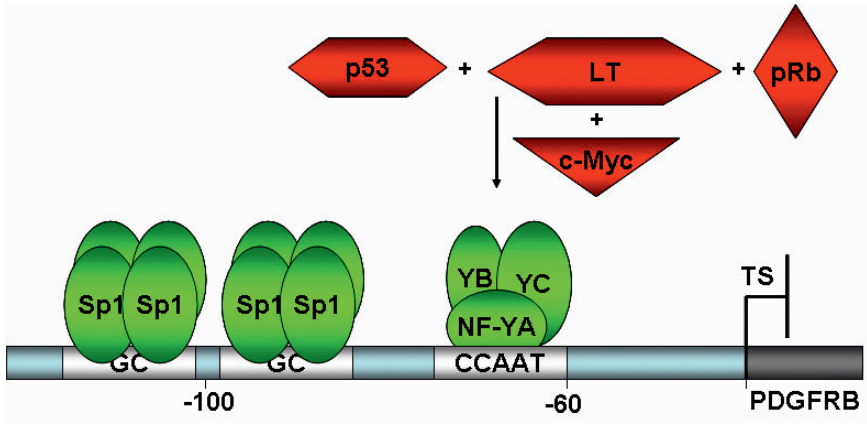

Figure 17. Illustration of LT-mediated repression of PDGFRB expression.

Paper III - Kinetics of repression by modified p53 on the PDGF β-receptor promoter

The tumor suppressor p53 plays an important role to induce cell cycle arrest and apoptosis. It is modified by posttranslational modifications such as phosphorylation of the N-terminus and acetylation of the C-terminus, both of which affect stabilization, activation, and association to target gene promoters. The C-terminal region of p53 associates with NF-Y at the HFM, and this interaction plays a key role for repression of G2/M cell cycle genes (Imbriano et al., 2005). In our previous study, ChIP assays revealed that p53 bound the PDGFRB promoter but no repressive effect of p53 was seen (Paper II). Thus we have examined the mechanism and effect of p53 on PDGFRB.

Overexpression of p53 in MEF, $p53^{-/-}$ MEF, or Saos2 induced a clear repression of PDGFRB promoter activity and decreased its mRNA and protein expression in MEF and $p53^{-/-}$ MEF (figure 18). Our findings here were in contrast to our previous finding where p53 upregulated the PDGFRB promoter activity through Sp1 in NIH3T3 cells. This indicated that the cellular context is of importance for the actions of p53. Activating endogenous p53 by MMC treatment downregulated PDGFRB expression at both mRNA and protein levels in MEF, which was however seen even in $p53^{-/-}$MEF. The decrease observed in $p53^{-/-}$ MEF could be explained by the increased p73 expression. Silencing of p73 in $p53^{-/-}$ MEF indeed confirmed this hypothesis since almost no downregulation of PDGFRB expression was observed.

Since the region surrounding the CCAAT-motif has a sequence similar to the p53 consensus binding sequence, we did EMSA and showed that this PDGFRB promoter region could bind p53. Re-ChIP assays were performed and Sp1, NF-Y, and p53 were found bound to the same region of the PDGFRB promoter. It has previously been reported that p53 directly binds NF-Y, and for this reason it is likely that they bind the PDGFRB promoter as a complex, as reported for G2/M promoters (Imbriano et al., 2005).

In order to study the kinetics of PDGFRB expression upon p53 expression we used Tet-On-p53 Saos2 cells. Gradual increase of p53 expression initially upregulated the total and phosphorylated PDGFRB, which later became undetectable. The same kinetics was seen for PDGFRB promoter activity. To explain the dynamic changes in expression we performed ChIP assays and showed that p53 bound the promoter upon p53 induction. p73 was not found at the promoter when p53 was induced. Moreover, the co-repressor HDAC1 bound to, and the co-activator p300 was dismissed from, the PDGFRB promoter when PDGFRB expression was repressed.

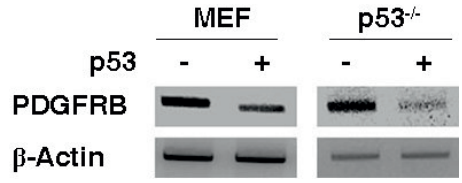

Figure 18. PDGFRB mRNA expression is downregulated after transfection with p53.

Regarding the role of posttranslational modifications of p53 for its actions of PDGFRB, promoter studies in MEF cells showed that the acetylation of the C-terminal lysines 370/372/373 was more important than that of 320 for the p53 effect on PDGFRB promoter activity. Also, K373R, unable to become acetylated, reduced p53 repression of the PDGFRB promoter. Chip assays were performed on MEF and on Tet-On-p53 Saos2 when p53 was activated by MMC. Binding of acetyl-K373-p53 and methyl-K370-p53 was observed following MMC treatment. In accordance with the strong repression on this promoter by the endogenously activated p53, both HDAC1 and HDAC4 bound the promoter following MMC-treatment in MEF cells. In Tet-On-p53 Saos2, the dynamic binding of HDAC4 was correlated with PDGFRB expression levels. These binding kinetics suggest that p53 determines the direction of its action by recruitment of corepressors and coactivators.

Moreover, immunofluorescence staining of Tet-On-p53 Saos2 showed that the frequency of nuclear p53 positive cells increased upon p53 induction. Nuclear NF-YB was observed only when the membranous expression of phospho-PDGFRB and cytoplasmic PDGFRB was increased. Also, immunofluorescence staining confirmed a strong nuclear accumulation of acetyl-K373-p53 upon p53 induction. Acetyl-K373-p53 localized in promyelocytic leukemia nuclear bodies (PML-NB) and MMC treatment increased the co-localization of acetyl-K373-p53 and PML.

With the purpose of understanding the biological consequence of PDGFRB downregulation we examined whether p53-induced apoptosis can be affected by PDGFRB inhibitors. Blockage of PDGFR signaling with the inhibitor STI-571 increased the fraction of apoptotic MMC-treated cells. In conclusion, p53 can bind and repress the PDGFRB promoter and epigenetic modifications of p53 affect its effects on the promoter (figure 19).

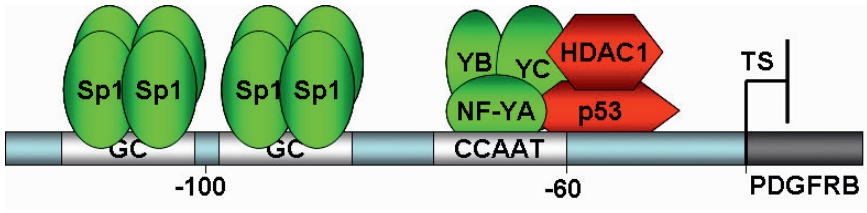

Figure 19. Illustration of p53 repression of PDGFRB transcription.

Paper IV - Dysregulation of PDGF β-receptor expression by ΔNp73 in neuroblastoma

ΔNp73 is a variant of p73 that lacks the N-terminal TAD and acts dominant negatively to the p53 and p73 tumor suppressors by competing with them for DNA-binding and oligomerization (Nakagawa et al., 2002; Zaika et al., 2002). A functional role of ΔNp73 in cancer progression is indicated by its expression in many human tumors including the most common solid tumor of early childhood, neuroblastoma, where ΔNp73 expression is often associated with poor outcome. We have previously characterized the transcriptional regulation of the PDGFRB by p53, p73 and ΔNp73 (Hackzell et al., 2002, Papers I-III). Dynamic interactions of p73 and ΔNp73 to the PDGFRB promoter were shown to be important for this regulation, where p73 mediated its downregulation and ΔNp73 its upregulation. In this study we examine whether the status of ΔNp73 and other p53-family members can explain the defective regulation of PDGFRB found in certain cancer.

We hypothesized that the high expression of PDGFRB in neuroblastoma might be due to dysfunctional p53 family proteins. Testing this assumption, serum-stimulation was used to induce and/or activate p53 family proteins in order to detect changes in PDGFRB expression in IMR-32 and SH-SY5Y neuroblastoma cell lines. Serum-stimulation of serum-starved SH-SY5Y cells resulted in an expected rapid decrease of PDGFRB protein and mRNA expression. However, in IMR-32 no such decrease was seen (figure 20). Overall, these results pointed to differences in the transcriptional regulation of PDGFRB. Endogenous expression of the transcriptional regulators of PDGFRB; p53, p73, and ΔNp73 and their response to serum-stimulation was seen not to differ between these two cell lines and FACS confirmed that serum-stimulation induced both cell lines to leave the G1-cell cycle phase.

Since the expression of p53 family members did not differ in the cell lines, we investigated the ability of exogenous p53, p73α, and ΔNp73α to affect PDGFRB transcription and found similar effects in both cell lines. p53 and p73α repressed and ΔNp73α increased PDGFRB promoter activity. However, SH-SY5Y responded more strongly to p53 and p73α and more weakly to ΔNp73α than IMR-32. This prompted us to examine the role of endogenous p53-family members in their ability to regulate PDGFRB transcription. We found that silencing of ΔNp73 reduced PDGFRB promoter activity and protein expression in IMR-32 but not SH-SY5Y, indicating a role of endogenous ΔNp73 in regulation of PDGFRB promoter activity in IMR-32.

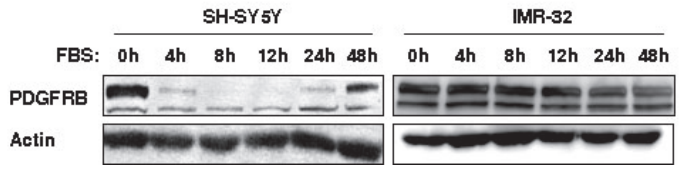

Figure 20. Serum-stimulation of serum-starved cells decreases PDGFRB protein expression in SH-SY5Y but not in IMR-32.

Moreover, ChIP assays were made for SH-SY5Y and IMR-32, using p53, p73, ΔNp73 and p300 antibodies to determine their *in vivo* recruitment to the PDGFRB promoter before and after serum addition. In SH-SY5Y, ΔNp73 was only found bound to the promoter in serum-starved cells. In contrast, in IMR-32, ΔNp73 was found bound both before and after serum-stimulation. Serum-stimulation induced binding of both p53 and p73 to the promoter in both cell lines. This supported our hypothesis that the PDGFRB promoter is continuously controlled by ΔNp73 in IMR-32 and that binding of p53 and/or p73 was not sufficient to downregulate PDGFRB protein expression.

In order to study the effect of posttranslational modifications of p53 and p73, we used cisplatin, a well-known anti-cancer drug that induces cell cycle arrest in G2/M or apoptosis through stabilization and activation of p53 and p73. Both IMR-32 and SH-SY5Y cells treated with cisplatin showed increased levels of p53, Ser-15 phosphorylation of p53, and induction of p21 mRNA. This verified that either p53 or p73 was functional. Interestingly, PDGFRB promoter activity and its protein and mRNA expression decreased in both cell lines with cisplatin treatment. This indicated that posttranslational modifications of p53 and p73 could block the control exerted by ΔNp73 on PDGFRB promoter activity. In support, silencing of ΔNp73 together with cisplatin treatment increased the repression of PDGFRB promoter activity. To see the endogenous recruitment of p53-family members to the PDGFRB promoter upon cisplatin treatment, we performed ChIP assays and found that ΔNp73 and p300 decreased their binding to the PDGFRB promoter and HDAC4 was recruited. This showed that ΔNp73 could be removed from the PDGFRB promoter upon cisplatin treatment thereby relieving the promoter from the effect of ΔNp73. In conclusion, results in this report implicate ΔNp73 binding to the PDGFRB promoter as a determinant of dysregulated PDGFRB expression and that posttranslational modifications of p53 and p73 can help restore the regulation (figure 21).

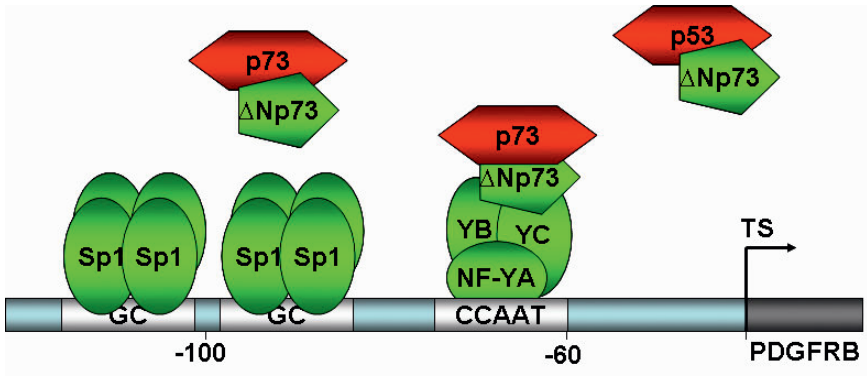

Figure 21. Possible actions of ΔNp73 in regulation of PDGFRB expression.

Discussion

Transcriptional regulation of PDGFRB expression

Since PDGF signaling is functionally involved in many diseases such as atherosclerosis, chronic inflammation, and cancer, it is important to understand how the expression of PDGF and its receptors is down-regulated to maintain normal growth control. A large body of evidence has already been accumulated regarding the importance of ligand-induced internalization and degradation of the receptors as regulatory mechanisms in response to PDGF signaling. However, the importance of transcriptional regulation as a negative feedback mechanism has not been addressed in the same degree. It is particularly important to understand the underlying regulatory mechanisms in PDGF-driven diseases where PDGFR expression is enhanced or dysregulated.

The focus of this thesis has been to clarify the transcriptional regulation of PDGFRB by p53 family members since both PDGFRB and p53 family members are intimately involved in cancer and development. In Papers I-IV, p53, p73 and ΔNp73, as well as the p53-interacting viral large T antigen were investigated for their roles in PDGFRB regulation. Our findings point to a direct role for p53 family members in PDGFRB downregulation in response to serum stimulation and DNA damage as well as a role for ΔNp73 in dysregulated PDGFRB expression in neuroblastoma.

It is known that p73 downregulates PDGFRB (Hackzell et al., 2002), but the mechanism remains to be clarified. In Paper I, we have provided insight into the mechanism of transcriptional repression of PDGFRB by p73. It seems clear that p73 in order to repress PDGFRB transcription competes with HATs for binding to NF-Y and recruits HDACs. Since PDGFRB promoter activity is upregulated by ΔNp73 the repressive effect of p73 likely depends on its N-terminal TAD. The fact that ΔNp73 is unable to bind HDACs while theoretically capable of binding to NF-Y, explains its inability of repression, but it does not explain its upregulation of PDGFRB promoter activity. One explanation for this could be that ΔNp73 allows simultaneous binding with p300 and P/CAF to NF-Y in order to upregulate PDGFRB expression (figure 22), as supported by our ChIP experiments. Furthermore, we have later determined that p53 binds the PDGFRB promoter, as described in Paper III. Since p73 can bind the same consensus sequence as p53 it is possible that ΔNp73 might compete with p53 and p73 for DNA binding. ΔNp73 might also bind to DNA as a heterotetramer with p53 or p73, to disable the effect of the full length members.

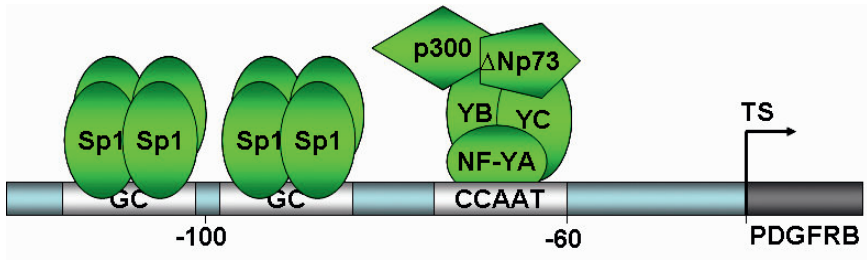

Figure 22. Possible mechanism of ΔNp73 on the PDGFRB regulation.

Viral oncogenes are important for cancer initiation and progression of many tumors (Dayaram and Marriott, 2008). The LT of SV40 interacts with and affects a large number of genes in the host cell. For PDGFRB, as seen in Paper II, the effect of LT is downregulation. It is difficult to envision why LT would need to downregulate PDGFRB, but in addition this was also seen for PDGFRA in certain fibroblasts (Wang et al., 1996). One would imagine that the induction of a competence factor would be beneficial for the invading virus in its efforts to transcribe its genome but this seems not to be the case.

Moreover, the complex dependence on c-Myc, pRb and p53 for LT downregulation of PDGFRB require a complex explanation. It is possible that the architecture of the PDGFRB promoter enables LT to interact with c-Myc bound to NF-Y, and p53 bound to Sp1. This, in turn, blocks access of p53 to p300 while the LT bound pRb might recruit HDACs to repress transcription (figure 23). The insect SL2 cells that lack NF-Y and Sp1 are often used in transcriptional studies to test the role of these factors. However, this system could be too simplified for studying mammalian cells, and the results drawn might depend on a lack of some other important factors as well. Nevertheless, in SL2 and NIH3T3, p53 induces PDGFRB promoter activity.

Since we had diverging results concerning the effect of p53 on transcriptional regulation of the PDGFRB (Papers II and III), we devoted our studies in Paper III to the effects of exogenous overexpression and endogenous activation of p53. There we show that p53 downregulates the expression of PDGFRB both at protein and mRNA levels in MEF cells. This effect is even greater in p53-negative cell lines as seen for $p53^{-/-}$ MEF, Saos2 and H1299 cells (unpublished observations). Interestingly, MMC, well known to stabilize and activate p53 (Fang et al., 1999), but not p73, is in our study able to stabilize p73 in $p53^{-/-}$ MEF. The finding that p73 is downregulated in MEF cells upon MMC treatment is in accordance with previous findings. However, in contrast to this previous report, our results in $p53^{-/-}$ MEF show that in the absence of p53, p73 can in fact become activated to repress target genes of p53. This explains why downregulation of PDGFRB expression is seen in $p53^{-/-}$ cells upon MMC treatment.

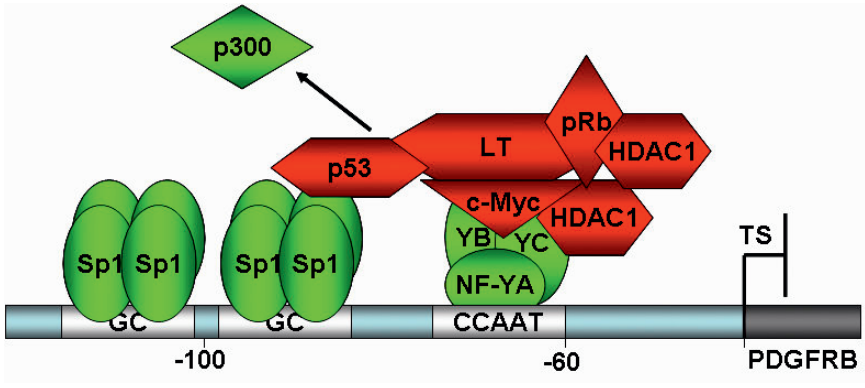

Figure 23. Possible mechanism of LT mediated downregulation of PDGFRB.

Both p73 and ΔNp73 have been suggested to have a role in neuroblastoma since the chromosomal region in which the p73 gene lies often is lost and overexpression of ΔNp73 is correlated with poor prognosis. Since PDGFRB regulation is disturbed in some neuroblastoma cell lines we wanted to investigate whether this was due to dysregulation by p53 family members. In Paper IV, two neuroblastoma cell lines were investigated as examples of regulated PDGFRB expression (SH-SY5Y) and dysregulated PDGFRB expression (IMR-32). The finding that ΔNp73 bound the dysregulated PDGFRB promoter in IMR-32 suggested that ΔNp73 allows binding of p300. In response to serum stimulation in IMR-32, neither p53 nor p73 oligomers were able to compete for binding to NF-Y, however, it is possible that they bind as heterooligomers with ΔNp73, but blocked to repress transcription. This reasoning is supported by the fact that both p53 and p73 binds the promoter upon serum stimulation without displacing ΔNp73 in IMR-32. This could also explain why overexpression of p53 and p73 enables overcoming any effect of endogenous ΔNp73, which cannot completely inactivate p53 and p73.

Three potential binding sites for p53 family members are present on the proximal PDGFRB promoter; NF-Y, Sp1 and the putative p53 binding site which overlaps the CCAAT sequence (figure 24). Of these, we have shown that interaction with NF-Y represses PDGFRB expression through competition with HATs for binding to NF-Y and recruitment of HDACs. Interaction with Sp1 alone, on the other hand, probably induces transcription through the formation of a complex with p300, thereby acting as a bridge over to members of the basal transcription machinery. Direct binding of p53 family members to DNA will either compete with NF-Y for binding or bind as a complex with NF-Y to DNA, to repress transcription.

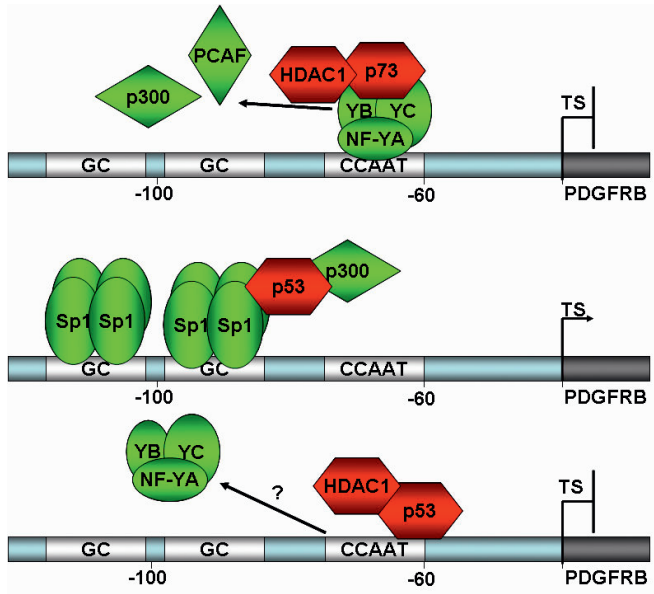

Figure 24. Binding sites for p53 family members on the PDGFRB promoter.

Posttranslational modifications

The role of posttranslational modifications of transcription factors on their activity and stability as well as on target gene association is currently under focus in many studies. Transcriptional regulation of PDGFRB is no exception, where important modifications of transcription factors are acetylation and phosphorylation of NF-Y and p53 family members.

Previous studies have shown that both HATs and NF-Y bind to G1 cell-cycle regulated genes, including PDGFRB (Caretti et al., 2003). Our studies in Paper I confirmed the role of acetylation in transcriptional regulation of the PDGFRB by using the HDAC inhibitor TSA which increased PDGFRB activity and p300 which activated the PDGFRB promoter. In agreement with previous findings (Li et al., 1998), p300 was able to further acetylate NF-YB. Additionally, we showed that p300 acetylated NF-YC. Interestingly, only the acetylation of NF-YC but not NF-YB, containing a very small TAD, could be outcompeted by p73. It is thus likely that NF-YC is the protein most important for transactivation. The acetylation of NF-Y indicates that HATs in addition to being bridging molecules to the basal transcriptional machinery also functions to activate NF-Y. p53 family members also reportedly increase their activity in response to binding of p300.

Differences between Paper II and III about the role of p53 in PDGFRB regulation indicate that the cellular context is important for the effect of p53 on its target genes. In response to DNA damage, p53 family members become activated by posttranslational modifications, including phosphorylation, acetylation and methylation. The level of DNA damage directs whether the cell needs to stop cell cycling to enable DNA repair or induce apoptosis. In response to DNA damage, different posttranslational modifications of p53 family members will target them to specific promoters. Studies have shown that if DNA damage is low, acetylation of Lys-320 by P/CAF directs p53 to initiate cell cycle stop thereby allowing repair to take effect. On the other hand, acetylation of Lys-373 and surrounding lysines of p53 will promote apoptosis (Knights et al., 2006). Whether p53 promotes apoptosis is decided through changes of what target genes p53 associates with, those acetylated at Lys-320 binds to high affinity p53 binding sites while those acetylated on Lys-373 binds low affinity binding sites on promoters of pro-apoptotic genes to activate them. Our results indicate a previously undescribed role for acetylated Lys-373 p53 to downregulate antiapoptotic genes. Only one putative p53 binding sequence was found on the PDGFRB promoter and is thus likely a low affinity p53 binding site. From our studies in Paper III on posttranslational modifications of p53 it seems that C-terminal lysines acetylated by p300 targets p53 to the PDGFRB promoter to downregulate it.

Since ΔNp73 can dysregulate PDGFRB it likely dysregulates the expression of many other genes. Interestingly, cisplatin overcomes dysregulation of PDGFRB expression by ΔNp73. However, it should be noted that cisplatin does not downregulate expression of ΔNp73 (Million et al., 2006). Serum-stimulation might activate p53 and p73 through Src-mediated activation of c-Abl and c-Abl-induced phosphorylation of p53 and p73. This is however not sufficient to enable p53 and p73 to compete with ΔNp73, at least not in IMR-32. Cisplatin, however, greatly affects the posttranslational modification of p53 and p73 with phosphorylation of Ser-15 on p53 leading to an association with p300 and subsequent acetylation of C-terminal lysines. These modifications might, as discussed above, target p53 to the PDGFRB promoter. Furthermore, it is possible that these modified p53 and p73 will not interact with ΔNp73 or be able to better compete for binding to NF-Y thus restoring downregulation of PDGFRB transcription.

Transcriptional regulation of other NF-Y controlled genes by p53 family members

Many cell cycle regulated genes are under the control of NF-Y. In response to DNA damage, some of these genes are downregulated by p53 family members in a NF-Y dependent manner. The mechanism of repression by the p53 family member p73 on NF-Y in regulation of PDGFRB described in Paper I has also been reported for p53 and p63 in their interaction of NF-Y in cell cycle regulated promoters (Imbriano et al., 2005; Testoni and Mantovani, 2006). In these studies it was reported that promoters containing double CCAAT boxes, separated by around 32 bp, were capable of binding p53, but promoters with a single CCAAT box were not. It was proposed that the tetrameric p53 was dependent on multiple interaction points on the promoter. Since the PDGFRB promoter, only contains one CCAAT box, the upstream GC boxes might function similarly to that of an upstream CCAAT box. In fact, since Sp1 itself binds as a tetramer and where every monomer can interact with p53 this would create even more interaction points for p53 than NF-Y thus further supporting this hypothesis.

Moreover, studies by Jung suggested that the mechanism of p53-mediated repression of cell cycle regulated genes was through inhibition of DNA binding capacity of NF-Y (Jung et al., 2001). Our studies in Paper III and those by Mantovani on p53 and p63 have shown that this is probably not the case since both molecules are found simultaneously bound to cell cycle regulated promoters (Imbriano et al., 2005; Testoni and Mantovani, 2006). However, in Paper I, NF-Y is displaced from the PDGFRB promoter upon serum stimulation. It might be so that local effects affect regulation, such as posttranslational modifications and/or association with HATs and HDACs on NF-Y and p53.

It has been reported that as many as 66% of NF-Y controlled genes are also targets for p53 family members (Ceribelli et al., 2006). Perhaps there has been an evolutionary bias or selective pressure for the introduction of CCAAT sites in promoters of cell cycle regulated genes in order for them to be simultaneously repressed by p53 family members. This might constitute a larger pattern which we are currently unable to determine, where a set of CCAAT-dependent genes are needed to be repressed for induction of apoptosis or cell cycle stop. It still remains to be elucidated how crucial posttranslational modifications and tetramer formation of p53 family members are for the association with NF-Y.

Transcriptional downregulation of PDGF receptors as a negative feedback mechanism

The negative feedback mechanisms that are initiated upon activation of PDGF receptors are crucial for controlled proliferation. While dephosphorylation and internalization attenuate signaling, it does not necessarily affect the amount of receptors presented on the cell surface and thus subsequent ligand-induced signaling. Transcriptional regulation, on the other hand, provides a way for the cell to adapt to its microenvironment by adjusting the amount of receptors it presents on the surface. Thus, lack of transcriptional regulation as a negative feedback mechanism would deprive the cell from an important regulatory mechanism.

Transcriptional downregulation can be initiated either by activation of already present transcription factors or through the induction of early response genes. For PDGFRB, the exact mechanism of how the receptor is downregulated in response to PDGF signaling is not known, but studies in this thesis indicate that posttranslational modifications of already present p53 family members might constitute a rapid way to decrease PDGFRB transcription. Another

scenario, although a slightly slower negative feedback mechanism, is the induction of c-Myc expression, possibly through Src-mediated c-Abl activation (Furstoss et al., 2002). Even if Src activates c-Abl, and c-Abl activates p53 and p73, it is unclear if this represents a functional pathway for negative feedback of PDGF signaling or if it instead is two pathways. Since the effects of c-Abl on p53 and p73 in response to DNA damage is a nuclear event (Agami et al., 1999) while the Src-mediated activation of c-Abl is located at the membrane or in the cytoplasm (Plattner et al., 1999), this indicates two separate pathways. However, c-Abl contains a NLS and might be shuttled to the nucleus in response to Src-mediated activation or affect p53 family members in the cytoplasm and induce their translocation to the nucleus. Interestingly, as mentioned earlier, the PI3K, Akt and mTOR pathway seems to represents a negative feedback mechanism for PDGF signaling, but how mTOR regulates PDGFRB transcription is still unknown (figure 25).

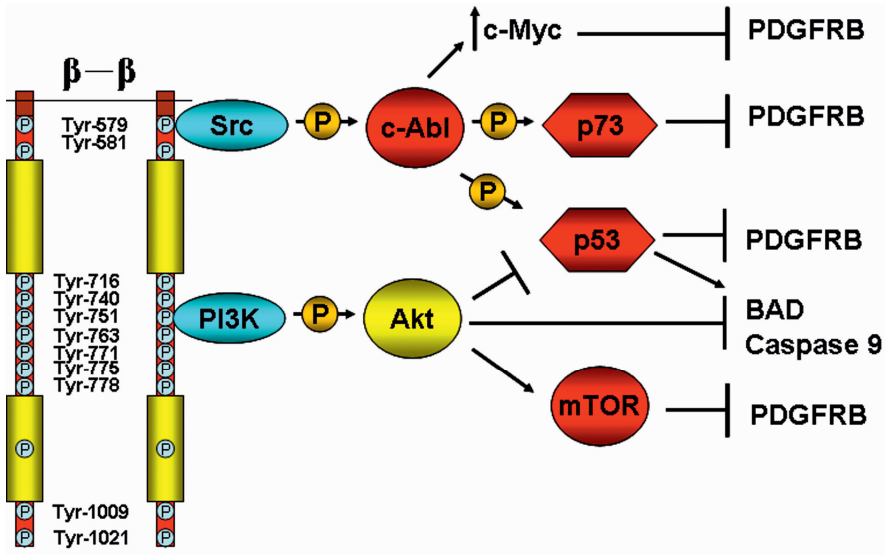

Figure 25. Effects and possible feedback mechanisms in response to PDGF signaling.

Role of transcriptional regulation of PDGFRB in cancer

PDGF signaling is involved in cancer progression where autocrine PDGF stimulation of tumor growth has been suggested. However, it is unlikely that dysregulated PDGF signaling alone enables uncontrolled growth of cancer as it is generally believed to act as a competence factor for cell cycling. Rather, autocrine signaling could provide an important step in cancer progression.

In support for such a role of autocrine PDGF signaling in cancer, recent findings showed that PDGFRA-expressing adult neural stem cells in the subventricular zone form atypical hyperplasia, in response to PDGF-A (Jackson et al., 2006). In that study, it was suggested that although not necessary for malignant transformation it represents an important step of such a formation. This was seen by others as a possible origin for glioma (Kesari and Stiles, 2006) since essentially all gliomas express PDGF ligands and receptor enabling an autocrine stimulation of growth.

Our findings in Paper IV indicate that neuroblastoma tumors with high expression of ΔNp73 might not respond by downregulating PDGF receptor expression in a microenvironment containing PDGF ligands, thereby providing a constitutive stimulus for cell proliferation. In addition, the dysregulated PDGFRB expression could indicate dysfunctional regulatory molecules. For example, functional inactivation of p53 and p73 would not only mean upregulated PDGFRB expression, it would also mean lack of important cell cycle regulators thus both providing competence for, and progression through, the cell cycle. The same reasoning could be argued for ΔNp73, where in addition to stimulating PDGFRB expression it could inhibit important cell cycle regulatory molecules. Based on these assumptions, the tyrosine kinase inhibitor imatinib might provide especially useful effects in treatment of patients with neuroblastoma with high ΔNp73 expression or with LOH of 1p.36.

The major treatment of PDGF-driven diseases are tyrosine kinase inhibitors. Targeting transcriptional regulation is today not really an alternative in treatment of PDGF-driven diseases. HDAC inhibitors can of course increase transcriptional activity but this activation is unspecific and the effects are difficult to predict. Expressing an important and specific transcriptional regulatory molecule might be a possible way of downregulating PDGF signaling but such molecules have yet to be identified.

Conclusions

I. The mechanism of downregulation of PDGFRB by p73 is due to competition with the HATs p300 and P/CAF for binding to NF-Y and recruitment of HDACs to the PDGFRB promoter. This greatly affects the acetylation status of NF-YC and the promoter activity. In support of this, *in vivo* binding of p73 to the PDGFRB promoter correlates with repression.

II. Overexpression of LT represses the PDGFRB promoter by interfering with the activation by NF-Y and Sp1. This repression depends on c-Myc, pRb and p53. Both LT and p53 bind the PDGFRB promoter where binding of LT reduces p53 binding.

III. p53 overexpression or endogenous p53 activation downregulates PDGFRB expression. Activation by MMC treatment induces acetylation of p53 enhancing its effects on PDGFRB. Lysines acetylated by p300 are of more importance for repression of PDGFRB than those acetylated by P/CAF.

IV. PDGFRB expression is dysregulated in IMR-32 where ΔNp73 binds constitutively to the PDGFRB promoter. In IMR-32, silencing of ΔNp73 represses PDGFRB expression. Activation of p53 family members by cisplatin treatment downregulates PDGFRB expression by dismissing ΔNp73 from the PDGFRB promoter and recruiting HDAC4 for repression.

Acknowledgements

This work has been carried out at the Institute of Biomedicine at the University of Gothenburg and at the Chiba Cancer Center Research Institute, Chiba, Japan. I would especially like to thank the following people who in different ways have contributed to the completion of this thesis.

My supervisor, **Keiko Funa** for accepting me as a PhD student and for giving support and encouragement during these years. Thank you also for giving me the opportunity to do part of my PhD in Japan!

Akira Nakagawara at the Chiba Cancer Center Research Institute for welcoming me at his lab.

Munemasa Ryu at the Chiba Cancer Center Hospital for making my stay in Japan an even greater experience. Thank you for all your help!

Special thanks to **Muna Elmi**, for being a great person and friend. It has been a true joy working beside you all these years. You have made a great and lasting impression on me, but it is nowhere close to the impressions you left in the sand when playing beach volleyball. Also, as a reminder, if you write another thesis, Chemistry is always spelled with an **r**.

Hidetaka Uramoto, for co-authorship, supervision and excellent teaching of methods.

My co-author and friend, **Weiwen Yang** for our fun and interesting conversations and for all your help with my project.

Yoshiki Matsumoto, co-author, for countless lunches and interesting discussions. A special thank you for all the help before and during my visit to Japan.

Anders Hackzell, for the co-authorship, coffee-table discussions and for making me feel welcome when I first came to the lab.

Abtin Moshiri, for being a great and positive guy, despite having endured my supervision. Thanks for all your help this past year!

Alicia Moshiri for creating a good mood in the lab and for laughing at almost everything I say.

Takeshi Yoshida, my fellow gambler and colleague, for good company at the lab and at numerous lunches. Good luck with your work in Japan!

Anke Brederlau (a.k.a Ankesaurus) for delivering and being the focus of so many jokes.

Karin Brandt for all the help when I first came to the lab, it was great fun working with you.

Former and present members of our lab, **Roland Faigle**, **Yvonne Arvidsson**, **Minoru Kawakami** and **Zeng Zhaojun** for being great colleagues.

All the members of the Chiba Cancer Center Research Institute for creating such a nice working environment. I would especially like to thank **Toshinori Ozaki** for helpful suggestions on my project and great supervision during my visit there.

Eva Lyche, **Laila Falk**, **Carina Ejdeholm** and **Minette Henriksson** for excellent administrative assistance

Erica Bäckström, good friend, fellow PhD student and coffee addict, for all the entertaining coffee breaks and fun conversations.

Anna Hogmalm, for being such a kind person and for all the helpful suggestions on my thesis, including what to write about you in the acknowledgement (about half a page I think).

Nicola Cavallini, fellow PhD student and a great guy, for pointing out that spending weekends at the lab is not necessarily a good thing for getting a PhD (or a life).

Daniel Hedberg, fellow PhD student, for including me in your project and for many interesting discussions.

Erik Johansson, a good friend and colleague. For all our movie-evenings, japanese classes and lunches. Good luck with your research in Japan!

Mikael Persson for being a good friend with an equally twisted sense of humor. Thank you for the stories about life as a post-doc in Germany, it helped during the writing of this thesis.

Claes Molin for being a great friend and person. Thank you for all the fun and non-scientific activities.

Anders Medegård, for long and lasting friendship. Thank you for all the midnight debriefing sessions.

Min mormor, för att du är en fantastisk person, både snäll och rolig. Att du sen är en klart sämre pokerspelare än mig gör dig bara till en ännu bättre mormor.

My mother, for always being there for me. Without your support this thesis would not have been possible.

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