

ANNUAL SCIENTIFIC REPORT

Uppsala Branch

2006

Staff

Heldin, Carl-Henrik, Director

PDGF Signaling Group

Heldin, Carl-Henrik, Member, Group Head

Section of Translational Research

Hellberg, Carina, Assistant Investigator, Section Head

Hasumi, Yoko, Postdoctoral Fellow, to June

Schmees, Christian, Postdoctoral Fellow, from July

Bäckström, Gudrun, Senior Technical Assistant, to September

Sandström Leppänen, Jill, Technical Assistant (on leave of absence)

Karlsson, Susann, Ph.D. Student

Klosowska-Wardega, Agnieszka, Ph.D. Student

Section for Signal Transduction

Lennartsson, Johan, Postdoctoral Fellow, Section Head

Amagasaki, Kenichi, Postdoctoral Fellow, to September

Jurek, Alexandra, Postdoctoral Fellow, from March

Witek-Zawada, Barbara, Postdoctoral Fellow, from November

Rorsman, Charlotte, Senior Technical Assistant

Johansson, Ann-Sofi, Ph.D. Student, to April

Wardega, Piotr, Ph.D. Student

Perić, Aleksandar, Student, from September

Cytoskeletal Regulation Group

Aspenström, Pontus, Associate Investigator, Group Head

Pacholsky, Dirk, Postdoctoral Fellow

Richnau, Ninna, Postdoctoral Fellow, to August

Ruusala, Aino, Senior Technical Assistant

Fransson, Åsa, Ph.D. Student (on leave of absence from August)

Gene Targeting Group

Heuchel, Rainer, Associate Investigator, Group Head
 Krampert, Monika, Postdoctoral Fellow
 Looman, Camilla, Postdoctoral Fellow, to July
 Suzuki, Shio, Postdoctoral Fellow
 Åhgren, Aive, Senior Technical Assistant
 Zurawski, Marek, Student, from February to July

TGF- β Signaling Group

Moustakas, Aristidis, Associate Member, Group Head
 Huminiecki, Lukasz, Postdoctoral Fellow
 Thuault, Sylvie, Postdoctoral Fellow
 Morén, Anita, Senior Technical Assistant
 Bergström, Rosita, Ph.D. Student, from July
 Lönn, Peter, Ph.D. Student
 Vanlandewijck, Michael, Ph.D. Student
 Dahl, Markus, Student, from March
 Tan, E-Jean, Student, from December

Integrated Signaling Group

Souchelnytskyi, Serhiy, Associate Member, Group Head, to December
 Dubrovskaya, Anna, Postdoctoral Fellow, to December
 Lomnytska, Marta, Postdoctoral Fellow, from April to December
 Yakymovych, Ihor, Visiting Scientist
 Yakymovych, Mariya, Visiting Scientist, to December (part time)
 Bhaskaran, Nimesh, Ph.D. Student
 Cunha, Sara, Ph.D. Student, from February to December
 Filyak, Yevhen, Ph.D. Student, to April
 Woksepp, Hanna, Ph.D. Student (on leave of absence from April)
 Zakharchenko, Olena, Ph.D. Student, from May to December
 Jia, Min, Student, from August to December
 Souchelnytskyi, Nazariy, Student, to December

Apoptotic Signaling Group

Landström, Maréne, Assistant Member, Group Head
 Sorrentino, Alessandro, Postdoctoral Fellow
 Thakur, Noopur, Postdoctoral Fellow, to June, from November
 von Bülow, Verena, Postdoctoral Fellow, from October

Grimsby, Susanne, Senior Technical Assistant, to October
 Ekman, Maria, Ph.D. Student
 Marcusson, Anders, Ph.D. Student, from March

Gene Expression Group

Ericsson, Johan, Associate Member, Group Head
 Grönroos, Eva, Assistant Investigator, to December
 Bengoechea Alonso, Maria Teresa, Postdoctoral Fellow
 Kanduri, Meena, Postdoctoral Fellow
 Punga, Tanel, Postdoctoral Fellow, to April
 Lukiyanchuk, Vasyl, Technician, to November
 Lopez Egido, Juan Ramon, Ph.D. Student, from July
 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)
 Karousou, Evgenia, Postdoctoral Fellow, from October
 Skandalis, Spyridon, Postdoctoral Fellow, from September
 Bernert, Berit, Ph.D. Student, from April
 Li, Lingli, Ph.D. Student, to October
 Nishitsuka, Koichi, Ph.D. Student, from June

Protein Structure Group

Hellman, Ulf, Member, Group Head
 Engström, Ulla, Senior Technical Assistant
 Wernstedt, Christer, Senior Technical Assistant

Technical Support

Hedberg, Ulf, IT-support
 Hermansson, Emeli, Laboratory Assistant, from January (part time)
 Hermansson, Lars-Erik, Service Engineer
 Pettersson, Gullbritt, Laboratory Assistant (part time)
 Schönquist, Inger, Laboratory Assistant (part time)

Administration

Hallin, Eva, Finance and Administration Manager

Secretariat

Schiller, Ingegård, Secretary

Introduction

Malignant cells are characterized by perturbations in signaling pathways that regulate cell growth, survival, differentiation and migration. The aim of the work at the Uppsala Branch is to elucidate the molecular mechanisms involved in the regulation of these events, and to use such knowledge for the development of useful treatment regimens for cancer patients.

Important themes of our research are, as before, platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β). PDGF is a family of dimeric isoforms of A-, B-, C- and D-polypeptide chains, which exert their cellular effects by binding to α - and β -tyrosine kinase receptors. 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 molecular mechanisms of signal transduction via PDGF receptors, and to explore the clinical utility of PDGF antagonists.

TGF- β family members act via heteromeric complexes of type I and type II serine/threonine kinase receptors. They inhibit the growth of most cell types and also affect apoptosis, matrix production and cell differentiation. TGF- β has a complicated role in cancer. Initially it is a tumor suppressor through its ability to inhibit cell growth and stimulate apoptosis of cells. However, at later stages of tumor progression, TGF- β promotes tumorigenesis 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 goal of our work is to explore whether selective TGF- β antagonists can be developed which inhibits the tumor promoting effects of TGF- β , while leaving the tumor suppressive effects unperturbed, and to investigate the possible clinical utility of such antagonists.

Our Branch is located at the Biomedical Center in Uppsala, using laboratory space provided by the University of Uppsala. The work has during 2006 been performed in 9 different groups, working on different aspects of signal transduction. The groups complement each other with regard to technical skills. Thus, in addition to conventional cell and molecular biology methods, we have access to technology in mass spectrometry and proteomics, micro array analyses, advanced microscopy and mouse genetics.

In January 2007, the Head of the Integrated Signaling Group, Serhiy Souchelnytskyi, will leave our Branch with his group for a professorship at the Karolinska Institute in Stockholm. We wish Serhiy and the members of his group the best of luck in their future work, and are looking forward to future interactions with them.

C.-H. Heldin

PDGF Signaling Group

Background

Tyrosine phosphorylation of proteins is an important posttranslational modification of components of signal transduction pathways that regulate cell growth, survival and death, as well as adhesion, migration and differentiation. Both protein tyrosine kinases and protein tyrosine phosphatases control cellular phosphotyrosine levels. Amplification of genes encoding tyrosine kinase receptors have been described in several forms of tumors. The increased expression of both ligand and receptor within a tumor suggests that tumor growth is at least partly due to autocrine receptor activation. It has also been suggested that growth factors synthesized by activated stromal cells participate in the growth and survival of tumor cells. In addition, tyrosine kinase receptors are involved in the signals inducing tumor vascularization, a process that is necessary for both growth and metastasis of tumors. We study platelet-derived growth factor (PDGF), an important growth regulatory factor for connective tissue cells and other cell types, which acts by binding to α - and β - tyrosine kinase receptors.

Section for Signal Transduction

The aim of the work performed in the PDGF Signal Transduction Section is to elucidate the molecular mechanisms whereby PDGF affects cell proliferation, migration, differentiation and survival, and to elucidate if perturbations of these pathways promote tumorigenesis.

PDGF-mediated chemotaxis

To shed light on the complex process of cell migration, we investigated the effect of a panel of low molecular weight kinase inhibitors on PDGF-mediated chemotaxis. From this analysis we found that the JNK MAP-kinase has a central role in cell migration (1). Studies on the molecular mechanism behind this observation revealed that JNK is activated in a phosphatidylinositol-3'-kinase (PI3-kinase)-dependent manner downstream of the PDGF β -receptor (PDGFR β) in fibroblasts. PI3-kinase has been shown by numerous investigators to be important for cell migration. Furthermore, we found that JNK localized to the actin dense membrane ruffles, in addition to the nucleus, after PDGF stimulation, consistent with a direct role in cell migration. Interfering with JNK activity using various approaches, strongly inhibited chemotaxis,

both in primary fibroblasts and in two glioblastoma cell lines. In conclusion, JNK is a critical component in the cell migration machinery employed by PDGFR β .

Function of the activation loop tyrosine residue 857 in PDGFR β

Tyrosine residue 857 (Y857) in the activation loop in the PDGFR β is believed to be involved in the regulation of the kinase activity. Consistently, we observed that the enzymatic activity of the mutant receptor *in vitro* was significantly reduced. However, when we expressed the tyrosine to phenylalanine mutant (Y857F) in PAE cells we observed a robust tyrosine phosphorylation of the mutant receptor comparable to that of the wild-type receptor (Wardega *et al.*, unpublished data). Thus, it appears that some compensatory mechanism *in vivo* mediates tyrosine phosphorylation of the kinase crippled Y857F receptor. Further work will aim to elucidate whether another kinase is responsible for this compensation. Interestingly, the Y857F mutant receptor was found to induce migration towards PDGF to the same extent as the wild-type receptor, but had no mitogenic ability. Thus, Y857F mutation separates the signaling pathways necessary for proliferation from those needed for migration. The molecular mechanism for this difference is under investigation.

Downregulation and sorting of PDGFR β

Recently it has become clear that receptor endocytosis and sorting have large impact on signal transduction in terms of controlling the half-life and intensity of the signal, but also through correct localization of the activated receptor complex. We are studying two proteins involved in these processes, *i.e.* the adaptor proteins Alix and Stam. We found that Alix affects downregulation of PDGFR β by modulating the interaction between the ubiquitin ligase c-Cbl and the receptor (20). To elucidate the role of Stam in sorting of PDGFR β , we are using mouse embryonic fibroblasts lacking Stam proteins. Our preliminary results indicate that Stam may be a key molecule regulating the ubiquitination level of PDGFR β .

The role of MAP kinase phosphatase 3 in PDGF-induced activation of MAP kinase

MAP kinase phosphatases (MKPs) are dual specificity phosphatases that dephosphorylate and thereby inactivate MAP kinases. We observed that the amount of MKP3, which is highly specific for Erk, was affected by PDGF stimulation (Jurek *et al.*, unpublished observations). The kinetics of MKP3 degradation and synthesis correspond in a reciprocal manner to the phosphorylation of Erk. Moreover, differences in MKP3

levels after activation of PDGFR α or β may explain differences in the kinetics of Erk phosphorylation induced by these receptor isoforms. The PDGF-induced degradation of MKP3 required a functional proteasome, and in fact, inhibition of the proteasome robustly blunted the ability of PDGF to induce Erk phosphorylation. Conversely, downregulation of MKP3 lead to an enhanced phosphorylation of Erk. This suggests that MKP3 is a key regulator of PDGF-induced Erk activation.

Purification and characterization of proteins interacting with the PDGFRs

The binding of SH2-domain-containing proteins relies on relatively short linear amino acid sequences. This makes it possible to synthesize peptides corresponding to the different autophosphorylation sites of the PDGFRs and to use these to find interaction partners. Our goal is to perform a screen for interacting proteins using peptides corresponding to all known autophosphorylation sites in the PDGFRs (11 in PDGFR β and 10 in PDGFR α). So far we have used a peptide with a sequence corresponding to the region around tyrosine residue 771 in PDGFR β , which is known to bind RasGAP. As expected, we were able to identify RasGAP as an interacting partner. In addition, we also found GAP-SH3 domain Binding Protein (G3BP), which probably interact indirectly through RasGAP. Interestingly, G3BP has been proposed to have nuclease activity and may link PDGFR signaling to RNA metabolism.

Section for Translational Research

The research in the Translational Research section is focused on the following two lines:

- PDGF receptors as cancer drug targets
- Termination of PDGF β -receptor signal transduction

PDGF receptors as cancer drug targets

PDGF receptor signaling is implicated in various cancer associated processes, including autocrine stimulation of growth, stimulation of tumor fibroblasts and promotion of tumor angiogenesis. During angiogenesis, PDGF receptor signaling is important for the recruitment of pericytes to the newly formed tumor vessels, a process needed for the stabilization and function of the newly formed vasculature. The presence of pericytes on tumor vessels has been proposed to protect endothelial cells from anti-angiogenic therapy targeting the vascular endothelial cell growth factor (VEGF) pathway. Thus, PDGF receptors on pericytes could provide a novel target for anti-angiogenic therapy.

To investigate this possibility, we used the B16 mouse melanoma tumor model where PDGF-dependent pericyte recruitment contributes to tumor growth. Treatment of B16 tumors with a combination of PDGF and VEGF receptor inhibitors, but not single agent treatment, significantly reduced tumor growth (Hasumi *et al.*, submitted for publication). This effect was more pronounced in the fast-growing tumors with a pericyte-rich vasculature, and coincided with a reduction in immature pericytes. Pericytes expressing the differentiation marker desmin were unaffected, identifying these cells as a subset of pericytes resistant to antiangiogenic therapy. The growth inhibition was associated with vascular remodeling, an increase in tumor cell apoptosis and a decrease in proliferation. This suggests that PDGF receptor antagonists could potentiate anti-angiogenic treatment in mature tumors, which are relatively insensitive to VEGF receptor inhibitors.

In experimental models, inhibition of PDGF receptors on stromal cells increases tumor uptake of low molecular weight chemotherapy drugs through a decrease of tumor interstitial fluid pressure (IFP). VEGF receptor inhibitors also lowers tumor IFP, and we are currently investigating the effects of the combination of PDGF and VEGF receptor inhibitors on IFP of Kat4 thyroid carcinomas. Both agents lowered the tumor IFP when given as monotherapies, with increasing effects over a four day treatment period (Kłosowska-Wardęga *et al.*, unpublished observations). Surprisingly, combination therapy given for four days displayed the same IFP as vehicle treated tumors. However, when PDGF receptor inhibitor was given for four days and the VEGF receptor inhibitor for the last two days of therapy, the tumor IFP was further reduced compared to the monotherapies, clearly demonstrating the need of identifying the optimal treatment regimes for combination therapies. In addition, we are continuing the studies on the mechanism(s) underlying the role of PDGF in the regulation of tumor IFP, focusing on the roles of the PI3-kinase and $\beta 3$ integrins. 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.

Ongoing clinical studies with targeted therapies 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. As a consequence of the discovery of PDGF-CC and -DD, we have also generated neutralizing PDGF-CC antisera to evaluate the role of PDGF-CC in disease processes.

Termination of PDGF β -receptor signal transduction

Following ligand stimulation, the PDGF β -receptor signaling is terminated by dephosphorylation of the receptor autophosphorylation sites in parallel with receptor internalization and subsequent degradation. We have identified the T-cell protein tyrosine phosphatase as a site-selective regulator of PDGF β -receptor phosphorylation. The screening for tyrosine phosphatases that are regulating PDGF β -receptor phosphorylation and signal transduction is being continued, with a current focus on the LAR phosphatase and PTP-L1.

The increased PDGF β -receptor phosphorylation observed in 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 (17). The induction of recycling is specific for the PDGF β -receptor, since neither the PDGF α -receptor nor the IGF-1 receptor display increased recycling in T-cell phosphatase $-/-$ fibroblasts. Since PDGF β -receptors do not normally recycle, these fibroblasts provide a unique model system for studying the regulation of the intracellular trafficking of the PDGF β -receptor. We are currently investigating the molecular mechanisms whereby the T-cell phosphatase regulates PDGF β -receptor trafficking.

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 GTPase family consists of 23 members in human cells which are key regulators of cell morphogenesis and cell migration. We have put efforts into the identification of the atypical Rho GTPases Miro-1-2, RhoBTB-1-3 and Wrch-1. It was shown that Miro binds mitochondria and has a role distinct from the classical Rho GTPases. Miro functions as a link between the mitochondria and the microtubular transport machinery by binding to the kinesin-interacting proteins OIP106 and GRIF1 (10).

Regulators and effectors of Rho GTPases

We have previously identified several proteins, which bind to the activated Rho GTPases and affect their biological activities. One such protein, the Cdc42-binding protein 4 (CIP4) was shown to have a role in the Cdc42-dependent regulation of the actin filament system (48). Interestingly, the Dishevelled-associated activator of morphogenesis 1 (DAAM1) was found to be a CIP4-binding protein. We propose a model in which DAAM1 collaborate with CIP4, Cdc42, RhoA and Src in the regulation of the actin filament system (3).

The Verprolins

The verprolins are pivotal modulators of signaling mediated by the WASP family of proteins. The Cdc42-binding protein WASP was originally identified as the gene defective in the severe X-linked immunodeficiency disorder Wiskott-Aldrich syndrome (WAS). The members of this family of proteins, which also includes N-WASP and Scar/WAVE 1-3, have been shown to be critical regulators of actin. The verprolins function to bring together SH3 domain-containing proteins, such as Nck and cortactin, with the WASP-dependent actin polymerization machinery.

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. Phil Soriano's group in Seattle, we generated signaling pathway-restricted mice. In these mice, PDGFR β carried point mutations, such that they were unable to bind and activate phosphatidylinositol-3'-kinase (PI3-kinase), or PI3-kinase and phospholipase C- γ (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 (D849N). Analogous gain of function 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. The D849N mutation in PDGFR β was found to confer 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. The GTPase-activating protein for Ras (RasGAP), a negative regulator of the Ras mitogenic pathway, displayed a delayed binding to the mutant receptor. Moreover, we have observed enhanced ligand-independent ERK1/2 activation and an increased proliferation of mutant cells. The p85 regulatory subunit of PI3-kinase is constitutively associated with the mutant receptor and this ligand-independent activation of the PI3-kinase 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 with oncogenic predisposition.

A hitherto unknown function of PDGFR β -signaling has been unraveled in the lab of Dr. Lena Claesson-Welsh (29). In an *in vitro* assay for differentiating embryonic stem cells, the activation of PDGFR β , which was found to be expressed on early hemangiopoietic precursor cells (hemangioblasts), led to developmental commitment towards the endothelial lineage at the expense of the hematopoietic lineage. By examining yolk sacs from mice carrying the activating D849N mutation in the PDGFR β , we could demonstrate that this is also true *in vivo* (29).

Recently, an activation loop mutation in PDGFR α was reported to be responsible for a certain percentage of gastrointestinal stromal tumors in human. We have generated embryonic stem (ES) cells with the identical mutation in the PDGFR β (D849V). In sharp contrast to the previously generated D849N mutant mice, which are fully viable, targeted ES cells carrying the D849V mutant PDGFR β do not generate viable chimeras. In addition, chimeric placentas display a largely disorganized labyrinthine layer, unable to support nutrient waste exchange between the maternal and fetal vascular systems (Looman *et al.*, submitted for publication). 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 *in vitro* (assay for differentiating ES cells) and *in vivo* (teratoma generated from embryonic stem cells injected into nude mice). This effect could be explained by increased VEGF-A expression in differentiating embryonic

stem cells. In addition, we found evidence that also the D849V mutant PDGFR β resulted in increased endothelial/perivascular lineage commitment of hemangioprecursors and thus contributed to the increased *in vitro* and *in vivo* vasulogenic/angiogenic effects observed (Magnusson *et al.*, submitted for publication).

Regulation and *in vivo* function of Smad7

The transforming growth factor- β (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. A particular member of the Smad family, namely Smad7, is induced by TGF- β itself and cause downregulation of TGF- β signaling, suggesting an auto-regulatory feedback mechanism.

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. Mutant animals showed severely reduced viability depending on the mouse strain background. On C57Bl/6 background homozygous animals die shortly after birth, whereas mutant mice are viable on CD-1 background. In general, we found that mutant mice were smaller than wild-type mice (24).

In line with the fact that TGF- β is 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 (24). In addition, we found skeletal anomalies, such as ectopic bone formation and a homeotic transformation of a cervical vertebra. Since the Smad7 mutation results in increased TGF- β signaling, these mice are ideally suited for disease models, where increased TGF- β signaling has an important influence on disease etiology and severity of disease as for instance cancerogenesis and fibroses.

TGF- β Signaling Group

The TGF- β Signaling Group centers its focus on epithelial cell biology and investigates how TGF- β regulates epithelial cell growth, differentiation and cellular processes of tumor invasiveness and metastasis.

Regulatory mechanisms in the TGF- β /Smad signaling pathway

TGF- β signals via type I and type II serine/threonine kinase receptors, which activate intracellular effectors that include Smad proteins and alternative signal transducers (50). The TGF- β type I receptor phosphorylates the R-Smads (Smad2 and Smad3), which form complexes with the Co-Smad (Smad4) and translocate to the nucleus, regulate gene expression and eventually exit from the nucleus and undergo ubiquitin-mediated degradation. We described a new mechanism in which Smad3 nuclear export is mediated by exportin-4 and the Ran GTPase (19).

The Co-Smad, Smad4, is regulated by ubiquitination. Smad4 in normal cells can be mono-ubiquitinated, while Smad4 carrying point mutations in various human tumor cells, undergoes proteasomal degradation. We study proteins that recognize this post-translational modification in Smad4 and attempt to understand the functional role of such protein complexes. In addition, we analyze the functional role of novel Smad4-interacting proteins that are implicated in the mechanism of regulation of the stability of this major signal transducer of TGF- β . Finally, via large scale gene expression analysis we identified a new TGF- β -responsive gene, whose serine/threonine kinase product regulates downregulation of the TGF- β receptor after ligand binding (Kowanetz *et al.*, submitted for publication). The latter two studies draw our focus towards the crosstalk between TGF- β /Smad signaling and the tumor suppressor, master kinase, LKB1. Our current evidence supports a mechanism whereby LKB1 negatively regulates TGF- β signaling components in human epithelial cells.

Regulation of epithelial cell proliferation and epithelial-mesenchymal transition by TGF- β

The TGF- β pathway acts as a tumor suppressor based on the ability of TGF- β to inhibit cell proliferation and induce apoptosis (67). TGF- β also promotes carcinoma cell invasiveness and metastasis, at least in part by inducing a differentiation switch called epithelial-mesenchymal transition (EMT) (55, 67, 68). In addition, TGF- β regulates the function of cancer-associated fibroblasts in the tumor stroma, thus modulating the tumor

suppressor and pro-metastatic actions of this pathway (65). An important gene target of the pathway involved in the tumor suppressor activities of TGF- β is the cell cycle inhibitor p21. Based on several previous transcriptomic screens, which led to new, refined analytical tools (2), we identified the homeobox transcription factor Meox2, which is responsible for the sustained and prolonged induction of p21 gene expression by TGF- β (45). In addition, we found that TGF- β regulates expression of Notch pathway ligands and receptors. Notch regulation by TGF- β is important during epithelial cell cycle arrest, as inhibition of Notch signaling abrogates growth inhibition by TGF- β (42). This crosstalk mechanism depends on cooperative regulation of p21 gene expression by the TGF- β and Notch pathways.

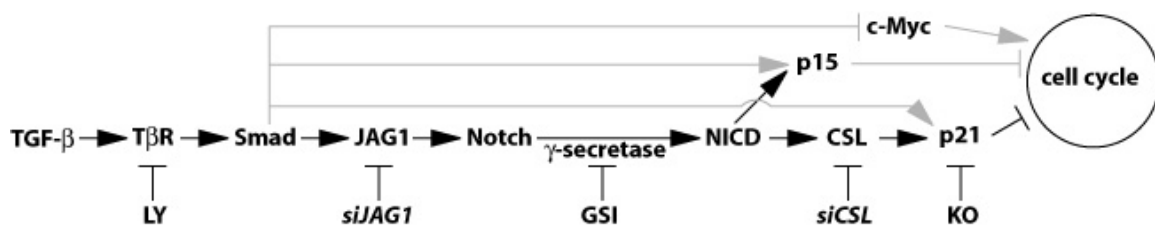


Fig. 1. A new signaling pathway downstream of TGF- β that mediates epithelial cytotaxis involves Notch receptor activation by its ligand Jagged1 and its downstream transcriptional effector CSL. Previously established pathways of Smad signaling are indicated with grey arrows. Inhibitors of the pathway are indicated below.

In an effort to understand the process of EMT deeper, we analyzed the effects of long-term exposure of mammary epithelial cells to TGF- β on proliferation and EMT (Gal *et al.*, submitted for publication). We identified new mechanisms of modulation of various non-Smad signaling effectors and uncovered an enhanced tumorigenic potential of the chronically exposed mammary cells. Furthermore, we established that the transcription factor of the high mobility group (HMG) family, HMGA2, induces EMT by regulating the expression of many other transcriptional regulators of EMT, such as Twist, Snail and Slug (33). We currently dissect the transcriptional mechanisms by which HMGA2 mediates its effects on various regulators of EMT. In addition, we analyze the function of several other immediate-early gene targets of the TGF- β pathway and their involvement in the process of EMT. We have therefore unraveled a complex regulatory network that defines the cytotstatic and EMT programs of epithelial cells in response to TGF- β . Our findings have direct relevance for the mechanisms by which TGF- β acts as a tumor suppressor or a pro-metastatic factor and direct our research towards novel therapeutic approaches.

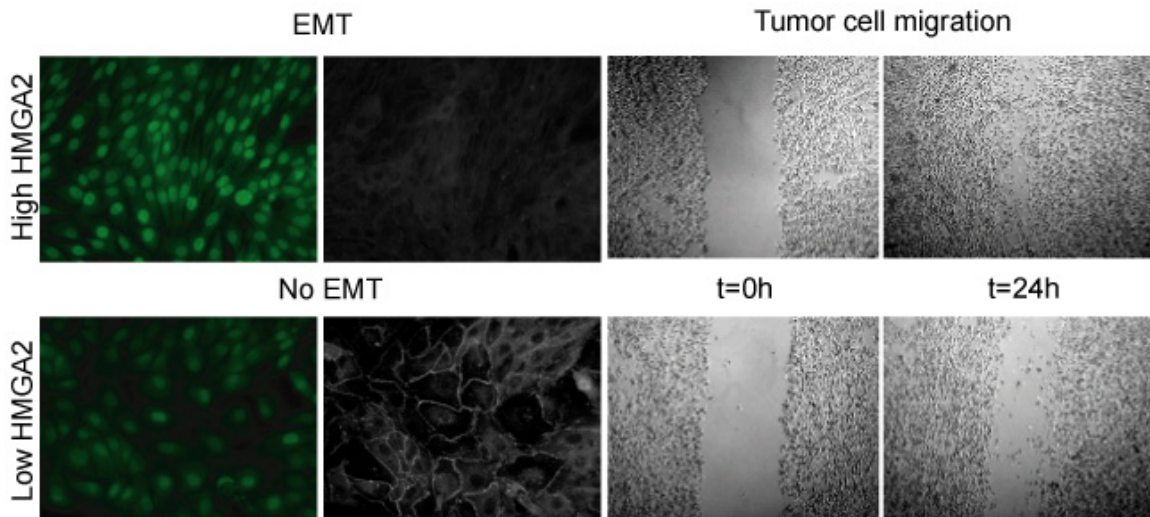


Fig. 2. Nuclear HMGA2 staining (green) correlates with EMT in mammary epithelial cells (High HMGA2, top), where loss of the tight junction marker ZO-1 is illustrated. The high HMGA2 nuclear content also correlates with mammary carcinoma cell migration in vitro. When endogenous HMGA2 is depleted using RNAi vectors (Low HMGA2, bottom), no EMT is observed, the tight junctions and epithelial polarity is preserved, and a significant reduction in carcinoma cell migration is scored.

Mathematical modeling of TGF- β signaling

Using bioinformatics and mathematical modeling we analyze the kinetics of TGF- β signaling and downstream target gene regulation in epithelial cells. We combine Boolean logic and ordinary differential equation analysis in order to make new predictions of nodal regulatory events in this pathway. In addition, we analyze the evolutionary origins of the TGF- β superfamily pathways based on new algorithms and sequence data of all available metazoan genomes. More critical for cancer research, using sophisticated meta-analysis of large scale transcriptomic data we attempt to model mathematically the tumor suppressor versus pro-metastatic actions of TGF- β in order to understand the factors behind this binary decision logic of the pathway.

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. We aim at identifying proteins which can be targeted for treatment and monitoring of human breast cancer. We also participate in development of inhibitors of TGF- β receptor kinase(s), which are to be used in treatment of cancer.

Proteomics and systems biology in studies of carcinogenesis

We perform proteome profiling of human breast cancer, using human breast epithelial cells, tumorigenesis studies with mice, and clinical samples of human breast tumors and non-carcinous samples. Protein expression maps (PEMs) of 184A, MCF10A and MCF7 cells are generated, and studies are ongoing to explore the role of observed differential protein expression in cell proliferation and apoptosis (Woksepp *et al.*, work in progress; Bhaskaran *et al.*, work in progress). We are also working with conditionally immortalized primary human breast epithelial cells. We identified a number of proteins previously not known to be involved in immortalization of cells (Min Jia *et al.*, work in progress). We have generated human breast epithelial cells of various degree of transformation with regard to enhanced proliferation, requirement of substrate and responsiveness to estrogen, epidermal growth factor (EGF) and TGF- β (Cunha *et al.*, work in progress). PEMs of samples from invasive ductal carcinomas and invasive lobular carcinomas are under construction, and will be compared to PEMs of non-carcinous human breast tissue (Zakharchenko *et al.*, work in progress; Lomnytska *et al.*, work in progress).

Identified proteins are characterized using tools for deposition, clustering analysis and exploration of functional connections and dependencies between identified proteins and cancer-related activities. The emphasis in extraction of hubs and dependencies are made on regulators of cell proliferation, apoptosis and DNA damage repair (Bhaskaran, Woksepp *et al.*, manuscript in preparation). In 2006, we introduced novel tools for a large-scale analysis and computing of models developed in our group.

Proteomics and TGF- β signaling

TGF- β is a potent regulator of carcinogenesis. It acts as an inhibitor of tumorigenesis at early stages, and promotes tumor growth at later stages of cancer. We use TGF- β signaling as a model to test systemic approaches to studies of regulatory processes in cells.

O-glycosylation is a dynamic post-translational modification which has been observed in many regulatory proteins, but has not been studied in the context of TGF- β signaling. To explore the effect of TGF- β 1 on protein O-glycosylation in human breast epithelial cells, we performed analyses of proteins which were affinity purified with *Helix pomatia* agglutinin (HPA). Using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI

TOF MS), we identified 21 HPA-precipitated proteins, which were affected by treatment of cells with TGF- β 1 (15). Among these proteins, regulators of cell survival, apoptosis, trafficking and RNA processing were identified. We found that TGF- β 1 inhibited the appearance of cell death-inducing DFF-like effector A (CIDE-A) in 2-D gels with HPA-precipitated proteins. CIDE-A is a cell death activator which promotes DNA fragmentation. We observed that TGF- β 1 did not affect expression of CIDE-A, but inhibited its glycosylation. We found that de-glycosylation of CIDE-A correlated with enhanced nuclear export of the protein, and that high level of non-glycosylated CIDE-A inhibited TGF- β 1-dependent cell death. Thus, inhibition of the glycosylation of CIDE-A may be a mechanism to protect cells from apoptosis (15).

Type I TGF- β receptor (T β RI) is the key receptor for initiation of intracellular signaling by TGF- β . We have reported proteomics-based identification of proteins which form a complex with T β RI. Using 2D-GE and MALDI TOF mass spectrometry, we identified 16 proteins which specifically interacted with a GST-fused T β RI Thr204Asp construct with constitutively active serine/threonine kinase. We confirmed interactions of the receptor with cAMP regulated guanine nucleotide exchange factor 1 (Epac1) (38). TGF- β 1-induced C-terminal phosphorylation of Smad2 was inhibited *in vivo* and *in vitro* in the presence of Epac1. Epac1 inhibited also TGF- β 1/T β RI-dependent transcriptional activation. We observed that expression of Epac1 counteracted TGF- β /T β RI-dependent decrease of cell adhesion and TGF- β /T β RI-induced stimulation of cell migration (38).

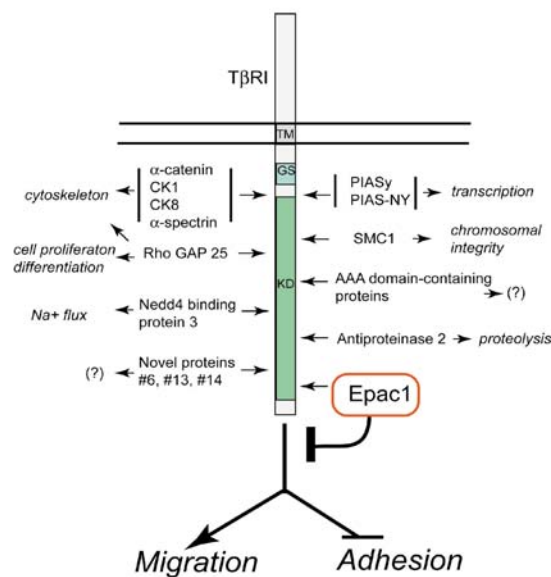


Fig. 3. T β RI-interacting proteins may provide links to a number of activities which can be triggered on the level of TGF β receptors (38).

We explored functional importance of the interaction between BMPRII and c-Kit (11). This introduced a new concept of direct interaction between serine/threonine and tyrosine kinase receptors.

We showed that anti-cancer drug doxorubicin inhibited early events in TGF- β signaling, *i.e.* Smad3-dependent transcriptional activation (Filyak *et al.*, manuscript submitted). This indicates a cross-talk between TGF- β signaling and a frequently used anti-cancer drug.

Inhibitors of TGF- β signaling

We 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, diabetes, nephrosis and fibrotic conditions. Previously we reported that type I TGF- β receptor kinase can be inhibited by low molecular weight compounds which interfere with binding of ATP or substrate to the kinase. We continue this work in collaboration with an industrial partner. We have developed assays for screening of chemical libraries for TGF- β receptor specific inhibitors. We also contribute to development of tests to monitor efficiency of potential drugs in animal tests and in clinical trials (Yakymovych M. *et al.*, work in progress).

Search for cancer markers using proteomics

Resolution power and sensitivity of protein identification by proteomics provide possibilities for successful search for markers which can be used for prediction, detection and monitoring of cancer. We have identified 3 proteins whose expression in plasma of breast and ovarian cancer patients correlated with stage and aggressiveness of the disease (25, 58). We continue this work aiming to development of a kit which will be used at sites of primary health care, thus initiating clinical use of our findings.

Apoptotic Signaling Group

TGF- β inhibits proliferation and induces apoptosis in most normal cell types, however, during tumor progression TGF- β instead promotes tumor growth due to its effects on transdifferentiation of cells, angiogenesis and immuno-surveillance. Smad7 is a target gene for TGF- β and we have demonstrated that Smad7 is required for TGF- β -induced apoptosis in prostate cancer cells as well as in human keratinocytes. Furthermore, we have reported that Smad7 facilitates the TGF- β -activating kinase 1 (TAK1), mitogen

activated protein kinase kinase 3 (MKK3) and p38 mitogen activated protein (MAP) kinase pathway, presumably by acting as an adaptor protein bringing the kinases close to each other. The aim of our work is to in detail investigate the molecular mechanisms for the activation by TGF- β of the TAK1 – p38 MAPK pathway. We also continue to investigate the targets for Smad7 and p38 in the TGF- β -induced apoptotic signaling pathway.

By which molecular mechanisms do Smad7 and p38 regulate apoptosis in prostate cancer cells?

We previously discovered that Smad7 acts as an adaptor protein in the TAK1-MKK3-p38 MAPK pathway. We are currently, in more detail, investigating how TGF- β and Smad7 can activate this pathway (Sorrentino, Grimsby *et al.*, submitted for publication).

Interestingly, Smad7 is predominantly localized in the nucleus of resting 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. After longer time-periods of TGF- β -stimulation of cells, Smad7 accumulates in the nucleus again. We have identified the tumor suppressor p53 as one target downstream of the active Smad7-p38 complex (36). We found that Smad7, as well as p38 MAPK, is required for TGF- β -induced phosphorylation of p53 on Ser15 in a number of human epithelial cells preceding apoptosis. Interestingly, Smad7 was also required for TGF- β -induced activation of the ataxia teleangiectasia mutated (ATM) kinase. As we observed that Smad7 interacts with p53, as well as the ATM kinase which is known to activate p53, we suggest that Smad7 can act as an adaptor protein in this pathway (36). We also observed that Smad7 might play an important role to prevent genomic instability in prostate cancer cells. We therefore plan to further investigate this novel role for Smad7 in coming *in vivo* experiments, by investigations of the expression of Smad7 in human prostate cancer tissues as well as the role for Smad7 in tumor growth in prostate cancer cells where the expression of Smad7 has been silenced with siRNA.

Does Smad7 act in a cross-talk between TGF- β and Wnt signaling?

Signaling molecules downstream of TGF- β and Wnt receptors regulate both 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. Furthermore, by the use of siRNA and anti-sense techniques, we have shown 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 regulates the activity of Akt and glycogen synthetase kinase-3 β (GSK-3) which in turns leads to the stabilization of β -catenin. We are now exploring by which molecular mechanisms TGF- β regulates the activity of Akt and GSK-3 β (Ekman, Lee, *et al.*, manuscript in preparation). We will also continue to further examine the underlying molecular mechanisms by which Smad7 and p38 MAPK affect the components in the Wnt-signaling pathway.

In collaboration with Dr. Aspenström, Cytoskeleton Regulation Group LICR, Uppsala, we have found that Smad7 expression is required for TGF- β -mediated cyto-skeletal regulation which occurs mainly via the small GTP-ase Cdc42 . We have investigated the role of Smad7 in TGF- β -dependent regulation of cytoskeletal processes, such as migration of cells. We have found that Smad7 interacts with components in the Wnt-signaling pathway and is crucial for TGF- β -induced migration of prostate cancer cells (Ekman *et al.*, manuscript in preparation).

The role of Smad7 in apoptosis induced by 2-methoxyestradiol (2-ME)

2-ME is an endogenous metabolite of estradiol-17 β . We and others have previously shown that 2-ME has both anti-angiogenic and direct cytotoxic effects on several investigated tumor cells *in vitro* and *in vivo* (30). We have investigated the role of Smad7 in the apoptotic pathway induced by 2-ME in human prostate cancer cells. Our data shows 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. 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 Drs. Nils-Erik Heldin and Michael Welsh.

Gene Expression Group

Our group is interested in how post-translational modifications regulate the activity of transcription factors. As model proteins, we have selected certain transcription factors involved in the regulation of cell growth and proliferation.

SMADs - Mediators of transforming growth factor- 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. The regulated expression of specific genes in response to TGF- β signaling is controlled, at least in part, by specific Smad proteins.

We previously demonstrated that Smad7 is acetylated and deacetylated in a dynamic fashion. We recently found that both Smad2 and Smad3 are acetylated by the coactivators p300 and CBP in a TGF- β -dependent manner (31). The major acetylated residue was mapped to lysine 19 (Lys19) in the MH1 domain of both the long and short isoform of Smad2. By generating acetyl-Lys19-specific antibodies, we could demonstrate that endogenous Smad2 becomes acetylated on this residue in response to TGF- β signaling. Acetylation of the short isoform of Smad2 improved its DNA binding activity *in vitro* and enhanced its association with target promoters *in vivo*, thereby augmenting its transcriptional activity. Acetylation of Lys19 also enhanced the DNA binding activity of Smad3. Our data indicated that the acetylation of Lys19 induces a conformational change in the MH1 domain of the short isoform of Smad2, thereby making its DNA binding domain accessible for interactions with DNA. Thus, coactivator-mediated acetylation of receptor-activated Smad molecules could represent a novel way to regulate TGF- β signaling.

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. 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.

Transcriptionally active SREBP molecules are rapidly degraded by the ubiquitin-proteasome pathway. The ubiquitin ligase Fbw7 has been shown to control several proteins critical for cell cycle control, and is inactivated in cancers of the breast, endometrium, ovary and colon. We previously demonstrated that Fbw7 enhances the ubiquitination and degradation of SREBP following GSK-3-mediated phosphorylation of two specific Ser and Thr residues in SREBP, T426 and S430. We have now found that

the GSK-3 β -dependent phosphorylation of these residues in SREBP1 is enhanced in response to DNA binding (28). DNA binding enhanced the direct interaction between the C-terminal domain of SREBP1 and GSK-3 β . Accordingly, we could demonstrate that GSK-3 β was recruited to the promoters of SREBP target genes *in vivo*. As a result of the phosphorylation of Thr426 and Ser430 in response to DNA binding, Fbw7 was recruited to SREBP molecules associated with target promoters. Using a reconstituted ubiquitination system, we could demonstrate that Fbw7-mediated ubiquitination of SREBP1 was dependent on its DNA binding activity. Thus, DNA binding could provide a link between the phosphorylation, ubiquitination and degradation of active transcription factors. Fbw7 and its interaction with SREBPs may be attractive targets for developing new cholesterol-lowering therapies for the fight against cardiovascular disease.

The SREBP family of transcription factors regulates genes involved in the synthesis of lipids, which are required for the formation of cell membranes. We recently found that SREBP-mediated transcription is regulated during the cell cycle as a result of specific modifications of the SREBP proteins. We found that the mature forms of SREBP1a and SREBP1c are hyperphosphorylated in mitotic cells, giving rise to a phosphoepitope recognized by the mitotic protein monoclonal-2 (MPM-2) antibody. We also found that mature SREBP1 was stabilized in a phosphorylation-dependent manner during mitosis. We have now mapped the major MPM-2 epitope to a serine residue, S439, in the C terminus of mature SREBP1 (4). Using phosphorylation-specific antibodies, we could demonstrate that endogenous SREBP1 is phosphorylated on S439 during mitosis. Mature SREBP1 interacts with the Cdk1/cyclin B complex in mitotic cells and we found that Cdk1 phosphorylates S439, both *in vitro* and *in vivo*. Taken together, our results suggested that Cdk1-mediated phosphorylation of S439 stabilizes mature SREBP1 during mitosis, thereby preserving a critical pool of active transcription factors to support lipid synthesis. Thus, our work suggests that SREBP1 may provide a link between lipid synthesis, proliferation and cell growth. This hypothesis was supported by our observation that siRNA-mediated inactivation of SREBP1 arrested cells in the G1 phase of the cell cycle, thereby attenuating cell growth. Altogether, our data support the hypothesis that deregulation of lipid synthesis could facilitate the growth and proliferation of cancer cells.

Matrix Biology Group

Our research is focused on understanding how the stromal microenvironment influences the proliferative and invasive behavior of malignant cells. In particular, we investigate the role of hyaluronan and its receptor CD44 in the progression of breast tumors, using

3D experimental models in order to recapitulate the *in vivo* 3D milieu. Furthermore, we investigate how hyaluronan-CD44 and growth factor receptors, including PDGF and TGF- β receptors, cross-talk and convey signals into the cells.

Studies on the role of hyaluronan-CD44 interactions in regulating the malignant properties of tumor cells

Stromal hyaluronan levels are regulated by the balanced activity of hyaluronan synthesizing (HAS) and hyaluronan degrading (HYAL) enzymes. Hyaluronan-CD44 interactions in normal tissues play important roles in determining tissue architecture and homeostasis. Upon cell transformation, hyaluronan synthesis is often increased resulting in its aberrant accumulation in various types of human tumors. We have demonstrated that there is a close correlation between excessive hyaluronan production and the malignant phenotype of tumor cells. Using a colon carcinoma model, we have demonstrated that expression of *HAS2* gene enhances tumor growth, whereas expression of *HYAL1* gene delays tumor development and frequency.

To investigate further the importance of *HAS2* in the maintenance of the malignant and invasive phenotype of the Hs578T breast cancer cells, we investigated the consequences of *HAS2* gene suppression by specific silencing using siRNAs. Interestingly, suppression of *HAS2* slows down the growth as well as the migratory and invasive capacities of Hs578T cells (41). Our data show that *HAS2* expression and endogenously synthesized hyaluronan promote breast cancer progression. Furthermore, *HAS2* cooperates with CD44 and *HYAL2*, and most likely these molecules are crucial for the aggressive character of breast tumor cells. Notably, breast cancer cells exhibiting low aggressive phenotype express very low amounts of *HAS2*, but relatively high levels of the *HAS3* isoform and even higher levels of *HYAL2* mRNA (41). More extensive studies of hyaluronan, HAS and HYALs as well as CD44 is presently performed in order to elucidate their role in the progression to the malignant phenotype of breast cancer cells. For this purpose, we have developed multicellular 3D models to recapitulate the 3D breast tumor / basement matrices and breast tumor / endothelial barrier.

Cross-talk between growth factor receptors and CD44

Previous studies by us and others revealed that growth factors, such as PDGF-BB and TGF- β , are potent stimulators of hyaluronan synthesis. Hyaluronan has a high rate of turnover, and thus impairment in the regulation of HAS and HYAL isoform activities

kan cause great tissue havoc. To date, the signaling pathways that regulate the hyaluronan biosynthesis in response to PDGF-BB and TGF- β have not yet been clarified. Recent studies using inhibitors of different signaling pathways, revealed that the Erk MAP kinase and PI3 kinase signaling pathways are necessary for the regulation of hyaluronan synthesis by PDGF-BB, and that prevention of its binding to CD44 inhibits PDGF-BB-induced cell growth (Li et al., submitted for publication). This finding suggests that induction of hyaluronan is involved in the mitogenic response of PDGF-BB.

Hyaluronan is known to mediate its cellular functions in a size- and concentration-dependent manner. Tissues containing hyaluronan of high molecular weight at high concentrations, such as the vitrous of the eye and articular cartilage, are avascular. However, hyaluronan fragmentation compromises tissue integrity and stimulates the growth of new blood vessels. We have studied how hyaluronan fragments affect neovascularization at the molecular level, in comparison to the known angiogenic factor FGF-2. A large number of commonly upregulated and suppressed genes, as well as differentially induced genes in response to either FGF-2 or hyaluronan fragments were observed, demonstrating the importance of both growth factors and matrix molecules in endothelial cell differentiation.

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 demonstrated that CD44 forms a complex with the PDGF β -receptor and that hyaluronan suppresses the PDGF-BB-mediated activation of the β -receptor and migration of human dermal fibroblasts. The suppressive effect of hyaluronan is mediated by CD44 and most likely involve the recruitment of a CD44-associated tyrosine phosphatase to the receptor (23).

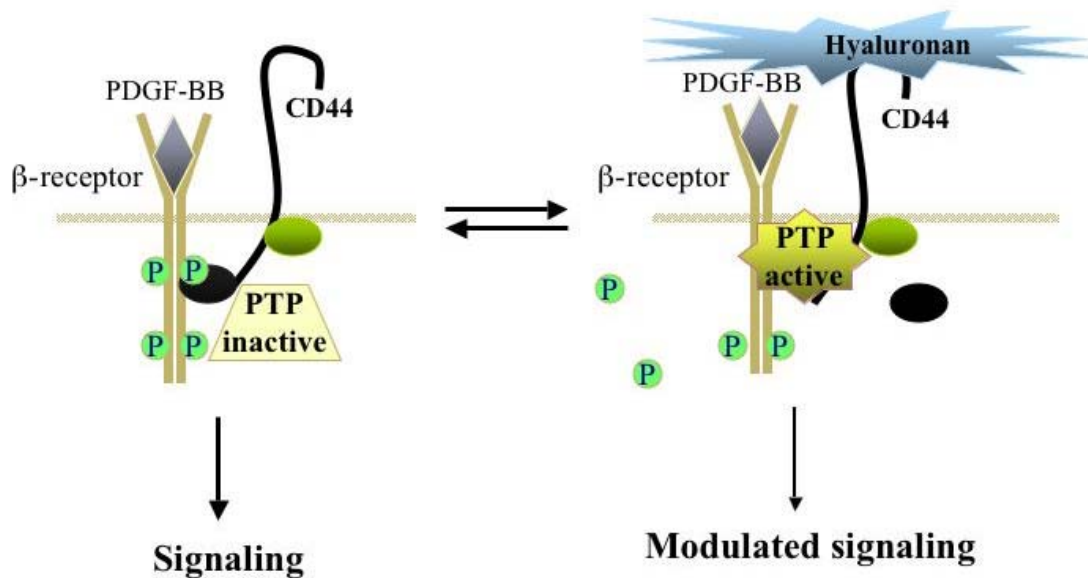


Fig. 4. Cross-talk between CD44 and PDGF β -receptor

Our current work is aiming at elucidating the functional consequences of the cross-talk between hyaluronan-CD44 and growth factor receptors, and its importance for pathophysiological conditions, such as cancer and inflammation. Increased understanding of the molecular mechanisms involved may help to design therapeutic interventions to prevent tumor invasiveness and metastasis.

Protein Structure Group

The Protein Structure Group has a solid experience in peptide synthesis, radio-labeled amino acid sequencing and mass spectrometry. For the latter activity, we use a top of the line matrix-assisted-laser-desorption-ionization-time-of-flight-mass-spectrometer (MALDI-TOF/TOF-MS). The present instrument (Bruker Ultraflex), installed in September 2003, has led to a dramatic increase in accuracy, user friendliness and sequence possibilities. Our three main activities are described in more details below.

Peptide synthesis

Contrary to the trend of outsourcing this highly specialized technique, we prefer to take advantage of our long gained know-how. Our synthesizer, a seven-year-old Applied Biosystems 433A instrument, is operated with Fmoc chemistry, and produces high quality peptides. The synthesis of peptides modified with phosphorylations, acetylations, oxidations at a given amino acid residue, has been most useful for the different groups at our Branch. All peptides are worked up manually and often, depending on the intended use, purified to homogeneity by liquid chromatography. The products are quality controlled using MALDI-TOF/TOF-MS. The peptides produced are used to generate anti-peptide antibodies, as substrates or inhibitors, or as ligands in affinity chromatography experiments. An important extension has developed over the last year – we now also carry out affinity purification of anti-peptide antibodies in collaboration with other groups of our Branch.

Radiolabeled phosphopeptide mapping

The introduction in our group of MALDI-ToF-MS nine years ago has made protein sequencing faster, more sensitive and less expensive, compared to classical chemical amino acid sequence analysis. We therefore do not use our ABI 494 peptide sequencer (from Applied Biosystems) for classical sequencing anymore. However, it is still being used for the important positioning of phosphorylated Tyr, Ser and Thr residues in phosphorylated proteins.

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. With Coomassie-visible material only a few percent is needed 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 micro RPC columns (*i.e.* μ ZipTips). Much of our group's work is manual, however, the Branch has a robot for high through put proteomics work and many samples for the MALDI are also generated using the robot.

Peptide mass fingerprinting (PMF)

Determining protein identity by PMF is a routine procedure for known proteins. After generation of a proteolytic digest and determination of peptide masses by

MALDI-TOF/TOF-MS, we utilize a search engine (ProFound or MASCOT are preferred) to make a match with a known protein. If a significant mass spectrum is obtained, we are practically always able to identify the protein with high confidence. Should this not be the case, the 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. We have taken up a technique of Lys-modification (an imidazol derivative), which renders those peptides more basic and therefore increases the sensitivity resulting in higher sequence coverage. As a bonus, such peptides are easy to fragment for sequence analysis. Furthermore, the Lys-Tag reagent may be used to label a set of proteins with deuterium for relative quantifications. Over the last years, we have carried out several interesting proteomics projects with groups the Karolinska Hospital and at the Dept. of Pathology, Uppsala University, looking for proteins that are significantly changed in various tumors (21, 22).

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

Fragment analysis of peptides by MALDI-TOF/TOF-MS using PSD is a straightforward technique. The resulting fragment spectra are commonly used for protein identification, but are difficult to use for *de novo* sequencing. The TOF/TOF technology allows complete PSD spectra to be generated in seconds, so it is possible to scan through several peptides from one digest in a short time. This easy and quick PSD combines extremely well with the Chemically Assisted Fragmentation (CAF) derivatization protocol, leading to easily interpreted spectra, as they comprise a unique series of γ -ions. Hence, an amino acid sequence can unambiguously (with the single exception of the isobaric Leu/Ile) be determined faster, cheaper and more sensitive than is possible using chemical Edman degradation. We use CAF-PSD for identification of un-characterized species, as well as for analysis of modified peptides (12).

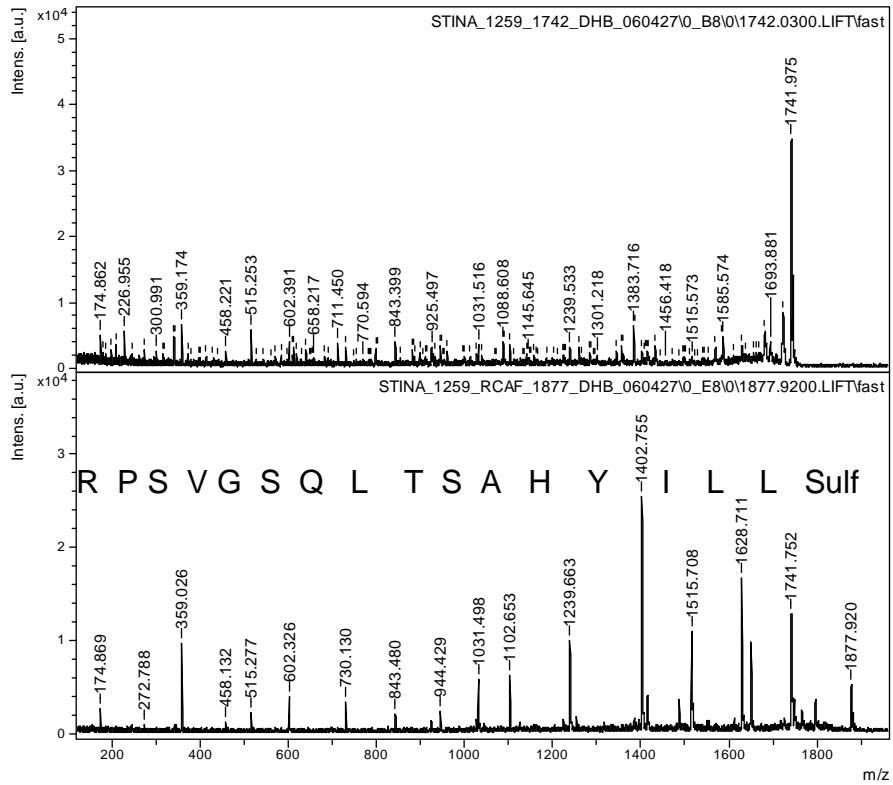


Fig. 5. A peptide from a tryptic digest was analyzed by Post Source Decay before (top panel) and after sulfonation. In the latter case, an amino acid sequence is easily called.

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