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Staff

Heldin, Carl-Henrik, Director

PDGF Signaling Group

Heldin, Carl-Henrik, Group Head

Section of Translational Research

Hellberg, Carina, Assistant Investigator, Section Head

Furuhashi, Masao, Postdoctoral Fellow, to June

Hasumi, Yoko, Postdoctoral Fellow, from April

Kowanetz, Katarzyna, Postdoctoral Fellow

Bäckström, Gudrun, Senior Technical Assistant

Sandström Leppänen, Jill, Technical Assistant

Karlsson, Susann, Ph.D. Student, from April

Klosowska-Wardeg, Agnieszka, Ph.D. Student, from July

Section for Signal Transduction

Lennartsson, Johan, Postdoctoral Fellow, Section Head, from January

Amagasaki, Kenichi, Postdoctoral Fellow, from September

Chiara, Federica, Postdoctoral Fellow, to February

Rorsman, Charlotte, Senior Technical Assistant

Kallin, Anders, Ph.D. Student, to January

Wardeg, Piotr, Ph.D. Student, from July

Gembarska, Agnieszka, Ph.D. Student, from March to December

Cytoskeletal Regulation Group

Aspenström, Pontus, Associate Investigator, Group Head

Hájková, Lucie, Postdoctoral Fellow, to April

Pacholsky, Dirk, Postdoctoral Fellow, from June

Ruusala, Aino, Senior Technical Assistant

Fransson, Åsa, Ph.D. Student

Johansson, Ann-Sofi, Ph.D. Student

Gene Targeting Group

Heuchel, Rainer, Assistant Member, Group Head

Li, Ronggui, Postdoctoral Fellow

Åhgren, Aive, Senior Technical Assistant

Zieba, Agata, Ph.D. Student, from September to December

TGF- β Signaling Group

Moustakas, Aristidis, Associate Member, Group Head

Niimi, Hideki, Postdoctoral Fellow

Thuault, Sylvie, Postdoctoral Fellow, from February

Valcourt, Ulrich, Postdoctoral Fellow, to June

Morén, Anita, Senior Technical Assistant

Kowanetz, Marcin, Ph.D. Student

Lönn, Peter, Ph.D. Student, from July

Pardali, Katerina, Ph.D. Student

Integrated Signaling Group

Souchelnytskyi, Serhiy, Assistant Member, Group Head

Dubrovskaya, Anna, Postdoctoral Fellow

Iwahana, Hiroyuki, Postdoctoral Fellow, to March

Yakymovych, Ihor, Visiting Scientist

Yakymovych, Mariya, Visiting Scientist

Bhaskaran, Nimesh, Ph.D. Student, from May

Woksepp, Hanna, Ph.D. Student, from February

Apoptotic Signaling Group

Landström, Maréne, Assistant Member, Group Head

Edlund, Sofia, Postdoctoral Fellow, to May

Lee, So Young, Postdoctoral Fellow

Zhang, Shouting, Postdoctoral Fellow

Grimsby, Susanne, Senior Technical Assistant

Ekman, Maria, Ph.D. Student, from August

Cavallini, Nicola, Student, from February to April

Gene Expression Group

Ericsson, Johan, Associate Member, Group Head

Grönroos, Eva, Assistant Investigator

Bengoechea Alonso, Maria Teresa, Postdoctoral Fellow

Kanduri, Meena, Postdoctoral Fellow, from May

Punga, Tanel, Postdoctoral Fellow, from April

Sundqvist, Anders, Postdoctoral Fellow

Lukiyanchuk, Vasyl, Ph.D. Student

Simonsson, Maria, Ph.D. Student

Matrix Biology Group

Heldin, Paraskevi, Associate Investigator, Group Head (joint appointment with
Department of Medical Biochemistry and Microbiology, Uppsala University)

Bagchi, Sonchita, Ph.D. Student, from August

Asteriou, Trias, Postdoctoral Fellow, to June

Li, Yuejuan, Postdoctoral Fellow

Kamiryo, Masaru, Ph.D. student

Li, Lingli, Ph.D. Student

Protein Structure Group

Hellman, Ulf, Member, Group Head

Conrotto, Paolo, Ph.D. Student, from April

Engström, Ulla, Senior Technical Assistant

Wernstedt, Christer, Senior Technical Assistant

Technical Support

Ejdesjö, Bengt, Purchasing Officer

Hedberg, Ulf, IT-support

Hermansson, Lars-Erik, Service Engineer

Pettersson, Gullbritt, Laboratory Assistant

Schönquist, Inger, Laboratory Assistant

Administration

Hallin, Eva, Finance and Administration Manager

Secretariat

Schiller, Ingegärd, Secretary

Introduction

The aim of the research at the Uppsala Branch is to elucidate the signaling pathways in cells that control cell growth and migration. As malignant cells show perturbations of such pathways, we hope that our results will reveal suitable targets for the development of signal transduction modulators, which can be used for treatment of cancer.

Important themes of our research are, as before, platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β). PDGF isoforms are major mitogens for connective tissue cells and certain other cell types, and are implicated in autocrine as well as paracrine stimulation in tumors. Important goals are to elucidate the mechanisms of signal transduction downstream of PDGF receptors and to explore the clinical utility of PDGF antagonists. TGF- β family members have important roles to control differentiation during the embryonal development; they inhibit the growth of most cell types. In cancer, the role of TGF- β is complicated; initially it is a tumor suppressor through its ability to inhibit growth and stimulate apoptosis of cells, but at later stages of tumor progression TGF- β promotes tumorigenesis, *e.g.* by changing the differentiated state of cells to a more invasive one and through indirect effects including stimulation of angiogenesis and suppression of the immune system. In addition to elucidating the molecular mechanism of TGF- β action, an important aim of our work is to explore whether TGF- β antagonists can be used for treatment of advanced cancer.

The work at our Branch is performed in 9 different groups, who work on various aspects of signal transduction. The groups complement each others in terms of technical skills. Thus, in addition to conventional cell and molecular biology methods, we have access to technology in proteomics and mass spectrometry, microarray analyses, advanced microscopy and mouse genetics.

C.-H. Heldin

PDGF Signaling Group

The platelet-derived growth factor (PDGF) isoforms (PDGF-AA, -AB, -BB, -CC and -DD) are major mitogens for connective tissue cells and certain other cell types, which exert their cellular effects via binding to α - and β -tyrosine kinase receptors (PDGFR α and β). We aim at elucidating the mechanisms of signal transduction via PDGF receptors, as well as exploring the clinical utility of PDGF antagonists.

Section for Signal Transduction

The work in the PDGF Signal Transduction Section aims at increasing our understanding of the molecular mechanisms underlying the diverse cellular effects of PDGFRs.

PDGF-mediated chemotaxis

The ability of cancer cells to form metastases in distant parts of the body is a major reason for cancer lethality. To clarify the role of different signal transduction molecules in directed cell movement mediated by PDGFRs, we study the migration of cells towards different PDGF isoforms in the presence or absence of interfering agents. Our results using both transfected porcine aortic endothelial (PAE) cells and primary foreskin fibroblasts suggest that JNK is critical for PDGF-induced chemotaxis. Current work seeks to identify the pathways mediating PDGF-dependent JNK activation and to pin-point downstream targets of JNK that regulate cell migration. A molecular understanding of the cell migration process may be valuable for the development of new strategies to interfere with the metastatic process.

Function of the activation loop tyrosine residue 857 in PDGFR β

In order to understand the role in regulation of the receptor kinase of Tyr857, located within the activation loop of PDGFR β , we have mutated this residue to phenylalanine (Y857F) and expressed the mutant receptor in PAE cells. Our results demonstrate that the Y857F receptor is kinase active and respond to various concentrations PDGF in a manner similar to the wild-type receptor. Furthermore, this mutant receptor can activate several signaling proteins, *e.g.* Erk and Akt. However, STAT3 and 5 show a significantly reduced phosphorylation in PDGF-stimulated Y857F cells compared to cells expressing the normal receptor, despite comparable receptor activation. Further

work aims to clarify the mechanism behind this selective defect in STAT phosphorylation and to identify its functional consequence.

Identification of novel proteins involved in PDGFR signal transduction

After activation of PDGFRs, many downstream proteins become tyrosine phosphorylated, which is critical for signal transduction to occur. In order to identify new phosphorylation targets downstream of the PDGFRs, we have stimulated cells expressing PDGFR α or PDGFR β and purified tyrosine phosphorylated proteins followed by mass spectroscopic identification. Using this approach, we have identified several proteins which have well characterized roles in PDGF signal transduction. In addition, other less well investigated proteins have been found; the functional role of these proteins are now being analyzed using transfected PAE cells and embryonic fibroblasts from knock-out mice.

Proteins interacting with activated PDGFRs do so via specialized domains called Src homology 2 (SH2) or phosphotyrosine binding (PTB) domains. We are in the process of identifying proteins interacting with the PDGFRs using phospho-peptides corresponding to the amino acid sequence of the 11 different autophosphorylation sites in PDGFR β and the 10 in PDGFR α . Our aim is to perform a complete and systematic analysis of interactions with each of the PDGFR autophosphorylation sites.

cDNA microarray analysis of gene induction by hetero- or homodimers of PDGFR

An important question is whether the different PDGFR homodimers have unique functional properties. In an attempt to address this question, we performed cDNA microarray analysis following induction of receptor homodimers. We have produced cDNA microarray profiles from ligand-stimulated PAE cells transfected with PDGFR β and compared this result with results from PAE cells expressing PDGFR α . Comparing our results from cells expressing only PDGFR α with results from cells expressing only PDGFR β , we indeed observed genes specifically induced by either receptor isoform. Currently, we are verifying these observations using RT-PCR, and are investigating the effects of PDGF on the levels of the corresponding proteins by Western blotting techniques. An interesting result has been obtained on the regulation of MAP-kinase phosphatases (MKPs) 1-3. The various MKPs dephosphorylate different types of MAP-kinases (Erk1/2, p38, JNK1/2); interestingly, they are differentially regulated by PDGF isoforms, and the expression of the different MKPs correlate with the different kinetics of MAP-kinase phosphorylation induced by the two PDGFR isoforms, *i.e.* high

MKP expression result in shorter duration of the corresponding MAP-kinase phosphorylation. These findings are now explored further.

Section for Translational Research

The research in the Translational Research Section follows two major lines, *i.e.* the exploration of PDGF receptors as cancer drug targets and investigations of the mechanisms for termination of PDGFR β signal transduction.

PDGF receptors as cancer drug targets

Activation of tyrosine kinase receptors through amplification or mutation of their genes have been described in several forms of tumors. Moreover, there are examples of autocrine receptor activation through increased expression of both ligand and receptor within a tumor cell. It has also been suggested that growth factors synthesized by activated stromal cells stimulate the growth and survival of tumor cells in a paracrine manner. In addition, tumor-derived factors induce tumor vascularization, a process that is necessary for both growth and metastasis of tumors. PDGF receptor signaling is implicated in various cancer associated processes, including autocrine stimulation of tumor growth, stimulation of tumor fibroblasts and promotion of tumor angiogenesis. Our aim is to investigate the role of PDGF and PDGF receptors in autocrine and paracrine stimulatory mechanisms in tumors, and to explore the possibility that PDGF antagonists can be used in therapy.

Ongoing clinical studies in which targeted therapies are explored have emphasized the need for activation-specific reagents to monitor target presence and activity. We are therefore developing novel methods for monitoring PDGF receptor status in tissues using different types of antibodies. As a consequence of the recent discovery of two novel PDGF ligands, PDGF-CC and -DD, we have also generated neutralizing antisera to evaluate the role of PDGF-CC and PDGF-DD in disease processes in preclinical models.

We have previously reported that inhibition of PDGF receptors on stromal cells, increases tumor uptake of low molecular weight chemotherapeutic drugs in experimental models. We are continuing these studies with the aim to elucidate mechanism(s) underlying this interesting effect of PDGF. Furthermore, the possibilities that targeting of PDGF receptors on stromal cells could increase the tumor uptake of therapeutic antibodies, as well as lowering tumor hypoxia, are under investigation.

We are also investigating the functional role of PDGF receptors on tumor pericytes (14). In the B16 melanoma mouse tumor model, PDGF-dependent pericyte recruitment contributes to tumor growth in a manner which is associated with reduced tumor cell apoptosis. This suggests that PDGF antagonist-mediated pericyte targeting could constitute an anti-angiogenic approach, either by itself or in combination with agents targeting endothelial cells. This notion is presently investigated in different mouse tumor models.

Termination of PDGF β -receptor signal transduction

Following ligand stimulation, the PDGFR β signaling is terminated by dephosphorylation of the receptor autophosphorylation sites, in parallel with receptor internalization and subsequent degradation.

PDGFR β contains eleven tyrosine residues that are autophosphorylated following receptor activation. Several tyrosine phosphatases that dephosphorylate specific tyrosine residues on PDGFR β have been identified. We have explored the exciting possibility that the activity of a phosphatase could modulate specific signaling pathways induced by receptor activation, rather than turning off the signaling completely. We found that PDGFR β phosphorylation was enhanced in T-cell phosphatase $-/-$ embryos as well as in cultured fibroblasts from these mice (37). The T-cell phosphatase preferentially dephosphorylated Y1021 of PDGFR β , thereby regulating the phospholipase- $C\gamma$ (PLC γ) signal transduction pathway.

Interestingly, the increased PDGFR β phosphorylation in the T-cell phosphatase $-/-$ fibroblasts is paralleled by a pronounced decrease in clearance of activated receptors from the cell surface. We found that this is due to an induction of receptor recycling, which occurs through Rab4 positive recycling endosomes. Preliminary data indicate that the induction of recycling is specific for PDGFR β , since neither PDGFR α nor the IGF-1 receptor display increased recycling in T-cell phosphatase $-/-$ fibroblasts. Since PDGFR β do not normally recycle, these fibroblasts provide a unique model system for studying the regulation of the intracellular trafficking of PDGFR β .

Cytoskeletal Regulation Group

The work within the Cytoskeletal Regulation Group is aimed at elucidating signaling pathways that control cell growth and cell migration during normal physiological conditions as well as during disease.

The Rho GTPases

The Rho GTPases is a group of small enzymes with homology to the proto-oncoprotein Ras. This family of proteins consists of 23 members in human cells, which can be further divided into 8 subgroups: **Cdc42** (Cdc42, TC10, TCL, Chp, Wrch1), **Rac** (Rac1-3, RhoG), **Rho** (Rho A-C), **Rnd** (Rnd1-3), **RhoD** (RhoD and Rif), **RhoH/TTF**, **RhoBTB** (RhoBTB1-3) and **Miro** (Miro 1-2) (60). The Rho GTPases are key regulators of cell morphogenesis and cell migration but they also participate in signal transduction pathways that regulate gene transcription, cell growth, cell cycle progression and cell survival. We have compared the effects on the organisation of the actin filament system triggered by the different Rho GTPases. Our studies have shown that the effects on the actin filament system evoked by the different Rho GTPases are more intricate than recognised before (5, 41).

Signaling to Rho GTPases

Several ligand:receptor systems have been found to activate the Rho GTPases. Our studies have shown that transforming growth factor- β (TGF- β) signaling induced a rapid reorganisation of the actin cytoskeleton. These responses were dependent on the activation of the Rho GTPases Cdc42 and RhoA. Interestingly, we found that Smad7 was required for the TGF- β induced activation of Cdc42. Thus, there seems to be a close correlation between the activity of the inhibitory Smad7 and the activation of Cdc42. In addition, we found that the TGF- β -dependent reorganisation of the actin filament system is dependent on the p38 MAPK and the phosphatidylinositol-3'-kinase (PI3K) signaling pathways (13).

Regulators and effectors of Rho GTPases

We have studied several proteins, which bind to RhoGTPases and affect their biological activities (32, 39, 40). The Cdc42-binding protein 4 (CIP4) was previously shown to have a role in the Cdc42-dependent regulation of the actin filament system. In a search for CIP4-binding proteins, the RhoGAP domain-containing protein RICH-1 (RhoGAP

interacting with CIP4 homologues-1) was identified. We have shown that RICH-1 is a RhoGAP for Cdc42 and Rac, and that it has a BAR (BIN/Amphiphysin/Rvsp) domain, a type of domain found in the endocytic proteins endophilin and amphiphysin. We showed that the BAR domain of RICH-1 binds to membrane lipids, and deforms spherical liposomes into tubes in a process mimicking the membrane shape changes that take place during, for example, endocytosis. These results suggest an involvement of RICH-1 in membrane trafficking events by virtue of its BAR domain (39).

The verprolins

The verprolins are pivotal modulators of signaling mediated by the WASP family of proteins. WASP was originally identified as the gene defective in the severe X-linked immunodeficiency disorder Wiskott-Aldrich syndrome (WAS). This family of proteins, which also includes N-WASP and Scar/WAVE 1-3, has been shown to be critical regulators of actin polymerisation via activation of the so-called Arp2/3 complex.

The verprolin family consists of 3 gene products in human cells: WIP, WIRE and CR16. The verprolins bind actin and the actin-regulating protein profilin via an N-terminal domain. The numerous proline-rich motifs bind to SH3 domain-containing proteins, such as Nck and cortactin, whereas the WASP-binding motif resides in the C-terminus of the verprolins. We have focused our studies on WIRE, and have found that ectopic expression of WIRE in mammalian cells results in reorganisation of actin filament into thick bundles. Interestingly, we found that WIRE mutants unable to bind WASP are still able to induce a reorganisation of the actin filament system, both in a manner dependent and independent of PDGF β -receptor, indicating that WASP was not necessary for the process. In cells ectopically expressing WIRE the endocytosis of the PDGF β -receptor was drastically reduced. In contrast to the effect on the actin filament system, the WIRE induced abrogation of the receptor endocytosis required the intact WASP-binding ability of WIRE (4).

Gene Targeting Group

This group uses gene targeting in the mouse in an effort to explore the *in vivo* importance of specific signaling pathways initiated by different growth factors.

Analysis of PDGFR- β function by the use of knock-in mice

In collaboration with Dr. Philippe Soriano's group in Seattle, we generated PDGF receptor signaling pathway-restricted mice. In these mice, PDGFR β carried point mutations of specific autophosphorylation sites, such that they were unable to bind and therefore activate PI3K, or PI3K and PLC γ upon ligand stimulation. In a model of subdermal edema formation, such mice showed a defect in the regulation of the interstitial fluid homeostasis, and in a model of experimental glomerulonephritis, mesangial cell defects during the wound healing process were observed.

In order to investigate the possible involvement of PDGFR β in disease, we generated a mouse with a point mutation in the activation loop of the kinase domain. Analogous mutations in the hepatocyte growth factor receptor and the stem cell factor receptor have been found in patients with hereditary papillary renal carcinoma and mastocytosis, respectively. In both cases, the mutations turned out to be of the gain of function type. The mutation we introduced by gene targeting into the activation loop of the murine PDGFR β in ES-cells, was an exchange of asparagine for aspartic acid at amino acid position 849 (D849N). This mutation conferred increased transforming characteristics to ligand-stimulated mouse embryonic fibroblasts derived from mutant mice. By comparing the enzymatic properties of the wild-type *vs* the mutant receptor protein, we demonstrated that the D849N mutation lowers the threshold for kinase activation, causes a dramatic alteration in the pattern of tyrosine phosphorylation kinetics following ligand-stimulation, and induces a ligand-independent phosphorylation of several tyrosine residues. These changes resulted in deregulated recruitment of specific signal transducers (9). The GTPase-activating protein for Ras (RasGAP), a negative regulator of the Ras mitogenic pathway, displays a delayed binding to the mutant receptor. Moreover, we observed enhanced ligand-independent ERK1/2 activation and an increased proliferation of mutant cells. The p85 regulatory subunit of PI3K was constitutively associated with the mutant receptor and this ligand-independent activation of the PI3K pathway may explain the observed strong protection against apoptosis and increased motility in cellular wounding assays. Our findings support a model whereby an activating point mutation results in a deregulated PDGFR β with oncogenic predisposition.

Recently, an activation loop mutation in the PDGFR α was reported to be responsible for a certain percentage of gastrointestinal stromal tumors in human. We have generated ES cells with an identical mutation in the PDGFR β (D849V). In sharp contrast to the previously generated D849N mutant mice, which are fully viable, the D849V mutation

has considerably stronger *in vivo* effect. Targeted ES-cells carrying the D849V mutant PDGFR β do not generate viable chimera. In collaboration with the group of Dr. Lena Claesson-Welsh, we observed that the D849V mutant PDGFR β exerts a strong vasculogenic and angiogenic effect. Experiments aiming at elucidating the difference(s) between the D849N and the D849V mutant PDGFR β with respect to the dramatic phenotypical *in vivo* difference, are under way.

Regulation and *in vivo* function of Smad7

TGF- β family members, which include TGF- β s, activins and bone morphogenetic proteins (BMPs), are secreted molecules that regulate a plethora of cellular responses, such as proliferation, differentiation, migration and apoptosis. Deregulated TGF- β family signaling has been implicated in multiple disorders and in various human diseases, including cancer, fibrosis and autoimmune diseases. TGF- β family members signal through specific type I and type II serine/threonine kinase receptors which in turn activate a subset of Smad proteins. These molecules relay signals into the nucleus where they direct transcriptional responses in concert with other proteins. The mRNA expression of a particular member of this family, namely Smad7, had been shown to be induced by TGF- β itself. Overexpression of Smad7 leads to downregulation of TGF- β signaling, suggesting an auto-regulatory feedback mechanism. We investigated the mouse Smad7 promoter and found not only an essential DNA binding site for the TGF- β activated Smads2, 3 and 4, but also the requirement for cooperation of these Smads with Sp1 and AP1 transcription factors in order to guarantee an efficient TGF- β response of the Smad7 promoter.

In order to learn more about the *in vivo* function of Smad7, we targeted the Smad7 gene in mice, in collaboration with Dr. Tony Pawson's lab in Toronto, Canada (Li *et al.*, submitted for publication). Mutant animals show severely reduced viability depending on the mouse strain background. On C57Bl/6 background, homozygous animals do not survive weaning, whereas mutant mice are viable on CD-1 background. In general, we found that mutant mice were smaller than wild-type mice.

In line with the role of TGF- β as a major player in the immune system, we observed increased immunoglobulin class switching activity towards IgA, as well as an elevated growth suppressing effect of TGF- β on B-cells, both of which can be explained by lack of Smad7 function. In addition, we found skeletal anomalies, such as ectopic bone formation and a homeotic transformation of a cervical vertebra. The Smad7 mutation results in an apparently increased TGF- β signaling, which makes these mice ideally

suitable for disease models, where increased TGF- β signaling is a major pathogenic factor, as for instance in fibrotic conditions.

TGF- β Signaling Group

The TGF- β Signaling Group investigates signaling pathways and gene networks that regulate cell growth and differentiation in response to TGF- β . Of special interest are TGF- β -mediated processes, which contribute to tumor cell invasiveness and metastasis.

TGF- β signaling and Smad regulation

The large superfamily of TGF- β morphogenic proteins signals via serine/threonine kinase receptors and Smad proteins, their cytoplasmic effectors (72). Smads, upon activation by the receptors, translocate to the nucleus and regulate gene expression. TGF- β induces phosphorylation of Smad3, a receptor-activated (R-) Smad, which is rapidly imported to the nucleus, regulates gene transcription and eventually is exported back to the cytoplasm. We have analyzed the mechanism of Smad3 nuclear export and identified exportin-4 and the Ran GTPase as the major transporting factors of this Smad (Kurisaki *et al.*, submitted for publication). In parallel, we study the regulation of the function of Smad4, which serves as the common effector for all TGF- β superfamily pathways. In carcinomas, specific amino acid substitutions in Smad4 lead to its enhanced poly-ubiquitination and proteolysis. Wild-type Smad4 can be mono- or oligo-ubiquitinated, which leads to efficient R-Smad/Smad4 oligomerization and enhanced transcriptional activity. We identified Smurf1 and Smurf2, WWP1 and NEDD4-2, as E3 ubiquitin ligases that induce poly-ubiquitination of wild-type Smad4 (Morén *et al.*, submitted for publication). All four E3 ligases have similar structural characteristics, including WW and HECT domains. We currently screen for additional ubiquitin ligases of Smad4 using RNAi library technology, and we also study the functional properties of mono-ubiquitinated Smad4, including specific protein partners that recognize this post-translational modification in Smad4. Finally, through microarray analysis, we identified a novel serine/threonine kinase, whose gene is directly and rapidly induced by TGF- β s and BMPs, and which regulates R-Smad, Smad4 and TGF- β receptor proteasomal degradation. Its role in TGF- β signaling is now being analyzed.

Transcriptional roles of Smads: Regulation of cell cycle, cell differentiation and the role of TGF- β as tumor suppressor or pro-metastatic factor

Upon signal-dependent translocation to the nucleus, Smad proteins cooperate with several transcription factors in order to regulate transcription (72). An important gene target of the pathway is the cell cycle inhibitor p21. We found that BMP-specific pathways and the corresponding R-Smad effectors show a stronger ability to induce p21 expression than their TGF- β -related counterparts (55). The BMP-induced p21 does not correlate with inhibition of proliferation. This is because BMPs also potently induce expression of Id proteins, thus leading to mitogenic signals. We demonstrated that Id2 specifically antagonizes p21 in regulation of epithelial cell proliferation. Through microarray analysis, we identified novel transcription factors which are directly regulated by the Smad pathway. We analyze the role of a homeobox transcription factor as a potential regulator of p21 expression in response to TGF- β , which may contribute to the sustained and prolonged induction of p21 gene expression by TGF- β . In addition, we scrutinize the tumor suppressor actions of TGF- β and Notch signaling pathways in mammary epithelial cells. Using microarray analysis we have identified a large number of genes that are co-regulated by the two pathways and we attempt to establish the molecular mechanism leading to synergistic suppression of cell proliferation by TGF-beta and Notch.

TGF- β plays a tumor suppressor role in early stages of carcinogenesis, yet it promotes carcinoma cell invasiveness and metastasis. We study mammary epithelial models that exhibit this dual response to TGF- β . Mouse NMuMG cells undergo epithelial to mesenchymal transition (EMT) *in vitro* in response to TGF- β , a change in cell differentiation important *in vivo* during tumor cell migration and metastasis. We have developed a two-cell model of CHO cells secreting latent TGF- β (the physiological form of this cytokine in the extracellular space) and responding NMuMG cells that leads to a significant increase in tumorigenesis in *SCID* mice (Gaal *et al.*, submitted for publication). Using a systematic approach of analyzing all receptors and Smads of the TGF- β superfamily, we established that TGF- β s but not BMPs are capable of inducing EMT (58). This selective epithelial response correlates strongly with the growth suppression response and we unequivocally demonstrated the role of Smad proteins in these processes. Using cDNA microarrays, we analyzed immediate-early and late gene responses to TGF- β during EMT and classified them functionally based on their response to TGF- β s versus BMPs. One of the top genes in this screen is a transcription factor of the high mobility group (HMG) family that seems to play the role of a master-

regulator of EMT, as it regulates the expression of many known regulators of EMT, such as Twist, Snail and Slug.

Human MDA-MB-468 cells are highly tumorigenic *in vivo* and lack critical tumor suppressors, such as Smad4 and p53. Using cDNA microarray analysis, we have identified specific gene targets whose regulation depends on the presence of Smad4. We have examined their relevance to the tumor suppressor and EMT pathways. One group of Smad4-dependent genes that scored highly in all of our microarray screens was the inhibitors of differentiation Id1, Id2 and Id3. We have shown that TGF- β downregulates Id2 and Id3 in order to elicit robust arrest of epithelial cell growth and potent EMT (28). In contrast, BMP fails to elicit potent epithelial growth inhibition or EMT, because it induces high levels of the same Id proteins. Thus, Id proteins define the ability of epithelial cells to give specific physiological responses to distinct TGF- β superfamily members. We currently investigate the mechanisms by which Id proteins define how epithelial cells will respond to TGF- β , BMP or other factors of the superfamily.

Integrated Signaling Group

The main objective in the Integrated Signaling Group is to explore molecular mechanisms of cellular carcinogenic transformation. Proteomics is being used to unveil cancer-related changes in cells, and intracellular signaling mechanisms of TGF- β are being explored.

Proteomics and cancer

Cancer is a systemic disease, with many genes and proteins being affected. Proteomics provides a possibility to get a comprehensive overview of changes in protein expression and activities during carcinogenesis. Proteomics also delivers markers to detect and monitor cancer. Our proteomics platform is based on two-dimensional gel electrophoresis for separation of proteins, dedicated computer-aided image analysis for detection of changes, and mass spectrometry for identification of selected proteins.

We have performed proteome profiling of human epithelial cells and explored TGF- β -dependent changes; more than 300 proteins regulated by TGF- β , have been identified. Proteome expression maps of human breast epithelial cells have been built, and significant changes in cell proteomes have been observed between primary, immortal and tumorigenic cells. Proteomics techniques have been used to identify more than 50 proteins which form complexes with components of TGF- β and bone

morphogenetic proteins (BMP) signaling pathways, *e.g.* BMPR-II and Smad3 (16, 20). The targets of TGF- β and BMP we have identified have unveiled novel signaling pathways of these growth factors, *e.g.* in the regulation of cell migration, cytoskeleton rearrangements and transcription. We described the formation of a complex between the BMPR-II serine/threonine kinase receptor and the c-Kit tyrosine kinase receptor (Hassel *et al.*, submitted for publication). We also described a complex between Smad3 and SREBP2, which indicates a cross-talk of TGF- β signaling with regulation of steroidogenesis (16).

Protein phosphorylation and glycosylation are post-translational modifications of crucial importance during the development of cancer. We use proteomics to study phosphorylation and glycosylation of proteins in cells treated with TGF- β . We have identified more than 50 proteins which change phosphorylation or glycosylation upon TGF- β treatment of cells. In-depth studies of two of the identified targets have provided insights into TGF- β -dependent regulation of transcription and apoptosis (Stasyk *et al.*, submitted for publication; Iwahana *et al.*, manuscript in preparation). We have developed a 2D gel-compatible immobilized metal-affinity chromatography technique for an efficient enrichment of phosphoproteins; phosphoproteome profiling of various human breast epithelial cells is on-going (Dubrovskaya *et al.*, submitted for publication).

We have detected aberrant expression of three proteins in human plasma of patients suffering from breast and ovarian cancer (Lomnytska *et al.*, submitted for publication). Expression of these proteins in plasma correlates with early stages of cancer, suggesting that they can be markers for early diagnostics of cancer.

TGF- β signaling and tumorigenesis

We continue to explore TGF- β signaling in regulation of DNA damage repair and DNA damage-induced signaling. We have described TGF- β - and Smad3-dependent inhibition of BRCA1-promoted repair of DNA damage, and a synergy between BRCA1 and Smad3 in transcriptional regulation (50). These findings, and our previous observation that Rad51 and BRCA2 are targets of TGF- β , define novel molecular mechanisms of TGF- β -dependent regulation of maintenance of integrity of genomic DNA.

In order to elucidate the roles of Smad2 and 3 in tumorigenesis *in vivo*, we performed studies in mice. Cells stably transfected with Smad2, Smad3 or empty control vector were inoculated in SCID mice, and the formed tumors were studied (43). We observed that even moderate increase of Smad2 expression is sufficient for an inhibition of tumor

growth. Smad2-dependent alterations in cell proliferation, apoptosis and angiogenesis were observed, as compared to control cells.

We continue our project on functional evaluation of novel substrates of type I TGF β receptor. These substrates were identified using a kinase-substrate screening of an expression library, which we have developed. The identified substrates are currently under investigation; most of them have not been described in relation to TGF β signaling.

Our results, and reports from other laboratories, indicate that manipulation of TGF- β signaling may have clinical benefits. We have explored mechanisms of substrate recognition by type I TGF β receptor, and identified key features required for specific recognition and phosphorylation of a substrate (45). Previously, we reported that imidazole and isoquinoline scaffolds can be used for development of specific ATP-competing TGF- β receptor inhibitors, and that small peptides can be used as substrate-mimicking inhibitors. We continue to explore the possibility of developing specific kinase inhibitors of TGF- β receptors. The aim of these studies is to develop compounds which will be useful in treatment of such diseases as cancer and fibrotic conditions.

Apoptotic Signaling Group

The aim of the work in the Apoptotic Signaling Group is to elucidate the molecular mechanisms for TGF- β -induced apoptosis.

Involvement of Smad7 and p38 in TGF- β -induced apoptosis in prostate cancer cells

We have observed increased expression of the receptor-activated Smads (Smad2, Smad3 and Smad4) as well as the inhibitory Smad6 and Smad7 in normal and malignant prostate epithelial cells *in vivo*, preceding apoptosis induced by androgen withdrawal. The presence of Smad7 in apoptotic cells *in vivo*, together with our previous observation that Smad7 is required for induction of apoptosis, encourage us to continue our search for the detailed molecular mechanisms for how Smad7 can act as a mediator for TGF- β -induced apoptosis in epithelial cells. As Smad7 is facilitating the activation of the p38 MAP kinase, we currently investigate possible novel target proteins downstream of the p38-Smad7 complex.

We have previously reported that Smad7 enhances the TGF- β -activating kinase 1 (TAK1), mitogen activated protein kinase kinase 3 (MKK3) and p38 MAP kinase pathway, presumably by acting as an adaptor protein bringing the kinases close to each other. We study now by which mechanisms TGF- β receptors activate this pathway.

Interestingly, Smad7 is predominantly localized in the nucleus of growth factor starved cells, while stimulation of cells with TGF- β causes a rapid export of Smad7 to the cytoplasm where it interacts with the TGF- β -activated receptor-complex. At longer time-points after TGF- β -stimulation of cells, Smad7 accumulates in the nucleus again. We continue to investigate whether the apoptotic effect of Smad7 is dependent on its possibility to interact with other pro-apoptotic proteins which can shuttle between the nucleus and the cytoplasm.

We have also demonstrated that Smad7 expression is required for TGF- β -mediated cytoskeletal regulation which occurs mainly via the small GTP-ase Cdc42, in a collaboration with Dr. Aspenström at our Institute. We will therefore investigate the role of Smad7 in TGF- β -dependent regulation of cytoskeletal processes, such as migration of cells.

A possible role for Smad7 as a bridge between the TGF- β and Wnt signaling pathways

Signaling molecules downstream of TGF- β and Wnt receptors regulate cell fate and proliferation during development and tissue homeostasis. We have recently reported that Smad7 interacts with β -catenin and lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF), transcriptional regulators in Wnt signaling, in a TGF- β -dependent manner (51). Furthermore, by the use of siRNA and anti-sense techniques, we show that Smad7 expression is required for TGF- β -induced stabilization of β -catenin, increase of c-Myc and subsequent apoptosis in human prostate cancer cells, as well as in immortalized human keratinocytes. Interestingly, we observed that Smad7 together with p38 regulate the activity of Akt and glycogen syntetase kinase-3 β (GSK-3 β), which in turns leads to the stabilization of β -catenin. We are now exploring the molecular mechanisms whereby TGF- β regulates the activity of Akt and GSK-3 β . We will also continue to further examine the underlying molecular mechanisms by which Smad7 and p38 MAPK affect components in the Wnt signaling pathway.

Smad7 target genes

Smad7 shuttles between the nucleus and the cytoplasm, as previously reported by us. In order to explore the potential effects of Smad7 on gene regulation, we have performed microarray analyses on cells with or without overexpression of Smad7. Possible candidate genes are currently being validated.

The role of Smad7 in apoptosis induced by 2-methoxyestradiol

2-Methoxyestradiol (2-ME) is an endogenous metabolite of estradiol-17 β . We and others have previously shown that 2-ME has anti-angiogenic and direct cytotoxic effects on several investigated tumor cells *in vitro* and *in vivo* (10, 30). We have investigated the role of Smad7 in the apoptotic pathway induced by 2-ME in human prostate cancer cells. Our data show that Smad7 expression is required also for 2-ME-induced p38 activation and apoptosis in prostate cancer cells, as cells transfected with an anti-sense Smad7 construct or siRNA for Smad7, are protected against apoptosis. Notably, the expression of Bim, a BH3 molecule in the Bcl-2 family is regulated by Smad7 (49). The apoptotic molecular pathway induced by 2-ME will be further examined by us also in collaboration with other groups at Uppsala university lead by Xin Fu, Nils-Erik Heldin and Michael Welsh.

Gene Expression Group

Most signal transduction pathways ultimately affect gene transcription and alter the expression of specific genes. Our group is interested in how post-translational modifications regulate the activity of transcription factors. As model proteins, we have selected a number of transcription factors involved in proliferation and the regulation of cell growth.

Smad molecules - mediators of TGF- β signaling

TGF- β belongs to a superfamily of cytokines that regulate diverse biological functions, ranging from differentiation, motility and apoptosis to the inhibition of cell growth. Inappropriate regulation of TGF- β signaling has been implicated in multiple human diseases, such as fibrosis, rheumatoid arthritis and carcinogenesis. Smad proteins regulate gene expression in response to TGF- β signaling. We have demonstrated that the inhibitory Smad7 interacts with the transcriptional coactivator p300, resulting in acetylation of Smad7 on two lysine residues in its N-terminus. Acetylation or mutation

of these lysine residues stabilizes Smad7 and protects it from TGF- β -induced degradation. Furthermore, we have demonstrated that the acetylated residues in Smad7 also are targeted by ubiquitination and that acetylation of these lysine residues prevents subsequent ubiquitination. Thus, our data suggest that competition between ubiquitination and acetylation of overlapping lysine residues constitute a novel mechanism to regulate protein stability. We are currently investigating the role of deacetylases in the regulation of protein stability, using Smad7 as a model protein. The ubiquitin-proteasome pathway regulates a large number of nuclear proteins and many of these are also acetylated. Therefore, it will be of utmost importance to determine if competition between acetylation and ubiquitination is a general mechanism to regulate protein stability.

SREBPs – key regulators of lipid metabolism

Members of the SREBP family of transcription factors control cholesterol and lipid homeostasis and play important roles during adipocyte differentiation. The transcriptional activities of SREBPs are dependent on the coactivators p300 and CBP. We have found that SREBPs are acetylated by the acetyltransferase activities of these coactivators. Coexpression with p300 dramatically increases the expression of both SREBP1a and SREBP2 and this effect is dependent on the acetyltransferase activity of p300, indicating that acetylation of SREBPs regulates their stability. Indeed, acetylation or mutation of the acetylated lysine residue in SREBP1a stabilized the protein. We have demonstrated that the acetylated residue in SREBP1a is also targeted by ubiquitination. Thus, our studies define acetylation-dependent stabilization of transcription factors as a novel mechanism for coactivators to regulate gene expression. We are currently using SREBP and Smads as model proteins to characterize the complex link between protein acetylation and the ubiquitin-proteasome pathway.

We have also demonstrated that the degradation of SREBP is dependent on its transcriptional activity, *i.e.* transcriptionally active molecules are degraded rapidly whereas transcriptionally inactive molecules are stable. We are currently investigating how the balance between coactivator-mediated acetylation and transcription-dependent degradation controls the stability of transcription factors, using SREBP as a model protein. In these studies, we focus on the identification of the signals and factors involved in the ubiquitination and degradation of SREBP.

The most common treatment for elevated cholesterol levels in humans is a group of drugs called statins. These compounds block cholesterol synthesis and, therefore,

activate SREBPs. Activation of SREBP leads to an enhanced expression of the LDL receptor gene and, thereby, increased clearance of LDL from the circulation. We hypothesize that compounds that enhance the stability of SREBPs should increase the levels of transcriptionally active SREBP in cells. Such compounds could potentially be used to enhance the cholesterol-lowering activities of statins.

Characterization of a new regulator of the p53 tumor suppressor

Mutations in the p53 gene or inactivation of the p53 protein are the most frequent alterations in cancer cells and are found in more than 50% of all human cancers. We have identified a nuclear protein, YY1, that binds to p53 and inhibits its transcriptional activity (17). In addition, we have demonstrated that overexpression of YY1 blocks the phosphorylation, stabilization and transcriptional activation of p53 in response to DNA damage. Furthermore, we have demonstrated that RNAi-mediated inactivation of YY1 promotes p53-dependent apoptosis in response to DNA damage, indicating that the endogenous protein is involved in the control of p53-dependent processes. It will, therefore, be of interest to determine if YY1 also regulates the tumor suppressor function of p53 *in vivo*.

Matrix Biology Group

Our research is focused on understanding the role of hyaluronan in the microenvironment of tumor cells during tumor progression. In particular, we study how the interaction between hyaluronan and CD44 affects cell signaling.

Studies on the effects of hyaluronan on the malignant properties of tumor cells

Several tumors are enriched in hyaluronan. However, until now it has not been clear how hyaluronan produced by tumor cells or adjacent non-cancer stromal cells affect cellular behavior in the tumor-host microenvironment. In an effort to explore the importance of hyaluronan production by tumor cells for their aggressiveness, we have compared the biological properties both *in vitro* and *in vivo* of a non-hyaluronan producing colon carcinoma cell line transfected with cDNA for the hyaluronan-degrading enzyme Hyal1 with those of the same cells made to produce hyaluronan by transfection with hyaluronan synthase 2 (Has2) cDNA. Interestingly Has2 overexpression promoted tumorigenicity, whereas Hyal1 overexpression suppressed tumor development.

To further investigate the importance of hyaluronan in the process of tumorigenicity, we investigated the consequences of Has2 gene suppression by silencing the Has2 protein using specific siRNAs. In these studies we have used the breast cancer cell line HS-578 which produces high amounts of hyaluronan; these cells express the Has2 gene, and also express the hyaluronan receptor CD44. Using a specific siRNA for Has2, we could suppress Has2 mRNA levels more than 70%. However, the hyaluronan released into the media was only about 50% lower than in the mock-transfected cells. Interestingly, using real-time PCR we found that the down-regulation of Has2 mRNA level was accompanied by an increase of Has1 (about 20-fold) and Has3 (about 2-fold) transcripts. This observation suggests that compensatory mechanisms were activated in the invasive breast cancer cells analyzed, in order to maintain the hyaluronan production. Furthermore, studies on the anchorage-independent growth of the cells in soft agar revealed that Has2 siRNA transfectants formed smaller colonies than the cells transfected with control siRNA. These observations indicate that Has2 inhibition suppresses the malignant phenotype of the aggressive breast cell types investigated. Furthermore, the Has2 siRNA transfectants migrated much slower than the control transfectants, suggesting a role of hyaluronan in the motility of breast cancer cells.

Growth factor regulation of hyaluronan turnover

Studies by us and other laboratories revealed that the expression of Has and Hyal isoforms are cell specific, and that their inductions in response to external stimuli are distinct. Based on this knowledge, we aim to investigate which signaling pathways are involved in the mechanism whereby growth factors, such as PDGF-BB and TGF- β , affect hyaluronan synthesis. Recent studies on the downstream signaling pathways through which PDGF-BB stimulate hyaluronan synthesis in skin fibroblasts, using specific inhibitors, revealed that inhibition of MEK1/2 and PI3K pathways completely abolished the synthesis of hyaluronan. Similarly, the SN50 inhibitor, which inhibits translocation of the NF- κ B active complex into the nucleus, also completely suppressed hyaluronan production.

Recent studies have shown that CD44, through its interactions with extracellular matrix molecules and crosstalk with tyrosine kinase receptors, influences cellular behavior and functions as a signaling regulator. We have found that PDGF-BB signaling induces a co-localization of CD44 with PDGFR β in membrane ruffles of skin fibroblasts. Furthermore, addition of exogenous hyaluronan lead to modulation of PDGFR β activity.

Studies on the mechanisms through which hyaluronan fragments induce endothelial cell differentiation

Tissues containing high amounts of hyaluronan, such as the vitreous of the eye and articular cartilage, are avascular. However, hyaluronan fragments have been shown to stimulate new blood vessel growth. In an attempt to investigate how hyaluronan fragments affect neovascularization at the molecular level, we have compared the gene expression profile of a capillary endothelial cell line(s) in response to hyaluronan fragments (HA12) and the known angiogenic factor FGF-2, using a microarray approach.

The analysis revealed that several genes were upregulated both after stimulation with HA12 and FGF-2, but there were also several genes which were distinctly up-regulated by HA12 or FGF-2. Among the early up-regulated genes in response to HA12 was the growth regulated oncogene 1 (Gro1), which was also confirmed by examination of its mRNA expression using real-time PCR. Furthermore, blocking antibodies against the Gro1/KC protein, and against CD44, inhibited endothelial cell differentiation induced by hyaluronan fragments. Among the commonly upregulated genes, several genes which have been implicated in the regulation of angiogenesis, such as ornithine decarboxylase gene (Odc) and Oazi, were detected. Our findings demonstrate the importance of both growth factors and matrix molecules, such as HA12, in endothelial cell differentiation.

Protein Structure Group

The activities in the Protein Structure Group include peptide synthesis, radio-labelled amino acid sequencing and protein identification by mass spectrometry using a top of the line matrix-assisted-laser-desorption-ionization-time-of-flight-mass-spectrometer, Bruker Ultraflex MALDI-TOF/TOF-MS.

Peptide synthesis

Our synthesizer, a five-year-old Applied Biosystems 433A instrument, is operated with Fmoc chemistry, and produces high quality peptides. These are worked up manually and often, depending on the intended use, purified to homogeneity by reversed phase liquid chromatography. Peptides carrying various modifications, *i.e.* phosphorylations, acetylations, oxidations etc., can be produced, which is most useful for the different Groups at our Branch. All peptide products are quality controlled analyzed by the

MALDI-TOF/TOF-MS. The uses of the peptides produced vary from generation of anti-peptide antibodies, as substrates or inhibitors, or as ligands in affinity chromatography experiments.

Radiolabelled phosphopeptide mapping

Since the introduction of MALDI-TOF-MS in our Group seven years ago, classical amino acid sequence analysis by Edman degradation has gone out of fashion. However, our peptide sequencer (ABI 494 from Applied Biosystems) is still being used, after a small rebuild, for identification of the important positions of phosphorylated Tyr, Ser and Thr residues within a peptide sequence. Combining ^{32}P -labeled substrates and the shielded phosphorimager from Fuji, we are able to perform analyses of phosphorylation sites at higher sensitivities than we can achieve using mass spectrometry.

Sample preparation for mass spectrometry

Over 95% of the samples for analysis by MALDI-TOF/TOF-MS come as bands or spots from one- or two-dimensional gels followed by in-gel tryptic digestion. With Coomassie-visible material, only a few percent need to be used for analysis; with silver stained material, often all the sample must be applied, and with weak silver stain, the sample must be concentrated and desalted on a hand-made reversed phase nano column. We have also taken up a technique of Lys-modification (using an imidazol derivative), which make it possible to detect Lys containing peptides with higher sensitivity and results in higher sequence coverage.

Peptide mass fingerprinting (PMF)

Determining protein identity by PMF is a routine procedure for known proteins. After generation of a proteolytic digest and analysis by MALDI-TOF/TOF-MS, we utilize a search engine (ProFound or MASCOT, are preferred). If a clear mass spectrum, with only few contaminating mass peaks, is obtained, we usually get the protein identity with high significance. When this is not the case, a protein's identity may be established by obtaining the amino acid sequence of one or more tryptic peptides (see below). A sequence homology search is, in contrast to PMF, tolerant to amino acid substitutions.

Post Source Decay (PSD) sequencing by MALDI-TOF/TOF-MS

Fragment analysis of peptides in MALDI-TOF/TOF-MS by PSD is an established procedure for MALDI instruments equipped with reflector. The process in standard MALDIs is, however, time consuming, rather un-precise and very difficult to interpret, and therefore not often used. Our new Ultraflex, on the other hand, is a dedicated top-of-the-line instrument with a special feature for PSD called “LIFT” by Bruker, in addition to an increased precision (low ppm range). The TOF/TOF technology allows PSD spectra to be generated within seconds, so it is possible to scan through several peptides from one digest in a short time. The quick and easy PSD combines extremely well with the Chemically Assisted Fragmentation (CAF) derivatization protocol, leading to easily interpreted spectra, as they comprise a unique series of y-ions. We use CAF-PSD for identification of un-characterized species, as well as for analysis of modified peptides.

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