# ANNUAL SCIENTIFIC REPORT

# Uppsala Branch

# 2010

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### PDGF Translational Research Group

Hellberg, Carina, Associate Investigator, Group Head
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Schmees, Christian, Postdoctoral Fellow, to August
Van Wieringen, Tijs, Postdoctoral Fellow, from February
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Klosowska-Wardega, Agnieszka, Ph.D. Student, to June
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### PDGF Signal Transduction Group

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Masaud, David, Student, from August

## TGFβ Signaling Group

Moustakas, Aristidis, Member, Associate Director, Group Head (co-affilitated with Department of Medical Biochemistry and Microbiology, Uppsala University)
Huminiecki, Lukasz, Postdoctoral Fellow, to November
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## Apoptotic Signaling Group

Landström, Maréne, Group Head (co-affiliated with Department of Pathology, Umeå University)
von Bülow, Verena, Postdoctoral Fellow, from October to December
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## Molecular Pathology Group

Miyazono, Kohei, Group Head (co-affilitated with Department of Pathology, Tokyo University), from January
Vasilaki, Eleftheria, Postdoctoral Fellow, from February

## Cancer Signaling Group

ten Dijke, Peter, Group Head (co-affiliated with Department of Molecular Cell Biology, University of Leiden), from January
Sundqvist, Anders, Postdoctoral Fellow, from March

## Matrix Biology Group

Heldin, Paraskevi, Associate Investigator, Group Head
Kozlova, Inna, Postdoctoral Fellow
Skandalis, Spyridon, Postdoctoral Fellow, to July
Voytyuk, Oleksandr, Postdoctoral Fellow, from May
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Bernert, Berit, Ph.D. Student
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## Protein Structure Group

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

## Technical Support

Hedberg, Ulf, IT-support

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## Administration

Hallin, Eva, Finance and Administration Manager

## Secretariat

Schiller, Ingegärd, Secretary

## Introduction

Cancer 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 that regulate these events. The expectation is that such knowledge will make it possible to develop means for better diagnosis, prognosis and treatment of cancer patients.

Researchers at our Branch study in particular two growth regulatory factors, *i.e.* platelet-derived growth factor (PDGF) and transforming growth factor-β (TGFβ). 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 have important roles during the embryonal development in the formation of different kinds of mesenchymal cell types. Overactivity of PDGF is implicated in autocrine as well as paracrine stimulation of tumors. Our aim is to elucidate the molecular mechanisms of signal transduction via PDGF receptors, and to explore the clinical utility of PDGF antagonists.

TGFβ family members act via heteromeric complexes of type I and type II serine/threonine kinase receptors, and have important roles during the embryonal development. Most often, the members of this family inhibit cell growth, but they also stimulate matrix production and induce apoptosis. In cancer, TGFβ is therefore initially a tumor suppressor. However, at later stages of tumor progression TGFβ becomes a tumor promoter through its ability to induce epithelial-mesenchymal transition which links to increased migration and metastasis. Additional pro-tumorigenic effects of TGFβ include its suppressive effect on the immune system and its ability to stimulate angiogenesis. Our aim is to explore the molecular mechanisms whereby TGFβ acts, and to investigate the possibility that selective TGFβ antagonists can be made that inhibit the protumorigenic effects of TGFβ while leaving its tumor suppressor effects unperturbed. The ultimate goal is to explore the clinical utility of such antagonists.

Another line of the research at our Branch is the role of the microenvironment in tumorigenesis. In particular, the role in tumor progression of the large polysaccharide hyaluronan and its receptor CD44, and their interacting with growth factor receptors, is explored. As before, the groups of our Branch are supported by expertise in proteomics and mass spectrometry.

During the year, two former Group Leaders of our Branch who recently were appointed Guest Professors at Uppsala University, *i.e.* Kohei Miyazono, Tokyo University, and Peter ten Dijke, Leiden University, have established small satellite groups at our Branch. We hope that this arrangement will further promote interactions between scientists of our Branch and the groups of Kohei and Peter. In addition, the Group Leader for the TGFβ Signaling Group Aristidis Moustakas obtained a Senior Investigator position from the Swedish Cancer Society, which is placed at the Department of Medical Biochemistry and Microbiology at Uppsala University. However, Aristidis and his group will be affiliated with our Branch where his group will continue to be located. Finally, the Group Leader for the Apoptotic Signaling Group Maréne Landström obtained a position as Professor in Pathology at Umeå University. Maréne will move to Umeå during 2011, but part of her group will remain at our Branch.

Our Institute is located at the Biomedical Center in Uppsala, using laboratory space provided by the University of Uppsala. Some of our progress during 2010 is described on the following pages.

*C.-H. Heldin*

# PDGF Translational Research Group

The aim of the research in the PDGF Translational Research Group is to explore PDGF receptors as cancer drug targets, and to elucidate mechanisms that modulate PDGF β-receptor signal transduction.

**Background**

Tyrosine phosphorylation of proteins is an essential component 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. Growth factors synthesized by activated stromal cells affect the growth and survival of tumor cells, often by signaling through receptor tyrosine kinases. 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. The appreciation of the role of tyrosine kinases in the generation and progression of cancer has led to development of a number of anti-tumor drugs that specifically targets tyrosine kinases. Further understanding of the molecular mechanisms underlying tumor formation should generate a new wave of target-specific drugs.

**Receptor tyrosine kinase inhibitors sensitize melanoma tumors to taxol**

We previously showed that targeting tumor vasculature by combining inhibition of PDGF receptors and VEGF receptors reduced the growth of B16 tumors (Hasumi *et al.*, Int. J. Cancer *121*: 2606-2614, 2007). We now demonstrate that inhibition of the receptor for stem cell factor (c-Kit), by imatinib (Fig. 1) or by siRNA knockdown, sensitizes both mouse melanoma cells and human melanoma cells to taxol (36). The effect was selective for taxol, as the cells were not sensitized to 5-FU.

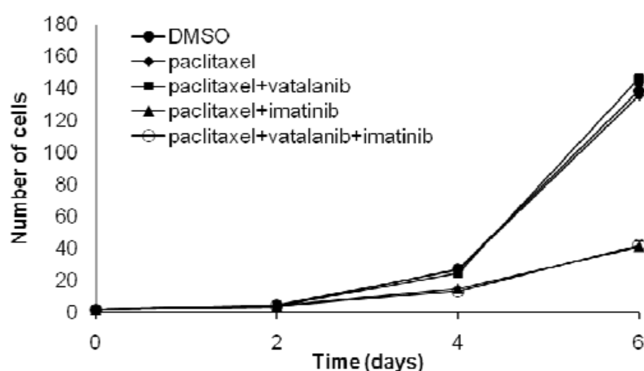


**Figure 1.** Imatinib sensitizes melanoma cells grown *in vitro* to paclitaxel (taken from ref. (36)).

When grown *in vivo*, B16 tumors do not respond to paclitaxel treatment, not even when combined with imatinib treatment. Instead, a combination of imatinib and vatalanib, a VEGF receptor inhibitor, sensitized these tumors to low doses of paclitaxel (Fig. 2).

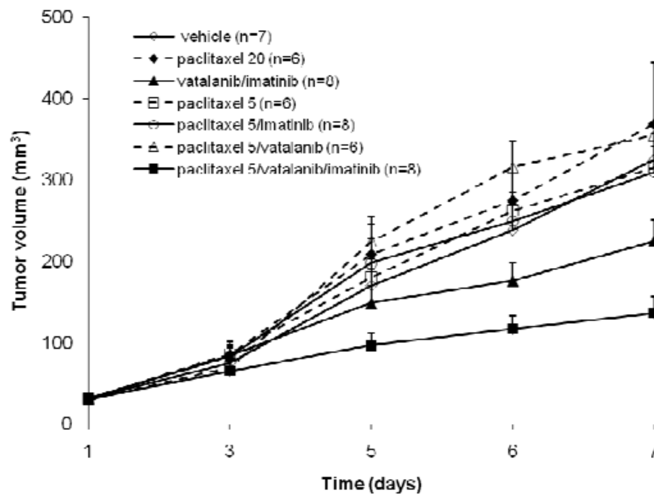


**Figure 2.** A combination of imatinib and vatalanib sensitizes B16 melanoma tumors to paclitaxel (taken from ref. (36)).

## Modulation of PDGF β-receptor signal transduction

Some time after ligand stimulation, PDGF β-receptor signaling is terminated by dephosphorylation of the receptor autophosphorylation sites in parallel with receptor internalization and subsequent degradation. Receptor signaling can therefore be modulated by altering the rate of receptor dephosphorylation or trafficking. We have now identified oncogenic H-Ras as a regulator of receptor internalization and signal transduction (Schmees *et al.*, submitted for publication). Expression of oncogenic H-Ras altered the route of ligand-induced internalization of PDGF receptors and caused an increase in both the amplitude and duration of receptor phosphorylation. The increased PDGF receptor phosphorylation was associated with increased survival signals. Since PDGF-BB also increased the anchorage-independent growth of H-Ras transformed fibroblasts, it is possible that the altered receptor trafficking augments cell transformation. This notion is currently being investigated.

## Involvement of protein tyrosine phosphatases in cell regulation

Screening for protein tyrosine phosphatases (PTPs) that regulate PDGF β-receptor phosphorylation and signal transduction have identified the receptor-like PTP LAR as a positive regulator of PDGF β-receptor phosphorylation. Inhibition of c-Abl kinase in

LAR ko fibroblasts reverted this phenotype, indicating that LAR promotes PDGF β-receptor activation by inhibiting c-Abl, which can act as a negative regulator for PDGF receptor activation (Zheng *et al.*, submitted for publication).

To investigate the role of T-cell phosphatase in tumor cell biology, novel substrates were identified using a substrate trapping mutant. The glycolytic enzyme pyruvate kinase M2 (PKM2) interacted with the T-cell phosphatase in a growth factor-dependent manner. Since PKM2 is a key mediator in the shift to aerobic glycolysis in tumor cells, "the Warburg effect", the possibility that T-cell phosphatase regulates tumor cell metabolism was investigated. Tyrosine phosphorylation decreased the activity of PKM2, and T-cell phosphatase-mediated dephosphorylation restored the activity *in vitro* (Karlsson *et al.*, submitted for publication). HeLa cells in which the T-cell phosphatase had been knocked down displayed increased PKM2 activity compared to the control cells, due to an increased enzymatic activity of the kinase. Downregulation of T-cell phosphatase affected the intracellular concentration of several intermediates in the glycolysis, indicating that this phosphatase could participate in the regulation of aerobic glycolysis.

# PDGF Signal Transduction Group

In our group the aim is to elucidate signaling mechanisms of PDGF receptors.

**Cross-talk between the Erk1/2 MAP kinase pathway and other pathways activated by PDGF receptors**

A major goal of our work is to understand the complex network of regulatory events that control PDGF-induced activation of different MAP kinase pathways, in particular the Erk1/2 and Erk5 pathways. The biological consequence of Erk1/2 activation is dependent on the magnitude as well as the temporal pattern of activation. Therefore, we have investigated if the major signaling pathways activated by the PDGFR can modulate the activation of Erk1/2 (35). We found that Src kinase activity was important for the initial phosphorylation of Erk1/2; inhibiting Src kinases caused a delay in Erk1/2 activation. Similarly, inhibition of phospholipase Cγ (PLCγ) also caused a delayed onset of Erk1/2 phosphorylation, which was found to be caused by lack of protein kinase C activation. However, interfering with phosphatidylinositol (PI) 3-kinase or the GTPase activating protein (GAP) for Ras increased the strength of Erk1/2 activation, suggesting that these pathways normally function to dampen Erk phosphorylation. When we investigated at which step in the Erk1/2 MAP kinase cascade the various pathways

influenced the activation, we found that in all cases this occurred at the level of Ras activation. The c-*fos* gene is an established downstream target of Erk1/2; notably we observed that the effects on Erk1/2 activation, caused by interfering with different signaling pathways, correlated well with changed kinetics or amplitude of c-*fos* gene expression. In conclusion, cross-talk with other PDGFRβ-induced signaling pathways is important for fine-tuning of the pattern of Erk1/2 activation.

**MKP3 negatively modulates PDGF-induced Akt and Erk5 phosphorylation as well as chemotaxis**

MAP kinase phosphatase-3 (MKP3), also known as DUSP6, is a dual specificity phosphatase considered to selectively dephosphorylate Erk1/2. We found that in NIH3T3 fibroblasts, MKP3 levels are regulated in an Erk1/2 and PI3-kinase dependent manner in response to PDGF treatment, but independently of Erk5 expression . Silencing of MKP3 expression did not affect PDGF-BB-induced Erk1/2 or p38 phosphorylation; however, the basal levels of Erk1/2 and p38 phosphorylation were elevated. Furthermore, we found that the PDGF-BB-mediated activation of Erk5 and Akt were enhanced when the MKP3 expression was reduced. In concurrence, inhibition of Mek1/2 using the inhibitor CI-1040 blocked the PDGF-BB-induced MKP3 expression, while it enhanced Akt and Erk5 phosphorylation. Functionally, we found that MKP3 silencing did not affect cell proliferation, but did enhance the chemotactic response towards PDGF-BB. Although both Akt and Erk5 have been linked to increased cell survival we were unable to detect any change in the ability of PDGF-BB to protect the NIH3T3 cells from starvation-induced apoptosis. However, we observed an increase in apoptosis in untreated cells with reduced MKP3 expression. Our data indicates that there is negative cross-talk between Erk1/2 and Erk5 that involves MKP3, and that PI3K in addition to promoting Akt phosphorylation also negatively modulate Akt, directly or indirectly, through MKP3 expression.

**Fer is necessary for PDGF-induced Stat3 phosphorylation and colony formation in soft agar**

In a screen for proteins interacting with phosphopeptides derived from the PDGFR sequence we found that the cytoplasmic tyrosine kinase Fer interacted with multiple autophosphorylation sites, primarily Tyr579 and Tyr581 in the juxtamembrane region of the receptor. This *in vitro* interaction was confirmed by co-immunoprecipitation experiments and the interaction was found to be PDGF-dependent. Downregulation of Fer by siRNA caused a diminished PDGF-induced Stat3 phosphorylation, suggesting

that Stat3 is a substrate for Fer kinase (Lennartsson *et al.*, submitted for publication). Silencing of Fer did not affect the ability of PDGF to promote cell migration or DNA synthesis; however, in the absence of Fer expression, transformed fibroblasts could not form colonies in soft agar. In support of the notion that Stat3 is an important effector of Fer, knock-down of Stat3 prevented colony formation in soft agar.

**The role of HD-PTP in PDGF receptor downregulation**

In order for a cell to respond in a suitable manner to PDGFR activation the process of receptor downregulation is important. One protein that has been implicated in this process, at least in the context of the epidermal growth factor receptors (EGFR), is HD-PTP. Despite its name it is unclear whether HD-PTP contains a phosphatase activity or if it functions as an adaptor or scaffold protein. Downregulation of HD-PTP by siRNA resulted in a loss of phosphorylation of the ubiquitin ligase c-Cbl in response to PDGF stimulation, which correlated with reduced PDGFR ubiquitination (Wardega *et al.*, submitted for publication). Whereas depletion of HD-PTP did not affect PDGFR internalization, the degradation of the receptor was inhibited.

Depletion of HD-PTP did not result in any change in PDGFR or PLCγ phosphorylation, but a decrease in the prolonged signaling through Akt, Erk1/2, p38 and SHP2 was observed. The chemotactic ability of fibroblasts was not affected by reducing the HD-PTP levels, but there was a small but reproducible increase in DNA synthesis. In summary, our data suggests that HD-PTP functions downstream of PDGFR, affecting c-Cbl phosphorylation, PDGFR degradation and signaling.

**Function of the activation loop tyrosine residue 857 in PDGFRβ**

To investigate the importance of Tyr857 in the activation loop of the PDGFR, we mutated this residue into phenylalanine (Y857F). Despite the fact that the Y857F PDGFR mutant lacked *in vitro* kinase activity, the receptor was normally phosphorylated *in vivo* and Tyr771 was even more strongly phosphorylated (30). Presumably the mutant PDGFR was phosphorylated by another yet unidentified tyrosine kinase. Analysis of signal transduction pathways revealed that the Y857F receptor had a reduced activation of Akt and Erk1/2. Functionally, Y857F PDGFR was unable to promote proliferation, whereas the chemotactic response was normal.

# TGFβ Signaling Group

Scientists in the TGFβ Signaling Group investigate the signaling pathways of transforming growth factor β (TGFβ) and of the related polypeptide factor bone morphogenetic protein (BMP). A central aim of the group is to elucidate new mechanisms of regulation of these pathways. More recent research lines explore the AMP-regulated kinase (AMPK) family, transcription factors and micro-RNAs that are involved in processes such as epithelial-mesenchymal transition (EMT), tumor cell invasiveness, metastasis and cancer stem cell self-renewal.

**Regulation of TGFβ/BMP/Smad signaling**

The signaling pathways that are initiated by TGFβs, BMPs and their family members via their cell surface receptor serine/threonine kinases include members of the Smad, MAP kinase and Rho GTPase families of signaling proteins. TGFβ family ligands mediate their biological effects by regulating expression of diverse sets of genes via the cooperation of Smads with other transcription factors, in a cell type-dependent manner. TGFβ signaling can also be negatively regulated by diverse molecular mechanisms, such as receptor and Smad de-phosphorylation and ubiquitination, leading to a fine-tuning of the signaling flow.

We have reported on new regulatory mechanisms of Smad function that operate at the chromatin level. PARP-1 (poly(ADP-ribose) polymerase 1) is a nuclear enzyme involved in gene transcription by ADP-ribosylating several transcription factors and histones, regulating their function. Our work has shown that TGFβ signaling leads to the rapid formation of nuclear complexes between PARP-1 and Smads (17). When PARP-1 ADP-ribosylates Smad3 and Smad4, it can regulate the dissociation of Smads from DNA. In this manner, PARP-1 controls the time that Smad complexes reside on DNA.

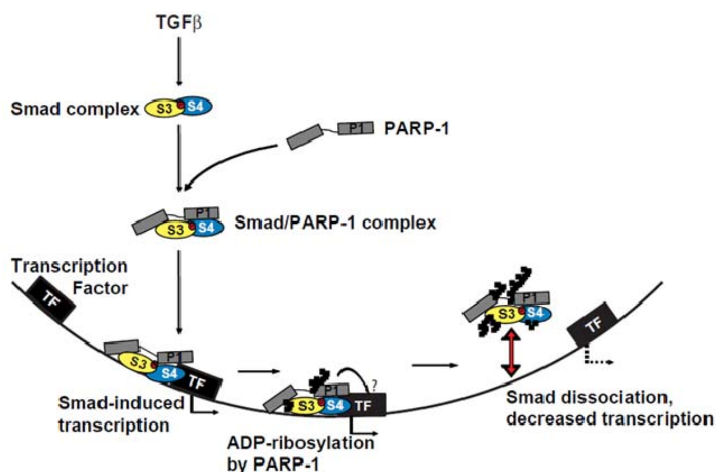


**Figure 3. Model of PARP-1 action during TGFβ signaling.** PARP-1 (P1) binds to TGFβ-induced Smad3/Smad4 (S3, S4) complexes after TGFβ stimulation. The Smad complex binds to DNA together with additional transcription factors (TF) and gene transcription starts. PARylation of Smad3/Smad4 by PARP-1 leads to dislocation (double arrow) of the Smad3/Smad4/PARP-1 complex from DNA, inhibiting gene transcription (dotted arrow). Red circles show Smad3 C-terminal phosphorylation and black squares represent poly-ADP-ribose chains.

In addition, we reported that Smads can associate with the chromatin insulator protein CTCF in the nucleus (3). One of the genomic loci where we could observe the Smad-CTCF protein complex is the *H19/Igf2* imprinting control region, known to undergo genomic imprinting. Our work suggests that TGFβ signaling may be involved in the epigenetic control of gene expression.

We have also focused on regulatory mechanisms of TGFβ signaling localized in the cytoplasm. The tumor suppressor kinase LKB1 was shown to form complexes with Smad4 via the adaptor protein LIP1 (38). LKB1 could phosphorylate Smad4 in its N-terminal, DNA-binding domain, and inhibit binding of Smad4 to DNA. We thus explained how LKB1 can negatively regulate both TGFβ and BMP signaling, by acting on the common Smad of these pathways, Smad4. A new line of research in collaboration with Peter ten Dijke's laboratory at the University of Leiden, explains why TGFβ causes cell death in a cell type-dependent and growth condition-dependent manner (40). The key regulatory protein is the mitogen and stress-activated kinase (Msk) 1. While TGFβ can cause cell death in various epithelial cell types, it also provides a survival signal, by inducing the activity of Msk1, which counteracts the apoptotic response.

**Epithelial-mesenchymal transition and regulation of tumor-initiating cells**

In addition to regulating cell proliferation and death, TGFβ can positively contribute to cancer progression, by promoting EMT, tumor cell invasiveness and metastasis (52). Growing evidence links the process of EMT and the generation of mesenchymal cells with the presence of unique cell types, carrying stem-like capacities within tumors.

Based on the establishment of a molecular pathway downstream of TGFβ, which involves the nuclear factor HMGA2 and its downstream targets Snail and Twist, we extend our work on EMT and tumor-initiating cells, by engaging the activities of additional nuclear factors. Our new work has established that PARP-1 regulates the EMT response to TGFβ (17), while LKB1 contributes to the epithelial phenotype as it counteracts the mesenchymal transition promoted by TGFβ (38). In collaboration with the laboratory of Vassilis Gorgoulis at the University of Athens, we analyzed the crosstalk of such new mechanisms that control EMT, with molecules that regulate genomic stability during cancer progression. Accordingly, we explore the role of p53 during EMT, and independently we study the role of the AMPK family member salt-inducible kinase 1 (SIK1) in controlling tight junction dissolution during EMT.

We have also progressed well into the mechanisms by which HMGA2 and Snail regulate the survival and tumor-initiating potential of cancer cells of the breast and brain (glioblastoma multiforme). Furthermore, we extended our previous work on the crosstalk of TGFβ and Notch pathways during epithelial cytostasis, by establishing a novel role of these two pathways during invasiveness of renal carcinomas in collaboration with Håkan Axelsson's laboratory at the University of Lund. Finally, we have completed two lines of collaboration with Christos Stournaras's laboratory at the University of Crete, analyzing detailed mechanisms by which TGFβ and BMP regulate actin dynamics during EMT. All the above findings have direct relevance to the mechanisms by which TGFβ acts as a pro-metastatic factor and direct our research towards novel therapeutic approaches.

**Systems biology analysis of TGFβ signaling and pathway modeling**

In collaboration with the laboratories of Zhike Zi, University of Freiburg, Edda Klipp at the Humboldt University Berlin, and Xuedong Liu at the University of Colorado at Boulder, we have performed quantitative analysis of Smad signaling in response to TGFβ and managed to provide novel insight as to how the initial phosphorylation of Smads by TGFβ receptors is directly linked to the output of this pathway in regulating mRNA synthesis. The importance of threshold ligand levels has been modelled and the oscillatory behaviour of the pathway was measured. This project is operating under the auspices of the EU-funded network of excellence "ENFIN" (www.enfin.org).

# Apoptotic Signaling Group

High levels of TGFβ promote tumor progression of several types of advanced cancers, including prostate cancer, while they induce apoptosis in normal epithelial cells and highly differentiated tumors. The aim of the scientific work in the Apoptotic Signaling Group is to elucidate the molecular mechanisms whereby TGFβ activates non-Smad signaling pathways leading to migration, invasion and apoptosis. Our long-term goal is to develop novel therapeutic strategies and to identify potential novel biomarkers, particularly in prostate cancer.

### The type I TGFβ receptor recruits the E3-ligase TRAF6 leading to apoptosis

We have reported that the TGFβ receptor interacts with TAK1, and that activation of TAK1 requires Lys63-dependent ubiquitination by the E3-ligase TRAF6 (Sorrentino *et al.*, Nature Cell Biol. *10*: 1199-1207, 2008). This post-translational modification of TAK1 determines its biological responses downstream of the active receptor complex. This finding provides a molecular mechanism for a non-Smad signaling pathway initiated by the TGFβ receptors. Interestingly, we found that TGFβ-induced activation of the p38 MAP-kinase pathway is not dependent on the kinase-activity in the type I TGFβ receptor (TβRI). Intriguingly, we have also identified a consensus binding site for TRAF6 in TβRI, which explains how TRAF6 associates with the receptor. Ligand-induced oligomerization of the receptor-complex thus causes auto-ubiquitination and activation of TRAF6, followed by Lys63-dependent poly-ubiquitination and activation of the TAK1 – p38 MAP-kinase pathway.

We are currently investigating the detailed molecular mechanisms whereby TRAF6 determines the specificity of cellular responses induced by TGFβ.

### Inflammation in relation to prostate cancer biology

There is a close link between chronic inflammation and tumor promotion in several cancers, including prostate cancer. We therefore want to understand the underlying molecular mechanisms for how inflammatory cytokines can promote tumor progression.

Another important aim of our work is thus to investigate the detailed molecular mechanisms for activation of the TAK1 – p38 MAP-kinsae pathway by other inflammatory cytokines, such as TNF-α and IL-1. We have further explored the importance of Lys63-linked polyubiquitination of TAK1, for its activation by TNF-α

and IL-1β (von Bülow, Hamidi *et al.* manuscript in revision). We have found that Lys34 in TAK1 is a major acceptor for Lys63-linked polyubiquitin chains also in these pathways, leading to activation of NFκB and induction of an inflammatory response which would promote tumor progression *in vivo*.

**Identification and development of novel treatment strategies for patients with advanced prostate cancer**

For patients with advanced and metastatic prostate cancer there is an urgent need to develop improved therapeutic strategies, as no efficient therapy exists today. A universal hallmark for tumors is aberrations in their expression of proteins implicated in regulation of cell polarity which might cause activation of oncogenic signalling pathways leading to a growth advantage for tumor cells.

The small gold compound aurothiomalate (ATM) is already in use for treatment of rheumatoid arthritis and has been demonstrated to cause growth inhibition of ovarian and non-small-cell lung cancer. In our search for novel options for treatment of advanced prostate cancer, we have investigated possible effects of ATM. Interestingly, ATM can specifically induce apoptosis of prostate cancer cells while normal primary prostate epithelial cells are not affected when exposed to ATM. We found that ATM disrupted the association between proteins in the cell polarity complex (Par6 and aPKC) which maintain pro-survival signals initiated by the Akt kinase. Treatment with ATM induces apoptosis of tumor cells via its inhibitory effects on Akt and activation of the MAP-kinase family members, p38 and Erk. We are currently investigating the putative antitumoral effects of ATM on additional prostate cancer cell lines. We hope that our studies will contribute to add ATM to our future therapeutic options to successfully treat patients with advanced prostate cancer.

We are also currently investigating whether inhibition of specific kinases, activated by TGFβ in the non-canonical Smad signalling pathway could be used to treat prostate cancer.

**Does Smad7 act as a bridge between TGFβ and Wnt signaling?**

Signaling molecules downstream of TGFβ and Wnt receptors regulate cell fate and proliferation during development and tissue homeostasis. We have previously reported that Smad7 interacts with components in the Wnt signaling pathway, *i.e.* β-catenin and lymphoid enhancer binding factor 1/T-cell-specific factor (LEF1/TCF). Furthermore, by

the use of siRNA and anti-sense techniques, we have shown that Smad7 expression is required for TGFβ-induced stabilization of β-catenin, in human prostate cancer cells and keratinocytes. Interestingly, we have identified Smad7 and p38 as regulators of the activity of glycogen synthase kinase-3β (GSK-3β), which causes stabilization of β-catenin. This event is crucial for TGFβ-induced cell migration of prostate cancer cells (Ekman *et al.* submitted for publication).

We will continue to further explore the underlying molecular mechanisms by which Smad7 and p38 MAP-kinase affect other key components in the Wnt-signaling pathway.

**Smad7 target genes**

Smad7 is a nuclear protein and TGFβ stimulation of cells leads to export of Smad7 to the cytoplasm, where it binds to the activated TGFβ receptor complex. Thereby, Smad7 inhibits further activation of the canonical Smad pathway, while it also acts as an adaptor protein for activation of the p38 MAP-kinase pathway, as described above. We are interested in the possibility that Smad7 has direct effects in the nucleus by regulating gene transcription, and have therefore performed microarray analyses on cells over-expressing Smad7. Candidate genes have been validated by RT-PCR and chromatin immunoprecipitation analysis (Thakur *et al.*, manuscript in preparation). The function(s) in TGFβ signaling of the identified Smad7 target genes, are currently investigated.

# Molecular Pathology Group

We are investigating the mechanisms of regulation of gene expression by TGFβ family members. TGFβ family cytokines regulate a variety of cellular processes, including differentiation, proliferation, migration and cell death in a cell-type specific and context-dependent manner. Recent technological advances in high-throughput analyses of transcriptional regulation by use of massively parallel sequencing and tiling microarrays enable us to determine regulatory mechanisms of such context-specific transcriptional regulation. We focus on the differences in Smad family binding genomic regions in different cell types, and analysis of angiogenesis-related factor(s) downstream of TGFβ family signaling pathways.

**Analysis of Smad family binding regions by ChIP-chip/ChIP-sequencing**

We have reported Smad2/3 binding regions in normal human epidermal keratinocyte cell line HaCaT using promoter tiling array (Koinuma *et al.*, Mol. Cell Biol. *29*: 172-186, 2009). We identified several co-regulatory factors which bound to Smad2/3 binding genomic regions and affected global TGFβ-induced transcription. We extended the analysis to determine the differences in Smad2/3 binding regions in some other cell lines, and successfully determined Smad2/3 binding regions in HepG2 hepatoblastoma cells (Fig. 4). We analyzed the extent of overlap of Smad2/3 binding regions between HepG2 and HaCaT cells, and found a HepG2-specific regulatory factor which cooperated with Smad2/3 by helping their target promoter recognition (Mizutani *et al.*, submitted for publication). Based on these findings, we will continue to reveal the mechanisms of changes in TGFβ responses in the cells in some pathological situations, including cancer.

**Regulatory mechanisms of angiogenesis by TGFβ family signaling**

Perturbations of TGFβ family signaling pathways have been implicated in diverse developmental changes and some diseases, including cancer, ectopic ossification, and cardiovascular diseases. Mutations in *ENG*, *ACVRL1* (also known as ALK-1) or *SMAD4* have been shown to cause hereditary hemorrhagic telangiectasia (HHT), which is a multisystemic vascular disorder characterized by epistaxis, telangiectasia, and arteriovenous malformation. *ACVRL1* and *ENG* encode endothelial-specific receptors for TGFβ and BMP-9/10 and transduce their signals through BMP-specific receptor-regulated Smads (Smad1/5/8). These facts indicate that BMP signaling in endothelial cells is implicated in pathogenesis of HHT. However, molecular mechanisms of BMP signaling in endothelial cells and its direct target genes have not been characterized. We determined Smad1/5 binding genomic regions in endothelial cells by ChIP-sequencing, and analyzed their characteristics. We identified novel target molecules of Smad1/5, and analyzed their roles in endothelial cells (Morikawa *et al.*, in preparation). We are currently collaborating with the group of Dr. Johan Ledin at the Evolutionary Biology Centre (EBC) of Uppsala University to reveal the *in vivo* relevance of our findings using a zebrafish angiogenesis model.

We work in close cooperation with the Department of Physiology and Developmental Biology, EBC, Uppsala University; Genome Science Division, Research Center for Advanced Science and Technology (RCAST), University of Tokyo; Department of Molecular Pathology, Graduate School of Medicine, University of Tokyo; and the TGFβ

Signaling Group, the Protein Structure Group and the Cancer Signaling Group at the Uppsala Branch of LICR.

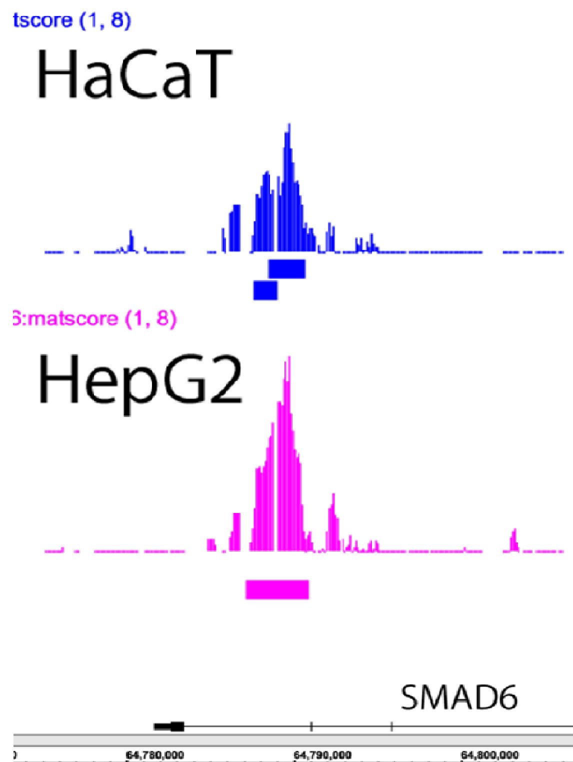


**Figure 4. A Smad2/3 binding region at Smad6 locus.** Smad2/3 binding regions were determined in HaCaT and HepG2 cells using Affymetrix human promoter 1.0 R array. MAT algorithm was used to calculate Smad2/3 binding signals and graphically represented by integrated genome browser (Affymetrix, Blue: HaCaT, Magenta: HepG2). Bars represent significant binding region by $p<10^{-4}$.

# Cancer Signaling Group

In order to properly respond to growth-stimulatory and growth-inhibitory signals, cells posses multiple signal transduction pathways, consisting of specific molecules that transmit these signals from their receptors to the nucleus, where transcription factors convert them into specific gene expression programs. To be able to integrate information from different types and doses of stimuli that can reach a cell simultaneously or within short (repeated) intervals, signaling molecules from distinct pathways interact on many levels, both on the membrane, in the cytoplasm and in the nucleus, thereby forming large cellular networks which are often localized to specific sub-cellular structures. During carcinogenesis and cancer progression cells loose their ability to correctly respond to growth inhibitory signals, and/or show increased sensitivity to growth-stimulatory and pro-invasive signals. This is the result of genetic defects, such as mutation, deletion or amplification of specific signaling molecules, or due to epigenetic events such as gene silencing.

We are interested in the detection, identification and functional characterization of signaling complexes and intermediates that distinguish cancer cells from normal cells, or that differ during the different stages of tumor progression. These complexes might function as diagnostic and/or prognostic markers and become targets for therapeutic intervention. In our current work, we focus on components of the TGFβ-Smad and MAP-kinase — AP-1 pathways, two pathways that play critical roles in tumor progression and interact on multiple levels.

**Induction of breast cancer cell invasion by TGFβ**

TGFβ has opposing roles in breast cancer by acting as a tumor suppressor in the initial phase, but stimulating invasion and metastasis at later stages. In contrast to the mechanisms by which TGFβ induces growth arrest, the pathways by which it mediates tumor invasion are not well understood. We have therefore developed a TGFβ-dependent invasion assay system consisting of spheroids of MCF10A1 normal breast epithelial cells (M1) and RAS-transformed (pre-)malignant derivatives (M2 and M4) embedded in collagen gels. Both basal and TGFβ-induced invasion of these cell lines were found to correlate with their tumorigenic potential; M4 showing the most aggressive behavior and M1 showing the least. Basal invasion was strongly inhibited by the TGFβ receptor kinase inhibitor SB-431542, indicating the involvement of autocrine TGFβ activity. TGFβ-induced invasion in premalignant M2 and highly malignant M4 cells was also inhibited upon specific knockdown of Smad3 or Smad4. Interestingly, both a broad spectrum matrix metalloproteinase (MMP) inhibitor and a selective MMP2 and MMP9 inhibitor mitigated TGFβ-induced invasion of M4 cells, while leaving basal invasion intact (41). In line with this, TGFβ was found to strongly induce MMP2 and MMP9 expression in a Smad3- and Smad4-dependent manner. This collagen-embedded spheroid system therefore offers a valuable screening model for TGFβ/Smad- and MMP2- and MMP9-dependent breast cancer invasion.

**TGFβ activates mitogen- and stress-activated protein kinase-1 (MSK1) to attenuate cell death**

We observed that TGFβ can activate mitogen- and stress-activated kinase 1 (MSK1) (40). Knockdown of GADD45, a Smad4-induced upstream regulator of p38 MAP-kinase prevented TGFβ-induced p38 and MSK1 activity. MSK1 functionally regulated pro-apoptotic BH3-only BCL2 proteins, as MSK1 knockdown reduced Bad phosphorylation and enhanced Noxa and Bim expression, leading to enhanced TGFβ-induced caspase-3 activity and cell death. This finding suggested that MSK1 represents

a pro-survival pathway bifurcating downstream of p38 and antagonizes the established pro-apoptotic p38 MAP-kinase function.

**In situ proximity ligation detection of c-Jun/AP-1 dimers in breast cancer cells**

Genetic and biochemical studies have shown that selective interactions between the Jun, Fos, and activating transcription factor (ATF) components of transcription factor activating protein 1 (AP-1) exhibit specific and critical functions in the regulation of cell proliferation, differentiation, and survival. For instance, the ratio between c-Jun/c-Fos and c-Jun/ATF2 dimers in the cell can be a determining factor in the cellular response to oncogenic or apoptotic stimuli. Until recently, no methods were available to detect endogenous AP-1 complexes in cells and tissues in situ. Therefore, we validated the proximity ligation assay (PLA) for its ability to specifically visualize and quantify changes in endogenous c-Jun/c-Fos, c-Jun/ATF2, and c-Jun/Fra1 complexes (2). We used among others, partner-selective c-Jun mutants. Furthermore, we examined the levels of c-Jun/AP-1 dimers in cell lines representing different types of human breast cancer and found that aggressive basal-like breast cancer cells can be discriminated from much less invasive luminal-like cells by PLA detection of c-Jun/Fra1 rather than of c-Jun/ATF2 and c-Jun/c-Fos (Fig. 5). Also in tumor tissue derived from highly metastatic basal-like MDA-MB231 cells, high levels of c-Jun/Fra1 complexes were detected. Together, these results demonstrate that in situ PLA is a powerful diagnostic tool to analyze and quantify the amounts of biologically critical AP-1 dimers in fixed cells and tissue material.

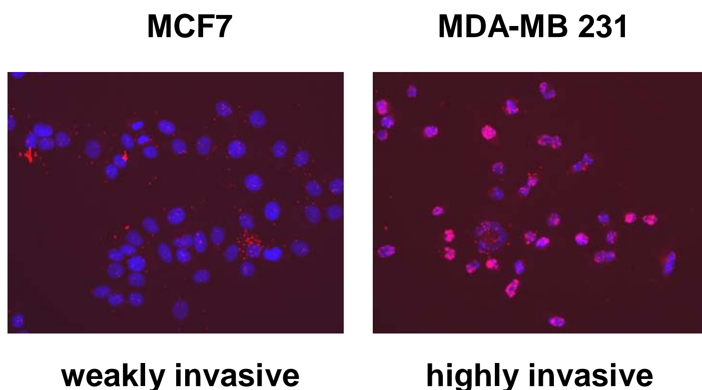


**Figure 5** In situ PLA with antibodies against c-Jun and Fra1 for weakly invasive and highly invasive breast cancer cell lines. The interaction events are visible as red dots (nuclear staining in blue (DAPI).

Currently, we are studying the role of Smad and AP-1 complexes in TGFβ-dependent breast cancer invasion by a combination of biochemical and genetic methods, including proximity ligation. The work is done in close cooperation with the Signal Transduction Section of the Department of Molecular Cell Biology at the Leiden University Medical

Center in the Netherlands, the Department of Genetics and Pathology of the Rudbeck Laboratory of Uppsala University, and the TGFβ Signaling Group and the Laboratory of Molecular Pathology at the Uppsala Branch of the LICR.

# Matrix Biology Group

The aim of our project is to explore the mechanism whereby hyaluronan via its receptor CD44 promotes tumorigenesis, including the regulation of hyaluronan synthases (HASs) by posttranslational modifications, the molecular mechanism of signaling via CD44 and the functional importance of its interactions with growth factor receptors, and the significance of hyaluronan-CD44 interactions for breast cancer cell metastasis.

**Regulation of HAS by posttranslational modifications**

The extensive accumulation of hyaluronan that occurs in various malignant and inflammatory conditions is correlated to the severity of the pathological condition. In order to develop novel treatment strategies, we need to understand the signaling mechanisms involved in the control of HAS gene expression and the events that regulate HAS activities. Recently, we have delineated the downstream signaling pathways through which PDGF-BB stimulates hyaluronan synthesis in human dermal fibroblasts, and now focus on regulation of the activity of HAS proteins.

We have demonstrated that HAS activation and stability is regulated by ubiquitination (12). By transfection of pairs of differently tagged HAS isoforms in COS-1 and CHO cells, followed by immunoprecipitation and immunoblotting, we showed that HAS2 forms both homodimers and heterodimers with other HAS isoforms, and the dimeric configuration is important for the activity of HAS2. We also demonstrated that HAS2 is mono-ubiquitinated at Lys190 (K190), which is conserved among all HAS isoforms and resides in their glycosyltransferase domain and that mutation of K190 inactivates the enzymatic activity of HAS2. These findings demonstrate for the first time that HAS2 activity is regulated through mono-ubiquitination at K190 and oligomerization

**Elucidation of the molecular mechanism of signaling via CD44**

CD44s is an adhesion receptor with an extracellular hyaluronan binding domain, a stalk region which is of variable length because of differential splicing, and an intracellular part with FERM-, ankyrin- and PDZ-binding motifs. CD44 has been proposed as an important marker for breast cancer-initiating cells, and its aberrant expression is

associated with persistent inflammation and malignant transformation. We and others have demonstrated that there is a cross-talk between CD44 and growth factor receptors, including the receptors for PDGF-BB, TGFβ, hepatocyte growth factor and epidermal growth factor.

To elucidate the molecular mechanisms underlying its multiple functions, we used a peptide-based pull-down assay to identify proteins that interact with CD44. Several interaction partners were identified, including proteins involved in cytoskeletal re-organization, transcription, endocytosis and intracellular transport (23). An endogenous complex between CD44 and one of the interacting proteins, the actin binding protein IQGAP1, was demonstrated in several normal and transformed cell types.

**Role of hyaluronan-CD44 interactions for breast cancer metastasis**

The adhesion of tumor cells to microvascular and lymphatic endothelium is a pre-requisite for their intravasation into vasculature and subsequent extravasation into secondary organs. Bone metastasis is prevalent for advanced breast cancer. A critical step in the metastatic process of breast cancer cells, and any other carcinoma, is remodeling of the basement membrane. Our aim is to explore the role of hyaluronan in the molecular mechanisms that underlie the degradation of basement membrane and translocation of tumor cells.

To study whether endogenous hyaluronan production is involved in the invasion of a clone of the breast cancer cell line MDA-MB-231 that forms metastasis in bone, we used of an *in vivo*-like basement membrane model. We found that knockdown of HAS2 completely suppressed the invasive capability of these cells, by the induction of tissue metalloproteinase inhibitor (TIMP)-1 and dephosphorylation of focal adhesion kinase (Bernert and Heldin, submitted for publication). This study provides new insights into a possible mechanism whereby HAS2 and hyaluronan production can promote breast cancer invasion.

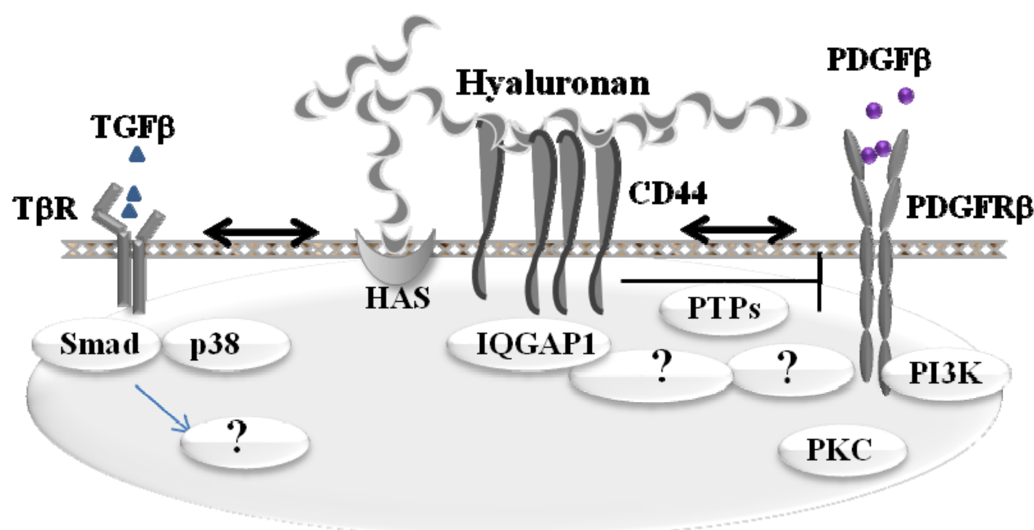


**Figure 6.** The hyaluronan receptor CD44 interacts with TGFβ and PDGF receptors, and may affect signaling via these receptors.

# Protein Structure Group

Our main mission is to support the activities within the Branch with our specialized techniques, and when appropriate to interact with other Branches in Sweden and abroad. Also, when time allows, we gladly collaborate with other research groups. This has over the years provided a broad experience with applications onto various types of projects.

The Protein Structure Group has a solid experience in peptide synthesis, general chromatography work and MALDI TOF mass spectrometry. In previous years, we have also offered radio-labeled amino acid sequencing, but this activity is discontinued from 2010 due to vanishing interest. Our main activities are described in more details below.

**Peptide synthesis and connected affinity chromatography**

Our peptide synthesizer, an eleven-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 or other chemical groups 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-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 years – we now also carry out affinity purification of anti-peptide antibodies in collaboration with other

groups of our Branch. We also perform affinity based searches for interacting partners using immobilized ligands (*i.e.* a modified synthetic peptide carrying a given motif). The combination of the biotin-streptavidin system and magnet beads has proved to be very powerful. Promising results are now being confirmed in *in vivo* cell experiments.

**MALDI mass spectrometry**

We use a top of the line matrix-assisted-laser-desorption-ionization-time-of-flight-mass-spectrometer (MALDI-TOF-MS). The present instrument (Bruker Ultraflex III TOF/TOF), installed in March 2007 offers a high sensitivity and accuracy for both MS and especially for MS/MS, user friendliness and peptide sequencing possibilities. This latter property is a consequence of the extra "TOF" which allows the development of full fragment spectra in few seconds.

**Sample preparation for mass spectrometry**

Practically all of the samples for analysis by MALDI-TOF/TOF-MS are delivered as bands or spots from one- or two-dimensional SDS-PAGE gels. The gel separated proteins are prepared for MS analysis by in-gel tryptic (almost always) digestion. With Coomassie-visible material only a few percent of the digest is needed for analysis; with silver-stained material, often the entire sample must be applied after concentration and desalting on micro RPC columns (*i.e.* C18 µZipTip). This, combined with other small improvements, allows us now to get significant identities of very minute amounts of sample – typically we often identify proteins represented by single very weak silver stained spots from one 2D gel.

We have introduced a proven approach to enrich for acidic, especially phosphorylated peptides, highly important but notorious for low sensitivity by MALDI TOF MS. This is based on home-made micro-columns comprising TiO2 particles, or, recently, pipette tips covered with $TiO_2/ZrO_2$ particles. These have proven to increase the sensitivity significantly for this group of highly relevant peptides.

**Peptide mass fingerprinting (PMF) and analysis of Post Translational Modification (PTM)**

Determining protein identity by PMF is a routine procedure for known proteins. After the generation of a proteolytic digest and determination of peptide masses by MALDI-TOF-MS, we employ a search engine (ProFound or MASCOT are preferred) to

search a match with a protein in the sequence databases. If a significant mass spectrum is obtained, we are practically always able to identify the protein with high confidence. Even when a significant match is found, we often confirm the identity by subjecting a few peptides to fragment analysis by Post Source Decay (see below) followed by MS/MS search via MASCOT. Should we fail to identify the target protein (*i.e.* if the searched protein is not found in any database or if that particular protein has an unfavorable distribution of target amino acids), the identity may be established by obtaining the amino acid sequence of one or more tryptic peptides (see below). A sequence homology search by BLAST is, in contrast to PMF, tolerant to amino acid substitutions. We have taken up a technique of Lys-modification (an imidazol derivative), which renders lysine containing 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 year, we have carried out interesting proteomics projects with groups at *e.g.* the Karolinska Hospital looking for proteins that are significantly changed in various tumors. We also spend a fair amount of effort to study PTMs – an important branch of proteomics. The nature of the modification determines the difficulty involved – we have been successful with *e.g.* methylation, acetylation, ubiquitinylation and phosphorylation.

**Post Source Decay (PSD) based peptide 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, rendering tryptic peptides acidified at the N-terminus by sulfonation, leading to easily interpreted spectra, as they comprise a unique series of y-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 was ever possible using chemical Edman degradation. We use CAF-PSD for identification of un-characterized species, as well as for analysis of modified peptides. As the CAF reagent was discontinued early 2008, we have started to replace it by SPITC (4-sulfophenyl-isothiocyanate), which is a much cheaper compound with similar positive effects on peptide fragmentation by MALDI-TOF/TOF.

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