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Introduction

The growth, migration, differentiation and apoptosis of cells are in part regulated by signals the cell receives from its environment. Important signals come from soluble growth regulatory factors which bind to specific receptors at the cell surface, thereby initiating a cascade of intracellular signaling pathways. Such pathways are subverted in tumor cells contributing to their lack of growth control and invasive behaviour. The aim of the work at the Uppsala Branch is to elucidate the character of such signaling pathways and their involvement in cancer and other diseases.

A lot of effort is devoted to studies of platelet derived growth factor (PDGF), which is a mitogen mainly for connective tissue cells, and transforming growth factor- β (TGF- β), which inhibits the growth of most cell types and has important roles during embryonal development, but other factors are also studied. In addition to elucidating the molecular mechanism of action of these factors, an important aspect of our work is to explore the possible use of inhibitors of signal transduction in the treatment of cancer.

Some highlights of our recent work are described on the following pages.

C.-H. Heldin

Growth Regulation Group

The Growth Regulation Group explores the role of PDGF and its tyrosine kinase receptors in disease, the involvement of protein tyrosine phosphatases (PTPs) in control of receptor tyrosine kinase signaling, and the function and regulation of the receptor-like PTP DEP-1. The role of PDGF in disease is studied mostly by using available PDGF antagonists in animal models of cancer and restenosis, but also includes development of antagonists to novel PDGF isoforms (31, 61). PTPs are natural tyrosine kinase antagonists and might be targets for novel strategies for inhibition of disease-associated tyrosine kinase signaling.

Effects of PDGF antagonists in animal models of cancer and restenosis

Autocrine PDGF stimulation occurs in *e.g.* brain and skin tumors (80). We have shown that a chromosomal translocation associated with dermatofibrosarcoma protuberans (DFSP), a skin tumor of intermediate malignancy, results in dysregulated PDGF-BB production (Shimizu *et al.* (1999) *Cancer Res.* 59, 3719-3723). Studies on DFSP in tissue culture and tumor xenografts also revealed that PDGF receptor inhibition blocks the growth of DFSP cells (Sjöblom *et al.*, submitted for publication). Our findings propose that PDGF antagonists can be used as a novel treatment regimen for DFSP.

PDGF receptors are expressed in the stromal compartment in most common solid tumors. Inhibition of stromally expressed PDGF receptors in a rat colon carcinoma model reduces the tumor interstitial fluid pressure and increases tumor transcapillary transport (64). To explore if this property of PDGF antagonists increases tumor uptake and therapeutic efficiency of conventional anti-cancer drugs, we investigated the effects of PDGF antagonists on Taxol and 5-fluoro-uracil responses in collaboration with K. Rubin, Uppsala University. In two different animal tumor models, which did not respond to PDGF antagonists only, PDGF antagonists increased the effects of the drugs (Pietras *et al.*, submitted for publication). These results suggest the highly interesting possibility that PDGF antagonists might increase drug-uptake and therapeutic effects of a broad range of anti-tumor agents in common solid tumors.

Arterial restenosis, which involves PDGF stimulation of smooth muscle cells, is a frequent complication after surgical treatment of atherosclerosis. In a rat model of restenosis we observed initial beneficial effects of PDGF antagonists but also found

that restenosis recurs after drug removal (29). To identify a treatment strategy that overcome this rebound effect, we performed together with S. Ylä-Herttuala, Kuopio University, a study in a rabbit restenosis model where a PDGF antagonists was combined with VEGF-C gene transfer to promote re-endothelialization of the damaged vessel. Interestingly, only the combination treatment led to a persistent reduction of restenosis (Leppänen *et al.*, submitted for publication). Whether these promising observations can be reproduced in *e.g.* models that include the use of stents will be investigated.

Involvement of PTPs in control of receptor tyrosine kinase signaling

It is well established that receptor tyrosine kinases (RTKs) are subject to negative regulation by PTPs (Östman and Böhmer, submitted for publication). The details and functional importance of these interactions are largely unknown. In a study on the effects of DEP-1 overexpression on PDGF β -receptor signaling we observed site-selective PDGF β -receptor dephosphorylation (25). This suggests that in some instances PTPs modulate rather than turn off RTKs. To what extent this contributes to RTK signaling specificity will be investigated by analyzing PDGF receptor signaling in cell lines that lack or overexpress individual PTPs. A novel role of PTPs in RTK activation was also indicated by the observation that PDGF receptor dimerization is associated with reduced susceptibility to receptor dephosphorylation (Shimizu *et al.*, submitted for publication). Efforts to identify the molecular mechanisms underlying this phenomenon are ongoing.

Most receptor-like PTPs are still orphan receptors. A Biacore based assay for detection of DEP-ligands has been established and is used to screen for potential DEP-1 ligands. In a complementary approach to identify DEP-1 ligands, we have analyzed the effects of various biological preparations on DEP-1 specific activity. Matrigel, a preparation of extracellular matrix components, was found to contain a DEP-1 agonist acting through binding to the DEP-1 extracellular domain (Sörby *et al.*, submitted for publication). Molecular identification of DEP-1 ligands is thus of high priority. Procedures for expression and purification of recombinant forms of the DEP-1 catalytic domain have also been developed and are used to obtain material for crystallographic analysis. Finally, fluorescence resonance energy transfer based analysis of interactions between DEP-1 and signaling proteins have been initiated.

Signal Transduction Group

The Signal Transduction Group is interested in elucidating the mechanisms by which the receptors for PDGF and stem cell factor (SCF) elicit their biological responses. This involves identification of phosphorylation sites on the respective receptors, identification of the proteins dependent on these sites for their binding and/or activation, and, furthermore, identification of downstream targets of these signal transduction molecules.

Signaling through heteromeric PDGF receptor complexes

Ligand-stimulation of the PDGF receptors leads to dimerization and activation of their intrinsic kinase activity, which in turn leads to phosphorylation of downstream signal transduction molecules. Five different isoforms of PDGF exist: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. Depending on which isoform is used to stimulate cells, either homo- or heterodimers of the structurally related α - and β -receptors, are formed. Stimulation with PDGF-AB leads to preferential formation of heterodimers of PDGF α - and β -receptors, which have been found to mediate a stronger mitogenic response than homodimeric receptor complexes. We have investigated the possible mechanisms behind this difference in signaling capability and found that Tyr771 in the PDGF β -receptor was significantly less phosphorylated in the heterodimeric β -receptor compared to the homodimeric receptor, and this correlated with reduced binding of RasGAP and decreased activation of the Ras/MAP kinase pathway. The mechanism behind the lowered phosphorylation of Tyr771 in the heterodimeric PDGF β -receptor was investigated. It was found that the SH2-domain-containing tyrosine phosphatase SHP-2 was responsible, at least in part, for the dephosphorylation of Tyr771 in the heterodimeric β -receptor (73). Furthermore, we have identified two heterodimeric-specific autophosphorylation sites, Tyr692 (in the first part of the kinase domain) and Tyr970 (in the carboxyterminal part of the kinase domain), and have shown that these phosphorylated tyrosines bind the low molecular weight phosphatase LMW-PTP. Mutation of Tyr692 or Tyr970 did not affect PDGF-induced mitogenicity, but mutation of Tyr692 reduced the chemotactic response mediated by the heterodimeric PDGF receptor complex (73).

Role of the adapter protein APS in Kit/SCFR signaling

The adapter protein APS consists of an SH2 domain and a PH domain and has been found to bind to Kit/SCFR and the PDGF receptors upon ligand stimulation. Ligand-dependent phosphorylation of APS leads to recruitment of c-Cbl and to increased ubiquitination of the respective receptors. We have identified Tyr568 and Tyr936 in Kit/SCFR as the sites of association of APS (Wollberg *et al.*, manuscript in preparation). Interestingly, the viral form of Kit/SCFR, v-kit, involves two deletions that leads to loss of Tyr568 and Tyr936. This loss of APS binding in v-kit might lead to decreased ligand-dependent ubiquitination of v-kit and hence decreased degradation of the receptor in the proteasome compartment. This is likely to contribute to the transformation ability of v-kit. We are currently in the process of expressing Kit/SCFR with the APS binding sites mutated, either individually or in combination, in NIH3T3 cells and Ba/F3 cells. The resulting cell lines will be tested for transforming ability in the standard focus formation assay, for anchorage independent growth and for ability to induce tumors *in vivo*.

Splice-form specific signaling

Two splice-forms of Kit/SCFR exist, in which a four amino acid insert in the extracellular part of the juxtamembrane region either exists or is missing. The presence or absence of these four amino acids, GNNK, lead to dramatic differences in signaling. The GNNK⁻ form is activated very rapidly, and quickly becomes heavily polyubiquitinated and degraded, while the GNNK⁺ form shows slow kinetics of activation and degradation. The GNNK⁻ is more efficient than the GNNK⁺ form in inducing transformation of NIH3T3 cells. We have shown that several differences in downstream signaling exist. The Ras/MAP kinase pathway is more strongly activated by GNNK⁻ than GNNK⁺, while phosphatidylinositol 3'-kinase (PI3-kinase) is about equally activated by both isoforms. A major difference between the two splice-forms is that the GNNK⁻ form more efficiently binds to and activates members of the Src family of tyrosine kinases (Mogi *et al.*, manuscript in preparation). Incubation of cells expressing either isoform of Kit/SCFR with the Src specific inhibitor SU6656 leads to kinetics and magnitude of phosphorylation of the receptors that are very similar between the two receptor types.

Gene Targeting Group

The Gene Targeting Group uses gene inactivation techniques in the mouse to explore the *in vivo* importance of specific signaling pathways initiated by different growth factors.

The receptors for PDGF dimerize upon ligand binding, resulting in phosphorylation of specific tyrosine residues within the intracellular part of the receptor. These phospho-tyrosines provide docking sites for SH2-domain containing proteins initiating signals for mitogenesis and actin cytoskeletal rearrangements. In collaboration with Dr. Phil Soriano's group in Seattle, we generated mice bearing point mutated PDGF β -receptors, that are unable to bind and therefore activate PI3-kinase upon ligand stimulation. PI3-kinase is important for PDGF-induced actin cytoskeletal rearrangements, proliferation and inhibition of apoptosis in cell culture experiments. Surprisingly, these mice had no obvious phenotype, but showed a defect in the regulation of the interstitial fluid homeostasis after a challenge leading to edema formation (Heuchel *et al.* (1999) PNAS 96, 11410-11415). In order to further restrict signaling from the β -receptor, we introduced an additional point mutation, such that neither PI3-kinase nor phospholipase-C γ , PLC γ , were able to bind to the activated PDGF β -receptor. Although embryonic fibroblasts from double mutant mice exhibited reduced proliferation and migration in response to PDGF, mutant mice showed no overt phenotype (52). However, in a model of experimental glomerulonephritis, mesangial cell defects were observed. Furthermore, in chimeric analysis, *i.e.* an *in vivo* competition situation between mutant and wild type cells, it was observed that the double mutant cells were defective in colonizing the vascular smooth muscle cell compartment.

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. The mRNA expression of a particular member of this family, namely Smad7, had been shown to be induced by TGF- β itself. Overexpression of Smad7 lead to downregulation of TGF- β signaling,

suggesting an auto-regulatory feedback mechanism. We investigated the mouse Smad7 promoter and found not only an essential DNA binding site for the TGF- β activated Smads 2, 3 and 4, but also the requirement for cooperation of these Smads with Sp1 and AP1 transcription factors in order to guarantee an efficient TGF- β response of the Smad7 promoter (8).

Cytoskeletal Regulation Group

The studies in the Cytoskeletal Regulation Group aim at elucidating signaling pathways that control cell growth and cell migration under normal physiological conditions as well as during disease. We study the Rho family of small GTPases and have focused our studies on Cdc42-regulated pathways, employing the yeast two-hybrid system to isolate clones encoding proteins that specifically bind to the activated, GTP-bound, Cdc42. These proteins included CIP4, PAR-6 and the Wiskott-Aldrich syndrome protein (WASP).

CIP4 is a protein of 545 amino acid residues with a C-terminal SH3 domain and an N-terminal FER-like coiled-coil. Overexpression of CIP4 in Swiss 3T3 fibroblasts reduced the amount of actin filament bundles in these cells, which implicated a role for CIP4 in the regulation of the actin cytoskeleton. In order to find out more about the function of CIP4, the yeast two-hybrid system was used to identify proteins interacting with its SH3 domain. This resulted in the identification of a novel RhoGAP domain-containing protein. This protein was named RICH-1 (RhoGAP Interacting with CIP4 Homologues). *In vitro* as well as *in vivo* assays demonstrated that the RhoGAP domain of RICH-1 catalyzed GTP hydrolysis on Cdc42 and Rac1, but not on RhoA (Richnau *et al.*, submitted for publication).

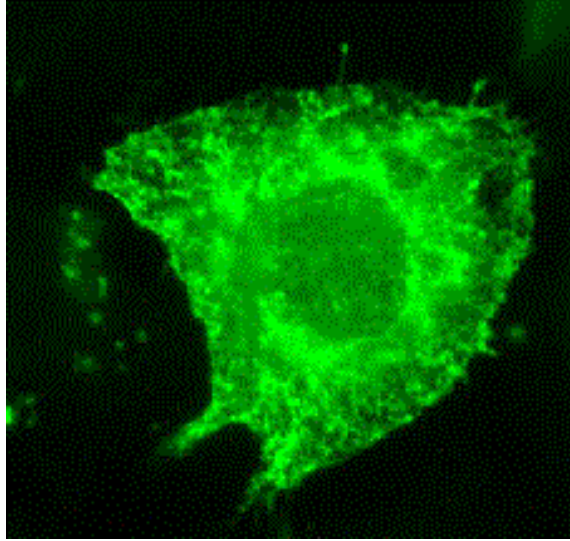


Figure 1. CIP4 in fibroblasts. Localization of HA-tagged CIP4 in NIH 3T3 fibroblasts. Cotransfected with HA-CIP4 and constitutively activated Cdc42. Ectopically expressed HA-CIP4 was visualized with a HA-specific antibody.

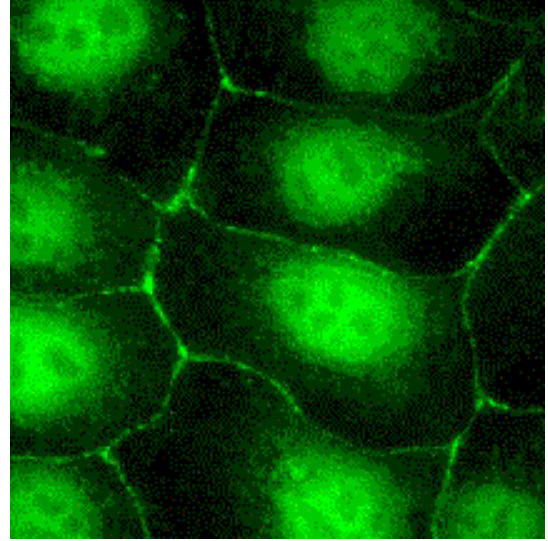


Figure 2. PAR-6 in MDCK cells. Localization of mammalian PAR-6 in Madine Darby canine kidney (MDCK) cells. PAR-6 was visualized with a PAR-6-specific antibody.

One Cdc42 interacting protein was the mammalian homologue of the PDZ domain containing *C. elegans* protein PAR-6. This protein has been shown to be crucial for the asymmetric cleavage and establishment of cell polarity during the first cell divisions in the growing worm embryo. In epithelial Madin-Darby canine kidney (MDCK) cells, endogenous PAR-6 was present in the tight junctions, however, it was also detected in the nucleus (21). Stimulation of MDCK cells with hepatocyte growth factor (HGF) induced a loss of PAR-6 from the areas of cell-cell contacts, in conformity with their progressive breakdown. In *C. elegans* PAR-6 co-localizes with PAR-3. Consequently, co-immunoprecipitation experiments demonstrated that mammalian PAR-6 and PAR-3 formed a direct complex (21). These findings suggest that Cdc42, Rac1 and PAR-6/PAR-3 are involved in the establishment of cell polarity in epithelial cells.

WASP was originally identified as the gene defective in the severe immunodeficiency disorder Wiskott-Aldrich syndrome (WAS). WASP is an adapter protein, with a phosphoinositide-binding domain, a Cdc42 binding domain, and an extended proline-rich domain, which binds SH3 domain-containing proteins. In addition, the WASP family of proteins, which also includes N-WASP and Scar/WAVE 1-3, binds directly to actin and to the so-called Arp2/3 complex. The interaction between WASP and the Arp2/3 complex appears to be crucial for regulating actin polymerization in cells. We are currently studying the signaling

mechanisms that result in the activation of members of the WASP family and, ultimately, in the mobilization of the actin filament system.

Molecular Signaling Group

The aim of the work in the Molecular Signaling Group is to characterize oncogenic regulatory networks during cancer development. Given the multigenic origin of cancer, we are focusing on various proteins that could participate in these networks including tyrosine kinase signaling modules, transcription factors and protein degradation modules. We use cDNA microarray screening and gene targeting to understand how components of these modules regulate cell transformation and metastasis.

Role of Pyk2/FAK tyrosine kinases in cell transformation and migration

Much of our current work is focused on the characterization of the Pyk2/FAK family of non-receptor tyrosine kinases in normal and transformed cells. Pyk2 and FAK contribute to cell transformation by co-ordinating signaling networks induced by adhesion, mitogenic signals and oncogenes. They are highly overexpressed in several tumor cell lines and primary human tumors and interact and co-operate with known oncogenes. In the last year, others and we have shown that FAK and Pyk2 act as proximal effectors of integrin and growth factor receptors important for the regulation of cell migration and neurite outgrowth as well as gene expression via MAP kinase signaling cascades (20; Blaukat et al. (1999) J. Biol. Chem. 274, 14893-14901). Moreover, we have studied Pyk2-independent signaling pathways linking bradykinin G protein-coupled receptors with MAP kinase cascades (6, 7). By using gene microarrays we are currently analyzing targets of Pyk2/FAK in transformed cell lines and primary tumors. This approach has a potential to identify specific genes controlled by Pyk2 and FAK during cell transformation. In addition, we are trying to develop novel methods to block functions of Pyk2/FAK *in vivo* and thus inhibit tumor growth and metastasis.

Cdx1-regulated genes during adenocarcinoma development

The *Caudal*-like homeobox gene Cdx1 is an intestine-specific transcription factor involved in the control of proliferation and differentiation of epithelial cells. Deregulation of homeobox gene expression was also implicated in uncontrolled cell growth and oncogenesis. We have recently shown that expression of Cdx1 in

intestinal epithelial cells (IEC-6) promotes anchorage-independent growth *in vitro* and adenocarcinoma development in nude mice (68). We are currently analyzing changes in gene expression during transformation of IEC-6/Cdx1 cells using cDNA microarrays. This approach has a potential to delineate Cdx1-induced factors that convert normal epithelial cells into tumor cells. The information thus gained will be compared with information on human susceptibility genes.

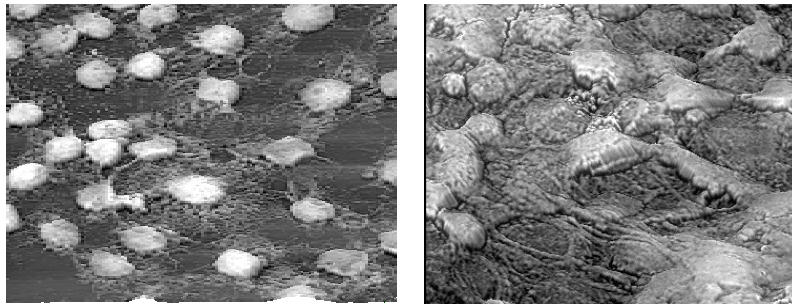


Figure 3. Atomic force microscopy of intestinal epithelial cells IEC-6 (left) and Cdx1-transformed IEC-6 cells (right).

Characterization of CASPs – Cbl-associated signaling proteins

Recent evidence indicates that c-Cbl acts as a E3-RING-dependent ubiquitin ligase that negatively regulates receptor tyrosine kinases by mediating their ubiquitination and degradation. Moreover, oncogenic forms of Cbl were shown to enhance ligand-independent signaling by growth factor receptors. By using the yeast two-hybrid system we cloned several Cbl associated signaling proteins (CASPs). We are currently focused on molecular characterization of CASPs in normal and transformed cells. In addition, CASP2-deficient mice will be created to study its function *in vivo*.

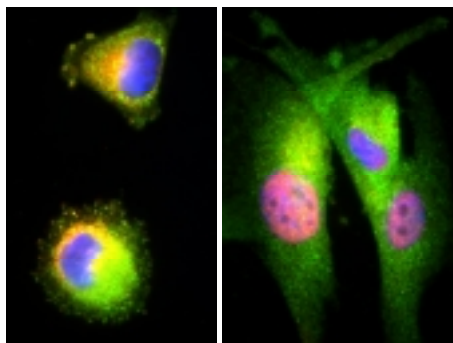


Figure 4. Colocalization of Cbl and CASP-2 in cultured fibroblasts.

TGF- β Signaling Group

The TGF- β Signaling Group investigates signaling pathways that regulate cell growth, differentiation and tumorigenesis in response to TGF- β .

TGF- β signaling and Smad regulation

TGF- β signals via plasma membrane serine/threonine kinase receptors and cytoplasmic effectors, the Smad proteins (Figure 5). The receptors activate the Smads, which move rapidly into the nucleus to regulate gene expression by associating with chromatin and cooperating transcription factors (88). Eventually, Smads exit the nucleus to be degraded in the cytoplasm.

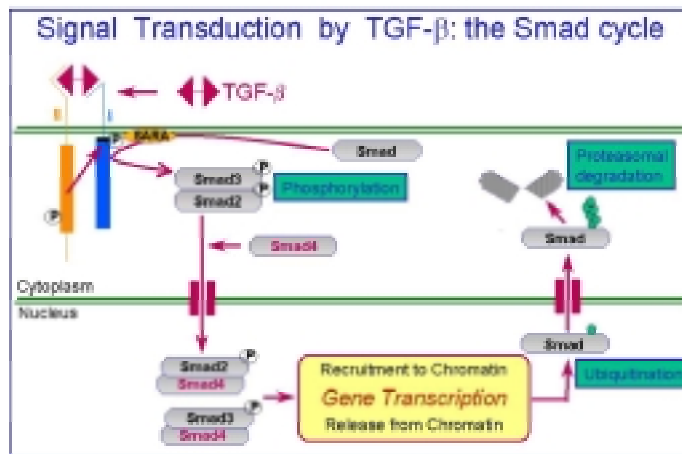


Figure 5

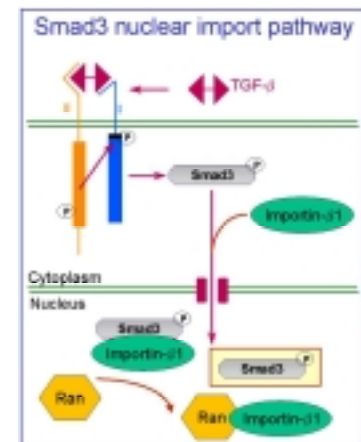


Figure 6

We defined the import mechanism of Smad3, a TGF- β pathway-specific Smad (63). The protein carrier importin- β 1 carries Smad3 to the nucleus, where the Ran GTPase disrupts the complex and causes release of Smad3 in the nucleoplasm (Figure 6). We investigate the nuclear export of Smads prior to their degradation. In parallel, we focus on the fate of Smad4, which is degraded by proteasomes. In human cancers, specific amino acid substitutions in Smad4 lead to its enhanced proteolysis (35). We analyze the molecular partners that regulate Smad4 ubiquitination and proteasomal degradation.

The Nuclear Role of Smads in Transcription and Cell Cycle Regulation

The activated, nuclear Smads specifically interact with several transcription factors. We have characterized novel Smad-interacting transcription factors. Moreover, we

have described the association of Smads with the transcription factor Sp1, a finding relevant to many TGF- β -responsive genes (40). TGF- β inhibits cell proliferation by repressing *c-myc* expression and inducing gene expression of cell cycle inhibitors p15 and p21 that lead to cell cycle arrest. We provided a mechanism by which TGF- β induces the *p21* gene, by activating Smad and Sp1 nuclear complexes (Figure 7; 40). We now study the *in vivo* and chromatin-dependent mode of regulation of the *p21* gene by TGF- β and aim at understanding how oncogenic stimuli such as *c-myc* and *c-ski* overexpression antagonize TGF- β -mediated growth inhibition.

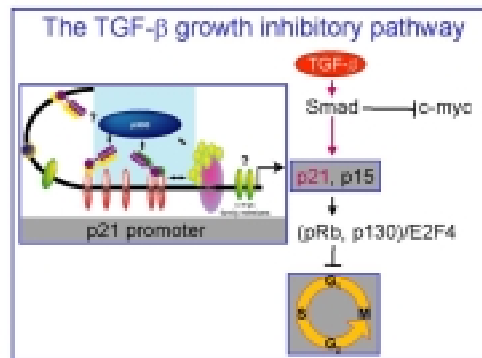


Figure 7

Selected view of the genomic response to TGF- β during growth inhibition and EMT

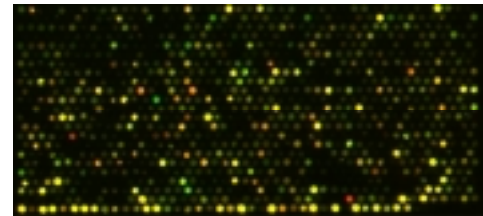


Figure 8

Role of TGF- β in Tumorigenesis and Global Gene Target Analysis

It is important to define the receptor-Smad modules activated in specific cell types. We focus on the model epithelial cell line NMuMG, because it undergoes epithelial to mesenchymal transition (EMT), a morphogenetic and differentiative alteration important *in vivo* during tumor cell migration and metastasis (44). We test whether TGF- β signaling is sufficient for EMT and tumorigenesis using a two-cell model of CHO cells secreting latent TGF- β (the physiological form of this cytokine in the extracellular space) and NMuMG cells.

The Smad pathway suggests that concerted regulation of gene expression might explain the complex physiological effects of the TGF- β superfamily. We acquired a cDNA microarray station and analyze the expressed genomic repertoire during TGF- β signaling. We find that a large proportion of the mammalian genome responds to TGF- β (Figure 8). We develop screens for novel TGF- β targets important in cancer development.

Integrated Signaling Group

The Integrated Signaling Group concentrates on TGF- β family signaling in normal and malignant cells and its cross-talk with other signaling pathways. We direct our efforts on the mechanisms of receptor activation, as well as Smad-dependent and Smad-independent signaling down-stream of the receptors. These studies aim at elucidating the role of TGF- β signaling in carcinogenesis. Proteomics techniques are used to investigate the complexity of integrated intracellular signaling.

In our studies of receptor activation, we have developed a system which allows us to identify specific regulators of TGF- β receptor kinase activity. These regulators will provide a basis for the development of drugs affecting TGF- β functions.

We have found that Smads are regulated by protein kinase C (PKC) (59); PKC directly phosphorylates receptor-regulated Smad proteins, which affects their ability to activate gene transcription. Interference with the PKC-Smad cross-talk results in an increased sensitivity of cells to the tumor promotor PMA, and to abrogation of the pro-apoptotic action of TGF- β 1. These findings provide additional insights into the interaction between signaling pathways that promotes tumor growth (PKC) and inhibit proliferation (TGF- β /Smad).

We have observed that all Smad proteins are phosphoproteins. We showed that the inhibitory Smad7 is phosphorylated on Ser249 by kinase(s) other than the TGF- β receptors, and in collaboration with the Apoptotic Signaling Group, we showed that this phosphorylation is important for a not previously recognized function of Smad7, *i.e.* its ability to act as a transcriptional regulator (65).

We have also described a functional interaction between osteogenic protein-1 (OP1) and vitamin D₃-regulated differentiation of human osteoblasts (Eichner *et al.*, submitted for publication). OP1, unlike TGF- β 1, inhibits vitamin D₃-induced differentiation; the possibility that this information can be used to improve the treatment of osteoporosis is currently explored.

In our studies of Smad-interacting proteins, we identified more than 15 candidates, which provides new insights into TGF- β signaling networks. The identified cross-talk between TGF- β signaling and products of human breast cancer susceptibility gene products BRCA1 and BRCA2 is under investigation.

We are completing an analysis of the proteome of Mv1Lu lung carcinoma cells upon TGF- β 1 treatment (Figure 9; Kanamoto *et al.*, in preparation). We have identified 28 new targets of TGF- β 1, and are currently investigating their functional role in TGF- β signaling. Our functional proteomics studies aim at creating a database of proteins targeted by TGF- β in normal and human breast cancer cells.

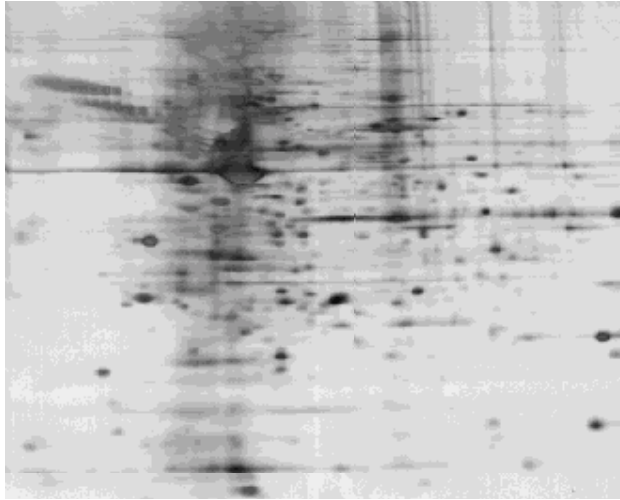


Figure 9. 2D proteome map of Mv1Lu cells

Apoptotic Signaling Group

By investigating the molecular mechanisms whereby TGF- β 1 and Smad7, as well as 2-methoxyestradiol (2-ME; an endogenous estrogen metabolite), transduce apoptotic signals, the Apoptotic Signaling Group hopes to obtain knowledge which can be used to improve treatment of common epithelial tumors.

TGF- β signaling in the prostate

TGF- β is an important regulator of proliferation, differentiation, migration and apoptosis in many different cell types. In the prostate, TGF- β has been shown to be directly linked to apoptosis of epithelial cells. In advanced prostate tumors, in which the tumor cells have become unresponsive to TGF- β , TGF- β favours tumor growth, due to its ability to stimulate angiogenesis and suppress the immune system.

We have earlier reported a strong correlation *in vivo* between apoptosis and increased expression of TGF- β 1, its receptors and the Smads, in the rat ventral prostate and a prostate tumor model early after castration. Notably, Smad7 was found in epithelial

cells showing morphological signs of apoptosis (Brodin *et al.* (1999) *Cancer Res.*59, 2731-2738).

We have recently reported that Smad7 acts as a mediator for TGF- β 1-induced, caspase-dependent apoptosis, in several tumor epithelial cell lines (PC-3U, DU 145) and in normal human keratinocytes (HaCAT), using antisense techniques (26). We have found that TGF- β 1, in a Smad7-dependent manner, selectively activates the p38 MAP-kinase pathway in PC-3U cells resulting in apoptosis. Moreover, a p38 inhibitor efficiently prevents Smad7-induced apoptosis. Since Smad7 binds to p38 and colocalizes with phosphorylated and activated p38 in the nucleus, they may act synergistically to regulate transcription of pro- or antiapoptotic genes (Bu *et al.*, in preparation).

An endogenous estrogen-metabolite, 2-ME, has potent cytotoxic effects on epithelial-derived common tumors

We have shown that 2-methoxyestradiol (2-ME), an endogenous metabolite of estrogen that does not bind to nuclear estrogen receptors, effectively induces apoptosis in Bcl-2 expressing human prostate and breast carcinoma cells *in vitro* and in a rat prostate tumor model *in vivo*. In contrast, 2-ME did not affect growth or survival of primary non-transformed mammary epithelial cells. 2-ME treatment of several cell lines derived from prostate, breast, liver and colorectal carcinomas, resulted in activation of c-Jun N-terminal kinase (JNK) and phosphorylation of Bcl-2, preceding apoptosis. Heterotrimeric G proteins were found to mediate 2-ME induced JNK activation and subsequent apoptosis (Bu *et al.*, submitted for publication).

Gene Expression Group

Most, if not all, signal transduction pathways ultimately affect gene transcription and alter the expression of specific genes. A novel posttranslational modification, protein acetylation, has been found to regulate the activity of many transcription factors.

YY1 – A multifunctional transcription factor

The transcription factor Yin Yang 1 (YY1) stimulates or represses gene expression by recruiting either coactivators (p300/CBP) or corepressors (HDACs) to targeted

promoters. In addition, YY1 acts as a repressor of human immunodeficiency virus type 1 (HIV-1) transcription and virus production in infected cells.

We have demonstrated that YY1 is acetylated by both p300 and P/CAF. Peptide mapping indicated that p300 acetylates a number of lysine residues, while P/CAF has a more restricted specificity. We have found that acetylation of YY1 by p300 enhances its DNA-binding activity and increases its ability to repress transcription from the HIV-1 LTR. Acetylation also enhances YY1-mediated repression of p53 (see below). We are currently using *in vitro* acetylation assays and site-directed mutagenesis to map the acetylated lysine residues. We have also demonstrated that YY1 is ubiquitinated *in vivo* and started to map the ubiquitinated residues, using our collection of lysine mutants.

Mutations in the p53 gene or inactivation of the p53 protein are the most frequent alterations in cancer cells and are found in more than 50% of all human cancers. We have demonstrated that YY1 binds to p53 and inhibits its transcriptional activity. We have mapped the interaction domains in both proteins and identified the repressor domain in YY1 (Terentiev *et al.*, manuscript in preparation). In addition, we have demonstrated that YY1 can interact with and inhibit a second member of the p53 family of proteins, *i.e.* p73. We are now trying to define the mechanisms of YY1-mediated repression of p53 and determine if YY1 affects any of the cellular responses to activated p53.

SREBPs – Key regulators of lipid metabolism

Members of the SREBP family of transcription factors are activated during adipocyte differentiation and when cells are starved for cholesterol. When cells accumulate high levels of cholesterol the active transcription factor is rapidly degraded.

We have shown that SREBPs are acetylated by p300 and we are currently mapping the acetylated lysine residues. We have also defined the p300-interaction domain in SREBP and found that mutations in this sequence blocks p300-mediated acetylation and inhibits the transcriptional activity of SREBP. We have shown that SREBPs are stabilized following acetylation by p300. Interestingly, p300 is unable to further stabilize SREBPs in the presence of proteasome inhibitors, indicating that p300 interferes with proteasome-mediated degradation (Giandomenico *et al.*, manuscript in preparation). We have also demonstrated that the stability of SREBP is correlated to its transcriptional activity, *i.e.* transcriptionally active molecules are degraded very

rapidly while transcriptionally inactive mutants are stabilized. These observations are of special interest, since both activation and inactivation of SREBPs are dependent on sterol-regulated proteolysis. In addition, one of the world's best-selling pharmaceutical drugs – statins – regulate the stability of SREBPs and thereby the metabolism of cholesterol and lipids. We propose to identify the acetylated residues that confer stability to SREBPs and determine if these lysine residues are also targets for ubiquitination. We also propose to determine if acetylation of SREBPs prevents sterol-regulated degradation.

Smads - Mediators of transforming growth factor- β signaling

We have demonstrated that Smads are acetylated by p300 *in vivo*. We have concentrated our efforts on Smad7, an inhibitory Smad. Smad7 resides in the nucleus of unstimulated cells. Following receptor activation, Smad7 translocates to the plasma membrane, where it interacts with TGF- β receptors and blocks further signaling. We have found that Smad7 interacts with the coactivator p300 in unstimulated cells and that Smad7 is acetylated under these conditions. TGF- β treatment inhibits both the formation of the p300-Smad7 complex and acetylation of Smad7. We have mapped the acetylated residues in Smad7 and generated acetylation-deficient mutants that are currently used to analyze the functional consequences of this modification (Grönroos *et al.*, manuscript in preparation).

Protein Structure Group

The aim of the Protein Structure Group is to support the other Groups at the Branch with the latest technologies in protein structure analysis as well as in peptide synthesis. Along with the gradual release of gene sequences from man and other species, the direction has changed towards identification of known proteins and the analysis of protein modifications. For these purposes we make good use of mass spectrometry and 2-D gel electrophoresis.

Peptide synthesis

Our new peptide synthesizer, an Applied Biosystems 433A, allows more flexibility in the design of modified peptides. Now there is a growing interest in acetylation of lysine residues in signaling molecules (see report from Gene Expression Group), and we are therefore gaining experience in this modification. Many of the peptides are used as ligands in affinity chromatography. Other important uses are as inhibitors

or substrates or the development of anti-peptide antibodies. A number of phosphorylation state specific antibodies have been generated by the use of synthetic phosphopeptides.

Amino acid sequencing

Classical Edman degradation. Since the introduction of mass spectrometry at our Branch three years ago, automated peptide sequencing has gradually decreased. This is because practically all sequencing leads to identification of a known protein, which is better done by MALDI-TOF-MS as shown in the comparison below:

	Edman-degradation	MALDI-TOF-MS
Sensitivity	1-10 pmol	0.01-0.1 pmol
Running cost per analysis	Ca 150 USD	A few cents
Analysis time	Overnight	A few minutes
Average number of cell culture flasks needed to prepare protein	100-150	1
Analysis of modifications	Only using standards	Possible

Radiolabeled amino acid sequencing. Identification of phosphorylation sites in growth factor receptors and signal transduction molecules continues to be an important activity in the Group.

Sample preparation for analysis by mass spectrometry

Since we are aiming at looking at samples in the fmole range it is difficult to avoid contaminating proteins. As the starting material is extremely complex, every sample must pass several enrichment steps prior to analysis. The last step is nearly always gel electrophoresis from which the gel piece containing the target protein is excised and subjected to in-gel digestion with a specific protease.

Peptide Mass Fingerprinting Using MALDI-TOF-MS

For the identification of proteins, peptide mass fingerprinting by MALDI-TOF-MS lends itself extremely well. The peptide masses are used to scan sequence databases and the list of matching proteins is returned within 20 seconds. To improve our throughput, we are replacing the present MALDI instrument with a more modern one.

Electrospray ionization mass spectrometry

A Bruker Esquire 3000 ESI-MS instrument was purchased in January 2001. It will complement the MALDI-MS in analysis of post-translational modifications, and hopefully allow *de novo* sequencing.

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