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With 59 figures

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EDITORS:

Associate Prof. Lin Wang
Biomedical center, School of Electronics & Engineering, Beijing Univ. of Post and Telecom. 100876, China
E-mail: wanglin98@tsinghua.org.cn

Prof. Minghu Jiang
Director of Lab of Computational Linguistics
School of Humanities and Social Sciences
Tsinghua Univ., 100084, China
E-mail: jiang.mh@mail.tsinghua.edu.cn

Prof. Stefan Wölfl
Institut für Pharmazie und Molekulare Biotechnologie (IPMB)
Abteilung Biologie
Ruprecht-Karls-Universität Heidelberg
69120 Heidelberg Jena
Email: wolfl@uni-hd.de

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Chapter 1 Introduction

1.1 Antibody Array Technologies and Applications

Protein array applications cover various fields, including protein-protein interaction, protein-RNA interaction, protein-DNA interaction, phosphorylation, dephosphorylation, proteolytic cleavage and dimerization, etc.

The number of antibodies, which are specific for proteins and protein modifications, including phosphorylation, is growing at a tremendous speed and in the future, it is very likely to obtain superior antibodies for most of the proteins and their modifications. To carry out research in a timely manner, suitable antibodies, combined on microarrays are used for initial protein-expression screening, very similar to the use of gene-expression profiling done today (Haab et al. 2001; Madoz-Gurpide et al. 2001).

Antibody microarrays hold potential promise for the high-throughput profiling of a smaller number of proteins (Fig. 1). Briefly, the antibodies (or other affinity reagents directed against defined proteins) are spotted onto a surface, such as a glass slide; a complex mixture, such as a cell lyzate or serum is passed over the surface to allow the antigens present to bind to their cognate antibodies (or targeted reagents). The bound antigen is detected either by using lyzates containing the fluorescently-tagged or radioactively-labeled proteins, or by using a secondary antibody against each antigen of interest. Low-density antibody arrays have been constructed that measure the levels of several proteins in blood and sera.

One important issue for antibody array is the matrix or surface used for immobilization. The golden standard in this respect are the porous membranes, such as nitrocellulose or nylon, which are used for a very long time in classical assays and have a very high capacity for protein binding. For the application in microarray technology, a combination of glass slides and nitrocellulose membranes is commercially available (Schleicher & Schüell) and is used for protein array applications (Knezevic et al. 2001). With a similar idea, this group earlier had developed an activated-agarose coating for slides that has a very good binding capacity for antibodies and ensures reliable and highly sensitive detection of protein antigens (Afanassiev et al. 2000). These and other microscopic glass-slide-based technologies require an optimized regime for handling, incubation, washing and a dedicated setup of incubation chambers.

Current methods of detecting protein activity by the array require the use of fluorescence and radioisotopes. To date, no protocol for antibody arrays has been

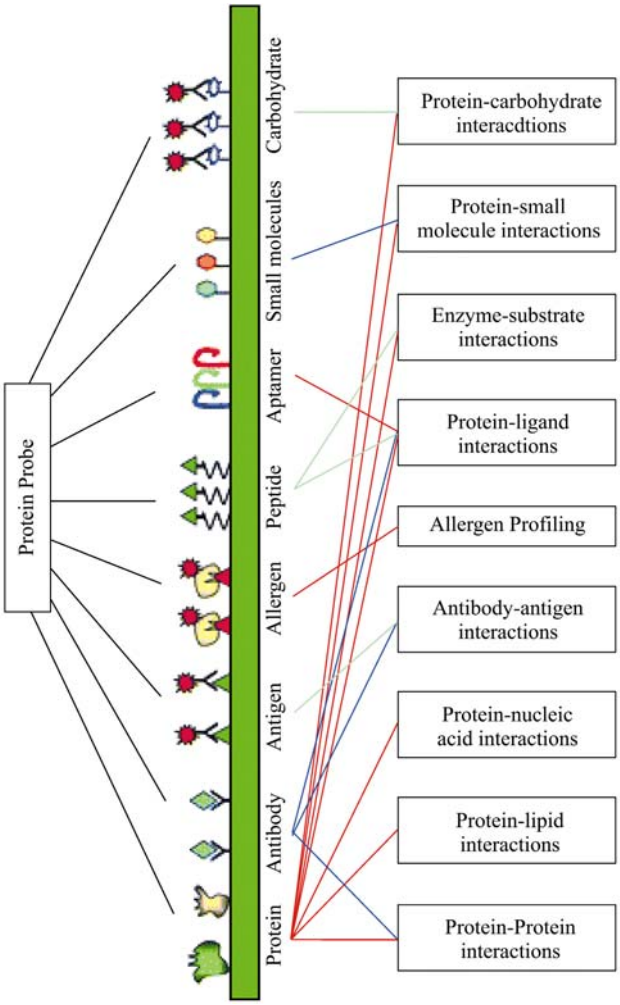

Figure 1 Protein microarrays and their applications. Ligands, such as proteins, peptides, antibodies, antigens, allergens and small molecules, are immobilized in high density on modified surfaces to form functional and analytical protein microarrays. These protein microarrays can also be used for various kinds of biochemical analysis (Zhu et al, 2003)

setup as a standard. Many problems need to be addressed, such as storage of antibody, protein purification, incubation and binding to arrays. This study attempts to analyze and compare the results in detail, under different conditions.

This experiment has developed the parameters, thresholds and testing conditions of a novel silver-staining system. Antibodies were selected with high specificity for phosphorylated proteins and these antibodies were combined on the microarrays in the ArrayTube™ platform. Nano-gold particles-mediated silver staining is a suitable technology for the detection of proteomics bound to an antibody array; quantification is possible using an online measurement of the silver precipitation step. The onset of precipitation and the speed of signal accumulation of the silver staining of the process enable the quantification, whereas endpoint signals only provide qualitative results. Combination of selective purification, e.g., cytoplasmic extract vs. nuclear extract with the antibody array technology, allows the phosphorylated proteins to the defined cellular compartments，the principle shown as in Fig. 2.

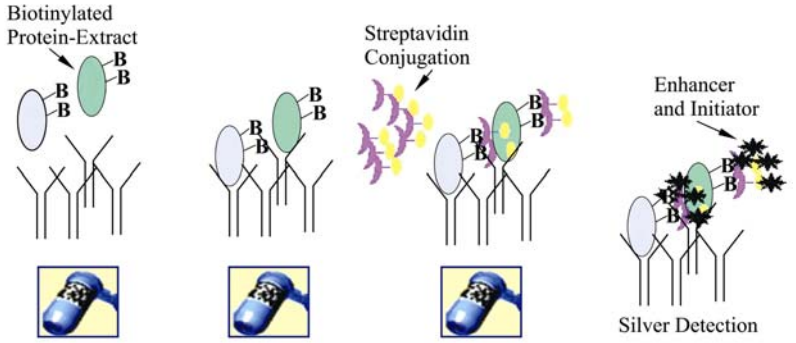

Figure 2 Nano-gold silver staining principle, including biotin-labeling protein, blocking Array-tube hybridization, nano-gold conjugation, enhancer and initiator

1.2 Protein Phosphorylation, Localization and Function

With the post-genome era, the proteomics study for direct analysis of proteins has become increasingly important (James et al. 1997). Identification of the type of modification and its location in a protein structure often provides crucial information for understanding the function or regulation of a given protein in the biological pathways (Zhu et al. 2003).

Phosphorylation is one of the most important protein modifications, by which many different cellular responses and biological processes are regulated.

Protein-localization data provide valuable information in elucidating eukaryotic protein function (Zhu et al. 2003). Protein trafficking between nucleus and

cytoplasm is fundamentally important in cell regulation. As such, the nuclear import and export are pivotal in orchestrating the activities of the key regulators of the cell cycle (Zhou et al. 2003), such as:

Smad nuclear translocation, which is a vital mechanism for the activin A-induced cell-death process in liver cells (Chen et al. 2000).

The extracellular signal-regulated protein kinase-1 (ERK1) and ERK2 nuclear translocation triggers cell proliferation in *in vitro* models (Tarnawski et al. 1998). The ERK activation plays an active role in mediating cisplatin-induced apoptosis of HeLa cells and functions upstream of caspase activation (cyto) to initiate the apoptotic signal (Wang et al. 2000). Lorenzini et al. (2002) verified in senescent cells that the ERK was unable to phosphorylate their nuclear targets, efficiently.

Dickens et al. (1997) inferred that the c-Jun amino-terminal kinase (JNK) cytoplasmic localization acts as an inhibitor of proliferation, by experimenting on murine cytoplasmic protein that binds specifically to JNK (the JNK interacting protein-1 [JIP-1]) causing cytoplasmic retention of JNK and inhibiting JNK-regulated gene expression.

The Fas induced MKK7-JNK/SAPK and MKK6-p38 pathways to cytoplasmic apoptotic activation (Toyoshima et al. 1997). The p38-MAPK induced cytoplasmic domain-dependent cellular migration (differentiation) of α-2 integrin subunit (Klekotka et al. 2001).

Phospho-tyrosine Rak (54 kDa) expressed in the nuclear and peri-nuclear regions of the cells in two different breast-cancer cell lines, inhibits their growth by arresting the G1 phase of the cell cycle (Meyer et al. 2003).

The STAT3 cytoplasmic localization was detected in the pathogenesis of mantle cell lymphoma (MCL) tumors. The nuclear localization of STAT3 was shown in node-negative breast cancer, which was associated with a better prognosis (differentiation and apoptosis) by tissue microarray analysis (Dolled-Filhart et al. 2003).

The Akt/PKB intra-nuclear translocation is an important step in signaling pathways that mediate cell proliferation (Borgatti et al. 2000). The Akt is not capable of phosphorylating its nuclear targets in senescent cells, efficiently (Lorenzini et al. 2002). The p70S6K is localized both in the cytosol as well as, after cytokine stimulation in nucleus, in the factor-dependent hematopoietic M-07e cells (Fleckenstein et al. 2003).

1.3 Interaction Proteomics and Pathway Building

It is widely acknowledged that the proteins rarely act as a single isolated species when performing their functions *in vivo*. The analysis of proteins with known functions indicates that proteins involved in the same cellular processes often

interact with each other. Following this observation, one valuable approach for elucidating the function of an unknown protein is to identify other proteins with which it interacts, some of which may have known activities. On a large scale, mapping protein-protein interactions has not only provided insight into protein function, but also facilitated the modeling of functional pathways to elucidate the molecular mechanisms of cellular processes (Zhu et al. 2003)

In this study, we attempted to elucidate some aspects of the regulatory network of signaling pathways and their role in the development of diseases. One of the most prevailing changes on the protein level is protein phosphorylation. In many cases, it reflects the activation state of the protein. It is controlled by kinase activities leading to phosphorylation and phosphatase activities, which in turn remove the phosphate groups. It is necessary to understand how a given activation signal growth factor, cytokine, morphogene or any drug, activates different signaling pathways. By classical methods, this would require an extremely large number of time-consuming experiments. Antibody array potentially allows the identification of all the proteins that carry those modifications in a single experiment.

1.3.1 BMP2 Signaling

The BMP2, member of the transforming growth factor-β (TGF-β) super family, is a multifunctional molecule regulating the growth, proliferation, differentiation, invasion and apoptosis in various target cells. Many researchers have shown that the BMP2 plays a vital role in cell proliferation and differentiation in development (Stoeger et al. 2002; Bain et al. 2003; Maguer-Satta et al. 2003; Nakamura et al. 2003; Pohl et al. 2003; Tsuda et al. 2003; Waite et al. 2003).

"The BMP2, as other members of the TGF-β family, elicits its cellular response through formation of heteromeric complexes of specific type-Ⅰ and Ⅱ serine/threonine kinase receptors. Five type Ⅱ receptors and seven type Ⅰ receptors, also termed as activin receptor-like kinases (ALKs) have so far been identified. The type Ⅱ receptor is a constitutively active kinase, which, upon ligand-mediated heteromeric complex formation, phosphorylates particular serine and threonine residues in the type Ⅰ receptor juxtamembrane region (also termed the GS-domain). The type Ⅰ serine/threonine kinase, thereby becomes activated and transduces signals downstream; type Ⅰ thus, acts downstream of type Ⅱ receptor and has been shown to determine signaling specificity within the heteromeric receptor complex" (Itoh et al. 2000), as shown in Fig. 3, Table 1.

"The Smads are pivotal intracellular nuclear effectors of the TGF-β family. Ligand-induced activation of the TGF-β family receptors with intrinsic serine / threonine kinase activity triggers phosphorylation of receptor-regulated Smads (R-Smads), whereas Smad2 and 3 are phosphorylated by TGF-β and activin-type Ⅰ

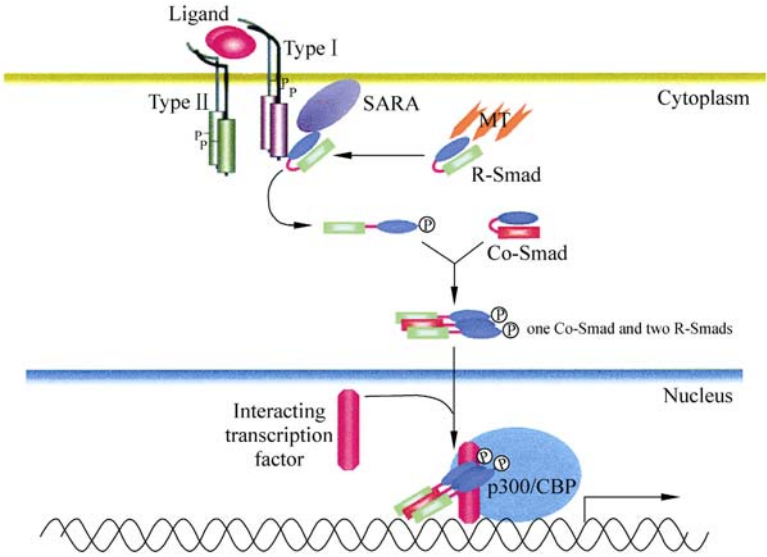

Figure 3 Activation of R- and Co-Smads. Upon ligand-induced heteromeric complex formation and activation of type Ⅰ and Ⅱ receptors, R-Smads are phosphorylated and form heteromeric complexes with Co-Smads that translocate into the nucleus, where they control the expression of target genes in a cell-type specific manner. Nonactivated Smads can be retained in cytoplasm through association with microtubules (MT). The recruitment of Smad2 to the TGF-β-receptor complex has been shown to involve a FYVE domain containing protein termed Smad anchor for receptor activation (SARA). Transcriptional modulation by Smads is achieved through complex formation with transcriptional coactivators like p300/CBP and interacting transcription factors. The R- and Co-Smads appear to form preferentially trimers, consisting of one Co-Smad and two R-Smads. However, Smad complexes with other stoichiometry cannot be excluded (Itoh et al. 2000)

receptors; Smad1, 5 and 8, act downstream of BMP-type Ⅰ receptors. Activated R-Smads form heteromeric complexes with common-partner Smads (Co-Smads), e.g., Smad4, which efficiently translocates to the nucleus, where they regulate, in cooperation with other transcription factors, coactivators and corepressors, the transcription of target genes. In most cases, inhibitory Smads act in a contrary manner from R- and Co-Smads. Like other components in the TGF-β-family signaling cascade, Smad activity is intricately regulated. The multifunctional and context dependency of TGF-β-family responses are reflected in the function of Smads, as signal integrators. Certain Smads are somatically mutated at high frequency in certain types of human cancers. Gene ablation of Smads in the mouse has revealed their critical roles during embryonic development and from Table 1, we can understand the binding partners of Smads (Itoh et al. 2000)."

The TGF-β-family factors have been shown to activate small GTP-binding proteins and MAP kinases in certain cells, (Itoh et al. 2000), as shown in Fig. 4.

Table 1 Binding partners of Smads

Binding proteins	Smad	domain	Functional properties
Membrane component			
ALK-1, 2, 3, 6	Smad1, 5, 6, 7, 8	MH2	Serine/threonine kinase
ALK-4, 5, 7	Smad2, 3, 6, 7	MH2	Serine/threonine kinase
SARA	Smad2, 3	MH2	FYVE finger anchor protein
Cytoskelton component Tubulin	Smad2, 3, 4	ND	Microtubule component
Nuclear transport protein Importin β	Smad3	MH1	Nuclear transporter
Cytoplasmic protein β-Catenin	Smad4	ND MH1	Wnt signal transducer; linking E-cadherin to the actin cytoskeleton
Calmodulin	Smad1, 2, 3, 4	PY motif	Calcium binding protein
Smurf1	Smad1,5	in linker	E3 ligase for ubiquitination
STRAP	Smad2, 3, 6, 7	MH2	WD repeat protein
TAK1	Smad6	ND	MAPKKK
Transcriptional coactivator			
MSG1	Smad4	MH2	Histone acetyltransferase
p300/CBP	Smad1, 2, 3, 4	MH2	
Transcriptional repressor	Smad1	MH1+ linker	Homeodomain containing protein
	Smad6	MH2	Zinc finger containing protein
Hoxc-8	Smad1, 2, 3, 5	MH2	Nuclear proto-oncogene product
	Smad2, 3, 4	MH2	Forkhead-associated domain
SIP1	Smad1, 2	ND	containing nuclear protein
Ski	Smad4	MH2	Nuclear proto-oncogene product
SNIP1	Smad2, 3, 4	MH2	Homeodomain containing protein
SnoN	Smad2	MH2	
TGIF			
Transcription factor			
ATF-2	Smad3, 4	MH1	b-ZIP containing protein
c-Fos	Smad3	MH2	Ap-1 family member
c-Jun, JunB, JunD	Smad3, 4	MH1, linker	Ap-1 family member
C-terminally truncated	Smad1, 2, 3, 4	ND	Zinc finger containing protein
Gli3	Smad3	MH2	Glucocorticoid receptor
GR	Smad3	MH1	Vitamin D receptor
VDR	Smad1, 2, 3	MH2	Adenoviral oncoprotein
E1A	Smad3	MH2	Zinc finger containing protein
Evi1	Smad2, 3	MH2	Winged-helix containing protein
FAST	Smad2, -3	MH1, MH2	HMG box containing protein
Lef1/Tcf	Smad4	MH1	Paired-like homeodomain

Continued

Binding proteins	Smad	domain	Functional properties
Transcription factor			
Milk	Smad2	MH2	containing protein
Mixer	Smad2	MH2	Paired-like homeodomain containing protein
	Smad1, 4	MH2	Zinc finger containing protein
OAZ	Smad1, 2, 3, 4	MH2	Runt-domain-containing
PEBP2/CBFA/AML	Smad3	ND	protein
p52	Smad2, 3, 4	MH1	NF-κB/Rel family
SP1, SP3			Transcription factor
TFE3 (μ E3)	Smad3, 4	MH1	Helix-loop-helix leucine zipper transcription factor

Abbreviations: ALK, activin-receptor-like kinase; AML, acute myeloid leukemia; AP, activator protein; ATF, activating transcription factor; CBFA, core-binding factor A; CBP, CREB-binding protein; CREB, cAMp-responsive element-binding protein; E1A, early region 1A; Evi, ecotropic virus integration; FAST, forkhead activin signal transducer; Gli, glioblastoma gene product; HMG, high mobility group; Hoxc-8, homeobox gene c-8; Lef, lymphoid enhancer factor; MH, Mad Homology; MSG, melanocyte-specific gene or mad-supporting gene; ND, not determined; OAZ, Olf1/EBF-associated zinc finger; PEBP2, polyomavirus-enhancer-binding protein; PY, PPXY motif; SARA, Smad anchor for activation; SIP, Smad-interacting protein; Ski/SnoN, Sloan-Kettering avian retrovirus/ski-related novel gene; Smurf, Smad ubiquination regulatory factor; STRAP, serine/threonine kinase receptor-associated protein; TAK, TGF-β activated kinase; Tcf, T-cell factor; TFE, transcription factor mu E3; TGIF, 5'TG'-interacting factor; VDR, vitamin D receptor. (Itoh et al. 2000).

"Crosstalk with Smad signaling may also result from the ability of TGF-β to activate signaling pathways, independent of Smads. The TGF-β can activate ERK-MAP kinase, p38-MAP kinase and JNK, although the extent and kinetics of activation differ among different cell lines and types. The MAP kinase TAK1, which is rapidly activated by TGF-β and which is also involved in other signaling pathways, could initiate these signaling cascades. The activation of p38-MAP kinase and JNK can enhance Smad signaling through either Smad phosphorylation or the phosphorylation of c-Jun and ATF-2, which are transcription factors that cooperate with Smad3, resulting in functional crosstalk with Smad-mediated transcription at the defined promoters" (Derynck et al. 2001).

"In addition, TGF-β can activate or stabilize the small GTPases, RhoA and RhoB; these may in turn have roles in several responses to TGF-β, for example, through the requirement of RhoB for activation of JNK. Finally, TGF-β induces an interaction of protein phosphatase 2A with S6 kinase, which regulates protein translation and growth control, decreasing its activity. Although, the mechanisms of activation by TGF-β and the roles of these non-Smad signaling cascades remain to be better characterized, these observations indicate that inactivation of

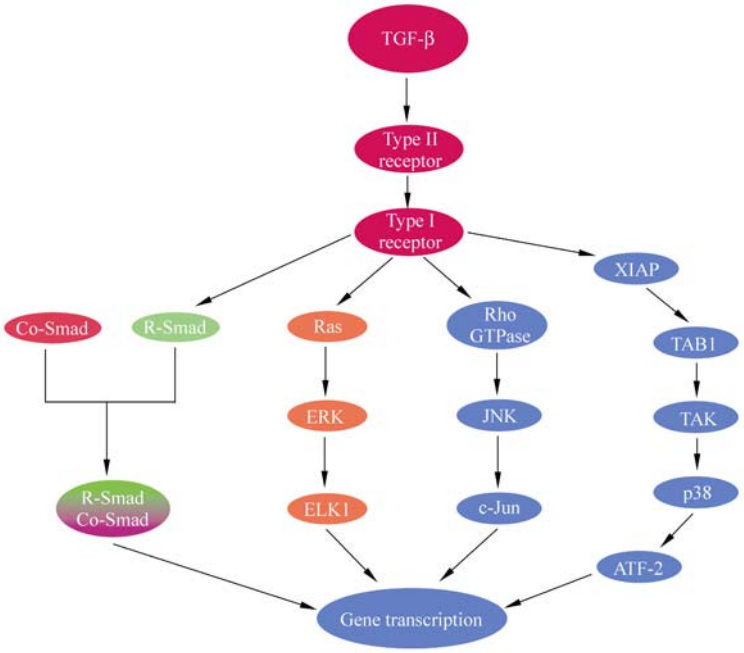

Figure 4 The TGF-β receptor-initiated signaling cascades. The TGF-β activates the Smad pathway as well as other signaling pathways. One example of a downstream transcription factor target for the different activated MAPK is indicated. Possible TGF-β receptor-induced responses that are independent of (Smad-mediated) transcription, as well as crosstalk between different downstream effector pathways are not indicated. Abbreviations: ATF, activating transcription factor; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; TAB, TAK1-binding protein; TAK, TGF-β activated kinase; TGF-β, transforming growth factor-β; XIAP, X-linked inhibitor of apoptosis protein (Itoh et al. 2000)

the Smad pathways may not leave the cell unresponsive to TGF-β" (Derynck et al. 2001), as shown in Fig. 5.

However, the molecular mechanisms of activation and inhibition of signal transduction from BMP2 to multifunctional positive and negative mediators of cells, such as p-p70S6 and p-Akt (PI3K signaling), p-p38 (p38 network), p-ERK and p-JNK (MAPK pathway), p-tyrosine (tyrosine-kinase network), p-STAT3 (JAK/STAT network) and p-Smad1, 2, 3 (Smad network) in U937 are not well characterized. More importantly, the modulation of phosphorylation and the subcellular localization have not been investigated. And also, while the role of BMP in development and bone formation is being well characterized, little information is known about its role in tumor.

In this study two different conditions of BMP2 signaling are compared by using antibody microarray for a long time and high concentration of BMP2 treatment of U937 cells. The first experiment verified the BMP2 induction of

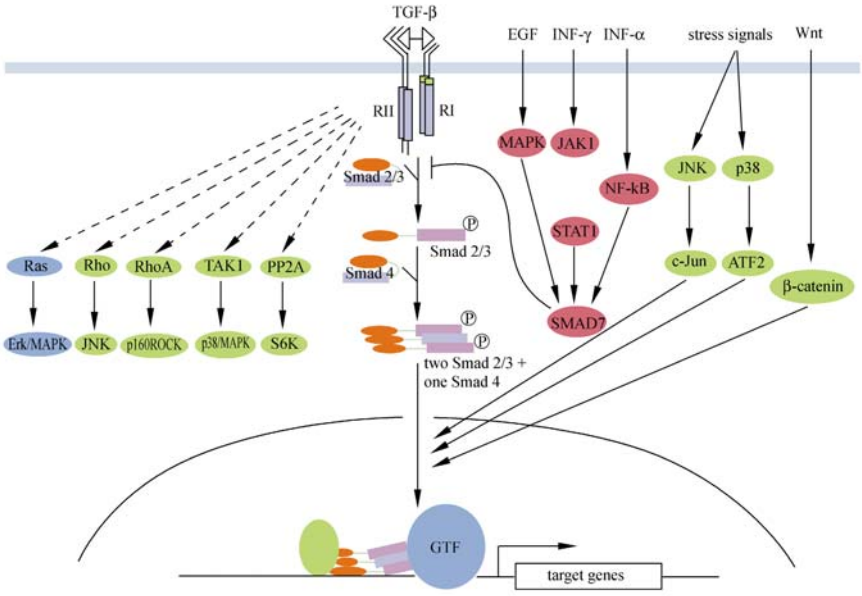

Figure 5 "The TGF-β-induced signaling through Smads and several non-Smad signaling mechanisms. After ligand-induced activation of the receptor, Smad2 and/or Smad3 interact transiently with the TRI receptor (RI) and this interaction is stabilized by the FYVE protein SARA. Smad2 and Smad3 are phosphorylated on their C-terminals by TRI, and then dissociate from the receptor to form a heterotrimeric complex, comprising two receptor-activated Smads and Smad4. This complex then translocates into the nucleus, where it interacts at the promoter with transcription factors, with sequence-specific DNA binding to regulate gene expression. The heteromeric Smad complex also interacts with the CBP/p300 transcriptional coactivator, which connects the Smad complex with the general transcription factors (GTF). Smad7 inhibits activation of Smad2 and/or Smad3 by the receptors and, its expression is induced on stimulation of one of several signaling pathways, for example, in response to EGF, interferon-β (IFN-β) or tumor necrosis factor-β (TNF-β). Several other signaling pathways also regulate both signaling by Smads and Smad-mediated gene expression, as exemplified here by the activation of JNK and p38-MAP kinase signaling in response to various stress signals and β-catenin signaling in response to Wnt proteins. The TGF-β also induces activation of Ras, RhoB and RhoA, as well as the TAK1 and protein phosphatase 2A, which leads to the activation of several MAP kinase pathways and the downregulation of S6 kinase activity. The mechanisms of activation of these non-Smad signaling events and how they connect to the heteromeric TGF-β receptor complex remain to be characterized (Derynck et al. 2001)"

apoptosis in U937 cells, by observing the cell number and FACS, after the cells were treated with 2000 ng/ml BMP2 for 3 days, which was considered as the optimal concentration and incubation time; the other selected short time and lower concentration of BMP2 treatment of MCF7 cells was performed by treating the MCF7 cells with lower concentration of BMP2 (100 ng/ml) for 4 h.

1.3.2 The Effect of STI571 on Cell Signaling

The STI571 that acts as an inhibitor of the Bcr/Abl, c-Kit and platelet-derived growth factor receptor kinases (Buchdunger et al. 2000), has been shown to inhibit the growth of Bcr/Abl-positive leukemic cells (Druker et al. 1996). However, (Yu et al. 2002) it has been indicated that the exposure of Bcr/Abl-positive cells to STI571, generally, has not been associated with downregulation of the Bcr/Abl protein and was reported that exposure of K562 cells to concentrations of STI571 that minimally induced apoptosis (200 nM), resulted in early suppression (i.e., at 6 h) of p42/44 MAPK phosphorylation followed ($\geqslant$24 h) by a marked increase in the p42/44 MAPK phosphorylation/activation at later intervals.

Importantly, clinical trials have now demonstrated that STI571, when administered at doses >300 mg/day, achieves clinical remissions in the large majority of patients with CML (e.g., 96%) (Druker et al. 2001). In addition, preclinical studies have demonstrated that the combination of STI571 with established chemotherapeutic drugs (e.g., Ara-C) results in enhanced toxicity in Bcr/Abl-positive leukemias (Thiesing et al. 2000). The findings of Fang et al. (2000) raised the possibility of combining STI571 with such agents, which might lead to an enhanced activity in CML and/or circumvention of drug resistance. In this context, Vigneri and Wang (2001) recently reported that coadministration of STI571 with leptomycin, an inhibitor of the nuclear export sequence receptor, resulted in increased killing of cells expressing Bcr/Abl. However, in this study optimal killing occurred in cells exposed to 10 μM of STI571, which is higher than the concentrations obtained in the plasma of patients receiving this agent (Druker et al. 2001).

In order to overcome the problems of STI-drug treatment as discussed earlier, various combination strategies may be possible. To identify important interactions relevant for STI571 treatment, it is necessary as well as interesting to study how different signal pathways are affected by STI571. Therefore, in this study we treated the human leukemia cell-line K562 with STI571 and studied the changes in signaling protein phosphorylation and localization, using the phosphorylation antibody array. As in the other examples, the cytoplasmic and nuclear extracts were analyzed independently.