Effects of nerve growth factor on TGF-β/Smad signal transduction in PC12 cells

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Marion Lutz
aus Nürnberg

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1. Gutachter: Prof. Dr. W. Sebald

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Meinen Eltern

‹‹ Das Glück deines Lebens
hängt von der Beschaffenheit
deiner Gedanken ab ››

Marc Aurel

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1 Introduction

The development and subsequent survival of multicellular organisms is a highly complex process that requires accurate regulation and coordinated interactions between a variety of different cells.

Communication between cells is mediated either through direct cell-cell contacts or through soluble mediators such as hormones, antigens, growth factors or cytokines.

Target cells receive the stimulus from the surrounding environment and rapidly transmit the signal into the cell where it is finally translated into biochemical responses such as modulation of enzymatic activities or regulation of gene expression. Initial ligand binding is mediated by specific receptors which are represented by intracellular transcription factors in the case of steroid hormones and by transmembrane receptors in the case of antigens, cytokines and growth factors.

Interestingly, despite the multitude of different stimulators, all of them use similar principles of intracellular signal transduction. Fundamental mechanisms are activation of kinases, phosphorylation and dephosphorylation, activation of G-proteins, oligomerization, activation of transcription factors and ligand-induced internalization of receptor-ligand complexes.

The complexity of intracellular signal propagation becomes evident if it is considered that signal transduction does not proceed in a linear stepwise fashion. Rather, various distinct signaling cascades mutually influence each other on different levels. Consequently, each living cell comprises a complex network of signaling pathways, all of which need to be tightly balanced and coordinated.

Elucidation of crosstalk between different pathways has taken on added importance as it substantially contributes to understand the nature of cell- and tissue-specific effects of distinct cytokines or growth factors.

The center of the present work, is the investigation of the effects of nerve growth factor (NGF) on the transforming growth factor-β (TGF-β)/Smad pathway. Therefore, the function and signal transduction of TGF-β and characteristics of NGF signaling will be described in the following.

1.1 TRANSFORMING GROWTH FACTOR-β (TGF-β)

1.1.1 TGF-β superfamily

TGF-β represents the prototype of a family of more that 30 structurally related but functionally distinct growth factors, known as the TGF-β superfamily [1, 2]. The latter includes bone morphogenetic proteins (BMPs), *drosophila* decapentaplegic (dpp), activins, inhibins, growth and differentiation factors (GDFs) and the more distantly related glial-derived neurotrophic factor (GDNF) and Müllerian inhibitory substance (MIS) which is also referred to as anti-Müllerian hormone (AMH) [3-5]. Members of this superfamily are conserved among organisms ranging from insects to human and they all share the conservation of seven or nine cysteine residues which form the characteristic “cystine-knot” structure. The existence of this structure allows to additionally assign the TGF-βs and its related molecules to the superfamily of cystine-knot containing growth factors which also comprises nerve growth factor (NGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and human chorionic gonadotrophin (HCG) [6-8].

1.1.2 Structure and functions of TGF-β

The founding member of the TGF-β superfamily was initially identified as a protein that induces transformation *i.e*. anchorage-independent growth of normal rat kidney fibroblasts [9]. Based on this property, the protein was named transforming growth factor-β (TGF-β). However, it became evident that TGF-β is a multifunctional protein that is ubiquitously secreted and acts on many different cell types to elicit a plethora of versatile effects which will be presented below (see Fig. 1.2).

Biologically active TGF-β is a homodimer of ~25 kDa consisting of two identical subunits of 112 amino acids which are linked by a single disulfide bridge. Nine cysteine residues are conserved among the TGF-β isoforms. Eight of them form the characteristic cystine-knot structure and one of them is engaged in forming the interchain disulfide bridge [10].

The three-dimensional structure of TGF-β2 which was determined by X-ray crystallography revealed an extended shape of the two monomers each of which consists of a central β-sheet with the cystine-knot on one end [10, 11] (Fig. 1.1). The two TGF-β monomers assemble to form an antiparallel dimer which is in contrast to the parallel dimerization mode of NGF. NGF also belongs to the cystine-knot containing growth factors and elicits similarity to TGF-β referring the monomer topology (see chapter 1.8.2).

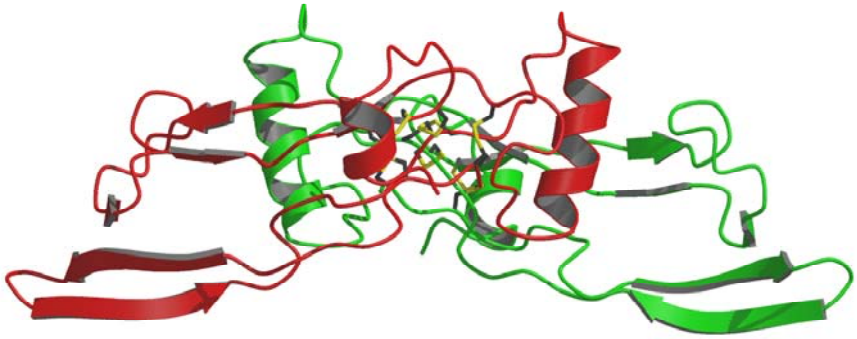


Fig. 1.1 Structure of TGF-β3
TGF-β3 represents an antiparallel dimer of two identical monomers which are depicted in red and in green, respectively. The cystine-knot structure is located in the center, indicated by yellow cystine-bridges. (According to Protein Data Bank; PDB ID: 1TGJ)

Five different isoforms of TGF-β are known. Three of them (TGF-β1, -β2 and -β3) exist in mammals whereas TGF-β4 and TGF-β5 have been proposed to represent chicken and *Xenopus* homologs, respectively, of the mammalian TGF-β1 [5]. Although the isoforms show similar functions in *in vitro* experiments, their effects *in vivo* are clearly distinct. This becomes evident (i) by the fact that different isoforms depict specific spatial and temporal expression patterns and (ii) by the distinct phenotypes of isoform-specific knock out mice [12-14].

As depicted in Fig. 1.2, TGF-β is capable of affecting a broad spectrum of cellular processes. The most prominent properties will be briefly described and will be connected with their potential pathological relevance.

TGF-β is implicated in mediating cell cycle arrest in most cells with the exception of some fibroblasts and some mesenchymal cells which are rather growth stimulated by TGF-β [15, 16]. Consequently, loss of TGF-β signaling is directly linked to transformation of cells and to tumorigenesis [17, 18]. Referring to the immune system, TGF-β is attributed to have potent regulatory properties [19, 20]. Based on immunosuppressive effects, therapeutic application of TGF-β in autoimmune diseases might be promising.

Furthermore, TGF-β is a potent inducer of extracellular matrix production and therefore plays a critical role in several fibrotic diseases [21, 22].

Worth mentioning is moreover the dual role of TGF-β in tumorigenesis. TGF-β is acting both, as suppressor and as promoter of tumorigenesis [23, 24]. In normal cells and in early stages of tumorigenesis, TGF-β mediates growth inhibition and thus prevents tumor progression. However, in the course of tumorigenesis, cells often become TGF-β resistant and thus escape from TGF-β-mediated growth arrest. By means of increased TGF-β production of the tumor cell, neighboring non-transformed cells become growth inhibited, angiogenesis is induced and epithelial to mesenchymal transition is supported. All these effects provide favorable conditions for the tumor cells and thus promote tumor progression and metastasis [24, 25].

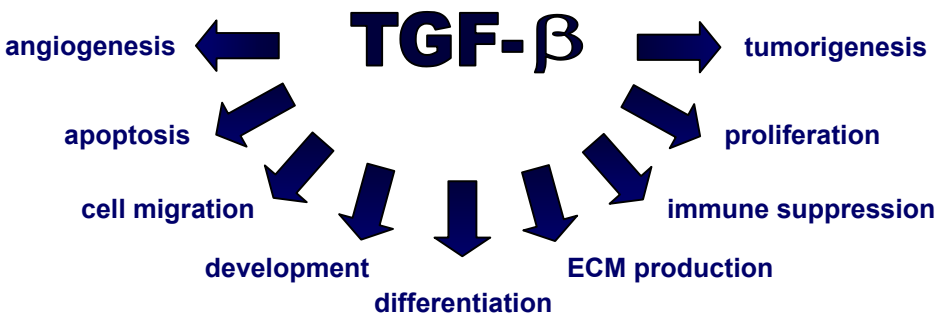


Fig. 1.2 Cellular processes that are affected by TGF-β
TGF-β is a multifunctional cytokine that is capable of regulating a tremendous array of cellular processes, spanning events from early development through homeostasis to programmed cell death.

1.2 TGF-β SIGNAL TRANSDUCTION

TGF-β homodimers signal through transmembrane serine/threonine kinase receptors designated as TGF-β type I (TβRI) [26] and type II (TβRII) receptors [27, 28] (see chapter 1.4). Initial ligand binding to the constitutively active TβRII, is followed by recruitment of TβRI into a heteromeric complex [29]. Subsequent transphosphorylation of TβRI at the juxtamembrane glycine/serine-rich region, the so-called GS-box, is mediated by TβRII and leads to activation of TβRI. TβRI in turn leads to activation of Smad proteins which act as intracellular signal mediators (see chapter 1.5). Members of the Smad family can be divided into three subfamilies: receptor-regulated Smads (R-Smads), common mediator Smads (Co-Smads) and inhibitory Smads (I-Smads) [30]. TβRI causes phosphorylation of TGF-β-specific R-Smads at their conserved C-terminal SSXS-motif thereby causing dissociation from the receptor and heteromeric complex formation with Smad4 [31-33]. Smad complexes translocate to the nucleus, assemble with specific DNA-binding cofactors and comodulators to finally activate transcription. The choice of target genes is thereby determined by the composition of the transcriptional complex [34].

This relatively simple general signaling pathway underlies tight and subtle regulation at various levels of the cascade and therefore becomes highly complex if all the potential regulatory elements are taken into account (Fig.1.3).

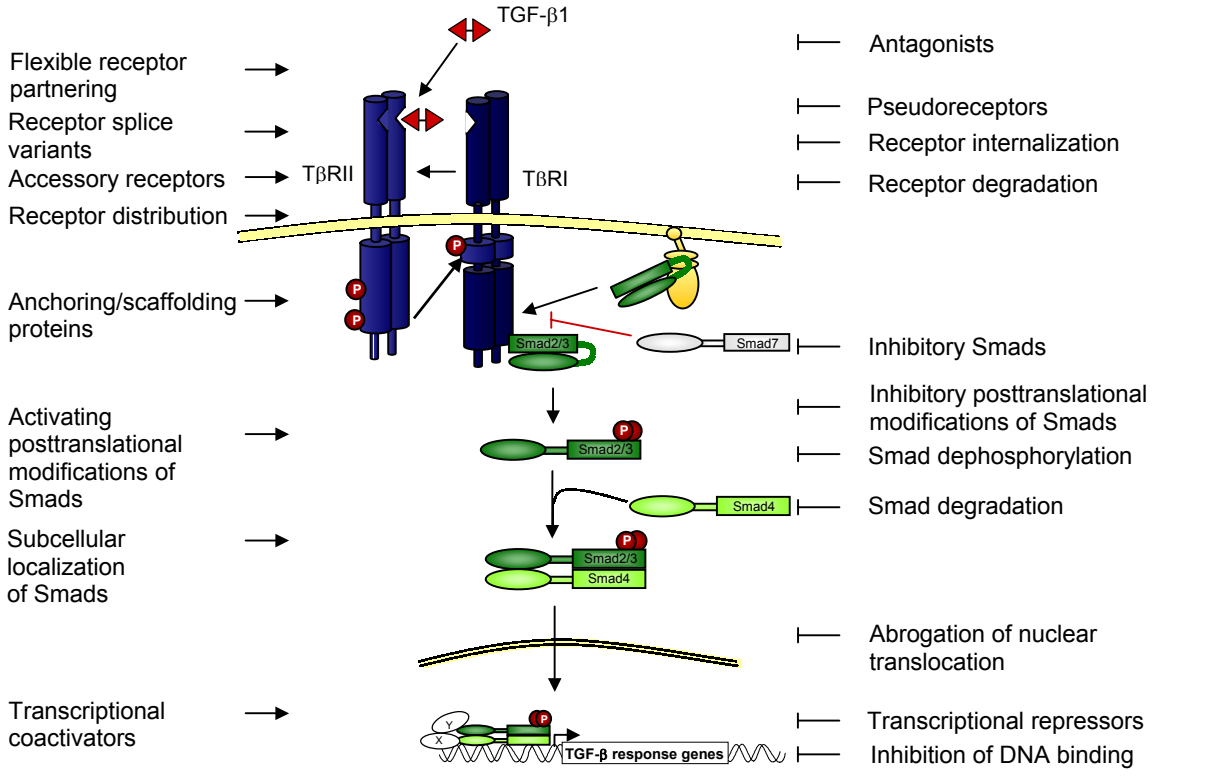


Fig. 1.3 TGF-β/Smad signal transduction and modulating inputs
The relatively simple model of signal transduction by TGF-β gets highly complex if all modulating inputs from other molecules are taken into account. From regulating the bioavailability of the ligand in the extracellular compartment to regulating gene expression in the nucleus, each step is tightly regulated. Signal promoting inputs are listed on the left side and signal inhibiting inputs on the right side.

1.3 TGF-β ACTIVATION

TGF-β is initially synthesized as a precursor proprotein which is cleaved during secretion. However, the mature disulfide-bonded TGF-β dimer remains non-covalently associated with the N-terminal propeptide which is referred to as latency associated protein (LAP) and prevents receptor binding of the mature TGF-β [35, 36]. In contrast to this “small latent complex”, the “large latent complex” differs in the additional association of a latent TGF-β binding protein (LTBP) which is linked to LAP via a disulfide bond. LTBPs, which were revealed to occur in four different isoforms, have no impact on TGF-β latency. Nevertheless, they are attributed to play a crucial role in folding and secretion of TGF-β. Furthermore, LTBPs are known to mediate deposition of the latent TGF-β complex to the extracellular matrix, thereby providing a reservoir for latent TGF-β [37, 38].

Given that the latent TGF-β complex is not capable of interacting with the TGF-β receptors, activation of TGF-β represents the key event in regulating the bioavailability of active TGF-β. Several mechanisms have been found to be responsible for TGF-β activation. *In vitro*, dissociation of LAP from TGF-β can be induced by heat, acidic pH or detergents [35]. *In vivo*, enzymatic processes such as deglycosylation of LAP or proteolytic degradation of LAP by

plasmin have been reported to play a central role. Further activators are thrombospondin-1 [39], integrin $\alpha_V\beta_6$ as well as hormones or other molecules that induce TGF-β activation by indirect mechanisms (*e.g.* Vitamin A derivatives which increase plasmin activity) [37 and references therein, 40]. It is likely that several proteins synergize in mediating TGF-β activation as this process requires two distinct steps of modification: first, the release from the extracellular matrix by degradation or cleavage of LTBP and second, the removal of LAP (Fig. 1.4).

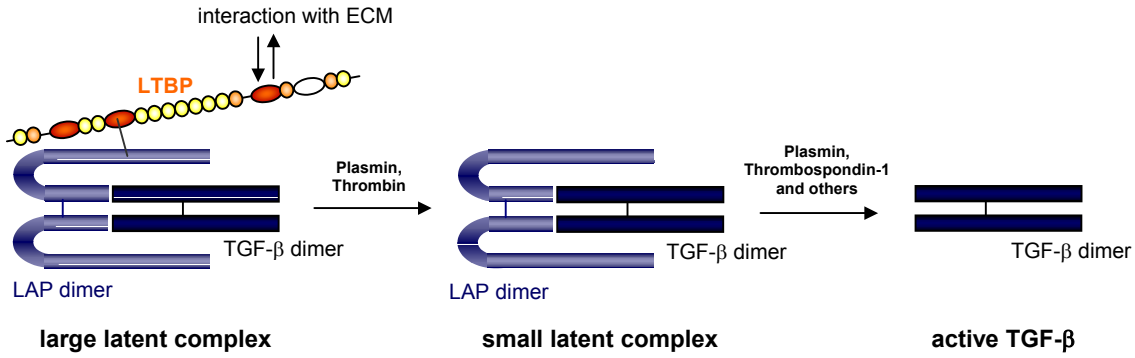


Fig. 1.4 Stepwise activation of TGF-β
Activation of TGF-β requires two sequential steps. First, TGF-β is released from the extracellular matrix by degradation or cleavage of LTBP and second, the LAP-dimer is removed by proteolytic cleavage.

1.4 RECEPTORS INVOLVED IN TGF-β SIGNALING

1.4.1 TGF-β receptor repertoire

1.4.1.1 Signaling receptors

In mammals, only five type II receptors and seven type I receptors have been identified for ligands belonging to the large TGF-β superfamily [4]. With the only exception of the GDNF receptor Ret, they all represent transmembrane receptors that contain an intracellular serine/threonine kinase domain.

The prototypic signaling receptors for TGF-β are the TGF-β type II receptor (TβRII) [27] and the TGF-β type I receptor (TβRI) [26]. In addition, two other TGF-β binding proteins designated betaglycan (or TβRIII) [41] and endoglin [42] are frequently involved in formation of receptor complexes and function predominantly in ligand presentation. However, the repertoire of TGF-β receptors is supplemented by splice variants of the receptors or by other receptors that are also capable of transducing signals in response to TGF-β.

In addition to TβRI, there are other type I receptors such as ALK1 and ActRI (ALK2) that transmit signals evoked by TGF-β.

Coexpression of ALK1 with TβRII and endoglin promotes efficient signaling in response to TGF-β1 and TGF-β3 but not TGF-β2 [43]. In endothelial cells, TGF-β1 was shown to mediate signals by either ALK1 or TβRI. ALK1 signals were found to trigger Smad1/5 activation

whereas TβRI phosphorylates Smad2/3 and the balance between these two pathways determines the signaling outcome *i.e.* causing distinct properties of the endothelium during angiogenesis [44].

ALK2 is similar to ALK1 in that it binds TGF-β following interaction with TβRII and an accessory receptor (see 1.4.1.2) and conveys the signal via the BMP-Smads, Smad1 and Smad5, respectively [45, 46]. ALK2 is postulated to promote epithelial-mesenchymal transition stimulated by TGF-β1 or TGF-β2 in murine mammary epithelial cells [47].

TβRI was found to appear in different variants arising from alternative splicing events. Agrotis and coworkers [48] found a splice variant, referred to as ALK5-S, which is competent in TGF-β1 signaling and even depicts a tendency to be more potent than the normally processed receptor.

ALK-7 is still an orphan member of the type I receptor family which is predominantly found in the nervous system [49, 50]. Nevertheless, there are some lines of evidence that hint to a functional role of ALK7 in TGF-β signaling [49, 51].

Referring to the type II receptors, there exists a splice variant of TβRII, TβRII-B [52], which contains an insertion of 25 amino acids in the extracellular part of the receptor and shows functional differences in isoform specificity. Whereas TβRII does not bind the TGF-β2 isoform unless it is presented by TβRIII, TβRII-B is able to bind all three TGF-β isoforms irrespective of the presence of TβRIII [53]. An outstanding physiological relevance for TβRII-B expression is therefore predicted for tissues such as bone, in which TGF-β2 represents the major TGF-β isoform.

1.4.1.2 Accessory receptors

The major TGF-β binding molecule on most cell types is TβRIII, also called betaglycan [41, 54]. TβRIII is a transmembrane proteoglycan that is able to bind all three TGF-β isoforms via two independent binding sites in the core protein [55, 56]. As mentioned above, particular importance is assigned to TβRIII in presenting TGF-β2 to TβRII which shows only low intrinsic affinity for TGF-β2 [57]. In contrast to the facilitation of ligand access to the receptors, the soluble secreted ectodomain of TβRIII [58], which results from proteolytic processing, accounts for antagonistic effects through binding and sequestrating the ligand [59]. Still puzzling is the function of the short cytoplasmic part with no discernible signaling entity. Distinctive for this domain is the high content of serines and threonines which represent suitable sites for phosphorylation [54]. Indeed, a previous report describes that phosphorylation of the cytoplasmic domain by autophosphorylated TβRII initiates the release of TβRIII from the active signaling complex, consisting of TβRI, TβRII and the bound ligand [60]. Thus, depending on the cellular context, betaglycan can act as a dual modulator – either promoting or inhibiting TGF-β signal transduction.

Endoglin, a homodimeric integral glycoprotein composed of disulfide-linked subunits is expressed at high levels on vascular endothelial cells. It shares regions of sequence similarity with TβRIII and its cytoplasmic domain likewise lacks known signaling motifs [61]. Similar to TβRII, endoglin interacts with TGF-β1 and -β3 but not with TGF-β2 [61, 62]. This restrictive isoform-specificity might be explained by the fact, that endoglin alone is not able to bind TGF-β but requires coexpression of the type II receptor to convey ligand binding [63]. In addition, endoglin binds activin-A, BMP-2 and BMP-7 only in the presence of their respective ligand binding receptors. Thus, unlike TβRIII, endoglin does not function in modulating ligand access to the receptors but probably contributes to recruit other proteins to the signaling complex.

1.4.1.3 Pseudoreceptors

A receptor variant that cross-regulates pathways of different members of the TGF-β family is represented by the BMP and activin membrane bound inhibitor (BAMBI) [64]. BAMBI is a naturally occurring pseudoreceptor which extracellularly resembles a type I receptor but lacks the cytoplasmic kinase domain. The capacity to associate with various type I receptors prevents the formation of functional homodimeric type I receptor complexes and thus causes abrogation of BMP- as well as TGF-β- and activin-mediated signaling.

1.4.2 Structure of TGF-β signaling receptors

The TGF-β type II receptor represents a 567 amino acid transmembrane glycoprotein which is composed of a relatively short cysteine-rich extracellular domain containing two potential N-glycosylation sites, a transmembrane domain and a cytoplasmic part consisting of a serine/threonine kinase domain and a short serine/threonine rich tail [27].

TβRII represents a constitutively active kinase that shows autophosphorylation. Autophosphorylation is not altered upon ligand binding, indicating that - in contrast to tyrosine kinase receptors - autophosphorylating activity of TβRII does not require ligand stimulation [29, 65]. The kinase was shown to undergo intra-molecular as well as inter-molecular autophosphorylation events that take place not only on serine and threonine but also on tyrosine residues [66]. Furthermore, autophosphorylation of specific residues was demonstrated to positively or negatively regulate receptor activity and downstream signaling [67].

TβRI also belongs to the family of serine/threonine kinase receptors and shows a similar overall structure to that of TβRII. In contrast to TβRII, TβRI contains a highly conserved serine/glycine rich sequence in the juxtamembrane region which is known as the “GS-box”. In addition, TβRI lacks the serine/threonine rich tail that is present in TβRII [68]. Besides the

GS-box, the so called L45 loop which determines the specificity for distinct R-Smads (see chapter 1.4.4) represents an important structural element of TβRI.

Ligand binding and ligand specificity is dependent on TβRII which is capable of binding TGF-β1 and TGF-β3 with high affinity. TGF-β2, however, does not bind to TβRII unless the ligand is presented by TβRIII [69, 70]. Alternatively, TGF-β2 is directly bound by the receptor splice variant TβRII-B [53].

Prerequisite for TGF-β induced signal transduction is the formation of a heteromeric receptor complex consisting of TβRI and TβRII because they are functionally dependent on each other: TβRI requires TβRII for ligand binding whereas TβRII requires TβRI to propagate the downstream signal [28, 65, 68].

1.4.3 Receptor oligomerization

At the cell surface, the overall distribution of the TGF-β receptors yields predominantly in a punctuate pattern and the receptors assemble to form different oligomeric units [71, 72]. TβRI, TβRII and TβRIII have all been found to be present as homo-oligomers in the absence of TGF-β [71, 73-75]. Based on their inherent affinity for each other, a small but detectable proportion of TβRI/TβRII heteromeric receptor complexes exists already in unstimulated cells [76, 77]. The fraction of these heteromeric complexes is significantly increased by ligand binding to TβRII which triggers recruitment of TβRI into a receptor complex and enables subsequent activation of TβRI and downstream molecules. This mechanism of sequential ligand binding is distinct from the cooperative binding mode that is known from BMP-receptors. BMP can directly bind to preformed complexes of BMP type I and type II receptors which exist to some extent at the cell surface already in the absence of ligand [78, 79].

The resulting TGF-β signaling complexes were unraveled to be tetrameric, consisting of two molecules each of TβRI and TβRII, and to represent the actual signaling entity [80, 81]. Homomeric receptor complexes, are not sufficient to propagate TGF-β responses but are proposed to be functionally important for regulating receptor kinase activity, as reported in the case of inter-molecular autophosphorylation at multiple serine residues of TβRII [67, 75].

1.4.4 TβRI activation

In the unphosphorylated state, interaction between the immunophilin FKBP12 and a helix that is located adjacent to the GS-motif of TβRI prevents phosphorylation by TβRII and thus represses catalytic activity of the kinase [82]. Activation of TβRI is achieved by ligand-induced conformational changes that trigger dissociation of FKBP12 and allow TβRII-mediated transphosphorylation at the conserved juxtamembrane GS-box [29]. Interestingly, TβRI activation is not due to an increase of the actual kinase activity but is rather based on

the creation of a binding site for R-Smad proteins which represent the substrates for TβRI. Along with formation of a binding surface for R-Smads, the docking site for the inhibitory protein FKBP12 is eliminated [83].

A key determinant of TβRI-Smad interaction is represented by a nine amino acid spanning region that is located between the kinase subdomains IV and V and is therefore termed the L45-loop [84]. Within the L45-loop, 4 amino acids that differ in TGF-β and BMP type I receptors, confer specificity for distinct Smad isoforms and thus separate TGF-β and BMP pathways [85, 86].

1.4.5 Receptor interacting proteins

Various receptor-interacting proteins have been identified that function to regulate TGF-β signaling by completely different mechanisms (Fig. 1.5).

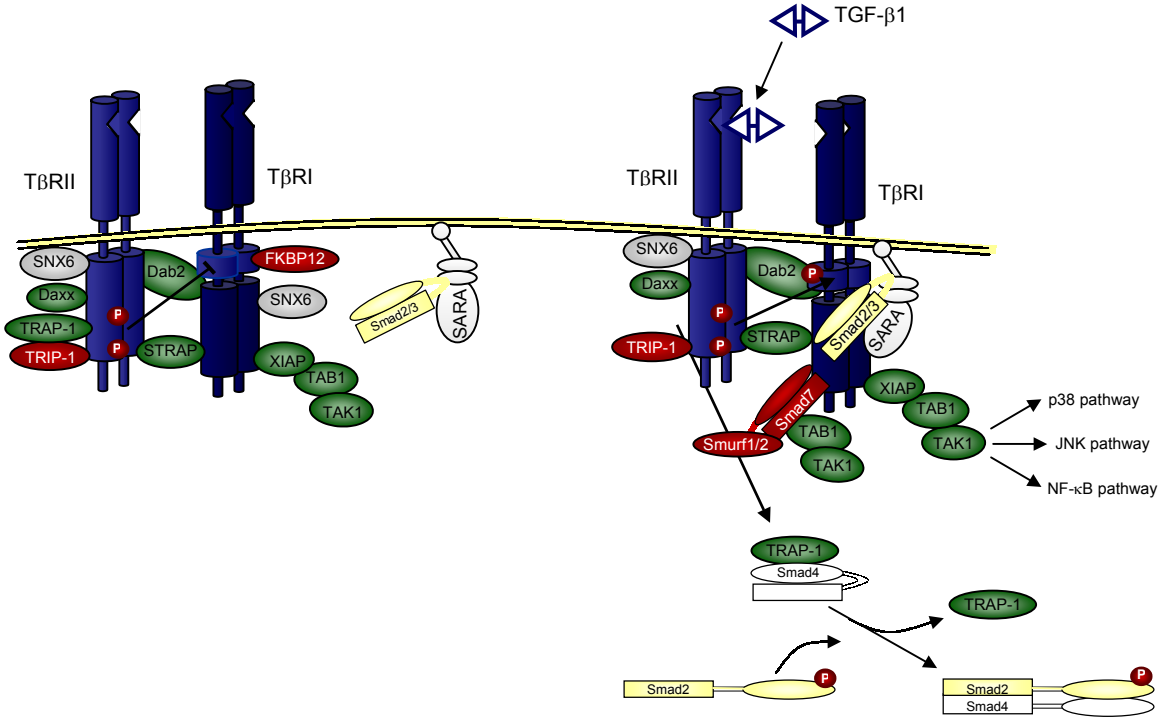


Fig. 1.5 Receptor interacting proteins
A number of proteins that positively or negatively influence TGF-β–induced signals were shown to interact with the cytoplasmic domain of the TGF-β receptors. Some of them (TRIP-1, Dab-2, XIAP, STRAP, SNX6 and Daxx) bind the receptors regardless of the kinase activity of the receptors, whereas others associate specifically with the activated receptor complex (SARA, Smad7, Smurf1/2) or are released from the receptor complex following ligand stimulation (TRAP-1, FKBP-12, SNX6). Antagonizing regulators are depicted in red, synergizing regulators are shown in green and proteins, the function of which is not yet known, are demonstrated in grey .

The basal receptor activity is kept under tight control by the immunophilin FKBP12 [87]. The inherent tendency of TβRI and TβRII to associate with each other, allows complex formation and TβRI activation already in absence of ligand. By binding to the unphosphorylated GS-box of TβRI [88, 89], FKBP12 stabilizes a conformation of TβRI that is incompetent of getting

transphosphorylated by TβRII, thus preventing ligand-independent signaling [90, 91]. Ligand binding to TβRII, however, induces conformational changes that lead to displacement of FKBP12 and subsequent TβRI activation by TβRII [83, 90].

Several receptor interacting proteins exert important functions in subcellular localization of Smad proteins. Among these, there are the Smad anchor for receptor activation (SARA) [72], disabled-2 (Dab2) [92] and the TβRI-associated protein-1 (TRAP-1) [93]. SARA contains a FYVE domain which mediates binding to phospholipids and consequently enables association with the cell membrane. The adjacent Smad binding domain allows interaction with Smad2 and Smad3 and the C-terminal part directly associates with the activated TβRI [72]. The cooperative binding of these proteins enables R-Smad phosphorylation by TβRI which is followed by dissociation of the R-Smad from SARA and formation of a heteromeric complex with Smad4. By masking the nuclear localization signal of Smad2, SARA functions also in retaining non-activated Smad2 in the cytoplasm [94]. Interestingly, SARA is required for Smad2 mediated signaling but is dispensable for Smad3 mediated signals [95]. The crystal structure of SARA binding to Smad2 was recently determined by Wu *et al.* [96].

Similar to the SARA protein, the member of the disabled gene family, Dab2, facilitates TGF-β signaling by bridging the receptor complex to the Smad proteins. Dab2 interacts with both, TβRI and TβRII in absence and in presence of TGF-β. The protein contains a C-terminal proline-rich domain (PRD) and an N-terminal phosphotyrosine binding site (PTB) that is likely to allow membrane association and binding to the MH2 domain of Smad2 and Smad3 in a ligand-dependent manner [92].

In contrast to SARA and Dab-2, TRAP-1 recruits the Co-Smad, Smad4, into the vicinity of the receptor complex. In absence of ligand, TRAP-1 interacts via multiple binding sites with TβRII. Ligand-induced changes in receptor conformation result in release of TRAP-1 from the complex. Transient interaction of TRAP-1 and Smad4 serves to attract the Co-Smad into the proximity of the receptor complex, thus facilitating heteromeric complex formation between activated R-Smads and Smad4 [97].

Negative regulation of TGF-β signaling is achieved by proteins such as the inhibitory Smad7 [98, 99], serine/threonine kinase receptor-associated protein (STRAP) [100] or the Smad ubiquitination regulatory factors (Smurf), Smurf1 [101] and Smurf2 [102].

Smad7 belongs to the subfamily of inhibitory Smads which were identified as potent inhibitors of TGF-β and BMP-signaling. Originally, Smad7 was described to block signal propagation by directly interacting with TβRI thereby preventing transient association and phosphorylation of R-Smads [98, 99]. Meanwhile, there are several lines of evidence that Smad7 can achieve signal abrogation by various mechanisms (see chapter 1.5.8.1)

In synergism with Smad7, the WD domain containing protein STRAP inhibits TGF-β mediated signals [100]. STRAP was described to interact with both, TβRI and TβRII, and

assists to stabilize the ternary complex consisting of Smad7, TβRI and STRAP. Moreover, STRAP is proposed to be involved in recruiting Smad7 to the TGF-β receptors [103].

E3 ubiquitin ligases of the HECT family, Smurf1 and Smurf2, are bridged to the receptor by Smad7. They possess two WW domains that mediate protein-protein interactions with the PPXY sequence (PY-motif) in the linker region of I-Smads as well as R-Smads. Induction of ubiquitination of Smad7 leads to subsequent proteasomal and lysosomal degradation of the complex containing Smad7 and receptor proteins [102, 104]. Based on their capacity to additionally interact with R-Smads, Smurf proteins can also modulate TGF-β signals by association with and degradation of R-Smads (see chapter 1.5.8.2).

An indirect interaction with the TβRI receptor can be assumed for TGF-β activated kinase-binding protein 1 (TAB1) [105]. TAB1 is an upstream signaling molecule of TGF-β activated kinase (TAK1) [106] which in turn triggers multiple other pathways such as the p38 mitogen-activated protein kinase (MAPK) and the c-Jun N-terminal kinase (JNK) cascade [107, 108]. Thus, the linkage of the TGF-β receptors to TAB1 and TAK1 represents a point of convergence with different other substantial signaling pathways. In addition, TAB1 can bind to the X-linked inhibitor of apoptosis (XIAP) [109] which serves as a cofactor in TGF-β signaling.

XIAP contains a C-terminal RING finger domain and three N-terminal baculovirus IAP repeats (BIR) whereby the latter represent the binding site for TβRI. Although XIAP serves as a bridging molecule between the receptors and TAB1, cooperative effects of XIAP and TGF-β are not necessarily mediated by TAB1/TAK1 dependent processes. The exact mechanism of how XIAP cooperates with TGF-β is not yet fully understood but activation of TGF-β responsive genes by XIAP was shown to be dependent on Smad4 whereas the anti-apoptotic effects of XIAP are elicited independent of Smad4 [110].

The TGF-β receptor interacting protein (TRIP-1) belongs to the WD40-repeat containing proteins and interacts with and gets phosphorylated by TβRII. By receptor-dependent as well as receptor-independent mechanisms, TRIP-1 specifically impedes the TGF-β induced gene expression from the PAI-1 promoter but does not repress TGF-β triggered growth inhibition [111].

Additional receptor interacting proteins, the functions and signaling mechanisms of which are not yet fully understood, are represented by the dimeric 14-3-3 protein, the sorting nexin 6 (SNX6) and the Daxx protein. 14-3-3 was first found in the parasitic trematode *Schistosoma mansoni*. It was shown to interact with TβRI and to act as a positive regulator of TGF-β mediated growth arrest probably through binding to Cdc25 [112]. SNX6 belongs to the family of sorting nexin proteins that interact with each other as well as with tyrosine kinase receptors and each of them can additionally bind to specific receptors of the TGF-β family.

SNX6 binds to TβRII and to the inactive TβRI. Functional relevance of sorting nexin proteins is proposed to reside in intracellular trafficking of receptors [113]. Daxx was originally identified as a Fas-receptor binding protein that triggers JNK activation and apoptosis [114]. Recently, it was demonstrated, that Daxx can interact with the constitutively active TβRII receptor and function as an intermediary between both, the Fas receptor and the TGF-β receptors on the one hand and the apoptotic machinery on the other hand.
Finally, the Bα subunit of protein phosphatase 2A [115] and the farnesyltransferase α (FT-α) should be mentioned [116]. These proteins belong to the class of WD-domain containing proteins and both of them were found to interact with and get phosphorylated by TβRI. Whereas Bα is known to potentiate the antiproliferative effects of TGF-β, the impact of FT-α on TGF-β signal transduction is not yet elucidated.

1.5 SMAD PROTEINS

The first members of the Smad family were discovered as the products of the "mothers against dpp" (Mad) gene in *Drosophila melanogaster* [117, 118] and the *sma-2*, *-3* and *-4* genes in *C. elegans* [119]. When homologs of Sma and Mad were identified in vertebrates, the names of their founder members were joined together to yield the name "Smads".

1.5.1 Classification of Smads

Smad proteins are so far the only known substrates for the TGF-β type I receptor that elicit signaling function in response to TGF-β. Although other receptor-interacting proteins can synergize with TGF-β signaling or mediate crosstalk, it is unique to the Smads that they transmit the signal directly from the receptors to the nuclear transcriptional machinery.
In vertebrates, eight members of the Smad family have been identified which can be further classified based on their structural and functional properties: (i) receptor-activated Smads (R-Smads) including BMP-activated Smads (Smad1, Smad5 and Smad8) and TGF-β-activated Smads (Smad2 and Smad3) which become phosphorylated by type I receptors, (ii) the common mediator Smad (Co-Smad: Smad4) which, as an obligate partner, oligomerizes with activated R-Smads and (iii) the inhibitory Smads (I-Smads: Smad6 and Smad7) which antagonize TGF-β or BMP signals by competing with R-Smads for type I receptor interaction (reviewed in [31, 120, 121]).

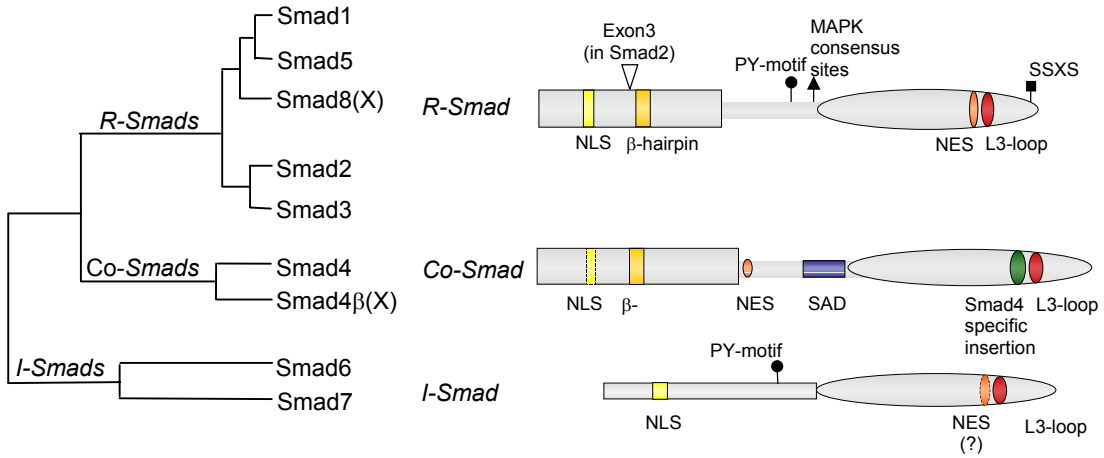


Fig. 1.6 The family of Smad proteins and their schematic structure
The subfamilies of Smad proteins are represented in a simplified dendrogram and the overall structures of the individual subfamily members are depicted. R-Smads and Co-Smads contain a nuclear localization signal (NLS) and a β-hairpin structure in the MH1 domain. In the MH2 domain, there is the L3-loop and a nuclear export signal (NES) which is located in the linker in the case of Smad4. Smad4 additionally contains the Smad activation domain (SAD) in the linker and a Smad4 specific insertion in the MH2 domain. The I-Smads consist of a conserved MH2 domain that contains a putative NES and the L3-loop and of a N-terminal part that differs from the MH1 domain of other Smad proteins. Smads signed with (X) are isoforms that are found in *Xenopus*.

1.5.2 Structure and functions of Smads

The overall structure of R-Smads and Co-Smads comprise the highly conserved N-terminal Mad homology 1 (MH1) and the C-terminal Mad homology 2 (MH2) domains which form globular structures and are linked by a divergent proline-rich region of variable length (Fig. 1.7). I-Smads likewise contain the conserved MH2 domain but show very little similarity to other Smads in their N-terminal part (reviewed in [30, 122]).

Lacking an intrinsic enzymatic activity, the Smad proteins exert their effects exclusively by protein-protein and protein-DNA interactions which are mediated by distinct functional regions within the proteins.

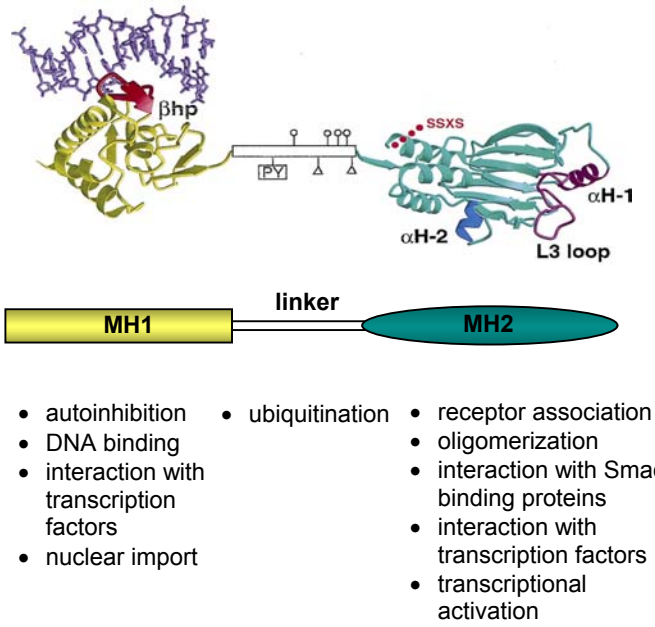


Fig. 1.7 Smad structural domains and their functions
The MH1 and MH2 domains form globular structures that contain several functional elements. The β-hairpin (βhp) in the MH1 domain enables DNA-binding. Interaction with the type I receptor is mediated by the L3 loop and the α-helix 2 (αH-2) in the MH2 domain. Cofactors such as FAST-1 associate with R-Smads via the α-helix 1 (αH-1). In the linker region, there are several consensus phosphorylation sites for MAP kinases and a proline-tyrosine motif (PY). (The Smad structure is taken from Massagué *et al*. [32]).

1.5.2.1 The MH1 domain

The MH1 domain was first characterized to mediate autoinhibition by physically interacting with the MH2 domain, thereby impeding its effector function in the absence of ligand [123]. Furthermore, the MH1 domain accounts for the ability to directly bind to DNA. Determination of the crystal structure of a Smad3 MH1 domain bound to DNA revealed that a conserved β-hairpin loop is responsible for binding to the major groove of the DNA helix [124]. An exception is represented by Smad2 which lacks DNA-binding capacity due to an insertion encoded by exon3 [125]. However, in cells that have removed exon3 from the *smad2* gene by alternative splicing, DNA binding of Smad2 is enabled [126]. Protein-protein interactions with transcription factors such as ATF-2 [127], c-Jun [128, 129], SP1 [130] or TFE3 [131] are also mediated by the MH1 domain.

1.5.2.2 The linker

The linker which connects MH1 and MH2 domains is less conserved between the different Smad classes but has been well conserved through evolution and contains several important regulatory peptide motifs. There are multiple consensus phosphorylation sites for mitogen-activated protein kinases (MAPK) [132] which upon phosphorylation cause sequestration of R-Smads in the cytoplasm by impeding nuclear translocation [132, 133]. Additionally, a proline-tyrosine (PY) motif present in R-Smads and I-Smads enables recognition by the WW domains of the E3 ubiquitin ligases, Smurf1 and Smurf2 [102, 104]. The Smad4 linker is

equipped with a nuclear export signal (NES) in the N-terminal part and with the Smad activation domain (SAD) in the C-terminal part of the linker which is essential for its transactivation activity [134].

1.5.2.3 The MH2 domain

The MH2 domain is known to mediate diverse protein-protein interactions, to exert effector function and, in case of the R-Smads, to provide receptor phosphorylation sites. In addition, an NES residing in the MH2 domain of R-Smads suggests a function of the MH2 domain in cytoplasmic targeting (see chapter 1.5.5). Protein-protein interactions mediated by the MH2 domain cover homomeric as well as heteromeric complex formation (see chapter 1.5.4). The MH2 domain is moreover involved in mediating Smad-receptor association. The specificity for distinct Smad isoforms is thereby determined by the L3 loop in the R-Smads and the L45 loop in the type I receptor [85, 86] (see chapter 1.4.4). The crystal structure of the Smad2 MH2 domain in complex with the Smad binding domain (SBD) of SARA pointed out that a basic patch adjacent to the L3 loop is likely to serve as a docking site for the receptor [96]. The activated type I receptor triggers phosphorylation of R-Smads and initiates the downstream cascade. In the nucleus, a multitude of coactivators, corepressors and DNA-binding partners can assemble with Smads to regulate expression of distinct target genes (see chapter 1.5.7).

1.5.3 Activation of Smads

TGF-β induced activation of TβRI is followed by transient interaction between TβRI and R-Smads which become phosphorylated at the last two serine residues within the C-terminal SSXS-motif [135-137]. Consequences of this phosphorylation event are the release of Smad autoinhibition [31, 123], the dissociation of the R-Smad from cytoplasmic retention proteins such as SARA [94], microtubuli [138] or potentially filamin [139] as well as from the type I receptor. At the same time, the affinity for Smad4 is increased [135, 136, 140].

1.5.4 Oligomerization of Smads

The stoichiometry is still a matter of controversy, for both, the homomeric [141, 142] and the heteromeric Smad complexes. In the absence of ligand, several distinct oligomeric states have been reported to occur. Kawabata *et al.* [141] postulated that all Smad proteins exist as monomers in the basal state. This seems to hold true for Smad2 in which the inserted exon3 precludes homo-oligomerization [142, 143]. Smad3 was found in varying oligomeric states [140, 142]. On the occurrence of Smad4, there are divergent opinions. Consistent with the crystal structure of the Smad4 MH2 domain [144], several groups propose Smad4 to exist as a homotrimer in the absence of ligand. Others, however, postulate Smad4 to be monomeric

since the Smad4 specific insertion in the MH2 domain (a structural extension referred to as the TOWER) prevents self-association [145, 146].

The heteromeric R-Smad/Smad4 complexes that assemble upon signal activation have been suggested to be hexameric [144], trimeric [140, 141, 145-147] or dimeric [142, 148]. Recent publications suggest for Smad1 [147] and Smad2 [143] that these R-Smads exist as monomers in the basal state. Upon ligand induced phosphorylation, they assemble to form a homotrimer which is stabilized by the subunit interface and by specific contacts between the phosphorylated tail and the L3 loop in the MH2 domain of the neighboring monomer [140, 149] (Fig. 1.8). However, formation of heterotrimers including Smad4 is energetically favored over the homotrimer formation.

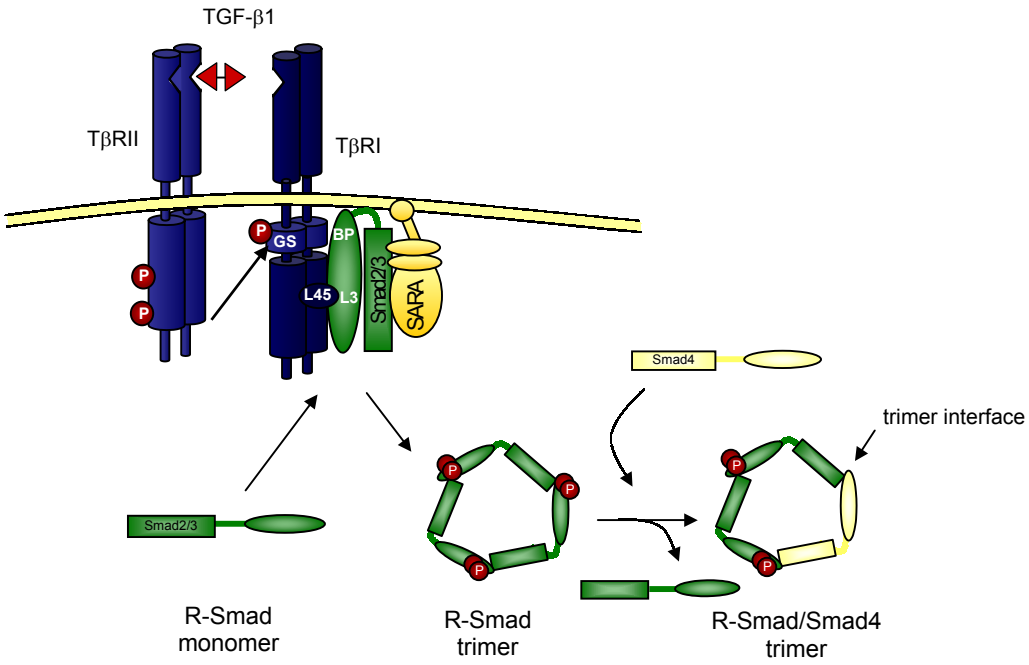


Fig. 1.8 Model for Smad activation and oligomerization
In the basal state, R-Smads are monomeric. Upon ligand binding, they transiently interact with the L45 loop (L45) of TβRI via its L3-loop and with the phosphorylated GS-box (GS) via a basic pocket (BP) in the MH2 domain. Phosphorylation of the R-Smad favors subunit trimerization and subsequently formation of a R-Smad/Smad4 heterotrimer which is energetically more stable than the homotrimer due to the unique favorable trimer interface in the R-Smad/Smad4 complex. (Adapted from Qin et al. [147])

1.5.5 Nucleoplasmic shuttling of Smads

In the absence of ligand, R-Smads as well as Smad4 are localized predominantly in the cytoplasm whereas I-Smads reside in the nucleus [94, 150-152].

Based on a basic nuclear localization signal (NLS)-like motif present in the MH1 domain, an intrinsic nuclear import activity is inherent to R-Smads. It is hypothesized for Smad3, that by virtue of the intra-molecular interaction of MH1 and MH2 the NLS-like motif is buried in the basal state. Phosphorylation of Smad3 causes conformational changes that expose the NLS-like motif, allowing interaction with importin-β1 and recognition by the nuclear import

machinery [150, 153]. Smad2 was shown to be retained in the cytoplasm by interaction with the anchor protein SARA. Ligand-induced release of SARA unmasks the NLS-like motif and leads to Smad2 nuclear translocation by a mechanism that is independent of importin-β1 but requires a specific region in the MH2 domain which elicits constitutive nuclear import function [94]. Although Smad4 contains only an incomplete NLS-like motif in its MH1 domain, it has the ability to constitutively enter the nucleus in absence of ligand. That yet cytoplasmic localization of Smad4 is observed under unstimulated conditions is based on active nuclear export mediated by the leucine-rich nuclear export signal (NES) that resides in the Smad4 linker region. Ligand-dependent nuclear entry of Smad4 presupposes inactivation of the NES which is achieved by hetero-oligomerization with R-Smads [151, 154].

In addition, an NES residing in the MH2 domain of R-Smads suggests a function of the MH2 domain in cytoplasmic targeting. Resembling Smad4, Smad1 was described to undergo constant nucleoplasmic shuttling conferred by the presence of both, functional NLS and NES. The four hydrophobic amino acids that are critical to convey nuclear export are conserved among all R-Smads, implying that they also trigger nuclear export of TGF-β specific R-Smads. However, despite the conserved sequence of the motif, structural and functional investigations rather hint to an executed activity of the NES only in BMP- but not TGF-β specific R-Smads [155].

In contrast to the R-Smads, the inhibitory Smad7 (but not Smad6) was found to be localized within the nucleus in the basal state and its nuclear export is triggered in response to ligand stimulation [152]. Although the mechanisms for both, nuclear localization and ligand induced nuclear export are not yet unraveled, there is evidence that an intact MH2 domain is critical for both events.

1.5.6 Smad interacting proteins

There is a constantly growing number of different proteins that interact with Smad proteins. They can be classified according to their function (*i.e.* receptors, coactivators, corepressors, transcription factors *etc.*) and according to the binding site in the Smad protein (Fig. 1.9). The individual proteins are not discussed in this chapter but detailed information on the proteins is given in the chapters of the respective protein type.

Function			Proteins
Regulatory phosphorylation	CamK II (S2)	CamK II (S2)	type I receptors (S1-3, S5, S8)
Receptors			ALK1-7
Oligomerization			R-Smads, Co-Smads
Cytoplasmic adaptors/ Cytoplasmic effectors	Filamin (S1-6) β-Importin (S3)	Filamin (S1-6)	SARA (S2, S3) STRAP (S2, S3, S6, S7) Dab2 (S2, S3)
		β-catenin (S4) Microtubules (S2-4) TAK1 (S6)	
Ubiquitination adaptors/ Ubiquitination substrates	HEF-1 (S3)	Smurf1 (S1, S5, S7) Smurf2 (S2, S3, S7)	HEF-1 (S3)
Transcriptional coactivators		MSG-1 (S4) p300/CBP (S1-4)	
Transcriptional corepressors	HDAC (S3)	Hoxc-8 (S1) Hoxc-8 (S1)	SIP-1 (S1-3, S5) Ski (S2-4) SnoN (S2-4) TGIF (S2) Tob (S1, S4, S5, S8)
		SNIP-1 (S1, S2, S4)	
Transcription factors	ATF-2 (S3, S4) Jun, JunB, JunC (S3, S4) Lef-1/Tcf (S2, S3) Sp1, Sp3 (S2-4) TFE3/μE3 (S3, S4) VDR (S3)		E1A (S1-3) Evi-1 (S1-3) FAST (S2, S3) Fos (S3) Lef-1/Tcf (S2, S3) OAZ (S1, S4) Runx/CBFα/AML (S1-4)

Fig. 1.9 Smad interacting proteins (according to Moustakas *et al*. [156])
An (incomplete) list of Smad interacting proteins is shown along with the interaction site in the Smad protein and with the function of the interacting proteins. The specific Smad members that are known to fulfill the indicated function are shown in parenthesis and are abbreviated with S1-S8 for Smad1 to Smad8, respectively. Proteins, for which the interaction site is not yet determined are listed in dashed boxes.

1.5.7 Transcriptional complexes

Following activation of R-Smads and association with Smad4, the heteromeric complexes are translocated to the nucleus to regulate transcription of Smad-dependent genes. Smad3 and Smad4 can directly bind to DNA [157, 158] whereas the inserted exon 3 in the Smad2 protein prevents DNA-binding [125, 126]. Transcriptional activity can be attributed to all Smad proteins [30].

However, as Smad proteins possess a relatively low affinity and specificity for Smad binding elements, transcriptional activation of Smad-responsive genes is regulated by functional cooperation with various transcription factors [159, 160]. Moreover, various transcriptional

coactivators and corepressors are involved in modulating the transcription of a specific gene. The outcome of the TGF-β response is thus reflected by the sum of effects mediated by coactivators and corepressors.

1.5.7.1 Transcription factors

A multitude of transcription factors was found to directly interact with Smad proteins and to be important for Smad-dependent transcriptional activation. Some of them will be introduced in the following.

The forkhead activin signal transducer (FAST-1) was initially identified in *Xenopus* and was described to bind to the activin response element (ARE) in the *Xenopus Mix.2* promoter [161, 162]. The mammalian homologs, FAST-1 and FAST-2, were shown to activate ARE and the goosecoid (gsc) promoter, respectively [163, 164]. FAST binds to DNA with high affinity and specificity but due to the lack of intrinsic transcriptional activity it is dependent on Smad activity to initiate transcription [160]. In contrast to FAST, most other transcription factors that synergize with Smad proteins can activate transcription by themselves.

Several TGF-β responsive genes contain AP1-binding sites which are activated by the heteromeric c-Jun/c-Fos complex. Smad3 can directly interact with c-Jun and the presence of active c-Jun/c-Fos dimers is essential to achieve efficient transcription from AP-1 containing elements [128, 129]. In the c-jun promoter, Smad and AP-1 complexes were found to bind to separate neighboring elements [165].

The activating transcription factor (ATF-2) binds to cAMP response elements (CRE). Smad3 and Smad4 associate and cooperate with ATF-2 to activate transcription [107]. As ATF-2 is attributed to be also a target of the p38 kinase cascade, ATF-2 represents a common nuclear target for both, the Smad and the p38 pathway [127].

Like ATF-2, the transcription factor TFE3/μE3 belongs to the family of basic helix-loop-helix proteins. TFE3 binds to an E-box sequence in the plasminogen-activator inhibitor (PAI-1) promoter and acts synergistically with Smad3 to activate transcription from this promoter. Thereby, phosphorylated Smads bind to a sequence that is adjacent to the TFE3-binding site [166].

Further DNA-binding partners for Smad proteins are members of the family of acute myelogenous leukemia (AML) proteins, which is also known as the family of core binding factors (CBFs) or polyoma enhancer binding proteins (PEPB2s). Although the relevance of the protein interactions still needs to be determined, simultaneous DNA-binding of Smad as well as AML proteins seems to be essential for transcriptional activation [167].

1.5.7.2 Coactivators

The coactivators p300 and CREB binding protein (CBP) play a central role in allowing transcriptional activation of TGF-β target genes [168, 169]. They promote transcription by (i) bridging transcription factors (*e.g*. Smads) to the basal transcriptional machinery and (ii) through their intrinsic histone acetyltransferase activity which triggers loosening of the chromatin structure. Ligand-induced phosphorylation of Smad3 facilitates interaction between Smad3 and p300/CBP [168, 170] and in addition, the unique SAD domain of Smad4 was proposed to enhance the association with p300/CBP [134]. Furthermore, p300/CBP is proposed to integrate TGF-β signaling with other cascades by simultaneous binding of Smads and other transcription factors as it was described for STAT3 and Smad1 [171].
The nuclear protein MSG1 which is not able to directly bind to DNA but elicits strong transcriptional activity was demonstrated to stabilize the interaction between Smad4 and p300/CBP [172].

1.5.7.3 Corepressors

Oncoproteins of the Ski/SnoN family prevent expression of TGF-β responsive genes by direct interaction with Smad proteins [173]. The cellular homolog of the v-ski oncoprotein, c-ski, associates with Smad proteins in the DNA-binding complex and thus impedes Smad3 interaction with the coactivator p300. Subsequently, c-ski interacts with N-CoR which in turn recruits histone deacetylase (HDAC) and thereby blocks transcriptional activation [174, 175]. Likewise, SnoN interferes with transcriptional activation by association with R-Smads and recruitment of the corepressor N-CoR [176]. By this mechanism, SnoN maintains the repressed state of transcription in absence of ligand and rapid degradation of SnoN following ligand-induced Smad nuclear accumulation allows transcription [177]. Later, upregulation of SnoN expression can provide a negative feedback and cause termination of transcription.
The homeodomain protein TG-interacting factor (TGIF) can also act as corepressor by either binding the corepressor mSin3 [178] or by recruiting HDAC [179, 180]. The Smad nuclear interacting protein (SNIP) harbors binding sites for Smad1 and Smad2 in the C-terminus and for both, Smad4 and the coactivator p300/CBP in the N-terminus. SNIP is capable to interfere with the formation of Smad4/p300 complexes and thus suppresses p300-dependent TGF-β signal transduction [181]. Further transcriptional repressors are the zinc finger proteins Smad-interacting protein (SIP1) and Evi-1. SIP1 binds to all R-Smads and was found to prevent expression of the *Xenopus Xbra2* gene [182]. Evi-1 interacts specifically with Smad3 and inhibits TGF-β mediated growth inhibition [183].

1.5.8 Termination of Smad activity

1.5.8.1 Inhibitory Smads

Smad7 belongs to the subfamily of inhibitory Smads which are highly divergent from R-Smads referring the N-terminal MH1 domain but contain conserved MH2 domains. Since inhibitory Smads lack the SSXS-motif, they do not serve as a substrate for TβRI. Smad7 stably interacts with the activated TβRI, thereby competing with the R-Smads for receptor association. As a consequence, phosphorylation of R-Smads and the succeeding downstream events are efficiently blocked by Smad7. Being an immediate early response gene for TGF-β, Smad7 triggers an efficient negative feedback [184, 185]. Recently, it was demonstrated, that physical interaction between the N-terminal domain of Smad7 and its MH2 domain enhances the inhibitory activity of Smad7, most likely by promoting receptor association [186].

In addition, several alternative mechanisms of Smad7-triggered signal abrogation have been recently described. STRAP was found to act synergistically with Smad7 by stabilizing the complex between TβRI and Smad7 [100, 103]. Moreover, by binding the E3 ubiquitin ligases Smurf1 and Smurf2, Smad7 bridges these proteins to the TGF-β receptors and promotes receptor degradation [102, 104]. Smad7 was also reported to associate with TAB1, thereby causing inhibition of TAK induced p38 activation and BMP-2 induced neurite outgrowth of PC12 cells [187]. In addition, there are evidences, that Smad7 might associate with other cellular kinases to achieve abrogation of Smad signaling [188].

Finally, based on the existence of a potential transcriptional activation domain located in the MH2 domain of Smad7 and given that Smad7 shows nuclear localization in the basal state, Smad7 was previously proposed to have additional nuclear functions [152].

1.5.8.2 Degradation of Smads

Smurf proteins have been implicated in proteasomal degradation of Smad proteins.

Degradation of cytoplasmic R-Smads, in particular Smad1, by Smurf1 or Smurf2 occurs regardless of receptor activation, indicating that Smurf proteins play an important role in controlling the steady-state levels of R-Smads [101, 189]. Phosphorylated Smad2 was described to preferably associate with Smurf2 which targets the ligand-activated nuclear Smad2 to proteasomal degradation [190]. Phosphorylated nuclear Smad3 is ubiquitinated by the SCF/Roc1 complex and exported from the nucleus prior to degradation [191]. Thus, Smurf proteins can specifically and selectively direct R-Smads for either cytoplasmic or nuclear degradation.

Furthermore, Smad proteins are involved in targeting other proteins such as the human enhancer of filamentation 1 (HEF-1) [192] or the transcriptional corepressor SnoN [193] for degradation.

1.6 CROSSTALK

1.6.1 Crosstalk with other signaling pathways

Originally, Smad proteins were only connected with TGF-β signal transduction but meanwhile they are known to function as important integrators of various signaling pathways.

Crosstalk events that are mediated by direct phosphorylation of Smad proteins by kinases of other cascades will be discussed in the succeeding section (see chapter 1.6.2).

Several molecules provoke inhibitory effects on Smad signaling by inducing expression of inhibitory Smad proteins. Interferon-γ (IFN-γ) is described to negatively affect TGF-β signal transduction by activation of the JAK1/STAT1 pathway which triggers expression of Smad7 and thus prevents phosphorylation of Smad3 [194]. Similarly, tumor necrosis factor a (TNFα) and interleukin 1 (IL-1) can interfere with TGF-β signaling by activating NF-κB which in turn activates Smad7 expression [195]. Although the mechanism is not yet defined, epidermal growth factor (EGF) was also found to induce expression of the inhibitory Smad proteins, Smad6 and Smad7 [184].

A growing number of publications provide evidence that TGF-β can mediate activation of several MAP kinase signaling cascades [196-198]. Activation of the p38 kinase pathway is likely to involve the TGF-β-activated kinase 1 (TAK1) and the TAK-binding protein 1 (TAB1) [107]. TAK1 directly activates MKK enzymes (*e.g*. MKK3, MKK6) which subsequently activate p38.

Crosstalk between components of the stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) cascade and the Smad pathway can lead to either activation or repression of Smad-dependent transcription. Abrogation of TGF-β mediated transcription in HepG2 cells was demonstrated to occur by direct interaction between Smad3 and c-Jun [199]. In contrast, activation of TGF-β induced transcription was reported to depend on Smad phosphorylation outside the C-terminal SSXS-motif by either JNK [197] or the MAP kinase kinase kinase MEKK-1 [188].

Signal convergence at the level of transcriptional complexes was observed for c-Jun complexes [128, 165] or ATF-2 complexes [127] which are both able to interact with Smad proteins.

Cooperation was also observed between TGF-β and Wnt/wingless signaling pathways in *Xenopus*. TGF-β and Wnt can independently or synergistically regulate the transcription factors lymphoid enhancer binding factor/T-cell-specific factor (LEF/TCF) which are usually activated by Wnt signaling. TGF-β induced LEF/TCF activation is due to direct physical interaction of Smad3 and LEF/TCF [200].

The crosstalk with vitamin D that is mediated by Smad3 should finally be mentioned in that context. Smad3 acts as a coactivator for vitamin D receptor-induced signals by interacting with a member of the steroid receptor coactivator-1 protein family [201].

1.6.2 Phosphorylation of Smads by proteins other than TβRI

Several lines of evidence reveal that Smad proteins are not solely phosphorylated by TβRI but additionally rank as substrates for a growing number of cytoplasmic kinases (Fig. 1.10).

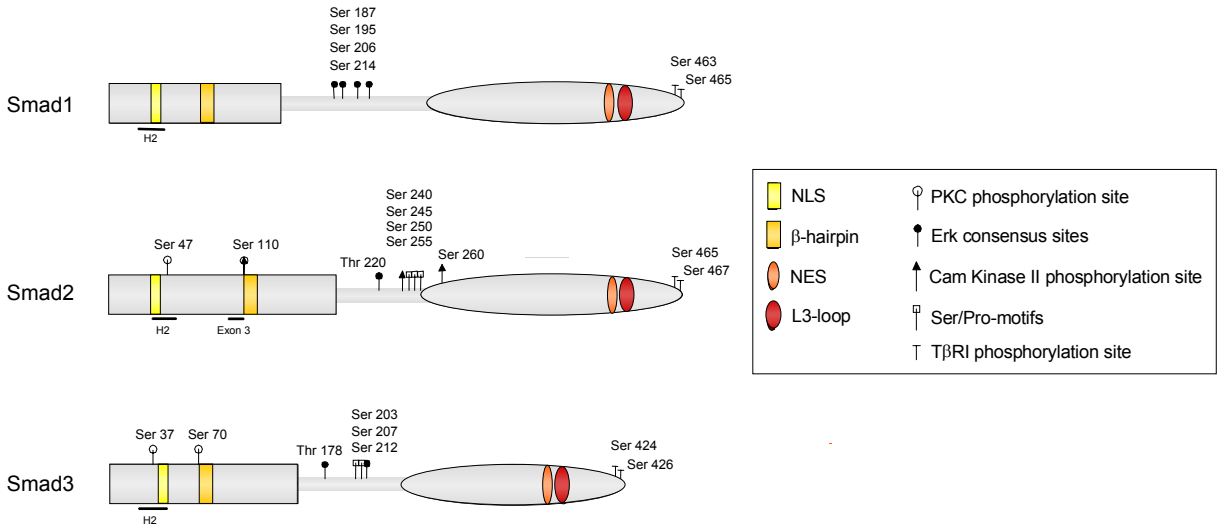


Fig. 1.10 Phosphorylation sites in R-Smad proteins that are used by diverse cellular kinases
R-Smad proteins get phosphorylated by the activated TβRI at the C-terminal serine residues. Other sites of phosphorylation are targeted by different kinases such as protein kinase C (PKC), Erk/MAP kinases or Ca^{2+}-calmodulin dependent protein kinase II (Cam kinase II), demonstrating that regulation of Smad activity by posttranslational modifications can be achieved by multiple proteins.

Members of the MAP kinase family are frequently involved in TGF-β/Smad signaling and several of them were shown to represent kinases for Smad proteins. Smad1 was found to be a substrate for the Erk subfamily of MAP kinases which becomes activated following binding of hepatocyte growth factor (HGF) or epidermal growth factor (EGF) to tyrosine kinase receptors [133]. Phosphorylation of four Erk consensus sites (PXSP motifs) located in the linker region of Smad1 results in prevention of nuclear accumulation of Smad1. Although the Erk consensus sites are not completely conserved in Smad2 and Smad3, EGF likewise induces linker phosphorylation in these R-Smads leading to their cytoplasmic retention. It is proposed that instead of the Erk sites, serine/proline motifs can equally function as targets for phosphorylation [132]. Oncogenic Ras was determined to initiate the same response but to produce stronger effects compared to EGF. Efficient abrogation of TGF-β signaling can account for the loss of TGF-β induced growth inhibition in tumors harboring oncogenic mutations in Ras [132]. The opposing effects of TGF-β and HGF/EGF demonstrate that Smad activity can be balanced depending on whether signals are emanating from receptor serine/threonine kinases (RSKs) or from receptor tyrosine kinases (RTKs).

Nuclear accumulation of Smad2 was demonstrated to be negatively affected by Ca^{2+}-calmodulin dependent protein kinase II (Cam kinase II) which triggers phosphorylation of several serine residues (Ser110, Ser240 and Ser260) within the Smad protein [202]. The

exact mechanism that results in inhibition of Smad signaling is not yet known but is likely to be complex due to the distinct sites that might convey signal regulation by different means.

Protein kinase C which is activated by TGF-β can provide a negative feedback by phosphorylating specific serine residues in the MH1 domain of Smad3 [203]. The phosphorylation sites reside on the surface that is facing the DNA and therefore preclude DNA-binding of Smad3. Although corresponding sites get phosphorylated in Smad2, the inhibitory effect is specific for Smad3 because Smad2 is not able to directly bind to DNA in any case.

Further cytoplasmic kinases that mediate Smad phosphorylation outside the C-terminal site that is used by TβRI, are the c-Jun N-terminal kinase (JNK) [197] and the MAP kinase kinase kinase (MEKK-1) [188]. In both cases, the exact positions of the target residues are not yet identified. TGF-β stimulated JNK activation was shown to be biphasic: the initial JNK activation leads to Smad3 phosphorylation at sites other than the SSXS motif, facilitating subsequent C-terminal phosphorylation by TβRI. Referring to the MEKK-1 kinase, it was reported that constitutively active MEKK-1 can mediate Smad2-dependent transcriptional responses in endothelial cells. The activating Smad2 phosphorylation induced by MEKK-1 occurs not on the C-terminal serine residues but is likely to target amino acids residing in the linker region or in the MH2 domain.

In conclusion, there is an increasing number of cellular kinases besides TβRI that can account for modulation of Smad activity via phosphorylation of distinct amino acid residues. Additionally, the interdependent relationship of JNK and Smad signaling demonstrates, that phosphorylation events can be critical to maintain the balance between different signaling pathways.

1.7 FUNCTION OF TGF-β IN THE NERVOUS SYSTEM

Being among the most abundant and versatile cytokines, TGF-β was discovered to have important roles in development and function of the nervous system [204-206].

Referring to the distribution of the three TGF-β isoforms in the nervous system, it is notable that TGF-β2 and TGF-β3 are the prominent isoforms. TGF-β2, TGF-β3 and their respective signaling receptors depict a widespread expression pattern in the developing and adult CNS and peripheral nervous system [207-209]. In contrast, TGF-β1 is usually restricted to a few single cell types such as mesangial cells. However, expression of TGF-β1 can be strongly induced in response to neuronal injury [205, 210].

Although TGF-β does not elicit neurotrophic functions on its own, it represents a potent cofactor for established neurotrophic factors such as neurotrophins (NGF, NT-3), fibroblast growth factor (FGF-2) or glial cell line-derived neurotrophic factor (GDNF). Already low

concentrations of these neurotrophic factors are sufficient to provoke the synergistic or potentiating effects of TGF-β [211, 212].

All TGF-β isoforms were implicated in promoting neuronal survival in various neuronal cells in culture [211, 213, 214].

TGF-β does not only reveal neurotrophic functions but rather mediates a large spectrum of actions in the nervous system. Exemplary, some of the functions of TGF-β in the nervous system will be listed.

TGF-β is known to act on astroglia, oligodendrocytes, microglia and neurons where it mainly regulates processes such as cell morphology, motility, differentiation and survival [206, 215].

An outstanding role is attributed to TGF-β in neuronal injury or neurodegenerative processes. Treatment of injured neuronal cultures with TGF-β revealed that TGF-β is capable to decrease the amount of damage and to maintain neuronal survival. Furthermore, TGF-β was found to accelerate tissue repair by attracting immune-cells and fibroblasts to the wound site and by induction of angiogenesis and extracellular matrix production [205].

1.8 NERVE GROWTH FACTOR (NGF)

1.8.1 The family of neurotrophins

Neurotrophins are secretory proteins that regulate differentiation, survival, growth and apoptosis of neuronal cells [216]. They constitute a family of proteins that are similar in sequence and in structure. In mammals, the neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) which are encoded by different genes but are suggested to be derived from a common ancestral gene [217]. The neurotrophins generally function as non-covalently associated homodimers, the monomers of which contain the characteristic cystine-knot motif which is also found in members of the TGF-β superfamily (see chapter 1.1.2). Ligand binding leads to activation of two different classes of receptors (see chapter 1.8.3) and triggers a multitude of signaling cascades, the most prominent ones being the pathways mediated by mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/Erk), phosphatidylinositol-3-kinase (PI3K) and Jun-N-terminal kinase (JNK) [218]. The $p75^{NTR}$ receptor binds all neurotrophins with similar nanomolar affinities. Despite this binding affinity for all neurotrophins, activation of $p75^{NTR}$ is exclusively triggered by NGF. The second class of receptors is the Trk family of tyrosine kinase receptors. The members of this family elicit specificity for the particular neurotrophins in that TrkA binds NGF, TrkB shows affinity for BDNF and NT-4 and TrkC signals in response to NT-3. However, NT-3 can also bind and signal through TrkA and TrkB [219].

1.8.2 Structure and functions of NGF

NGF was discovered in the 1950s [220] and represents the prototype and the best characterized member of the neurotrophin family.

The neurotrophically active NGF (β-NGF) is isolated from mouse submaxillary gland as part of a larger protein complex, the 7S NGF. Interestingly, the 7S complex is not found in other mouse tissues or in other species. 7S NGF consists of one β-NGF dimer and two copies each of α-NGF and γ-NGF subunits which belong to the serine proteases of the kallikrein family. The whole complex is stabilized by two zinc ions. As the α- and γ-subunits inhibit β-NGF activity by blocking the receptor binding sites, they must be removed to obtain bioactivity of β-NGF [7]. Efficient isolation of β-NGF from the 7S complex can be achieved for example by performing acid dissociation and subsequent FPLC ion exchange.

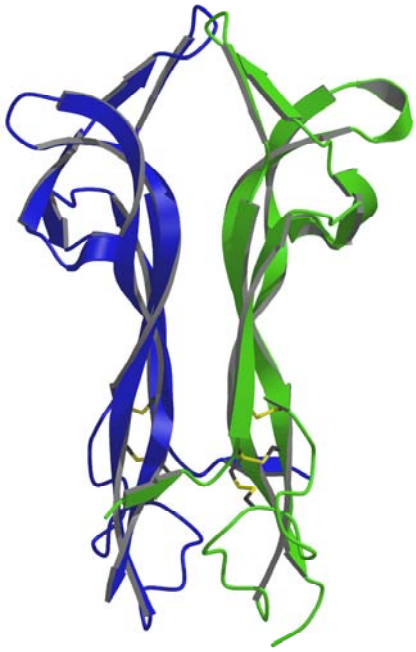


Fig. 1.11 Structure of NGF
NGF is composed of two identical monomers which are shown in blue and green, respectively. The monomers are arranged to form a parallel dimer and the cystine-knot is indicated in the lower part of the molecule by yellow cystine-bridges. (According to Protein Data Bank; PDB ID: 1BTG)

Determination of the structure of NGF by X-ray crystallography revealed that the biologically active form is composed of two non-covalently associated monomers that are arranged in a parallel manner [221]. The monomers resemble the topology of TGF-β monomers in that they have an elongated shape with a central β-sheet, the cystine-knot on one end and loops of varying lengths on the other end (see chapter 1.1.2 and Fig. 1.1). The cystine-knot of NGF is formed by six cysteine residues all of which form intermolecular disulfide bonds. Structural differences between the topology TGF-β and NGF are represented by three α-helices in TGF-β that are not present in NGF and three short β-strands in NGF that do not exist in TGF-β.

Despite the structural similarity between the monomers of TGF-β and NGF, the sequence homology is restricted to the pattern of disulfide bonding. Most striking is the profound difference in dimer formation. TGF-β dimerizes in an antiparallel manner whereas in NGF, the monomers assemble in a parallel fashion [11, 222, 223].

NGF possesses numerous functions in selected neurons and in certain non-neuronal cells. The most prominent properties include promotion of neuronal survival and neuronal differentiation, modulation of synaptic activity, production of neurotransmitters as well as induction of neurite outgrowth [224, 225]. Furthermore, NGF is implicated in preventing neuronal loss in neurodegenerative diseases and is thus considered as a potential therapeutic agent in neurodegenerative diseases such as Alzheimer's disease [226].

1.8.3 NGF receptors

NGF binds to two different classes of transmembrane receptors, the tyrosine kinase receptor TrkA [227, 228] and the $p75^{NTR}$ [229] receptor which belongs to the TNF receptor superfamily.

1.8.3.1 The low-affinity NGF receptor $p75^{NTR}$

$p75^{NTR}$ is a cell surface glycoprotein that binds each of the neurotrophins with similarly low affinity [230]. Structural motifs that are shared between $p75^{NTR}$ and other receptors of the TNF family are the cysteine-rich repeats in the extracellular part and the intracellular death domain [231] (Fig.1.12).

The function of $p75^{NTR}$ was for quite a long time enigmatic because deletions in the $p75^{NTR}$ receptor did not reveal significant phenotypic changes that would have allowed to draw conclusions concerning specific functional properties of the $p75^{NTR}$ receptor other than a modulatory role. Meanwhile, it is known that in addition to the ability to promote signaling via TrkA, $p75^{NTR}$ elicits distinct functions in absence of TrkA.

TrkA and $p75^{NTR}$ display direct physical interaction, thereby creating a receptor complex that enables high-affinity binding of NGF [232, 233]. Besides supporting NGF binding to TrkA, $p75^{NTR}$ enhances TrkA autophosphorylation [234]. Thus, $p75^{NTR}$ functions in promoting TrkA-mediated neuronal survival.

In absence of TrkA, $p75^{NTR}$ expression leads to induction of NGF-mediated apoptosis in neuronal cells [235]. However, coexpression of TrkA rescues neuronal cells from $p75^{NTR}$-induced cell death.

In conclusion, the decision between survival and death of neuronal cells is determined by the ratio of $p75^{NTR}$ to TrkA receptors [236, 237].

1.8.3.2 The high-affinity NGF receptor TrkA

The high-affinity receptor for NGF is represented by the cell surface receptor designated TrkA which mainly accounts for the trophic effects exerted by NGF. TrkA consists of an intracellular part that contains a tyrosine kinase and short C-terminal tail, a transmembrane domain and an extracellular part comprising several cysteine- and leucine-rich domains as well as two IgG-like domains [238] (Fig. 1.12).

The extracellular portion of TrkA contains several glycosylation sites. Receptor glycosylation was suggested to be important for correct membrane localization and for inhibition of spontaneous receptor activation [239]. Ligand-binding is mediated by the IgG-like domains, with the most membrane proximal IgG-like domain being the major ligand binding structural element [240].

Although it is known that homodimers of TrkA are essential for signal propagation [241], it is not yet elucidated whether homodimers assemble following ligand stimulation or whether they preexist and just require ligand binding to induce conformational changes.

The mechanism of TrkA activation in response to NGF binding follows the established scheme for tyrosine kinase receptors which describes a two step mechanism including ligand-induced receptor homodimerization and autophosphorylation which leads to activation of the kinase [242]. Initially, kinase activity is triggered by phosphorylation of three tyrosine residues within the activation loop of the receptor [243]. In the unstimulated state, the activation loop adopts a conformation that prevents binding of ATP and thus inhibits basal receptor activity. Ligand binding leads to conformational changes that stabilize an open conformation and thus allow substrate binding.

Full kinase activation is achieved by autophosphorylation of several other tyrosine residues that – upon phosphorylation – serve as docking sites for adaptor proteins or signaling molecules which in turn couple the receptors to diverse intracellular signaling cascades [224] (Fig. 1.13).

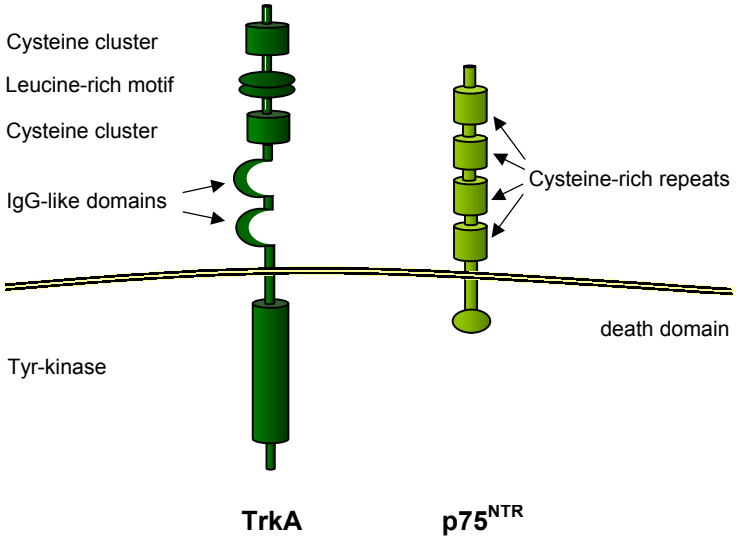


Fig. 1.12 Schematic representation of the structural motifs present in NGF receptors
TrkA belongs to the tyrosine kinase receptors and carries two IgG-like domains and several repeats rich in leucine and cysteine in its extracellular part. The $p75^{NTR}$ receptor belongs to the family of TNF receptors which share the canonical cysteine rich domains in the extracellular part. The cytoplasmic domain of $p75^{NTR}$, however, lacks known signaling motifs.

1.8.4 NGF signal transduction

As mentioned above, multiple tyrosine residues get phosphorylated in response to ligand binding. Besides the adaptor molecules, Shc, Grb2 and SOS, which are depicted in Fig. 1.13, a variety of other docking proteins have been identified [224]. However, in this section, only the major downstream pathways originating from TrkA will be briefly described.

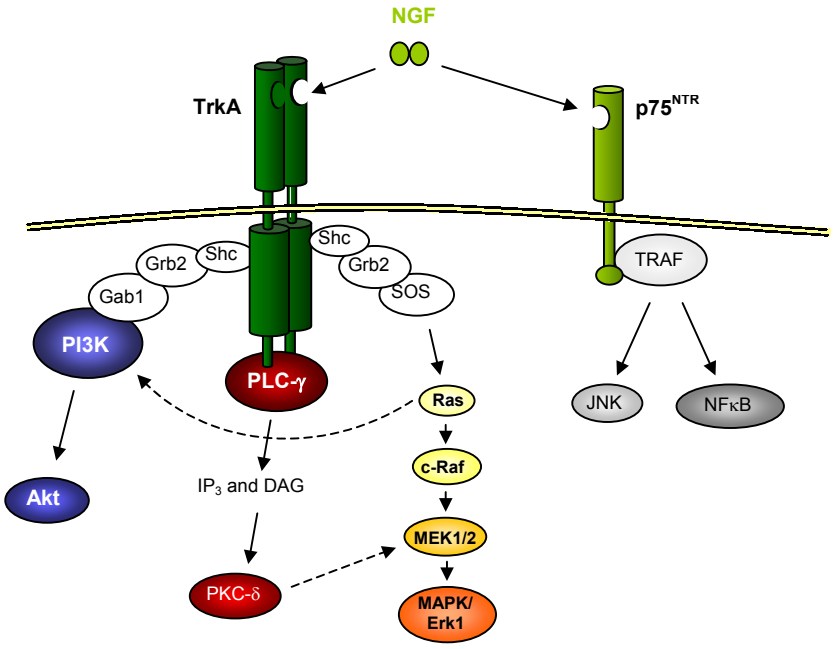


Fig. 1.13 Signal transduction pathways originating from TrkA and $p75^{NTR}$
The main signaling pathways activated by NGF binding to the TrkA receptors are the PI3 kinase pathway, signals transduced by PLC-γ and the MAPK/Erk cascade. For $p75^{NTR}$, TRAF6 was identified as a receptor-interacting protein which in turn activates either NFκB or the JNK pathway.

The most prominent signaling cascades downstream of TrkA are mediated by either MAPK/Erk, PI3K or phospholipase C (PLC-γ) (Fig. 1.13).

Signaling through the MAPK/Erk pathway is initiated by recruitment of the adaptor molecule Shc to the autophosphorylated TrkA receptor. Subsequently, the adaptor Grb-2 and the Ras exchange factor son of sevenless (SOS) are recruited which finally triggers transient phosphorylation of Ras. Ras can then activate the c-Raf/Erk pathway. Furthermore, Ras is also able to activate PI3 kinase.

Besides Ras-mediated activation, PI3K can be induced by the three adaptor proteins, Shc, Grb-2 and Gab-1.

PLC-γ is directly recruited to a phosphorylated tyrosine residue in the short tail of the TrkA receptor. PLC-γ functions in catalyzing the hydrolysis of phosphatidylinositides to yield inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The latter activates protein kinase C (PKC-δ) which in turn regulates MEK/Erk activity [244, 245].

Referring to $p75^{NTR}$, downstream signaling events are largely unknown because up to now there are only very few proteins identified that associate with the intracellular part of $p75^{NTR}$. The TNF receptor associated factor-6 (TRAF-6) was found to interact with $p75^{NTR}$. TRAF molecules in general are known to signal via JNK and NFκB. Indeed, TRAF-6 could be demonstrated to modulate NFκB mediated responses in Schwann cells [246]. An additional $p75^{NTR}$-associated protein is represented by the neurotrophin receptor interacting MAGE protein (NRAGE). NRAGE was found to block association of TrkA and $p75^{NTR}$, thereby promoting $p75^{NTR}$ mediated apoptosis [247]. Furthermore, caveolin-1 ranks as a $p75^{NTR}$-interacting protein. Caveolin-1 also binds to TrkA and thus functions in regulating signal transduction via both, $p75^{NTR}$ and TrkA in neuronal and glial cell populations [218, 248].

Cloning of further $p75^{NTR}$-associated proteins might lead to identification of novel signaling cascades originating from this receptor.

1.9 AIM OF THE PROJECT

Complex biological responses often require signals originating from more than one receptor system [249].

For cells of neuronal origin, it was shown that neurotrophic effects evoked by NGF or other neurotrophins highly depend on the cooperativity with cytokines belonging to the TGF-β superfamily [212, 250, 251].

In the present work, a potential co-dependency of NGF- and TGF-β signaling should be investigated in the rat pheochromocytoma cell line, PC12.

As PC12 cells are widely used as a model system for NGF-induced neuronal differentiation, they served as an adequate cell system to elucidate whether TGF-β1 is required as a crucial cofactor to trigger NGF-induced neurite outgrowth.

Furthermore, it should be assessed whether NGF-triggered signals are capable of influencing the TGF-β/Smad pathway. As previous observations demonstrated that NGF is indeed able to activate transcription from Smad-dependent reporter genes, the aim of the project was to characterize the mechanism of NGF-induced Smad activation.

2 Material and solutions

2.1 CHEMICALS

Basic chemicals of highest purity were purchased from the companies Merck, Roth, Serva and Sigma.

All solutions were prepared using deionized water or water of Millipore quality.

Cell culture reagents were obtained from Biochrom, Bio Whittaker, Falcon, Gibco BRL, Greiner, Nunc and Merck.

Substances used for preparation of liquid growth media for bacteria were supplied by Roth and Difco.

2.2 TECHNICAL DEVICES

Centrifuge	Sepatech Megafuge 1.0	Heraeus
Centrifuge	Beckman J2-21	Beckman
Clean bench	HLB 2472	Heraeus
Electrophoresis apparatus	Mini-V8.10	Gibco BRL
Electrophoresis apparatus for horizontal agarose gels		Workshop of the institute
Electrophoresis power supply	Phero-stab. 500	Biotech-Fischer
Flow cytometer	FACScan	Becton Dickinson
Fluorescence microscope	DM IRB	Leica
Gel Dryer	Model 483 SLAB DRYER	BIO-RAD
Incubator	HERA cell	Heraeus
Light microscope	Leitz DM IL	Leica
Luminometer	FB 12	Berthold
Microcentrifuge	Centrifuge 5417C	Eppendorf
PCR cycler	Cetus DNA Thermal Cycler	Perkin Elmer
Phosphor imager		Molecular Dynamics
Scale	1264 MP	Satorius
Scale	2001 MP2	Satorius
Scintillation counter	Wallac 1410	Pharmacia
Speed Vac Concentrator		Bachofer
Spectrophotometer	Kontron Uvicon 930	Kontron
UV-transilluminator		UVP Inc.
X-ray developing machine	Compact 35	Protec

2.3 ENZYMES

2.3.1 Restriction endonucleases

Restriction enzymes were purchased from MBI and New England Biolabs (NEB).

2.3.2 DNA- and RNA- modifying enzymes

RNaseA	Roth
RNAsin™	Promega
Shrimp alkaline phosphatase (SAP)	Roche
M-MLV reverse transcriptase	Promega
Superscript II RNaseH reverse transcriptase	Life Technologies
T4 DNA-ligase	Promega

2.3.3 Polymerases

DNA-Polymerase from *Pyrococcus furiosus* (Pfu)	Promega
DNA-Polymerase from *Thermus aquaticus* (Taq)	prepared by W. Hädelt (Würzburg)

2.4 KITS

CONCERT™ Rapid Gel Extraction System	Life Technologies
Dual Luciferase Assay Kit	Promega
CONCERT™ Rapid Plasmid Miniprep System	Life Technologies
RNeasy Midi-Kit	QIAGEN

2.5 OLIGONUCLEOTIDES

Oligonucleotides were synthesized and purchased by the company Interactiva. They were delivered following HPLC purification and lyophilisation.

Primers used for PCR mutagenesis, sequencing and PCR reactions are listed in the appendix (see appendix A.2).

2.6 PLASMIDS

2.6.1 Expression vectors

pcDNA1 (Invitrogen) mammalian expression vector for high level constitutive expression driven from a CMV promoter; the plasmid encodes the tRNA suppressor F gene (supF) which demands transformation in specific bacterial strains that harbor the P3 episome (*i.e.* MC1061/P3); sensitivity to tetracycline and ampicillin is generated by suppression of the amber mutations

pcDNA3 (Invitrogen)	mammalian expression vector for high-level constitutive expression driven from a CMV enhancer promoter
pcDNA3.1 (Invitrogen)	mammalian expression vector for high level constitutive expression driven from a CMV enhancer promoter; similar to pcDNA3 but differs in the multiple cloning site
pHIT60	mammalian expression vector containing a CMV promoter (should not be transformed in DH5α) (obtained from D. Lindemann, Würzburg)
pRC-CMV (Clontech)	expression vector for constitutive expression driven by a CMV promoter
pCMV5	mammalian expression vector that yields extremely high expression levels in COS cells (obtained from M. Chao, New York, USA)
pHIT60/gag-pol	expression vector for the gag-pol protein (obtained from D. Lindemann, Würzburg)
pczVSV-G wt	expression vector for the VSV-G envelope protein [252] (obtained from D. Lindemann, Würzburg)

2.6.2 Retroviral vectors

pLPCX	retroviral vector with puromycin resistance (obtained from R. Derynck, San Francisco, USA)
pLXSN	retroviral cloning and expression vector with neomycin resistance [253]
pMX	retroviral cloning and expression vector (obtained from Y. Henis, Tel Aviv, Israel)
pczCFG EGIRT iAhCD8α	retroviral vector containing a pcDNA3.1 backbone; the vector expresses the green fluorescent protein (GFP), an intervening ribosomal entry site (IRES) and the reverse transactivator (rtTA) under the control of a modified MuLV long terminal repeat (LTR). The expression of the gene of interest is driven by a tetracycline-inducible minimal CMV promoter (the vector was obtained from D. Lindemann, Würzburg)

2.7 BACTERIAL STRAINS

E. coli DH5α	*deo*R, *end*A1, *gyr*A96, *hsd*R17 (r_k^- m_k^+), *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ(*lac*ZYA-*arg*FV169)ϕ80*lac*ZΔM15, F^- [254]
E. coli C600	*lac*Y1, *leu*B6, *mcr*B+, *sup*E44, *thi*-1, *thr*-1, *ton*A21, F^- [255]
E. coli MC1061/P3	*ara*D139, *gal*K, *gal*U, *hsd*R2($r_k^-m_k^+$), *rps*L, *thi*-1, Δ(*ara*-*leu*)7696, Δ*lac*X74, F-[P3*kan*^{r} amber *amp*^{r} amber *tet*^{r}] [256]
E. coli XL1-Blue	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*, *supE44*, *relA1*, *lac* [*F'*, *proAB*, *lac1*^{q}*ZΔM15*, Tn*10*(Tet^r)] [257]

2.8 GROWTH MEDIA AND REAGENTS FOR CELL CULTURE

DMEM (Life Technologies)	Dulbecco's Modified Eagle Medium is prepared according to manufacturer's instructions and sterilized by filtration
EMEM (Life Technologies)	Essential Minimum Eagle Medium is prepared according to manufacturer's instructions and sterilized by filtration
Horse serum (Linaris)	serum is heat-inactivated for 30min at 56°C before use
FCS (PAA)	Fetal calf serum is heat-inactivated for 30min at 56°C before use
L-Glutamine (Biochrom KG)	is added to cell culture media to reach a final concentration of 1mM
NEAA (Biochrom)	non-essential amino acids are added to culture medium in a 1:100 dilution
Nu-Serum (Becton Dickinson)	serum is heat-inactivated for 30min at 56°C before use
Penicillin (Biochrom KG)	is added to the culture medium to reach a final concentration of 100U/ml
RPMI 1640 (Life Technologies)	is prepared according to manufacturer's instructions and sterilized by filtration
Streptomycinsulfat (Biochrom KG)	is added to the culture medium to reach a final concentration of 100µg/ml
Trypan blue (Biochrom KG)	is diluted 1:4 with PBS
Trypsin (Biochrom KG)	500mg Trypsin and 200mg EDTA are dissolved in PBS and sterilized by filtration

2.9 CELL LINES

293	human embryonic kidney cell line transformed with adenovirus 5 DNA (CRL-1573, ATCC)
293T	human embryonic kidney cell line transformed with the large T-antigen from the SV40 virus (from D. Lindemann, Würzburg) [258]
COS-7	african green monkey kidney fibroblast-like cell line (CRL-1651, ATCC)
Hep3B	human hepatoma cell line (HB-8064, ATCC)
Hep3B-TR	cell line derived from the Hep3B cell line that lacks both alleles of the *tβRII*-gene [18]
L6	rat myoblast cell line (CRL-1458, ATCC)
Lan-1	human neuroblastoma cell line (from K. Krieglstein, Homburg)
MCF-7	human breast cancer cell line (HTB-22, ATCC)
MDA-MB-468	human breast cancer cell line (HTB-132, ATCC)
MLEC	cell line derived from the mink lung epithelial cell line (Mv1Lu), which stably expresses a luciferase reporter gene under the control of a truncated PAI-1 promoter [259]
Mv1Lu	mink lung epithelial cell line (CCL-64, ATCC)
PC12	rat pheochromocytoma cell line (from K. Krieglstein, Homburg) [260]

2.10 GROWTH FACTORS

human TGF-β1 (carrier free)	Tebu
human TGF-β1 (with BSA)	R&D Systems
mouse NGF (2.5S)	Alomone Labs

2.11 ANTIBODIES

Antibodies used during these studies are listed below (Tab. 2.1). Buffers to be used for antibody dilution in Western blot are indicated by the abbreviations (B1-B4) and their corresponding components are listed in Tab. 2.2.

Antibody	Dilution for WB	Conc. for IP	Type, origin	Epitope, recognition	Reference
1° Antibody					
anti-Flag M2	1:450 in B2	2 µl/ml	monoclonal, mouse, IgG_1	recognizes the Flag epitope (DTLDDDDL)	Sigma (F3165)
anti-HA (12CA5)	1:200 in B1	20 µl/ml	monoclonal, mouse, was prepared by T. Lutz (Universität Würzburg) according to standard protocols using hybridoma cells	recognizes the haemagglutinin (HA) epitope (YPYDVPDYA)	
anti-P-Smad1/3	1:1000 in B4	-	polyclonal, rabbit, antiserum	recognizes the C-terminally phosphorylated form of Smad1 (KKKNPISSVS) and crossreacts with phosphorylated Smad3 [261]	from P. ten Dijke (Amsterdam) [262]
anti-P-Smad2	1:1000 in B4	-	polyclonal, rabbit, antiserum	recognizes the C-terminally phosphorylated form of Smad2 (KKKSSMS)	from P. ten Dijke (Amsterdam) [262]
anti-TrkA (B-3)	1:800 in B3	8 µl/ml	monoclonal, mouse, IgG_{2a}	recognizes the highly conserved C-terminus of the human TrkA gp140 precursor protein	Santa-Cruz (sc-7268)
anti-P-Tyr-100	1:2000 in B5	-	monoclonal, mouse, IgG_1	recognizes phosphotyrosine	Cell Signaling (#9411)
anti-Smad1 (QWL)	1:1000 in B4	-	polyclonal, rabbit, antiserum	recognizes a region in the MH2 domain of Smad1 (QWLDKLTQMGSP HNPISSVS)	from P. ten Dijke (Amsterdam) [263]
anti-Smad2 (SED)	1:1000 in B4	-	polyclonal, rabbit, antiserum	recognizes the Smad2 linker region (SEDGETSDQQLN QSMDTG)	from P. ten Dijke (Amsterdam) [263]

Antibody	Dilution for WB	Conc. for IP	Type, origin	Epitope, recognition	Reference
1° Antibodies					
anti-Smad3 (DHQ)	1:1000 in B4	-	polyclonal, rabbit, antiserum	recognizes the Smad3 linker region (DHQMNHSMDAG SPNPM)	from P. ten Dijke (Amsterdam) [263]
anti-VPN	-	15 µl/ml	polyclonal antiserum, rabbit	recognizes the juxtamembrane domain of TβRI (VPNEEDPSLDRP FISEGTTLKD)	[26]
anti-260	-	15 µl/ml	polyclonal antiserum, rabbit	recognizes the very C-terminal part of TβRII (CSEEKIPED GSLNTTK)	[57]
2° Antibodies					
Goat-anti-mouse IgG/HRP	1:20000 in B3	-	polyclonal, goat, HRP-conjugated	recognizes heavy and light chains of mouse IgGs	Dianova
Goat-anti-rabbit IgG/HRP	1:20000 in B4	-	polyclonal, goat, HRP-conjugated	recognizes heavy and light chains of rabbit IgGs	Dianova
Neutralizing Antibodies					
anti-TGF-β	-	20 µg/ml	polyclonal, rabbit, pan-specific		R&D Systems (AB-100-NA)
anti-TGF-β1, -β2, -β3	-	20 µg/ml	monoclonal, mouse, IgG_1	bovine TGF-β2 was used as Antigen	R&D Systems (MAB 1835)
FACS-Antibodies					
Donkey-anti-mouse IgG /PE	-	-	monoclonal, donkey, phycoerythrin (PE) conjugated	recognizes heavy and light chains of mouse IgGs	Jackson Immuno Research

Tab. 2.1 Antibodies

buffer	components
B1	PBS
B2	TBS
B3	Wash buffer + 0,1% Tween-20
B4	Wash buffer + 0,5% Tween-20
B5	Wash buffer + 0,1% Tween-20 + 5% BSA

Tab. 2.2 Buffers for antibody dilution

PBS
170 mM NaCl
3,3 mM KCl
10 mM Na_2HPO_4
1,8 mM KH_4PO_4
dissolve in dH_2O; adjust pH 7,4; autoclave

TBS
50 mM Tris/HCl (pH 7,4)
150 mM NaCl
dissolve in dH_2O; adjust pH 7,4; autoclave

Wash buffer
10 mM Tris/HCl (pH 8,0)
150 mM NaCl
0,1% or 0,5% Tween-20

2.12 MARKERS

2.12.1 DNA-markers

HMS (high molecular weight standard) λ-phage-DNA; *Hind*III digested

LMS (low molecular weight standard) pUC lac-DNA; *EcoR*I and *Hinf*I digested

These markers are loaded at a concentration of 25ng/µl and allow calculation of the amount of DNA present in the probe with the help of the specifications shown in Tab. 2.3.

HMS		LMS	
base pairs	**percentage**	**base pairs**	**percentage**
23130	48%	1608	21,2%
9416	19%	1201	15,9%
6557	14%	999	13,2%
4361	9%	711	9,4%
2322	5%	567	7,5%
2027	4%	517	6,8%
564	1%	396	5,2%
		360	4,8%
		222/219/214	8,6%
		132/120/112	4,8%
		75/65/30/27	2,6%

Tab. 2.3 DNA-markers (HMS and LMS)

1kb-marker (NEB) 10kb – 8kb – 6kb – 5kb – 4kb – 3kb – 2kb – 1,5kb – 1kb – 0,5kb

100bp-marker (NEB) 1500bp – 1200bp – 1000bp – 900bp – 800bp – 700bp – 600bp – 500bp – 400bp – 300bp – 200bp – 100bp

2.12.2 Protein markers

To identify proteins according to their molecular weight, prestained protein standards are used in SDS-PAGE.

The molecular weights of standard proteins vary slightly between different batches of the product.

SDS-7B ⇨ used for Western blot analysis (Sigma)

Protein	Molecular weight
α_2-macroglobulin	205 kDa
β-galactosidase	130 kDa
fructose-6-phosphate kinase	90 kDa
pyruvate kinase	64 kDa
fumarase	53 kDa
lactic dehydrogenase	37 kDa
triosephosphate isomerase	32 kDa

Tab. 2.4 Molecular weight standard (SDS-7B)

^{14}C-rainbow protein marker ⇨ used for experiments with radiolabeled proteins (Amersham)

Protein	Molecular weight
myosin	220 kDa
phosphorylase b	97,4 kDa
BSA	66 kDa
ovalbumine	46 kDa
carbonic anhydrase	30 kDa
trypsin inhibitor	21,5 kDa
lysozyme	14,3 kDa

Tab. 2.5 Molecular weight standard (^{14}C)

3 Methods

3.1 MICROBIOLOGICAL METHODS

3.1.1 Sterilization

All solutions, buffers and media are sterilized by autoclaving at 120°C and 2bar for 20min. Solutions that contain heat-sensitive components are sterilized by filtration using sterile 2µm filters.

3.1.2 Growth media

Luria-Broth (LB-medium)	10 g/l Trypton 5 g/l Yeast-extract 10 g/l NaCl adjust pH 7,4 and autoclave
SOB-medium	20 g/l Trypton 5 g/l Yeast-extract 0,5 g/l NaCl 0,83 mM KCl adjust pH 7,0 and autoclave
SOC-medium	100 ml SOB-medium + 1 ml 40% Glucose + 1 ml 1M $MgCl_2$ + 1 ml 1M $MgSO_4$
LB-agar plates	To prepare agar plates, LB-medium is supplemented with 15g/l agar (Difco). After autoclaving, the solution should cool down to approximately 40°C before ampicillin is added to a final concentration of 50µg/ml. The agar is poured into petri dishes and after solidification, plates are stored at 4°C.

3.1.3 Cultivation of bacteria

Bacterial cultures are grown in plain LB-medium (see chapter 3.1.2) or in LB-medium containing appropriate antibiotics. To inoculate the cultures, bacteria can be taken from existing bacterial cultures, from glycerin-stocks or by picking a single colony from a LB-agar plate, respectively. Cultivation is carried out over night at 37°C under permanent shaking.

3.1.4 Conservation of bacteria

3.1.4.1 Short-term conservation

For short-term conservation of bacterial cultures, bacteria are grown on agar-plates containing the appropriate antibiotics and are incubated over night at 37°C. Bacteria plated in this way can be stored for about 4 weeks at 4°C.

3.1.4.2 Long-term conservation

A single colony is inoculated and cultivated in LB-medium over night. For long-term cryo-conservation, 500µl of this fresh culture are mixed with 200µl sterile glycerin (86%) and stored in cryo-vials at –80°C.

3.1.5 Preparation of chemically competent *E.coli*

Prerequisite for transformation of *E.coli* via heat-shock is to render the bacteria chemically competent.

Therefor, 100ml LB-medium without antibiotics are inoculated with 1ml of a fresh bacteria over night culture and incubated at 37°C until an optical density of A_{550nm}= 0,2-0,3 is reached. Bacteria are then kept on ice for 5 min. Following centrifugation for 10min at 2500rpm and 4°C, the supernatant is discarded and the pellet is resuspended in 40ml ice-cold TFB1. Then, a second centrifugation step is carried out for 10min at 3000rpm and 4°C. Again, the supernatant is discarded and the pellet is resuspended thoroughly in 4ml ice-cold TFB2. The suspension is incubated on ice for additional 15min. Aliquots of 200µl are transferred to sterile cryo-vials and immediately frozen in liquid nitrogen. Competent bacteria can be stored at –80°C.

To determine the transformation rate, 1ng of a plasmid is transformed in 100µl of competent bacteria and the resulting amount of colonies is calculated as colonies per µg DNA.

The transformation rate should be > 10^7 colonies per µg plasmid DNA.

TFB1

30 mM K-Ac
100 mM RbCl
10 mM $CaCl_2$*$2H_2O$
50 mM $MnCl_2$*$4H_2O$

dissolve in 425ml dH_2O; adjust pH 5,8 and add
75ml glycerin (86%) to reach a final volume of 500 ml; autoclave

TFB2

10 mM MOPS
75 mM $CaCl_2$*$2H_2O$
10 mM RbCl

dissolve in 85ml dH_2O; adjust pH 6,5 and add
15ml glycerin (86%) to reach a final volume of 100 ml; autoclave

3.1.6 Transformation of competent *E. coli* via heat-shock

Competent bacteria of the appropriate strain are thawed on ice. 1-10ng of plasmid DNA or 100-200ng of a ligation mixture, respectively, are added to 100µl of competent bacteria. Following incubation on ice for 30min, a heat-shock is performed at 42°C for 90sec to allow DNA uptake into the bacteria. Immediately after the heat-shock, bacteria are resuspended in 1ml SOC-medium (see chapter 3.1.2) and incubated for 45-60min at 37°C to enable expression of the resistance gene. To select transformed bacteria, 100µl of the transformed bacteria suspension are plated on a LB-agar plate containing the appropriate antibiotics and incubated over night at 37°C.

3.2 MOLECULAR BIOLOGICAL METHODS

3.2.1 Standard DNA purification methods

There are different standard protocols that can be used to purify or concentrate DNA.

3.2.1.1 Phenol/chloroform extraction

To remove proteins from solutions containing nucleic acids, an equal volume of phenol (pH 8,2) is added and thoroughly mixed. Separation of the aqueous and the organic phase is achieved by centrifugation for 5min at 14000rpm. The upper aqueous phase is transferred to a fresh Eppendorf tube. Subsequently, an equal volume of chloroform/isoamylalcohol (24:1 v/v) is added, mixed and centrifuged as before. The upper phase is again transferred to a fresh tube and the procedure is repeated to completely remove residing amounts of phenol.
Phenol/chloroform extraction is usually followed by an ethanol or isopropanol precipitation (see chapter 3.2.1.2 or 3.2.1.3).

3.2.1.2 Ethanol precipitation

Concentration of nucleic acids can be achieved by ethanol precipitation. Therefore, the salt concentration is first adjusted to 0,3M by adding 1/10 volume of a 3M sodium acetate (pH 5,2) solution. After addition of 2,5 volumes of ice-cold 100% ethanol, the mixture is incubated for 30min at –20°C. The precipitated DNA is sedimented by centrifugation for 15min at 14000rpm and at 4°C. The pellet is then washed twice with 70% ethanol, dried in a speed-vac for 2min and finally dissolved in dH_2O.

3.2.1.3 Isopropanol precipitation

Alternatively, precipitation can be carried out using isopropanol instead of ethanol. First, 1/10 volume of 3M sodium acetate (pH 5,2) and 0,6 volumes of isopropanol are added. Then, the mixture is incubated for 10min at RT and subsequently centrifuged for 10min at 14000rpm at RT. The pellet is washed twice with 70% ethanol, dried and resuspended in dH_2O.

3.2.2 Plasmid minipreparation [264]

A 2ml over night bacterial culture is used to isolate plasmid DNA for analytical purposes. Bacteria are sedimented by centrifugation for 1min at 10000rpm at RT. The supernatant is discarded and the pellet is resuspended in 100µl solution I (see chapter 3.2.3). Addition of 3µl RNase A (10mg/ml) is followed by incubation for 5-10min on ice. Bacteria are lysed by addition of 200µl solution II (see chapter 3.2.3), gently mixing by inversion of the tube and incubation for 5min on ice. 150µl solution III (see chapter 3.2.3) are added to neutralize the solution and incubation is again carried out for 5-10min on ice. The supernatant is then transferred to a fresh tube and the DNA is precipitated with isopropanol as described under 3.2.1.3.

3.2.3 Plasmid midipreparation

To isolate larger amounts of DNA, 100ml of an over night bacterial culture are centrifuged for 5min at 6000rpm and at 4°C. The pellet is resuspended in 5ml of solution I and 50µl RNase A (10mg/ml) are added prior to incubation for 5-10min on ice. For lysis, 5ml of solution II are gently mixed in by inverting the tube, followed by incubation for 5min on ice. After adding 10ml of solution III, incubation is again carried out for 5-10min on ice. To remove any floating precipitate, the supernatant is transferred to a fresh Eppendorf tube by filtration. The DNA is then precipitated using isopropanol as described under 3.2.1.3 and finally resolved in 1ml dH_2O. To remove residing RNA, the solution is treated with 50µl RNase A (10mg/ml) for 30-60min at 37°C. Subsequently, the RNase A is removed by phenol/chloroform extraction (see chapter 3.2.1.1) and DNA is concentrated by isopropanol precipitation (see chapter 3.2.1.3) and dissolved in 100µl dH_2O. The yield of DNA can be determined by spectrophotometry as described in 3.2.4.

Solution I	25 mM Tris/HCl (pH 8,0) 50 mM Glucose 10 mM EDTA (pH 8,0) store at 4°C; add 5mg/ml lysozyme before use
Solution II	0,2 N NaOH 1% SDS prepare freshly before use
Solution III	3 M K-Ac (pH 4,8)
DNase-free RNase	dissolve 10mg/ml RNase A in 10 mM Tris/HCl (pH 7,5) 15 mM NaCl incubate 15min at 96°C; then, slowly cool down to RT; store in aliquots at –20°C

3.2.4 Determination of nucleic acid concentrations

The concentration of nucleic acids in aqueous solutions can be determined by measuring the absorption at a wavelength of 260nm using a spectrophotometer.
Therefor, DNA isolated by plasmid midipreparation is diluted 1:100 in dH_2O and the absorption is documented in the range of 240-320nm.

One absorption unit (A_{260nm}) corresponds to:
- 33µg/ml for oligonucleotides
- 40µg/ml for ssDNA or RNA
- 50µg/ml for dsDNA

Considering the factor of dilution, the concentration of nucleic acids can be calculated with the help of these equations.

3.2.5 Agarose gel electrophoresis

Separation of nucleic acids is achieved by horizontal gel electrophoresis. Depending on the size of the fragments that are analyzed, the concentration of agarose can be adjusted to achieve optimal separation. Predominantly, 1% agarose gels are used which are suitable for fragments of 400bp-7kb in size. Agarose is dissolved in 1x TAE buffer, brought to the boil and then stored at 65°C. Agarose gels are prepared according to standard protocols [264]. Nucleic acid containing samples are mixed with 1/5 volume of 6x DNA sample buffer before they are loaded onto the gel. To allow assignment of the fragment size, an appropriate DNA standard (see chapter 2.12.1) is loaded on the gel next to the samples. The gel run is carried out at 100-110V. Intercalation of EtBr allows to visualize the DNA-fragments by means of an UV-transilluminator.

10x TAE	400 mM Tris/acetate (pH 8,5) 10 mM EDTA
6x DNA sample buffer	0,25% Bromphenolblue 0,25% Xylencyanol 30% Glycerin dissolve in dH_2O; store in aliquots at 4°C
EtBr	5 mg/ml EtBr (= 10.000x stock solution)

3.2.6 Elution of DNA from agarose gels

Following preparative agarose gel electrophoresis, DNA-fragments can be visualized as described in 3.2.5 and specific bands can be excised using a scalpel. Subsequent elution of the DNA from the gel slice is performed by means of the CONCERT™ Rapid Gel Extraction System (Life Technologies) according to manufacturer's protocols.

3.2.7 Polymerase chain reaction (PCR)

The polymerase chain reaction is a tool to amplify small amounts of defined DNA- or cDNA-fragments. First, the double stranded DNA is denatured to single strands which can then function as a template for the synthesis of new DNA. Then, primers are allowed to bind to the template during the annealing phase and in the subsequent extension phase, the synthesis of DNA is completed.

Synthetic oligonucleotides are used as primers for the PCR reaction. They should be designed such that they have a length of approximately 20-25bp, a GC-content of 40-60% and a melting temperature of about 50-60°C. The melting temperature (T_D) can be calculated by the following formula, in which "n" represents the number of the respective bases:

$$T_D = [(C_n+G_n) \times 4 + (A_n+T_n) \times 2]°C$$

With an increasing GC-content of the primer, the annealing temperature should be raised. As the specificity of primer binding is increased at higher temperatures, variation of the annealing temperature determines and alters the stringency.

The duration of the extension phase is dependent on the length of the fragment which is amplified and on the processivity of the DNA-polymerase that is used.

The standard PCR reaction is performed by using the following reaction mixture and PCR-cycler program:

Reaction mix

10x *Pfu*-buffer	5 µl
10 mM dNTP	1 µl
100 pmol/µl 5'-primer	0,5 µl
100 pmol/µl 3'-primer	0,5 µl
Pfu-DNA polymerase (3U/µl)	0,5 µl
template DNA (~ 1-10 ng)	x µl
dH_2O	ad 50 µl

Cycler-program

Denaturation	95°C	5 min	
Denaturation	95°C	1 min	} 25-30 x
Annealing	52-55°C	1 min	
Extension	72°C	2 min	
Final extension	72°C	8 min	

3.2.7.1 Analytical PCR

To verify the success of a cloning procedure, either samples of a DNA-miniprep or directly single bacteria colonies from an agar plate are used as a template for the amplification using an appropriate primer pair. For this purpose, the reaction volume is kept to a minimum of 20µl and *Taq*-polymerase is used for amplification.

3.2.7.2 Preparative PCR

As the products of a preparative PCR are used for further cloning procedures, the reaction volume should be increased to 50µl to ensure higher yields and moreover, the error rate of the DNA-amplification should be minimized by using a polymerase that possesses a reliable proof-reading activity. Usually, *Pfu*-DNA polymerase is used for these purposes.

3.2.7.3 Recombinant PCR

Recombinant PCR is used for either PCR-mutagenesis (*e.g*. to introduce mutations or to generate/destroy restriction sites) or for introduction of epitope tags.

First, two overlapping fragments are generated in separate PCR reactions using primers carrying the mutation of choice. The resulting fragments serve as templates for the second PCR reaction in which the full length fragment is amplified by using the outer primers.

3.2.8 Reverse transcription

3.2.8.1 Isolation of total RNA from mammalian cells

Total RNA is isolated from mammalian cells by using the RNeasy Midi Kit (QIAGEN) according to manufacturer's instructions. Yield and quality of the RNA are verified by spectrophotometry and agarose gel electrophoresis, respectively.

3.2.8.2 Reverse transcription

Prior to amplification in a PCR-reaction, RNA has to be transcribed to cDNA by means of the retroviral enzyme "reverse transcriptase".

To generate cDNA, either random hexamers or primers specific for the gene of interest can be used. 5µg of total RNA are mixed with 50pmol of random hexamers or specific primers and incubated for 10min at 70°C. The reaction mix is cooled down on ice. The reaction buffer, DTT, RNasin and dNTPs are added and incubated for 2min at 42°C. Addition of 1µl Superscript II RNase H reverse transcriptase (Life Technologies) is followed by incubation for 10min at RT and for further 50min at 42°C. Finally, the enzyme is inactivated by incubation for 15min at 70°C.

The produced cDNA can be directly used for RT-PCR (see chapter 3.2.9).

Reaction mixture

5 µg total RNA	x µl
100 pmol/µl primer	0,5 µl
5x Superscript-RT buffer	4 µl
0,1 M DTT	2 µl
RNasin	0,5 µl
10 mM dNTP-mix	4 µl
Superscript II RT	1 µl
DEPC-H_2O	ad 20 µl

3.2.9 RT-PCR

For the RT-PCR reaction, the cDNA obtained from the reverse transcription reaction (see chapter 3.2.8.2) is used as template. Usually, 5µl of a 20µl reverse transcription reaction are taken for the RT-PCR and primers specific for the gene of interest are chosen.

3.2.10 DNA sequencing

Sequencing of DNA-constructs as well as DNA-fragments generated by PCR or RT-PCR was carried out by W. Hädelt (Biozentrum, Würzburg) using the DNA sequencer 373A (Applied Biosystems).

3.2.11 Digestion of DNA by restriction endonucleases

Restriction endonucleases recognize and cleave specific palindromic DNA-sequences. To digest isolated DNA, the optimal salt concentration is adjusted by adding the restriction buffer which is supplied with the enzyme and the DNA is incubated with the appropriate enzyme according to manufacturer's instructions. Usually, digestion is carried out for 1h at 37°C. The completeness of the restriction and the size of the resulting fragments can be verified by agarose gel electrophoresis (see chapter 3.2.5).

3.2.12 5'-Dephosphorylation of DNA

To avoid religation of vector-DNA during ligation, the 5'-phosphate residues should be removed by treatment with alkaline phosphatase. During these studies, shrimp alkaline phosphatase (Roche) was used. Dephosphorylation is carried out according to manufacturer's instructions and afterwards, the enzyme is removed by phenol/chloroform extraction and subsequent isopropanol precipitation (see chapter 3.2.1.1 and 3.2.1.3).

3.2.13 Ligation of DNA-fragments

Ligation of DNA-fragments is catalyzed by T4 DNA-ligase which enables formation of phosphodiester-bonds between the 3'-hydroxy group and the 5'-phosphate group of dsDNA. For ligation, vector DNA and insert DNA are generally used in a molar ratio of 1:4. Vector DNA is recommended to be dephosphorylated prior to ligation (see chapter 3.2.12).

The reaction volume should be minimized and following addition of the T4 DNA-ligase, the reaction mixture is incubated over night at 16°C. 5-10µl of the ligation mixture can then be used for transformation in *E. coli* (see chapter 3.1.6).

Reaction mix		
	10x T4 DNA-ligase buffer	1 µl
	T4 DNA-ligase	1 µl
	Insert DNA	x µl
	Vector DNA (50-100 ng)	x µl
	dH_2O	ad 10 µl

3.3 CELL BIOLOGICAL METHODS

3.3.1 Cultivation of mammalian cells

Mammalian cells are cultivated according to standard methods [265]. Depending on the respective cell line, cells are grown in DMEM, EMEM or RPMI supplemented with the appropriate amounts of FCS or horse serum, 1% glutamine and antibiotics. Cultivation is carried out at 37°C, 95% relative humidity and 5% or 10% CO_2, respectively.

For subcultivation, adherent cells are detached using trypsin whereas suspension cells are thoroughly resuspended and diluted to the optimal cell density.

3.3.2 Determination of cell numbers

A Neubauer counting chamber is used to determine cell numbers. The cell suspension is mixed with PBS containing 0,5% trypan-blue at a 1:1 ratio. As the trypan-blue dye is not taken up by living cells, dead cells can be identified by a blue staining of the cell body. Cells located in two out of the four big quadrants are counted and the resulting number is multiplied by the factor 10^4 to get the amount of cells present per ml of cell suspension.

3.3.3 Cryo-conservation of cells

For long-term conservation of mammalian cells, $1x10^6$-$1x10^7$ cells are resuspended in growth medium containing 10% DMSO and transferred to a cryo-vial. Cells are stored on ice for 1h, followed by storage at –80°C for 1-2 days and finally, they are kept at –180°C in liquid nitrogen.

Thawing of cells should be performed rapidly in a 37°C water bath. The cells are then transferred to prewarmed growth media, spinned down for 3min at 1100rpm and the cell pellet is resuspended in growth media and seeded in culture dishes.

3.3.4 Coating of cell culture dishes with collagen

Collagen is isolated from rat tails according to standard protocols and solubilized in 0,1% acetic acid. Isolated collagen was obtained from K. Krieglstein (Universität des Saarlandes, Homburg).

Cell culture dishes are coated with rat tail collagen by incubating the dishes with a solution of 38µg/ml collagen in 0,1% acetic acid for at least 1h at RT. Prior to use, plates are washed three times with sterile dH_2O and twice with medium without supplements.

3.3.5 Induction of neurite formation in PC12 cells

Neurite outgrowth of PC12 cells can be induced by treatment with NGF [260]. Cells are plated on collagen-coated 6-well plates at a density of $4x10^4$ cells per well. Neurite formation is triggered by addition of 2nM (50ng/ml) NGF to medium containing 1% horse serum. First

elongations can be observed after 24h. Following NGF treatment for 7 days, almost 100% of the cells show neurites and even networks of neurites have been formed.
Cell morphology can be monitored by phase contrast microscopy.

3.3.6 Transfection methods

Different methods are established to allow uptake of plasmid-DNA into mammalian cells which then express the gene of interest.

3.3.6.1 Calcium-phosphate coprecipitation [266]

During this study, the calcium-phosphate coprecipitation method was used to transfect 293 or 293T cells, respectively.
The day before transfection, $2x10^6$ cells are seeded on a 6cm plate. Prior to transfection, the medium is replaced by 4ml of fresh growth medium and the cells are returned to the incubator for 45min to allow equilibration of the pH which is a critical parameter. 15µg of DNA are mixed with dH_2O to reach a final volume of 438µl and 62µl of 2M $CaCl_2$ are added. After thoroughly mixing, 500µl of 2x HBS are added by bubbling and the solution is immediately distributed drop by drop on the cells. Following incubation for 7-10h at 37°C, the transfection medium is replaced by fresh growth medium and cells are returned to the incubator. 48h post transfection, cells can be used for further experiments.

2x HBS	50 mM HEPES 10 mM KCl 12 mM α-D-Glucose 280 mM NaCl 1,5 mM Na_2HPO_4 dissolve in dH_2O; adjust pH 7,05; autoclave and store in aliquots at –20°C
2M $CaCl_2$	dissolve $CaCl_2$ in dH_2O; autoclave and store in aliquots at –20°C

3.3.6.2 DEAE-dextran [267]

The DEAE-dextran method is the method of choice to efficiently transfect COS cells.
Transfection is performed in 10cm dishes and the confluence of the cells should be in the range of 50%-70%. 10-20µg of DNA are dissolved in 1140µl of transfection buffer and 60µl of DEAE-dextran (10mg/ml) are added. After washing three times with prewarmed transfection buffer, the cells are incubated with the DNA/DEAE-dextran mixture for 30min at 37°C. Subsequently, 14ml Chloroquine-NS medium are added to the cells without aspirating the transfection mixture and the cells are returned to the incubator for further 2,5-3h.
The DMSO-shock which is performed afterwards is supposed to facilitate DNA uptake. Therefor, the transfection medium is aspirated and cells are treated for 2,5min with 3ml of

growth medium containing 10% DMSO. Residing DMSO is removed by washing the cells with medium. The cells are then cultivated in normal growth medium until, 48h post transfection, they can be used for further experiments.

Transfection buffer	250 mM NaCl 2,3 mM NaH_2PO_4 7,7 mM Na_2HPO_4 dissolve in dH_2O; adjust pH 7,5; autoclave
DEAE-dextran	10mg/ml in dH_2O; sterilize by filtration
Chloroquine (40 mM)	20mg/ml in dH_2O; sterilize by filtration
Chloroquine-NS medium:	10% NU-Serum 80 µM Chloroquine in DMEM medium without supplements

3.3.6.3 Lipofectamine™

Lipofectamine™ transfection was basically performed as to manufacturer's instructions (Life Technologies). Briefly, cells were seeded on 6-well plates at a density of 1-2,5x10^5 cells per well, so that on the day of transfection, cell confluence reaches ~80%. A total of 3µg DNA is mixed with medium containing 0,2% serum to achieve a volume of 100µl. In parallel, 8-10µl of Lipofectamine™ are added to 90µl of medium without supplements, in a separate tube. The two solutions are combined, gently mixed and incubated for 30min at RT. Meanwhile, the cells are washed once with plain medium. 800µl of plain medium are added to the transfection mixture before it is distributed on the cells, drop by drop. Following incubation for 5-6h at 37°C, the transfection solution is replaced by 2ml of normal growth medium and 48h post transfection, cells can be used for further experiments.

3.3.7 β-Galactosidase assay

The β-galactosidase assay provides a tool to verify transfection efficiency in an assay. One plate of cells is transfected with a plasmid carrying the β-galactosidase construct. 48h post transfection, cells are fixed by addition of 200µl glutaraldehyde (25%) in 5,5ml PBS for 15min at 37°C. Subsequently, the fixation solution is removed and a mixture of 300µl 2% X-Gal and 2,7ml X-Gal incubation buffer is added to the cells. β-galactosidase is an enzyme that catalyzes hydrolysis of lactose. X-Gal, being a colorless lactose-analogue, can also be hydrolyzed by β-galactosidase, resulting in a blue staining of the β-galactosidase expressing cells. Transfection efficiency can thus be determined by estimating or counting the amount of blue stained cells.

PBS	170 mM NaCl 3,3 mM KCl 10 mM Na_2HPO_4 1,8 mM KH_4PO_4 dissolve in dH_2O adjust pH 7,4; autoclave	
Fixation solution	25% Glutaraldehyde PBS	0,2 ml 5,5 ml
X-Gal incubation buffer	1 mM $MgCl_2$ 3,3 mM $K_4Fe(CN)_6*3H_2O$ 3,3 mM $K_3F(CN)_6$ dissolve in PBS; store at 4°C	
2% X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactosid Dimethylformamid store in aliquots at –20°C	2 mg 1 ml

3.3.8 Retroviral infection using replication deficient pseudotyped MuLV-viruses

Retroviral infection is suitable to stably transfect different cell lines with high efficiency.

For production of high-titer replication deficient retroviruses, the packaging cell line, 293T, is used to produce the viral components and to assemble the virus particle.

293T cells are transfected with separate constructs encoding the essential viral proteins (envelope, gag-pol, retroviral DNA containing the gene of interest) by using the calcium-phosphate coprecipitation method (see chapter 3.3.6.1). One day post transfection, cells are treated with medium containing 10mM sodium butyrate (Merck) for 10h to increase expression [268, 269].

If target cells are adherent, they should be plated in 6-well plates at a density of $5x10^4$ per well the day before infection. For target cells that are cultured in suspension, $2x10^5$ cells are taken for infection. The first infection is carried out two days post transfection. The supernatant of the 293T cells is harvested and filtered through a 0,45µm syringe filter into a tube containing the appropriate amount of polybrene (Sigma) to reach a final concentration of 8µg/ml after adding the supernatant. The medium of the target cells is aspirated and the viral supernatant is added to the cells. Following incubation for 4-6h at 37°C, the viral supernatant is replaced by normal growth medium. If necessary to obtain higher infection efficiency, the infection procedure can be repeated the next day.

Gene expression in the target cells can be verified by Western blotting (see chapter 3.4.4) or FACS-analysis (see chapter 3.3.9)

Sodium-butyrate	500 mM n-Butyric acid sodium salt (= 50x stock solution) dissolve in PBS; sterilize by filtration

Polybrene	800 µg/ml Hexadimethrine bromide (= 100x stock solution) dissolve in PBS; sterilize by filtration

3.3.9 Characterization of cells by FACS analysis

Since the retroviral vectors used during these studies contain the *gfp*-gene, infected cells can be easily identified and selected by fluorescence-activated cell sorting (FACS). Moreover, expression of cell surface proteins can be detected by staining the proteins with specific fluorescent antibodies and subsequent FACS analysis.

3.3.9.1 Antibody staining for FACS analysis

For FACS analysis, approximately 2-5x10^5 should be used. Adherent cells are detached from the culture dish using 10mM EDTA because trypsin treatment would lead to digestion of the surface proteins. Detached cells are resuspended in 4ml PIN and centrifuged for 5min at 1200rpm and RT. The supernatant is discarded whereby the remaining liquid comes to ~100µl. Then, 1µg of the antibody specific for the protein of interest is diluted in 10µl PIN and added to the cells. Incubation is carried out in the dark for 30min on ice before cells are washed with 4ml PIN, centrifuged as before and the supernatant is discarded. If the first antibody is directly coupled to a fluorescent dye, 250µl PIN are added and the cells can be used for FACS analysis.

If the first antibody is not a fluorescent one, cells are subsequently incubated with a fluorescence-conjugated antibody directed against the first antibody. Staining is performed analogous to the first antibody.

EDTA	10mM EDTA 20 mM HEPES dissolve in dH_2O; adjust pH 7,0; sterilize by filtration
PIN	2% FCS 0,05% sodium azide dissolve in PBS

3.3.9.2 FACS analysis of cells

FACS analysis can be used to characterize living cells in respect of their size and their granularity. Above that, it allows simultaneous detection of the emission of three different fluorescences. Fluorescence can thereby derive either from specific antibodies coupled to the cells or from cellular fluorescent proteins such as GFP. FACS analysis was performed and evaluated as previously described [270].

3.3.9.3 Selection of GFP-positive cells by FACS sorting

Sorting of GFP-positive cells was performed by Sonja Rotzoll (Institut für Virologie und Immunologie, Würzburg). After sorting, the cells are thoroughly washed with PBS to avoid contamination. They can then be cultured and expanded in normal growth medium.

3.4 PROTEIN CHEMICAL METHODS

3.4.1 Cell lysis

Depending on the succeeding assay, different lysis buffers can be used for cell lysis. Mostly, the TNE- or the Tx-lysis buffers, which are listed below, were used during these studies. The use of diverging buffers is mentioned in the description of the respective methods.

To produce lysates from mammalian cells, cells are washed twice with cold PBS. An adequate amount of lysis buffer containing protease inhibitors (Complete^{TM}, Roche), PMSF and - if necessary - phosphatase inhibitors are added to the cells which are subsequently collected using a cell scraper and transferred to an Eppendorf tube. Lysis is performed by incubation for 40min at 4°C under rotation. Cell debris is sedimented by centrifugation for 10min at 14000rpm and 4°C. The supernatant is transferred to a fresh tube and can be used for further analysis.

TNE-buffer	⇨ used for Smad Western-Blot
	20mM Tris/HCl (pH7,4) 150mM NaCl 1mM EDTA 1% Triton-X100
Tx-Buffer	⇨ used for immunoprecipitation and binding and cross-linking assays
	0,5-1% Triton-X100 1mM EDTA
	dissolve in PBS
PMSF	100 mM Phenylmethylsulfonyl fluoride dissolve in isopropanol (= 100x stock solution), store at –20°C
Phosphatase inhibitors	200mM NaF (= 4x stock solution) dissolve in dH_2O; store at RT
	200mM $Na_4P_2O_7$ (= 20x stock solution) dissolve in dH_2O; store at RT
	200mM Na_3VO_4 (= 200x stock solution) dissolve in dH_2O; adjust pH 10 – the solution is yellow; boil the solution until it turns colorless; cool to RT; readjust pH 10 and repeat the procedure until the solution stays colorless and the pH stabilizes at 10

3.4.2 Immunoprecipitation

To immunoprecipitate specific proteins from total cell lysates, an antibody specific for the respective protein is added to the lysate at concentrations given in Tab. 2.1. Immunoprecipitation is carried out over night at 4°C under rotation. To enable precipitation of the formed immune-complexes, protein A-sepharose beads (Sigma), which efficiently bind IgG molecules via protein A, are added. Per ml of cell lysate, 50µl of protein A-sepharose are used. Following rotation for 2h at 4°C, sedimentation of immune-complexes is performed by centrifugation. Sepharose beads are washed twice with ice-cold lysis buffer and twice with ice-cold PBS. Then, proteins are detached from the affinity-matrix by adding 2x SDS-sample buffer (see chapter 3.4.3) and boiling for 5min at 95°C. After centrifugation for 2min at 14000rpm, the supernatant is transferred to a fresh Eppendorf tube and can be used for SDS-PAGE (see chapter 3.4.3).

3.4.3 SDS-polyacrylamid gel electrophoresis

SDS-PAGE was performed according to Laemmli [271].

Cell lysates that are supposed to be separated by SDS-PAGE are prepared as described above (see chapter 3.4.1). To denature the proteins, 2x or 6x SDS-sample buffer is added and the samples are boiled for 5min at 95°C.

7,5-12,5% polyacrylamid gels are prepared following the pipetting scheme shown in Tab. 3.1. After polymerization of the gels, they are put in the Mini-V8.10 chamber (Life Technologies). The chamber is filled with SDS-running buffer and the samples are loaded on the gel together with a standard protein marker (see chapter 2.12.2). The gel-run is carried out at 80V until the samples have reached the resolving gel. The voltage can then be increased to 180V. After separation of the proteins by SDS-PAGE, the gels can be either dried or used for Western blotting (see chapter 3.4.4).

reagents	stacking gel 3,75%	resolving gel 7,5%	resolving gel 10%	resolving gel 12,5%
AA/BAA	250 µl	1,5 ml	2,0 ml	2,5 ml
4x Lower Tris	-	1,5 ml	1,5 ml	1,5 ml
4x Upper Tris	500 µl	-	-	-
dH_2O	1,25 ml	3 ml	2,5 ml	2 ml
APS 40%	4 µl	12 µl	12 µl	12 µl
TEMED	4 µl	12 µl	12 µl	12 µl

Tab. 3.1 Pipetting scheme for SDS-PAGE

2x SDS-sample buffer	125mM Tris/HCl (pH 6,8) 4% SDS 10% β-Mercaptoethanol 0,02% Bromphenolblue
6x SDS-sample buffer	125 mM Tris/HCl (pH 6,8) 30% Glycerin 10% SDS 0,6 M DTT 0,012% Bromphenolblue
SDS-running buffer	25 mM Tris 190 mM Glycin 0,1% SDS dissolve in dH_2O; store at RT
AA/BAA	30% Acrylamid 1% N, N'-Methylenbisacrylamid
4x Lower Tris	1,5 M Tris 0,4% SDS dissolve in dH_2O; adjust pH 8,8
4x Upper Tris	0,5 M Tris 0,4% SDS dissolve in dH_2O; adjust pH 6,8

3.4.4 Western blot

To detect single proteins out of a total cell lysate, proteins are separated by SDS-PAGE and subsequently transferred to a nitrocellulose membrane where they can be detected by means of specific antibodies and visualized by enhanced chemiluminescence (ECL).

Following SDS-PAGE (see chapter 3.4.3), the Western blot is arranged according to standard protocols [272]. For the blotting procedure, Mini-V8.10 chambers (Life Technologies) are used. The chamber is filled with transfer buffer and transfer to the nitrocellulose membrane (Schleicher & Schüll) is carried out at 120V for 1h. Then, the membrane is blocked with either wash buffer or PBS containing 3%-5% low-fat dry milk for at least 1h. The first antibody is diluted as listed in Tab. 2.1 and Tab. 2.2, added to the membrane and incubated over night at 4°C. The antibody solution is removed and the membrane washed three times with wash buffer for 5min each. Thereafter, the membrane is covered with the respective dilution of the secondary HRP-coupled antibody and incubated for 1h at RT. Following three rounds of washing as described before, proteins are visualized by ECL detection.

Therefor, 1ml each, of the luminol solution and the H_2O_2 solution, are mixed immediately before use and distributed on the membrane. After 1min, the solution is aspirated and the

membrane covered with foil. An X-ray film (Konica) is then exposed and developed in the dark room.

To detect other proteins on the same membrane, the bound antibodies can be removed by incubation in stripping buffer for 30min at 65°C. Before blocking, the membrane has to be thoroughly washed with PBS to remove residual β-mercaptoethanol. Incubation with the appropriate antibodies is performed as described above.

WB-transfer buffer	25 mM Tris 190 mM Glycine 20% Methanol dissolve in dH_2O; store at RT
Wash buffer	10 mM Tris/HCl (pH 8,0) 150 mM NaCl 0,1% or 0,5% Tween-20 dissolve in dH_2O; store at RT
p-cumaric acid	90 mM p-cumaric acid dissolve in DMSO; store at 4°C in the dark
Luminol	2,5 mM 3-Aminophthalhydrazid (Luminol) 1% DMSO dissolve in 0,1 M Tris/HCl (pH 8,5); store at 4°C in the dark
Luminol solution	1ml Luminol 4,4 µl p-cumaric acid
H_2O_2 solution	1ml 0,1 M Tris/HCl (pH 8,5) 1 µl H_2O_2 (30%)
Stripping buffer	5 mM Phosphate buffer 2% SDS add 0,014% β-Mercaptoethanol before use

3.4.5 Detection of nuclear translocation

To detect nuclear translocation, cells are stably transfected with a GFP fusion protein by retroviral infection (see chapter 3.3.8). Thus, the localization of the protein of interest can be easily monitored by fluorescent microscopy.

Cells are seeded on collagen-coated 6-well plates (see chapter 3.3.4) at a density of 1-3x10^5 cells per well, depending on the cell line that is used. The cells are allowed to attach before they are starved for 4h in medium containing 0,2% serum. Subsequently they are stimulated for 24h with 200pM TGF-β1 or 2nM NGF, respectively. The medium is aspirated and the fixation solution is added to the cells and incubated for 15min at 37°C. The fixation solution is removed by aspirating and washing the cells three times with PBS. To stain the nuclei of the cells, cells are covered with a solution containing 1µg/ml Hoechst 33258 and stained for

10min in the incubator. Aspiration of the stain solution is followed by three washing steps with PBS and covering cells with fixation solution. In this way, cells can be used for fluorescent microscopy.

3.4.6 Reporter gene assay

Reporter gene assays are used to study the capacity of soluble ligands to activate specific promoter elements. Therefore, the responsive promoter element of choice is cloned in front of a luciferase gene (*e.g*. firefly luciferase as the experimental reporter) and transfected into cells. The "dual luciferase assay system" additionally includes an internal control which is represented by a constitutively active luciferase gene (*e.g*. *renilla* luciferase as the control reporter) which is cotransfected. Thus, the *renilla* signal serves as a baseline signal that can be used to normalize the activity of the firefly signal which itself is dependent on ligand stimulation.

For reporter gene assays, different TGF-β-responsive elements were used during these studies. The p3TP-luc(+) reporter is derived from the p3TP-luc construct [28] but contains a modified *luc*-gene (pSP-luc(+), Promega) [273]. The pSBE reporter [274] comprises four copies of the Smad binding element (SBE) and in $p(CAGA)_{12}$-luc, twelve repeats of the CAGA-box are cloned in front of the luciferase reporter. Whereas pSBE-luc serves as a readout for TGF-β as well as BMP signaling the p3TP-luc(+) and $p(CAGA)_{12}$-luc constructs respond specifically to TGF-β-mediated signals [158, 275]. Reporter constructs are described in more detail and are graphically depicted in the appendix (see appendix A.3). The pRL-TK construct (Promega), which encodes the *renilla* luciferase under the control of a thymidine kinase promoter, was used as control reporter.

Cells are plated on 6-well plates prior to cotransfection with 1µg reporter construct, 0,3µg pRL-TK and with 2µg of a gene of interest. The total amount of DNA is kept constant by addition of empty vector (pcDNA3). 24h post transfection, cells are starved in medium containing 0,2% serum for 4h, followed by stimulation with either 2nM NGF or 200pM TGF-β1 for additional 24h. Cell lysis and luciferase measurement are carried out according to manufacturer's instructions. The "Dual Luciferase Assay System" (Promega) is used for the assay and measurements are performed by a FB12 Luminometer (Berthold). Luciferase activity is measured in relative light units (RLU). The evaluation of the data is described below.

Ligand	Firefly luciferase			*Renilla* luciferase			Ratio (%)	Adapted ratio
	reporter signal	back-ground signal	actual signal	reporter signal	back-ground signal	actual signal		
- Ligand	1352	144	1208	40818	7033	37804	3,20	1
+ NGF	5199	144	5055			37804	13,37	4,185
+TGF-β1	1808	144	1664			37804	4,40	1,377

Tab. 3.2 Recorded data from a luciferase measurement
Cells were tested for reporter activation following stimulation with either NGF or TGF-β1. For each sample, the light units of both, the signal-dependent firefly luciferase and the constitutive *renilla* luciferase were determined. Data are normalized by calculating the ratio of firefly:*renilla* signals (ratio). For better comparison of different experiments, the value of unstimulated cells was set to one and all other data were calculated accordingly (adapted ratio).

Luciferase activity is determined from unstimulated cells as well as cells treated with NGF or TGF-β1. The background signal is derived from mock transfected cells, *i.e.* cells that were not transfected with any of the reporter plasmids. The relative light units measured in these cells (background signal) are subtracted from the respective reporter signals to yield the value for the actual signal. For the determination of the *renilla* signal, only untreated cells are examined, because (i) ligand induced effects on the *renilla* expression should be excluded and (ii) since cells are taken from the same transfection procedure, equalization of the *renilla* signals only compensates potential ligand mediated variations but does not falsify the determination of the transfection efficiency. To allow better comparison between different experiments, the value obtained from unstimulated cells that were transfected only with the reporter constructs and empty vector was always set to one and all other data were calculated accordingly. The adapted ratio of firefly:*renilla* signals gives rise to the normalized data which is used for graphic presentation. Error bars were calculated from three independent experiments if not mentioned otherwise.

3.4.7 Quantification of TGF-β1 in the supernatant using MLEC cells

The amount of TGF-β1 in the supernatant of cells is determined using mink lung epithelial cells (MLEC) that are stably transfected with a luciferase reporter gene under the control of a truncated PAI-1 promoter [259]. MLEC cells are plated in a 96-well plate at a density of $1{,}6 \times 10^4$ cells per well and allowed to attach for 3h. The medium is then replaced with 100µl of the supernatant that is to be investigated. Following incubation for 18h, MLEC cells are washed twice with PBS, lysed and luciferase activity is measured according to manufacturer's instructions (Promega). To evaluate the amount of total (active and latent) TGF-β1, aliquots of the respective supernatant are acidified with HCl for 3min and neutralized with NaOH prior to addition to the MLEC cells. Without acidification, only active - but not latent - TGF-β can be determined.

In parallel, a calibration curve is recorded using defined concentrations of TGF-β1.

3.4.8 Radioactive labeling of TGF-β1 and NGF with Na[^{125}I]

Iodination of TGF-β1 and NGF is performed according to the chloramine T method [276]. First, 2µg of the respective ligand are dissolved in 10µl 1M Na_3PO_4 (pH 7,2). Afterwards, the solution is carefully mixed with 2µl (0,2 mCi) of radiolabeled Na[^{125}I] (Amersham). Following addition of 2µl chloramine T, the reaction mix is incubated for 2min. Renewed addition of 2µl chloramine T is followed by a waiting period of 90sec and repeated addition of 2µl chloramine T. 60sec later, the reaction is quenched by using 10µl acetyl-tyrosine. Finally, 100µl of potassium iodine and 100µl of a saturated urea-acetic acid solution are added.

For TGF-β1, the reaction mix is subsequently loaded on a NAP 10 Sephadex-G-25 column (Pharmacia). After a 2min incubation time, bound proteins are removed from the column by adding elution buffer. The eluat is collected in several fractions and an aliquot of 1µl of each fraction is then subjected to thin-layer chromatography in 10% TCA to separate free [^{125}I] from protein bound [^{125}I]. The amount of radioactivity present in the particular fractions is then determined by means of a γ-counter. This allows to identify the fraction that contains the predominant amount of [^{125}I]-labeled TGF-β1 and to estimate iodination efficiency.

NGF is directly used for chromatography without fractionation by a sephadex column.

Phosphate buffer	1 M Na_3PO_4 (pH 7,2) dissolve in dH_2O; store at RT
Potassium iodine	100 mM KI dissolve in dH_2O; store in aliquots at –20°C
Acetyl tyrosine	100 mM Acetyl tyrosine dissolve in dH_2O; store in aliquots at –20°C
Elution buffer	4 mM HCl 75 mM NaCl dissolve in dH_2O; store at 4°C add 0,1% BSA freshly before use
Urea-acetic acid solution	1,2g urea dissolve in 1ml acetic acid

3.4.9 Binding and cross-linking assays

Binding and cross-linking allows to covalently bind a radiolabeled ligand to its appropriate receptors which can then be detected via autoradiography.

3.4.9.1 Binding and cross-linking of [^{125}I]-TGF-β1 [27]

For binding and cross-linking, cells are washed three times with prewarmed KRH containing 0,5% fatty acid free BSA (Sigma) and are then incubated in the same buffer for 30min at

37°C. After this time, cells are placed on ice. The buffer is replaced by binding buffer containing 500pM [^{125}I]-TGF-β1 and cells are kept at 4°C for 3-4h under gentle shaking. Afterwards, unbound ligand is thoroughly removed by washing the cells four times with ice cold KRH. Cross-linking is performed for 15min at 4°C by covering the cells with 270µM DSS in KRH. The cross-linking solution is aspirated and cells are lysed (see chapter 3.4.1) and respective receptors are immunoprecipitated with specific antibodies as described above (see chapter 3.4.2). Following separation of the proteins by SDS-PAGE (see chapter 3.4.3), the polyacrylamid gel is dried for 2h at 80°C using a gel dryer. Radioactive signals are visualized by exposure to a phosphor imager screen.

KRH buffer	50 mM HEPES (pH 7,5) 128 mM NaCl 5 mM $MgSO_4$ 1,3 mM $CaCl_2$ 5 mM KCl dissolve in dH_2O; sterilize by filtration
Binding buffer	KRH + 0,5% fatty acid free BSA
Cross-linking solution	10 mg/ml DSS dissolve in 1 ml DMSO dilute 1:100 in KRH

3.4.9.2 Binding and cross-linking of [^{125}I]-NGF

Binding and cross-linking is carried out following the method described by Meakin *et al*. [277]. Cells are washed once with cold binding buffer and placed on ice until the binding solution containing 1nM [^{125}I]-NGF is added to the cells for 2h at 4°C under gentle shaking. Unbound ligand is taken off by washing the cells four times with cold binding buffer. To cross-link the bound ligand, binding buffer containing 150µM DSS is added for 15min at RT and for further 15min at 4°C. The cross-linking reaction is quenched by the addition of 10mM Tris/HCl (pH 7,4). Finally, cells are lysed in RIPA buffer, receptors are immunoprecipitated and proteins are separated and visualized as described for binding and cross-linking with [^{125}I]-TGF-β1 (see chapter 3.4.9.1).

Binding buffer	137 mM NaCl 4,7 mM KCl 2,5 mM $CaCl_2$ 1,2 mM KH_2PO_4 (pH 7,4) 1,2 mM $MgSO_4$ 1 mg/ml Glucose 1 mg/ml BSA 10 mM HEPES (pH 7,0) 1 mM PMSF

Quenching reagent	10 mM Tris/HCl (pH 7,4)
RIPA buffer	300 mM NaCl 10 mM Tris/HCl (pH 8,0) 10 mM KCl 1 mM EDTA 1% NP-40 1% Sodium deoxycholate 0,1% SDS

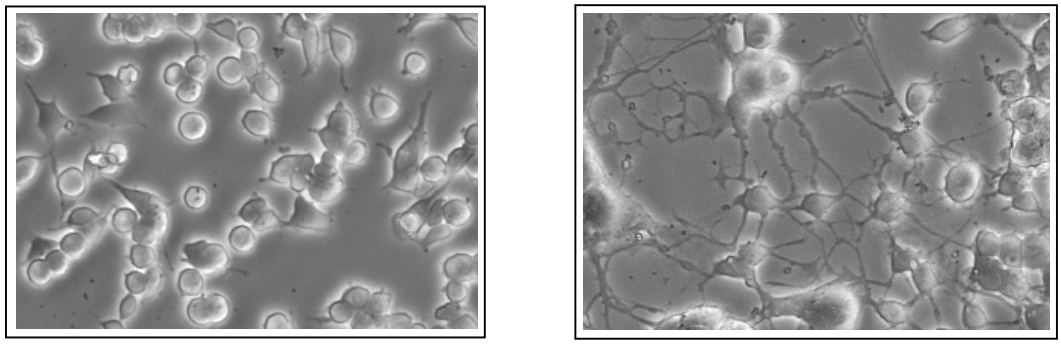
4 Results

During the past years, it became evident that signal transduction pathways in a cell do not only proceed in a linear stepwise fashion but rather form a complex network of cascades that mutually regulate each other [249]. Referring to cell populations of neuronal origin, members of the TGF-β superfamily are described to possess neurotrophic effects when acting in concert with other cytokines or neurotrophins [212, 215]. This suggests that there is convergence or "crosstalk" between the signaling pathways of TGF-βs and neurotrophins.

4.1 CHARACTERIZATION OF PC12 CELLS

Investigation of the potential interdependence between the signaling cascades of TGF-β and NGF was performed in rat pheochromocytoma cells (PC12) which represent a well established model system for neuronal differentiation [260].

As shown in Fig. 4.1, treatment of PC12 cells with NGF leads to initiation of neurite formation, thereby conferring a cell morphology that resembles a neuronal phenotype.



- NGF + NGF

Fig. 4.1 Neurite outgrowth of PC12 cells
PC12 cells were plated on collagen-coated 6-well plates at a density of $4x10^4$ cells per well. Cultivation was performed for 7 days in medium containing 1% horse serum and either in presence or in absence of 2nM NGF.

4.1.1 Receptor repertoire of PC12 cells

PC12 cells are known to express both types of NGF receptors, the high-affinity receptor TrkA and the low-affinity receptor $p75^{NTR}$ [277, 278]. However, the presence of TGF-β receptors, in particular TβRII, is controversially discussed in literature [51, 279]. To characterize PC12 cells in terms of TGF-β receptor expression, cells were investigated by RT-PCR as well as binding and cross-linking experiments using radiolabeled TGF-β1 to detect receptor mRNA or protein, respectively.

Fig. 4.2 shows, that TβRII mRNA can be detected in PC12 cells (Fig. 4.2, lanes 5 and 6) but not in Hep3B-TR cells (lane 4) that are derived from the human hepatoma cell line Hep3B

but lack both alleles of the *tβrII*-gene [18]. As positive control, L6 rat myoblasts were used which are known to express TβRII (lane 3) [53]. Moreover, it can be observed that treatment of PC12 cells with NGF does not alter the amount of TβRII mRNA (compare lanes 5 and 6).

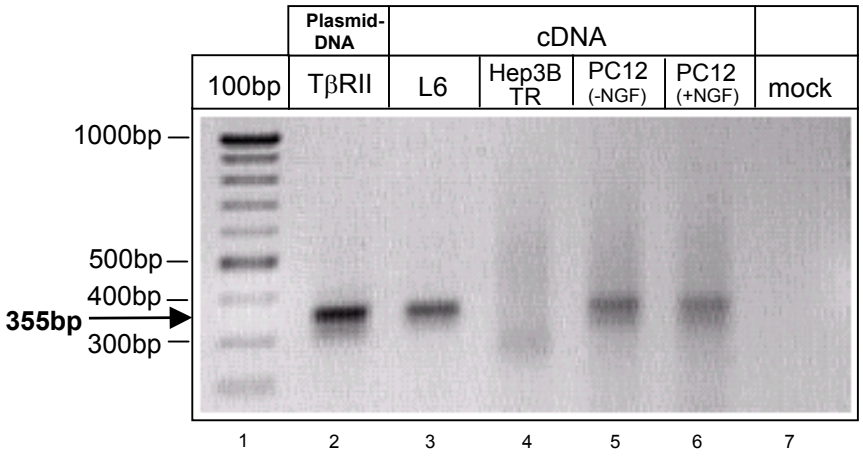


Fig. 4.2 Detection of TβRII mRNA by RT-PCR
Total RNA was isolated from the indicated cell lines, reverse transcribed to generate cDNA and the cDNA was finally amplified by PCR using primers specific for TβRII (P9/P11; sequences see appendix A.2). Due to low levels of TβRII mRNA in PC12 cells, two rounds of PCR amplification were necessary to yield a sufficient amount of DNA to visualize it on the agarose gel. All PCR-fragments were verified by sequencing.

Expression of TGF-β as well as NGF receptors in PC12 cells was confirmed by RT-PCR (Fig. 4.3). For amplification, primers specific for the respective receptors were chosen. The products generated by RT-PCR were verified by sequencing.

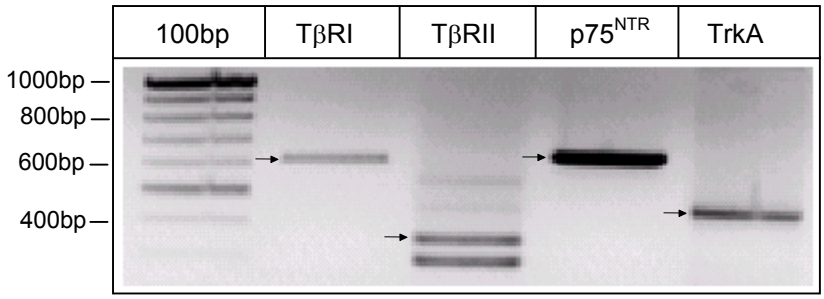


Fig. 4.3 Detection of TGF-β and NGF receptor mRNA by RT-PCR
Total RNA was isolated from PC12 cells, reverse transcribed to generate cDNA and finally amplified by PCR using primers specific for the respective receptor. For TβRI: TR1-4, TR1-9; for TβRII: P9, P11; for $p75^{NTR}$: p75-1, p75-2; for TrkA: F-TrkA2, F-TrkA3 (sequences of the oligonucleotides are listed in the appendix, see appendix A.2).

Besides mRNA detection, it was also tested whether the receptor proteins are indeed expressed and transported to the cell surface. For this purpose, PC12 cells were subjected to a binding and cross-linking experiment using [^{125}I]-TGF-β1. Following immunoprecipitation with antibodies recognizing TβRI or TβRII, respectively, receptors that were covalently linked to the radiolabeled ligand could be visualized by autoradiography (Fig. 4.4).

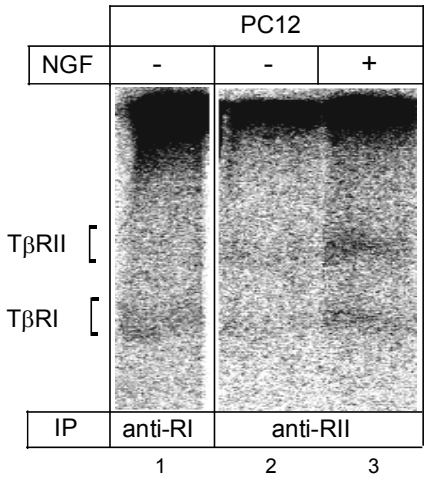


Fig. 4.4 Cell surface expression of TGF-β receptors on PC12 cells
Binding and cross-linking was performed as described in 3.4.9 using 500pM [^{125}I]-TGF-β1. Immunoprecipitation was performed with antibodies specific for TβRI (anti-RI) or TβRII (anti-RII).

Figure 4.4 shows that PC12 cells are endogenously equipped with both TGF-β signaling receptors, TβRI and TβRII. Following immunoprecipitation with an antibody against the type II receptor, bands corresponding to TβRII as well as TβRI are visible (Fig. 4.4, lanes 2 and 3). The appearance of TβRI is due to coimmunoprecipitation of the receptor which is recruited into a heteromeric complex with TβRII following ligand binding. Immunoprecipitation with an anti-RI antibody brings down the type I receptor, whereas the TβRII receptor can hardly be detected under these conditions (Fig. 4.4, lane 1). Although it appears that NGF treated PC12 cells express a higher amount of TGF-β receptors at the cell surface, this is rather an effect of unequal amounts of protein loaded onto the gel after immunoprecipitation. This is supported by the likewise weaker signal of the most upper band (lane2) that represents over-cross-linked proteins.

4.1.2 Investigation of TGF-β responsiveness of PC12 cells

The preceding experiments demonstrated that both types of TGF-β receptors are present in PC12 cells. However, TβRII seems to be expressed in very low amounts as the mRNA could be detected only after a second round of amplification in the PCR reaction.

TGF-β responsiveness of PC12 cells was evaluated by Western blotting to detect TGF-β induced Smad2 phosphorylation (Fig. 4.5) and by reporter gene assays to determine ligand mediated activation of Smad-dependent reporter genes (Fig. 4.6).

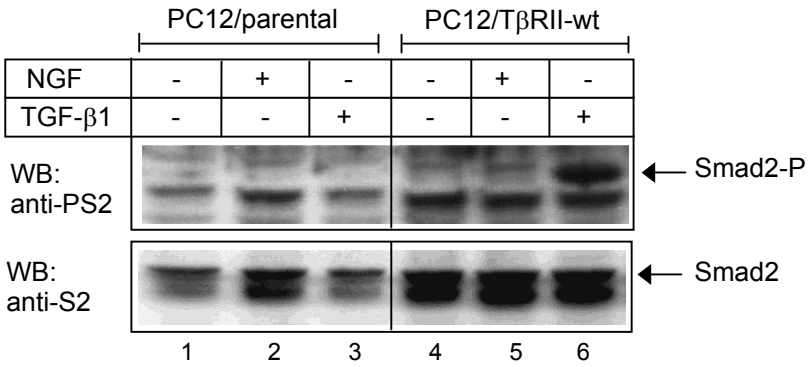


Fig. 4.5 Western blot analysis to detect phosphorylated Smad2 following TGF-β1 stimulation
Parental PC12 cells (lanes 1-3) or PC12 cells stably expressing TβRII (lanes 4-6) (see chapter 4.2 for description of generation) were stimulated with either 2nM NGF or 200pM TGF-β1 for 30min. Ligand-induced phosphorylation of Smad2 was investigated by immunoblotting using an antibody that is specific for C-terminal phosphorylated Smad2. Total amounts of Smad2 were detected in parallel by using an antibody that recognizes the linker region of Smad2.

Western blot analysis demonstrated, that TGF-β1 induces C-terminal phosphorylation of Smad2 only in cells that ectopically express TβRII (Fig. 4.5, lane 6) but not in parental cells (lane 3). NGF treatment in contrast does not result in Smad2 phosphorylation – neither in parental nor in TβRII expressing cells (lanes 2 and 5).

In accordance with these data, reporter gene assays revealed that TGF-β responsiveness of PC12 cells requires increased levels of TβRII. Whereas overexpression of TβRI is not sufficient to augment TGF-β1-mediated transcriptional activation of the pSBE-luc reporter (Fig. 4.6, lane 4), ectopic expression of TβRII leads to a 2-fold increase in TGF-β responsiveness (lane 6).

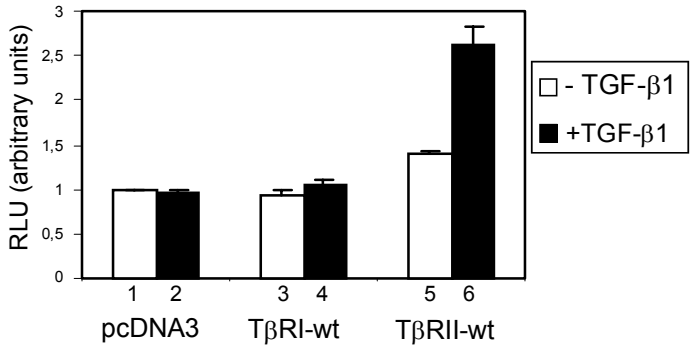


Fig. 4.6 TGF-β1 mediated reporter gene activation in PC12 cells
PC12 cells were plated on collagen-coated petri dishes, transfected with pSBE-luc, pRL-TK and the indicated receptor constructs or empty vector. 24h post transfection, cells were starved for 4h and subsequently stimulated with 200pM TGF-β1 for 24h. Cell lysis and determination of luciferase activity were performed according to manufacturer's instructions.

Collectively, data from phosphorylation analysis and reporter gene assays indicate that TβRII is the limiting factor for TGF-β signaling in PC12 cells.

4.2 GENERATION OF STABLE PC12 CELLS BY RETROVIRAL INFECTION

As the efficiency of transient transfection of PC12 cells was not high enough to obtain strong expression of receptor or Smad proteins in PC12 cells which is needed for several assays, stable transfection was performed using retroviral infection.

Therefor, DNA-fragments encoding (i) the wild-type TβRII (TβRII-wt), (ii) a mutant TβRII which lacks almost the complete intracellular domain (TβRII-Δcyt) and (iii) the inhibitory Smad7, were first cloned into a retroviral vector.

4.2.1 Cloning of retroviral vector constructs

The retroviral vector that was used to insert various TGF-β receptor- or Smad-constructs was obtained from D. Lindemann (Institut für Virologie und Immunologie, Würzburg) (Lindemann *et al*., in preparation) and is described in the following.

4.2.1.1 The retroviral vector

The retroviral vector contains a pcDNA3.1 backbone and carries modified long terminal repeats (LTRs) derived from the moloney retrovirus which belongs to the genus of murine leukemia viruses (MuLV). The LTRs flank the genes that are stably inserted into the genome of the target cell following infection (Fig. 4.7). Expression of the enhanced green fluorescent protein (EGFP) is driven by the 5'LTR. The succeeding intervening ribosomal entry site (IRES) functions to connect the translation of EGFP with the one of the reverse tetracycline-controlled transactivator (rtTA). The rtTA which is part of the tetracycline (tet) regulatory system [280] is a fusion protein composed of the repressor of the Tn*10*-tet resistance operon of *E.coli* and the C-terminal portion of protein 16 of the herpes simplex virus (VP16) which functions as a strong activator of transcription. This reverse transactivator can only bind to the tet-operator (tetO) in presence of the tetracycline-derivative doxycycline and subsequently activate transcription from the minimal cytomegalovirus promoter (CMV). Consequently, the expression of the "gene of interest" can be induced by doxycycline treatment of the cells that were infected with the retrovirus.

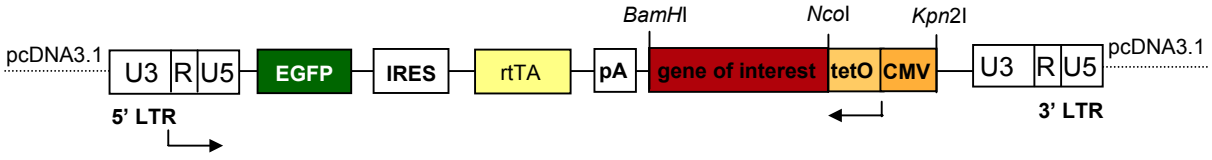


Fig. 4.7 Map of the retroviral vector pczCFG EGIRT

To insert the gene of interest, the retroviral vector construct was first digested with *BamH*I and *Kpn*2I (see Fig. 4.7) because the *Nco*I restriction site is not unique in this vector. The resulting fragments were separated by agarose gel electrophoresis and subsequently eluted from the gel. The larger fragment was dephosphorylated and the smaller fragment was digested with *Nco*I to isolate the 447bp-fragment consisting of the tet-operator sequence and the minimal CMV promoter. Then, the respective gene of interest was ligated with the retroviral vector fragment and the tetO/CMV-fragment.

4.2.1.2 Cloning of a retroviral vector containing the *TβRII-wt* gene

The *TβRII* gene was excised from the pLXSN/TβRII construct [281] by digestion with *BamH*I and *Nco*I. The TβRII construct contains a HA-epitope tag inserted 9 base pairs downstream of the signal peptide. Following purification of the TβRII-fragment, it was used for ligation with the tetO/CMV-fragment and the dephosphorylated retroviral vector.
The resulting plasmid was verified by restriction analysis and sequencing.

4.2.1.3 Cloning of a retroviral vector containing the *TβRII-Δcyt* mutant

To construct a mutant type II receptor that lacks the cytoplasmic domain, a stop codon was introduced such that in the resulting protein, 9 amino acids of the intracellular part are left to ensure proper anchorage in the plasma membrane. In addition, a *BamH*I restriction site was generated downstream of the stop-codon to enable subsequent cloning into the retroviral vector. Required mutations were achieved by a PCR-mutagenesis approach (Fig. 4.8) in which the LXSN/TβRII [281] construct served as template. In the first reaction, the DC-1 primer introduced the stop-codon, whereas in the second reaction, the complementary primer, DC-2 created the stop-codon and the DC-3 primer generated the *BamH*I restriction site. The resulting PCR-fragments were then applied to obtain the full length construct using the flanking primers, 5'LXSN and DC-3. Prior to ligation with the tetO/CMV fragment and the retroviral vector, the PCR-fragment was digested with *Nco*I and *BamH*I and purified.

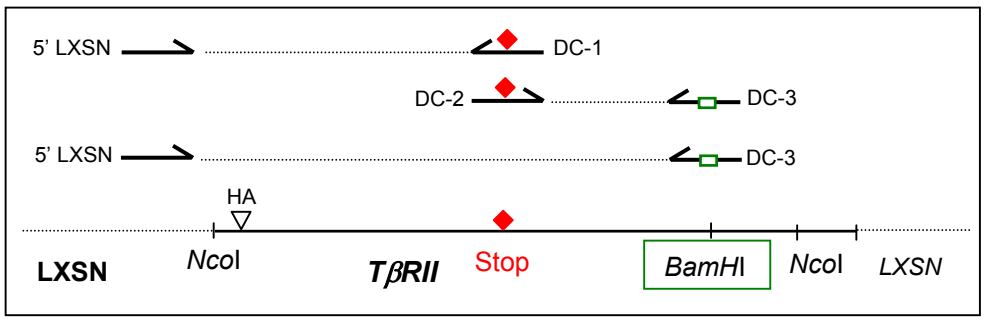


Fig. 4.8 Schematic illustration of the generation of the TβRII-Δcyt mutant

4.2.1.4 Cloning of a retroviral vector containing the *Smad7* gene

To insert the *Smad7* gene into the retroviral vector, it was first necessary to delete an internal *Nco*I restriction site and to generate a *BamH*I restriction site at the 3'-end of the construct. Both changes were introduced by PCR mutagenesis as shown in the scheme below (Fig. 4.9). The Flag-tagged Smad7 construct that was used as template was obtained from P. ten Dijke (Amsterdam, The Netherlands) and was previously described [99]. The *Nco*I restriction site was destroyed by introduction of a silent mutation to avoid changes in the amino acid composition. Following mutagenesis, the PCR-fragment was digested with *Nco*I and *BamH*I, purified and used for ligation with tetO/CMV and the retroviral vector. Restriction analysis and sequencing were performed to verify the success of the cloning procedure.

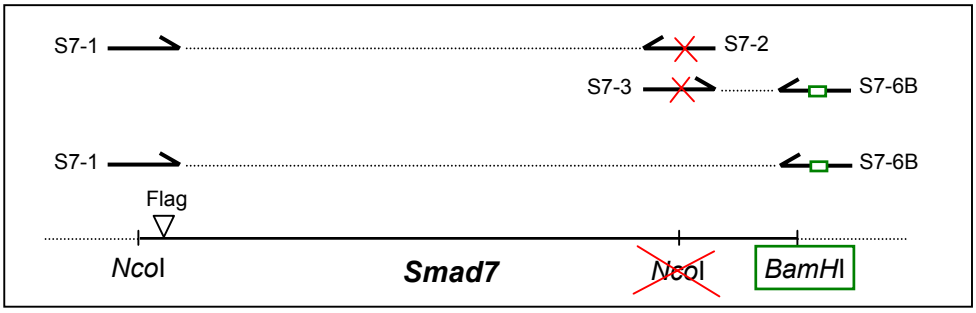


Fig. 4.9 Schematic illustration of the mutagenesis of the Smad7 construct

4.2.2 Production of replication-deficient VSV-G pseudotyped retroviral particles

For infection of the target cells, VSV-G pseudotyped virus particles were used. The characteristic of pseudotyped viruses is that the envelope protein (env, *e.g.* VSV-G) originates from an other virus strain than the retroviral vector (*e.g.* MuLV-derived retroviral vector) [282, 283]. Advantages of pseudotyped viruses are that they give rise to very high virus titers and moreover allow multiple integration events in the genome of the target cell [252].

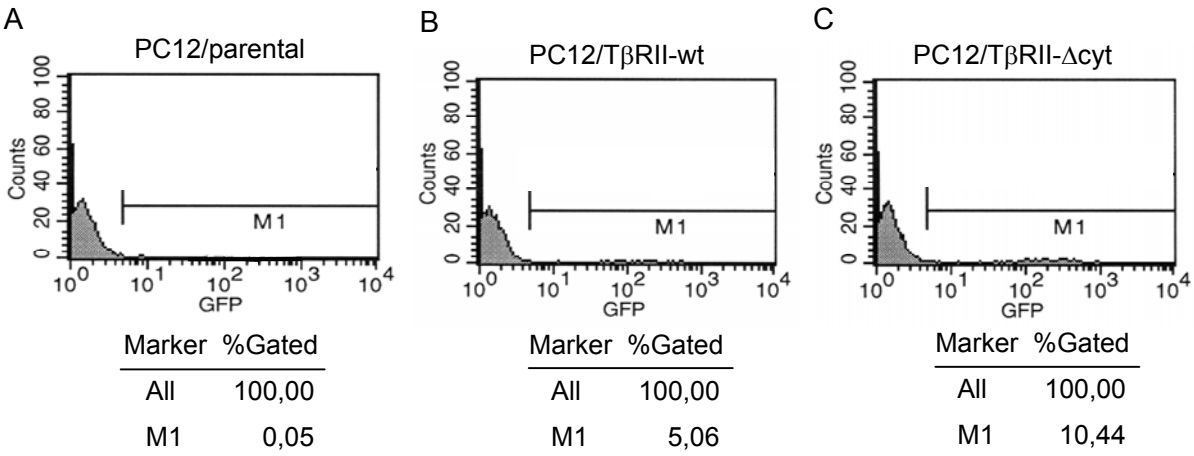
Following cloning of the retroviral constructs, 293T cells which served as a packaging cell line were transiently transfected to produce infectious virus particles. The cells were cotransfected with the retroviral DNA as well as with separate expression vectors for gag-pol and env. Since the coding sequences for the viral structure proteins (gag-pol, env) are separated from the retroviral DNA, they are exclusively used for production of infectious virus particles by the packaging cell line but the resulting virus particles themselves are replication-deficient due to the lack of the genetic information for the gag-pol and env proteins. Harvest of the virus containing supernatant and infection of the target cells were carried out as described in 3.3.8.

4.2.3 Characterization of stable PC12 cells

4.2.3.1 Characterization by FACS analysis

Several days after infection and expansion of the cells, FACS analysis was performed to verify infection efficiency and doxycycline-inducibility of the gene of interest.

Infection efficiency can be easily quantified by counting the percentage of GFP-positive cells (Fig. 4.10a). For testing doxycycline-induced expression of the HA-tagged receptors at the cell surface (Fig. 4.10c), cells were cultured in absence or in presence of 1µg/ml doxycycline for 3 days. Prior to FACS analysis, cells were stained with anti-HA antibodies and with secondary phycoerythrin (PE)-conjugated donkey-anti-mouse antibodies as described in 3.3.9.1.



Marker	%Gated
All	100,00
M1	0,05

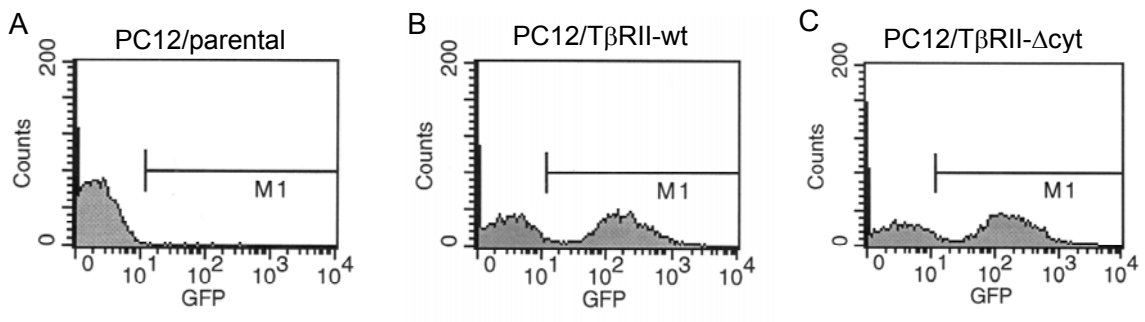
Marker	%Gated
All	100,00
M1	5,06

Marker	%Gated
All	100,00
M1	10,44

Fig. 4.10a Infection efficiency of stable PC12 cells quantified by FACS analysis
Stable PC12 cells were subjected to FACS analysis and the percentage of GFP-positive (*i.e.* cells infected with the retroviral DNA) was determined. On the basis of parental, GFP-negative PC12 cells, the gate M1 could be defined. All cells appearing in gate M1 are GFP-positive.

Fluorescence measurement of the infected cells revealed an infection efficiency of 5% and 10% for PC12/TβRII-wt and PC12/TβRII-Δcyt, respectively.

To enrich the population of infected cells, GFP-positive cells were separated by FACS sorting.



Marker	%Gated
All	100,00
M1	0,07

Marker	%Gated
All	100,00
M1	54,05

Marker	%Gated
All	100,00
M1	63,16

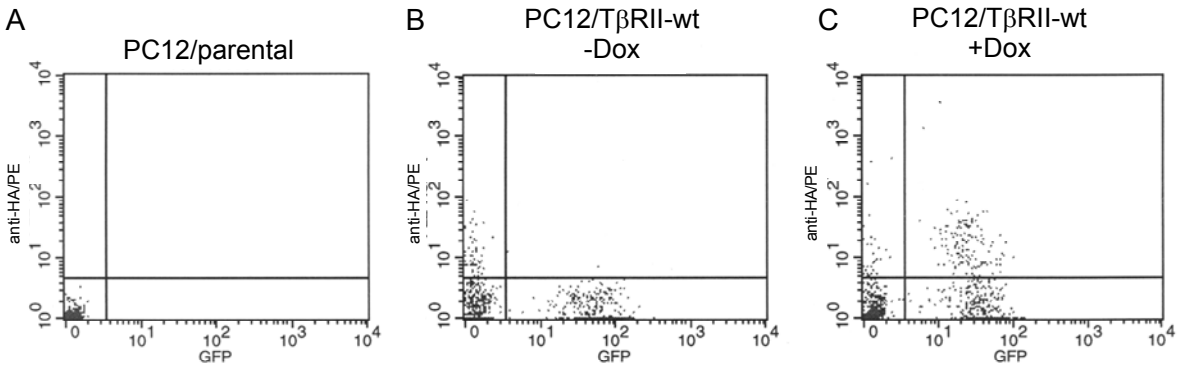
Fig. 4.10b Infection efficiency of stable PC12 cells quantified by FACS analysis after sorting
Following sorting the stable PC12 cells for GFP-positive cells, the percentage of GFP-positive cells was again determined by FACS analysis to control the efficiency of the sorting procedure.

Renewed quantification by FACS analysis confirmed the enrichment of infected cells which resulted in 54% and 63% of GFP-positive cells for PC12/TβRII-wt and PC12/TβRII-Δcyt, respectively (Fig. 4.10b).

Fig. 4.10c exemplary demonstrates the investigation of doxycycline-inducibility of infected PC/12TβRII-wt cells. Again, parental cells which are negative for both, the GFP- and the PE-fluorescence as well as single positive cells showing either GFP- or PE-fluorescence (data not shown) were used to define the threshold that marks the region of GFP-positive cells on the one hand and PE-positive cells on the other hand.

The percentage of GFP-positive cells can be calculated by adding the values measured for the lower right (LR) and upper right (UR) quadrants. Thus, panels B and C show that the percentage of GFP-positive *i.e.* infected cells reaches 46%-50% of the PC12/TβRII-wt cells.

Comparison between unstimulated (Fig. 4.10c, panel B) and doxycycline stimulated (panel C) PC12/TβRII-wt cells demonstrates, that expression of the receptor, which is indicated by a shift of the cells from the lower right (LR) quadrant to the upper right (UR) quadrant, only occurs in presence of doxycycline. Panel C shows that 19% of all cells are infected and express the TβRII-wt receptor following addition of doxycycline. However, 30% of all cells are infected but do not express the receptor even in presence of doxycycline. Consequently, one can calculate that approximately 38% of the GFP-positive cells are doxycycline-inducible. The remaining cells in the lower left (LL) and the upper left (UL) quadrant represent GFP-negative, *i.e.* uninfected cells.



Quadrant	%Gated
UL	0,00
UR	0,00
LL	100,00
LR	0,00

Quadrant	%Gated
UL	7,09
UR	0,45
LL	46,53
LR	45,93

Quadrant	%Gated
UL	4,13
UR	19,46
LL	46,03
LR	30,38

Fig. 4.10c FACS analysis to verify the doxycycline-inducibility of TβRII-wt in PC12 cells
Stable PC12 cells were cultured in absence or in presence of 1µg/ml doxycycline for three days. Then, cells were stained with anti-HA and a PE-conjugated secondary antibodies and analyzed by FACS. The lower left (LL) quadrant shows cells that are double-negative, in the lower right quadrant (LR) cells appear that are GFP-positive but negative for the PE-conjugated antibody (which is bound to the receptors via the HA-antibody). The cells in this quadrant are thus infected cells that do not express the TβRII-wt receptor. The upper left quadrant (UL) contains FL2-positive but GFP-negative cells and the upper right (UR) quadrant shows double positive cells, *i.e*. infected cells that express the receptor.

Cells infected with the TβRII-Δcyt construct (data not shown), showed a doxycycline-inducibility of 6% of all cells and approximately 9% of the GFP-positive cells. Cells infected with the Smad7 construct were not investigated by FACS analysis because Smad7 is a cytoplasmic protein which requires a different staining procedures to allow FACS analysis. Smad7 expression was therefore verified only by Western blot (see Fig. 4.11b).

4.2.3.2 Characterization by Western blot

Following FACS sorting, lysates of the cells were additionally subjected to Western blotting to verify the doxycycline-inducible expression of receptors or Smad7 protein, respectively.

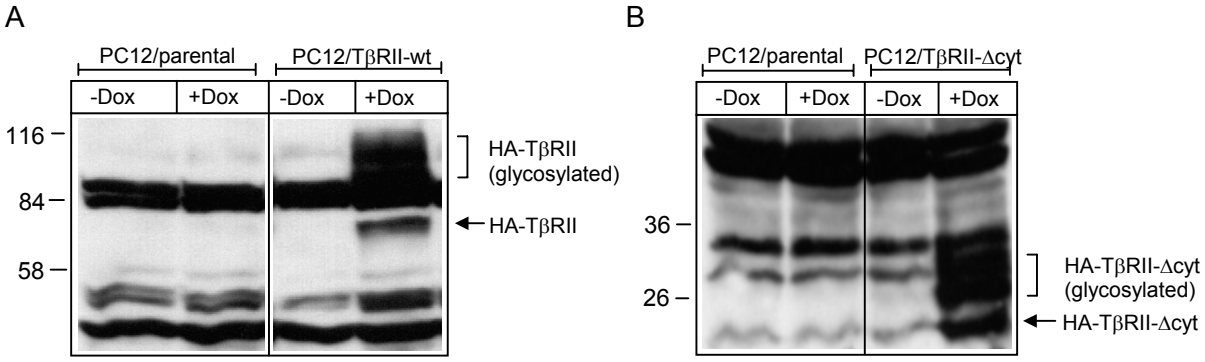


Fig. 4.11a Doxycycline-inducible expression of TβRII-wt and TβRII-Δcyt in PC12 cells
Parental PC12 cells as well as PC12 cells transduced with either TβRII-wt (panel A) or TβRII-Δcyt (panel B) were cultured in absence or in presence of 1µg/ml doxycycline for 3 days. Cell lysates were prepared and analyzed for receptor expression by immunoblotting using antibodies that detect the HA-epitope of the receptors.

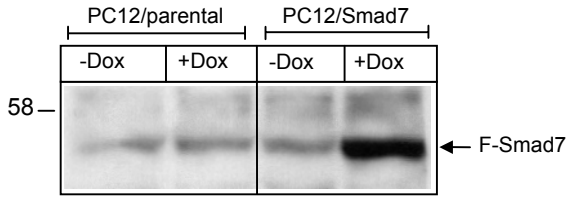


Fig. 4.11b Doxycycline-inducible expression of Smad7 in PC12 cells
Stable PC12 cells were cultured in absence or in presence of 1µg/ml doxycycline for 3 days. Cell lysates were analyzed by Western blot for Smad7 expression using antibodies that detect the Flag-epitope of the Smad protein.

Immunoblotting confirmed that TβRII-wt, TβRII-Δcyt and also Smad7 were only expressed in the infected cells following stimulation with doxycycline. Neither parental cells nor untreated infected cells show ectopic expression of the receptor or Smad proteins, respectively, demonstrating that there is no leakiness of the inducible promoter elements.

4.2.3.3 Characterization by binding and cross-linking

For the heterologous cell surface receptor, TβRII-wt, expression and ligand binding capacity could also be demonstrated by binding and cross-linking experiments.

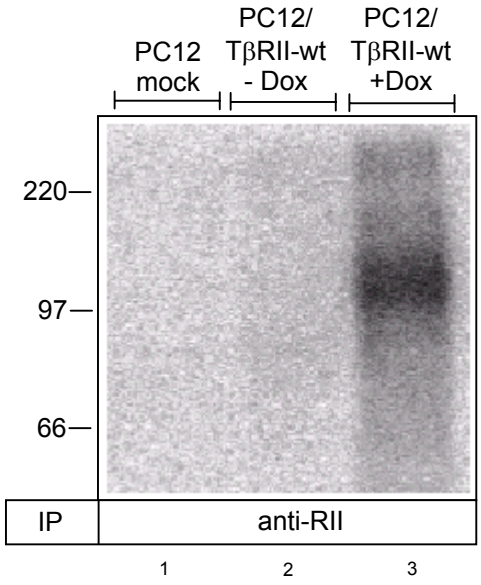


Fig. 4.12 Binding and cross-linking shows inducible expression of TβRII-wt in stable PC12 cells
Parental PC12 cells (lane 1) and PC12 cells stably transfected with TβRII-wt (lanes 2 and 3) were analyzed in a binding and cross-linking assay using [^{125}I]-TGF-β1. The cells were cultured for three days in absence or in presence of 1µg/ml doxycycline to regulate TβRII-wt expression. After chemically cross-linking the radiolabeled ligand to the receptors, TβRII was immunoprecipitated using anti-RII. Proteins were separated by SDS-PAGE and signals were visualized by autoradiography.

Fig. 4.12 reveals that following treatment with doxycycline, PC12/TβRII-wt cells express the TβRII-wt receptor at the cell surface and that this receptor is indeed capable of binding its

ligand, TGF-β1. As expected, there is no detectable expression of TβRII-wt in absence of doxycycline.

4.3 EXAMINATION OF THE INVOLVEMENT OF THE TGF-β/SMAD PATHWAY IN NEURITE OUTGROWTH

Given that TGF-β superfamily members were shown to synergize with several neurotrophins in eliciting neurotrophic effects [211, 212], the impact of the TGF-β/Smad pathway on NGF induced neurite formation of PC12 cells was investigated. Therefor, stable PC12 cells, inducibly expressing either TβRII-wt or proteins that block the TGF-β signaling cascade such as the dominant-negative mutant TβRII-Δcyt or the inhibitory Smad7 protein were tested in a neurite outgrowth assay (see chapter 3.3.5).

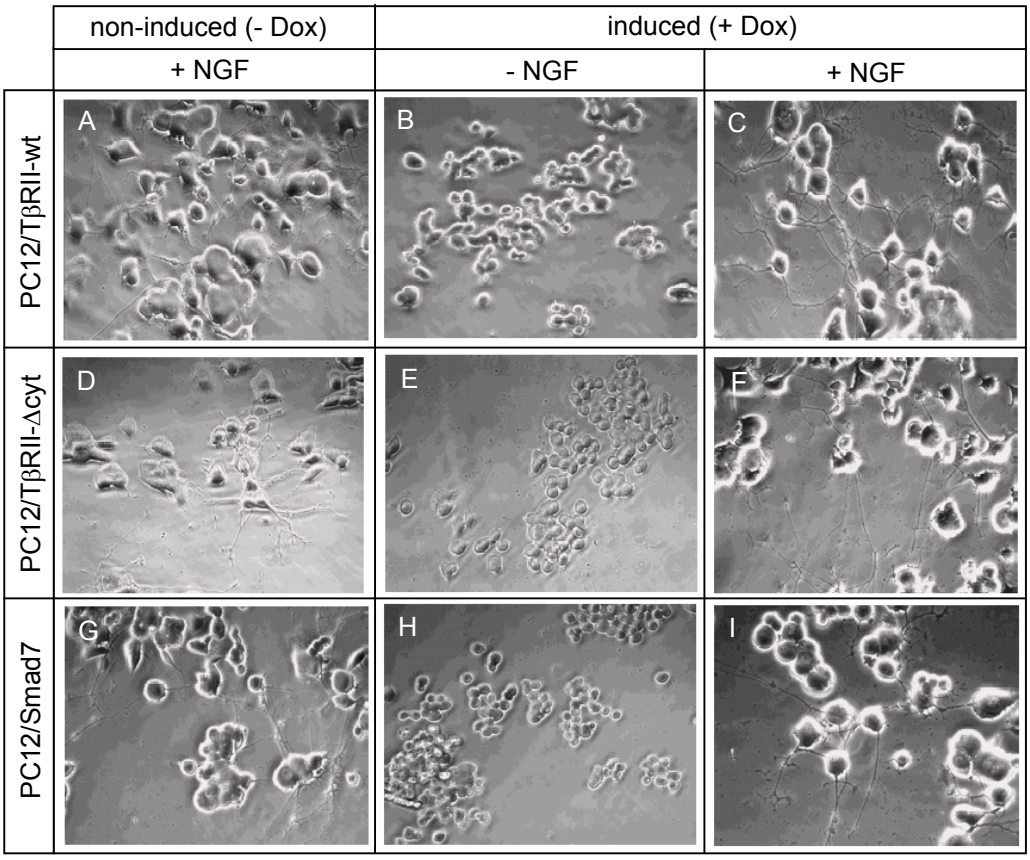


Fig. 4.13a Neurite outgrowth of stable PC12 cells
Stable PC12 cells were cultured in presence or in absence of 1µg/ml doxycycline for 3 days. Then, they were plated on collagen-coated petri dishes in medium containing 1% horse serum. Neurite formation was induced by addition of 2nM NGF as indicated. Cell morphology was monitored by phase contrast microscopy after a period of 7 days.

Fig. 4.13a shows that neurite formation is absolutely dependent on NGF since in absence of NGF, PC12 cells retain their round shaped cell morphology and do not exhibit any elongations (panels B, E and H). The TGF-β pathway, however, does not seem to play a

crucial role because neurite outgrowth is not impaired by expression of TβRII-Δcyt (panel F) or Smad7 (panel I) which are known to potently inhibit the TGF-β pathway [99, 284].
In addition to expression of dominant-negative molecules, the TGF-β cascade was also blocked by adding TGF-β neutralizing antibodies (Fig. 4.13b).

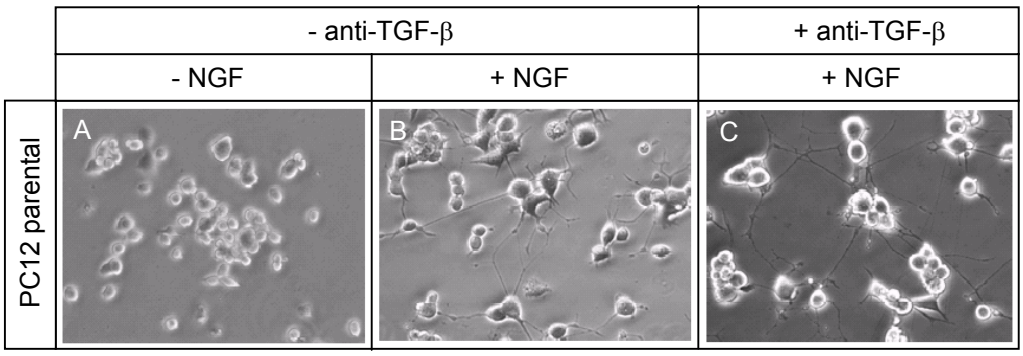


Fig. 4.13b Neurite outgrowth of PC12 cells in absence or in presence of TGF-β antibodies
Parental PC12 cells were seeded on collagen-coated petri dishes in medium containing 1% horse serum. Neurite formation was induced by addition of 2nM NGF as indicated and cells were cultured either in absence or in presence of 20µg/ml neutralizing TGF-β antibodies (anti-TGF-β). Cell morphology was monitored by phase contrast microscopy after 7 days of NGF treatment.

Experiments using neutralizing TGF-β antibodies (Fig. 4.13b) confirmed, that the TGF-β pathway is not necessarily required for neurite formation of PC12 cells because even in presence of the neutralizing antibodies, PC12 cells elicit neurite-like elongations (panel C).
These results suggest, that the potential synergism or interdependence between NGF and TGF-β is important for cellular processes of PC12 cells other than neurite outgrowth.

4.4 EXAMINATION OF THE EFFECTS OF NGF ON SMAD-DEPENDENT REPORTER CONSTRUCTS

Given that TGF-β was found to be dispensable for NGF-mediated effects on PC12 cells, it was further tested, whether – vice versa - NGF can influence the TGF-β/Smad pathway.
For this purpose, PC12 cells were transiently cotransfected with the *renilla* control reporter (pRL-TK) and different Smad-dependent reporter constructs by using lipofectamine™ (see appendix A.3 for description of reporter constructs). Luciferase activity was recorded in response to stimulation with either NGF or TGF-β1 and data were evaluated as described in 3.4.6.

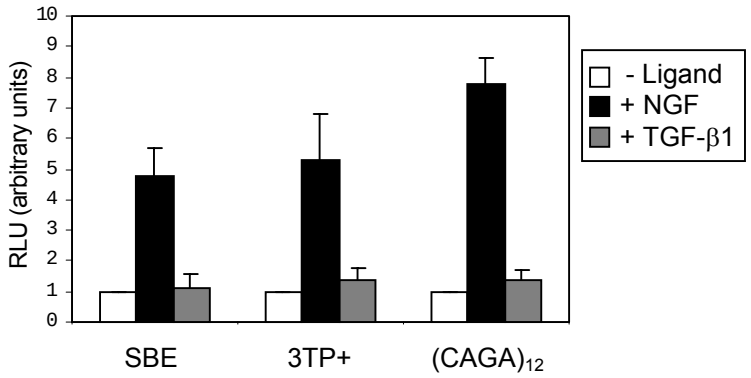


Fig. 4.14 NGF-induced activation of different Smad-dependent reporter constructs in PC12 cells
PC12 cells were transfected with *renilla* luciferase and the indicated reporter plasmids. 24h post transfection, cells were starved and stimulated with either 2nM NGF or 200pM TGF-β1. Cell lysates were prepared and luciferase activity was measured. Standard deviations were calculated from three independent experiments.

The experiment depicted in Fig. 4.14 shows that NGF leads to a significant increase of transcriptional activity on all tested Smad-dependent reporter constructs. TGF-β1, in contrast, is not able to activate transcription from these reporters in PC12 cells, which is in accordance with previous results indicating that low expression of TβRII represents the limiting factor for proper TGF-β signaling in PC12 cells (see chapter 4.1.2).

To investigate, whether TGF-β can synergize with NGF in that it supports or enhances the NGF mediated reporter activation, both ligands were tested either individually or in combination for their capacity to induce pSBE-luc reporter activation.

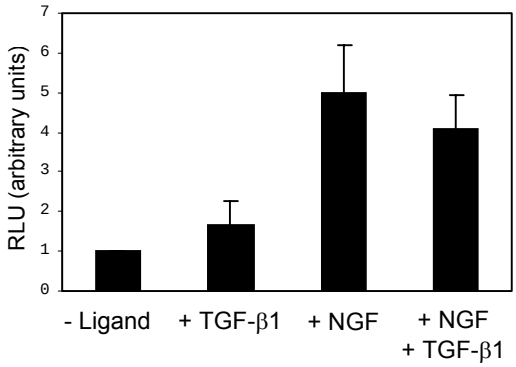


Fig. 4.15 Investigation of synergistic effects of NGF and TGF-β by reporter gene assay
PC12 cells were cotransfected with pSBE-luc and pRL-TK prior to starvation and stimulation with either 200pM TGF-β1, 2nM NGF or both, 200pM TGF-β1 and 2nM NGF.

TGF-β1 does not significantly induce luciferase activity, whereas NGF is capable to evoke a 5-fold increase in reporter activation (Fig. 4.15). However, simultaneous stimulation with both growth factors demonstrates that TGF-β1 and NGF do not show synergistic effects, rather the NGF-mediated signal shows a tendency to be decreased in the presence of TGF-β1.

4.5 VERIFICATION OF THE TGF-β INDEPENDENCE OF THE OBSERVED NGF EFFECTS

Although preceding experiments point to an incapability of exogenous TGF-β1 to activate transcription from Smad-dependent reporters in PC12 cells (Fig. 4.14 and 4.15), it can not be excluded, that the NGF-mediated transcriptional response is a secondary effect, caused for instance by NGF-triggered secretion of TGF-β1. As it was previously published that NGF induces transcription of TGF-β1 in PC12 cells [285], different approaches were used to show that the observed NGF effect is independent of TGF-β1.

On the one hand, TGF-β1 in the medium was neutralized by adding either anti-TGF-β antibodies (Fig. 4.16) or the extracellular domain of TβRII-B (TβRII-B-ECD) (Fig. 4.18) and on the other hand, the amount of active TGF-β1 present in the cell supernatant was determined (Fig. 4.19 and Tab. 4.1).

4.5.1 Neutralization of TGF-β1 in the medium

4.5.1.1 Neutralization by TGF-β antibodies

In this experiment, monoclonal anti-TGF-β1, -β2, -β3 antibodies were used to neutralize all TGF-β isoforms that are potentially present in the medium.

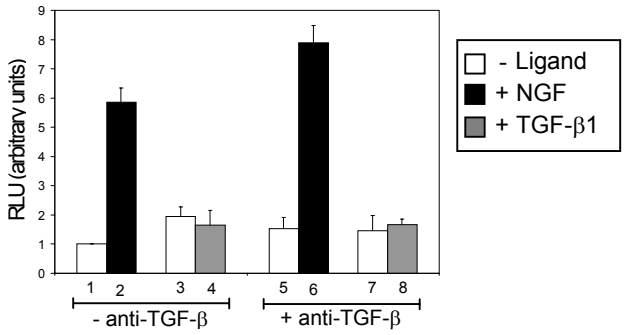


Fig. 4.16 Effects of neutralizing TGF-β antibodies on growth factor-mediated reporter activation
PC12 cells were cotransfected with pSBE-luc and pRL-TK. Following starvation, either control medium or medium containing 20µg/ml anti-TGF-β antibodies was added to the cells 1h prior to stimulation with medium supplemented with 2nM NGF or 200pM TGF-β1, respectively. Cell lysates were prepared and utilized for determination of luciferase activity.

NGF treatment of PC12 cells resulted in a significant induction of transcriptional activity (Fig. 4.16, bar 2) which is not impaired by the presence of neutralizing TGF-β antibodies (bar 6). TGF-β1 in contrast does not provoke activation of transcription, neither in absence nor in presence of antibodies (bars 4 and 8).

To verify the neutralizing capacity of the antibody, TGF-β1-mediated Smad3 phosphorylation was monitored by Western blot using the TGF-β responsive L6 cell line. Stable L6 cells expressing a GFP-Smad3 fusion protein were generated by retroviral gene transfer of the pMX-GFP-Smad3 construct (obtained from Y. Henis, Tel Aviv) as described in 3.3.8. The cells were then starved and treated with control medium or with medium containing TGF-β1,

both, in absence and in presence of neutralizing TGF-β antibodies. Phosphorylated Smad3 was detected by an antibody that specifically recognizes the C-terminally phosphorylated form of Smad3 (anti-PS1/3, see Tab. 2.1) whereas total amounts of Smad3 were recognized by an antibody raised against the linker region of Smad3 (anti-S3).

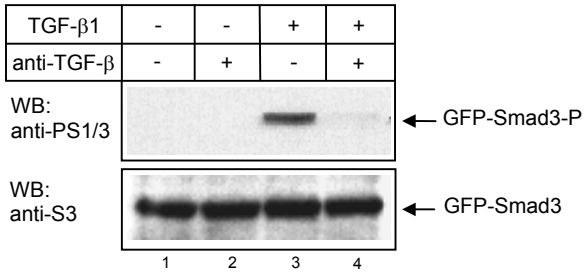


Fig. 4.17 Abrogation of TGF-β signaling by neutralizing TGF-β antibodies
Stable L6 cells expressing GFP-Smad3 were stimulated with 200pM TGF-β1 for 30min either in presence or in absence of neutralizing TGF-β antibodies. Cells were lysed and phosphorylated Smad3 (upper panel) as well as total amounts of Smad3 (lower panel) were detected with anti-PS1/3 or anti-S3 antibodies, respectively.

In the highly TGF-β1 responsive L6 cells, Smad3 becomes phosphorylated at the very C-terminal two serine residues in response to TGF-β stimulation (Fig. 4.17, lane 3). However, simultaneous treatment with TGF-β1 and TGF-β neutralizing antibodies leads to complete abrogation of Smad3 phosphorylation (lane 4), confirming that the antibody potently captures TGF-β. Without TGF-β induction, there is no Smad phosphorylation at all (lanes 1 and 2).

4.5.1.2 Neutralization by the TβRII-B-ECD

In a complementary experiment, TGF-β1 was neutralized using TβRII-B-ECD instead of the TGF-β antibodies. TβRII-B-ECD is the soluble extracellular domain of TβRII-B which represents a splice variant of TβRII that binds all three TGF-β isoforms [53]. PC12 cells, transiently transfected with pSBE-luc and pRL-TK, were incubated with control medium or medium containing two different concentrations of TβRII-B-ECD (100- and 1000-fold molar excess). Additionally, cells were either left untreated or were stimulated with NGF or TGF-β1, respectively (Fig. 4.18, upper panel). Prior to cell lysis, the supernatant of thus treated PC12 cells was harvested and transferred to L6 cells which were equally transfected with pSBE-luc and pRL-TK (Fig. 4.18, lower panel).

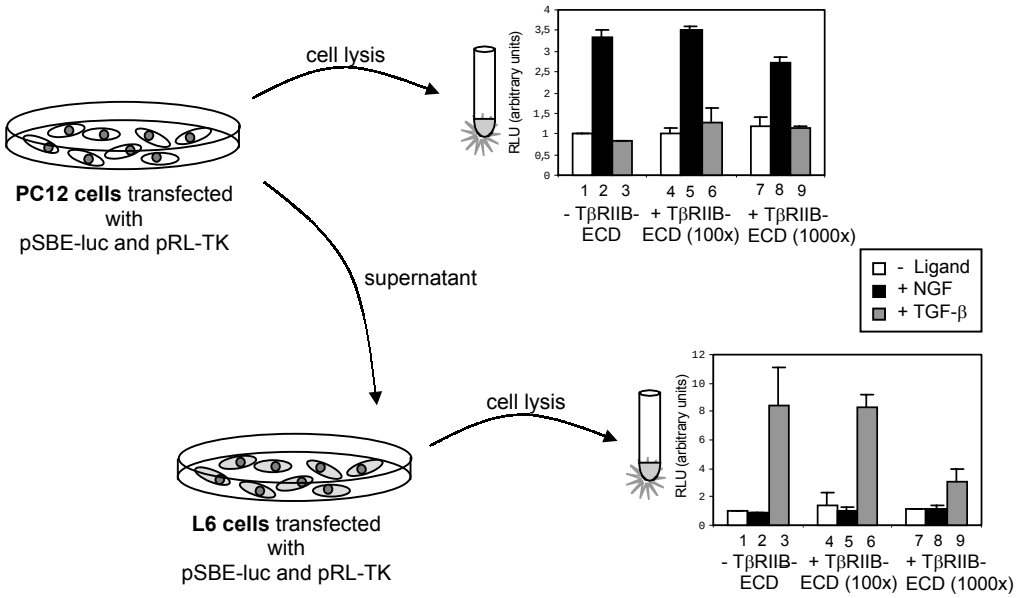


Fig. 4.18 Reporter activation following neutralization of TGF-β by TβRII-B-ECD
PC12 cells as well as L6 cells were cotransfected with pSBE-luc and pRL-TK. TβRII-B-ECD was added in different concentrations (100- and 1000-fold molar excess) to the PC12 cells and luciferase activity was determined in response to either 2nM NGF or 200pM TGF-β1. The supernatant of the PC12 cells was placed on transfected L6 cells and reporter activation was likewise determined.

Although there is a slight decrease of luciferase activity in presence of a 1000-fold molar excess of TβRII-B-ECD (Fig. 4.18, upper panel, compare bar 2 and bar 8), TβRII-B-ECD does not significantly impair NGF-mediated reporter activation in PC12 cells. Referring to L6 cells stimulated with the PC12 cell supernatant, the results confirm that TβRII-B-ECD is functional in neutralizing TGF-β1 because the strong reporter activation evoked by TGF-β1 (Fig. 4.18, lower panel, bar 3) can be efficiently blocked by a 1000-fold molar excess of TβRII-B-ECD (Fig. 4.18, lower panel, bar 9). Furthermore, there is no transcriptional activation detectable in TGF-β sensitive L6 cells that were treated with the supernatant of NGF stimulated PC12 cells (Fig. 4.18, lower panel, bar 2), indicating that NGF treatment of PC12 cells does not lead to production of sufficient amounts of active TGF-β1.

4.5.2 Quantification of the amount of secreted TGF-β1 in the supernatant

The amount of TGF-β1 that is secreted in response to NGF stimulation of PC12 cells was determined by means of the MLEC cell line which stably expresses a luciferase reporter gene under the control of a truncated PAI-1 promoter [259]. MLEC cells were seeded on 96-well plates as described in 3.4.7 and were stimulated by adding the supernatant of PC12 cells that were cultured in absence or in presence of 2nM NGF for 24h. In parallel, a calibration curve was recorded by stimulating MLEC cells with defined concentrations of TGF-β1.

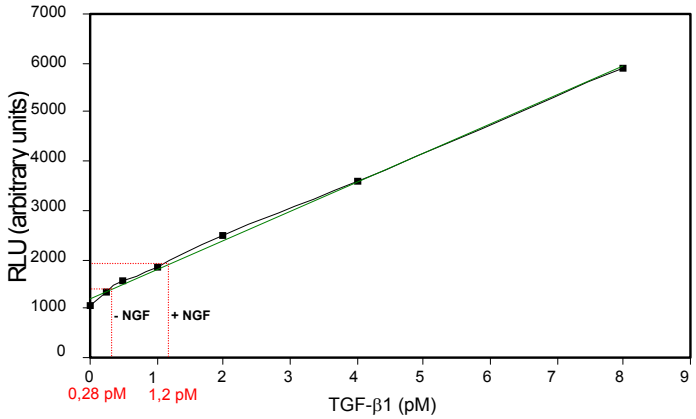


Fig. 4.19 Quantification of active TGF-β1 in the supernatant of PC12 cells
Luciferase activity was measured in MLEC cells that were stimulated with the supernatant of either untreated PC12 cells or PC12 cells that were cultured in presence of 2nM NGF for 24h. The calibration curve was generated by treating MLEC cells with defined concentrations of TGF-β1.

The concentration of active TGF-β1 present in the supernatant of PC12 cells could be determined to the amount of 0,28pM for untreated PC12 cells and to 1,2pM for NGF treated PC12 cells (Fig. 4.19a).

In addition, the assay allows to distinguish between active and latent forms of TGF-β1. Usually, only the active form of TGF-β1 can be determined by reporter gene assays. However, activation of latent TGF-β can be achieved by acidification and subsequent neutralization of the supernatant before transfer to the MLEC cells. Thus, activation enables to quantify the total amount (*i.e.* active and latent) TGF-β. The results obtained from the MLEC assay are listed in Tab. 4.1.

	active TGF-β	total TGF-β
- NGF	0,28 pM	18,2 pM
+ NGF	1,12 pM	~ 40 pM

Tab. 4.1 Quantification of active and latent TGF-β in the supernatant of PC12 cells
MLEC cells were stimulated with the supernatant of PC12 cells that were cultured in absence or in presence of NGF for 24h. To evaluate the amount of total (*i.e*. latent and active) TGF-β, aliquots of the PC12 cell supernatant were acidified as described in 3.4.7 prior to addition to the MLEC cells. The calibration curve that was recorded by using defined concentrations of TGF-β1 reached saturation at about 40pM TGF-β1.

4.6 INVESTIGATION OF THE SIGNAL TRANSDUCTION MECHANISM INDUCED BY NGF

The preceding experiments revealed that NGF can activate the Smad pathway independent of TGF-β1. From TGF-β signal transduction it is known that ligand stimulation leads to phosphorylation of the C-terminal SSXS-motif of R-Smads, heteromeric complex formation of R-Smads with Smad4 and finally nuclear translocation and gene expression [286]. The following experiments were performed to investigate whether signals mediated by NGF are transduced via the same mode of Smad activation.

4.6.1 R-Smads involved in NGF-induced reporter activation

The reporter gene assay shown in Fig. 4.14 proved that NGF is capable of activating transcription from different Smad-dependent promoter elements. The distinct specificity of the reporter constructs that were used allows to distinguish between signals originating from different R-Smads: whereas pSBE-luc serves as a readout for TGF-β- as well as BMP-activated R-Smads (Smad2/3 as well as Smad1/5/8), p3TP-luc(+) and p(CAGA)$_{12}$-luc are restricted to TGF-β-activated Smads (Smad2/3), whereby p(CAGA)$_{12}$-luc particularly responds to Smad3 (see appendix A.3 for detailed description of the reporter constructs). Consequently, the results represented in Fig. 4.14, showing activation of all three reporter constructs, already hint to an involvement of TGF-β R-Smads, in particular Smad3. To verify this hypothesis, all R-Smads were tested for activation by NGF in PC12 cells.

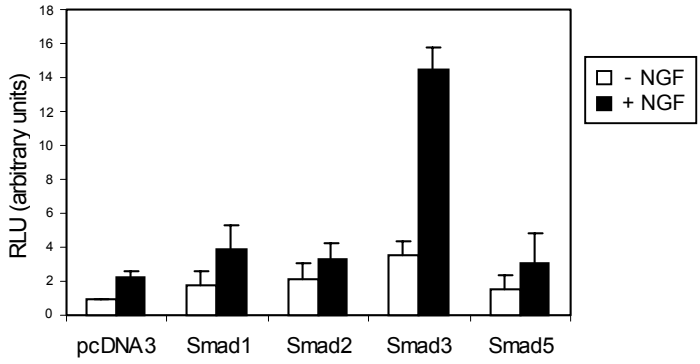


Fig. 4.20 Identification of R-Smads that become activated in response to NGF stimulation
PC12 cells were transfected with p3TP-luc(+), pRL-TK and the indicated Smad constructs. Following starvation and stimulation with 2nM NGF, cells were lysed and luciferase activity was determined. Error bars were calculated from three independent experiments.

Ectopic expression of various R-Smads demonstrates that Smad3 provokes the most prominent induction of transcription following NGF stimulation (Fig. 4.20). Similar results were obtained with the p(CAGA)$_{12}$-luc reporter (data not shown).

Based on these results, investigations concerning phosphorylation and nuclear translocation were focused on Smad3. First, Smad3 and a mutant of Smad3 lacking the C-terminal SSXS-motif (Smad3ΔSSXS) were compared in respect to their potential to activate the Smad3 specific reporter p(CAGA)$_{12}$-luc (Fig. 4.21).

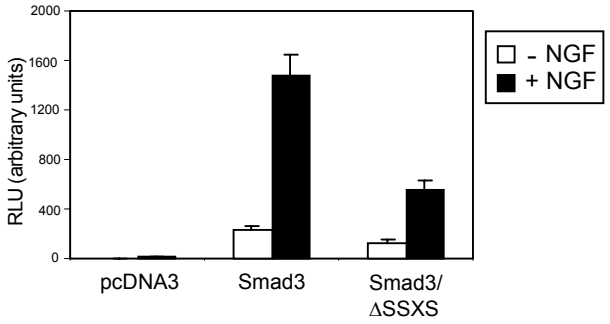


Fig. 4.21 Studies on the role of the C-terminal SSXS-motif of Smad3
PC12 cells were cotransfected with p(CAGA)$_{12}$-luc, pRL-TK and either empty vector or the indicated Smad3 variants. Luciferase activity was measured in untreated and NGF stimulated cells.

Expression of the wild-type Smad3 protein shows a very strong activation capacity, whereas reporter activation is markedly impaired in presence of the Smad3 mutant (Smad3ΔSSXS) (Fig. 4.21). However, even the truncated Smad3 protein, which cannot be phosphorylated at the C-terminus, mediates a significant induction of the luciferase reporter compared to the signal of mock transfected cells. These results propose a supportive but not a necessary role of the C-terminal SSXS-motif in transmitting NGF-induced signals.

4.6.1.1 Examination of C-terminal phosphorylation of Smad3

Whether phosphorylation of the SSXS-motif is involved in NGF-triggered Smad activation was investigated by immunoblotting using antibodies that specifically detect the C-terminally phosphorylated Smad3 protein (anti-PS1/3).

Stable cell lines of L6 myoblasts as well as PC12 cells were generated by retroviral transfer of a GFP-Smad3 fusion protein (see chapter 4.5.1.1 and 3.3.8). C-terminal phosphorylation of Smad3 was monitored following treatment with either NGF or TGF-β1 in both cell lines.

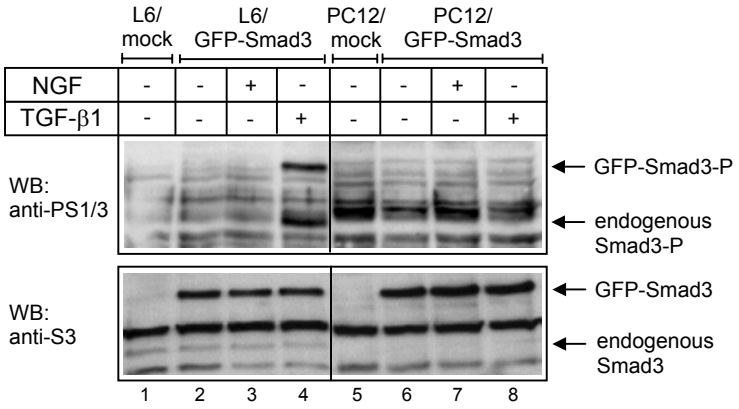


Fig. 4.22 Investigation of C-terminal phosphorylation of Smad3
TGF-β responsive L6 rat myoblasts (lanes 1-4) and PC12 cells (lanes 5-8) were transduced with the pMX-GFP-Smad3 construct (lanes 2-4 and 6-8). Stable cells were starved and subsequently stimulated with either 2nM NGF or with 200pM TGF-β1 and cell lysates were analyzed for C-terminally phosphorylated Smad3 (upper panel) or for total amounts of Smad3 (lower panel) using the respective antibodies.

The phosphorylation pattern of stable L6 cells shows that TGF-β1 stimulation leads to phosphorylation of both, heterologous GFP-Smad3 and endogenous Smad3 proteins (Fig. 4.22, lane 4), whereas NGF does not trigger C-terminal phosphorylation of Smad3 (lane 3). In stable PC12 cells, however, neither NGF nor TGF-β1 treatment gives rise to phosphorylation of the Smad3 SSXS-motif (Fig. 4.22, lanes 7 and 8). It should be noted that the lack of Smad3 phosphorylation in PC12 cells following TGF-β1 treatment is likely to be due to the low amount of TβRII receptors as demonstrated for Smad2 in Fig. 4.5.

4.6.1.2 Examination of nuclear localization of Smad3

In the current model of TGF-β signal transduction, Smad phosphorylation is necessary to allow dissociation of the R-Smad from the type I receptor and to form heteromeric complexes with Smad4 which subsequently enter the nucleus [135, 136]. However, it was also previously published that a constitutively active form of the MAP kinase kinase kinase, MEKK-1, is able to activate Smad-dependent transcription in endothelial cells [188]. The underlying mechanism was described to involve phosphorylation events outside the SSXS-motif but yet results in enhanced association with Smad4 and nuclear translocation.

The following experiment was performed to investigate, whether Smad3 enters the nucleus in response to NGF stimulation although (according to Fig. 4.22) NGF cannot trigger phosphorylation at the C-terminus.

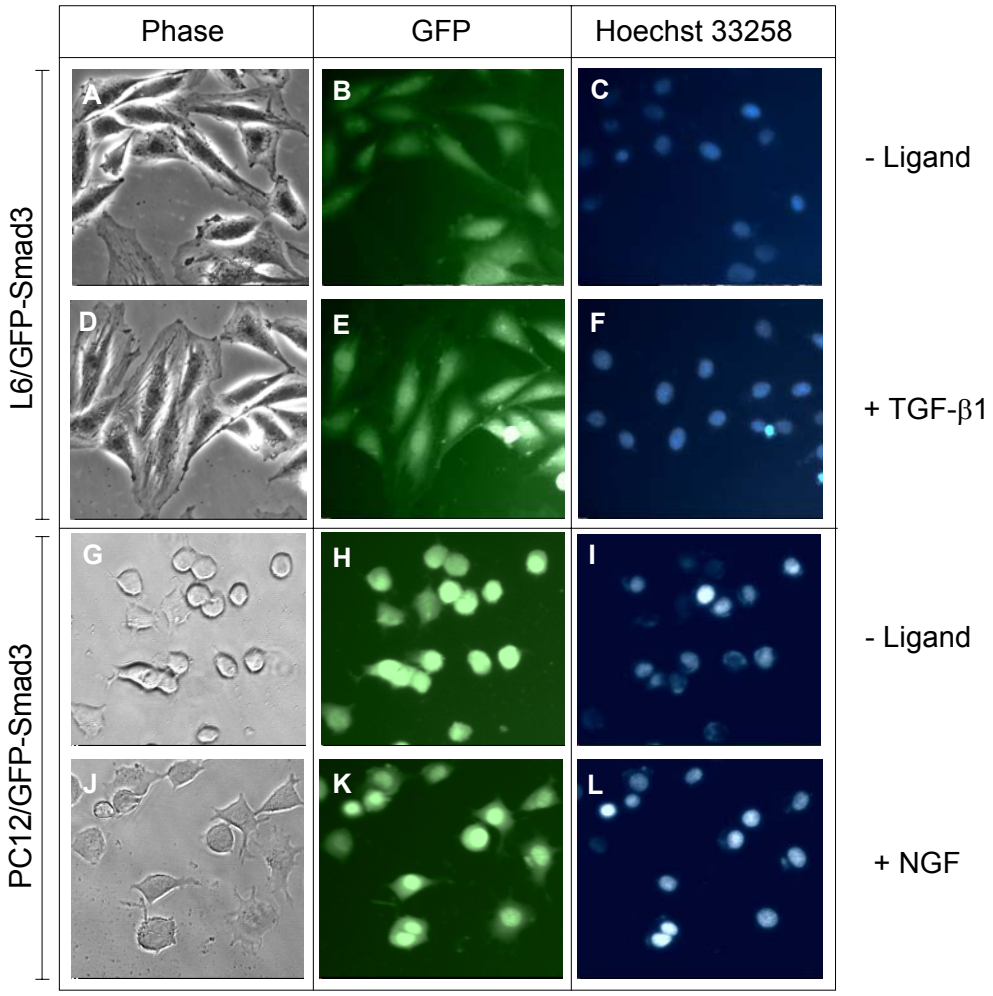


Fig. 4.23 Nuclear translocation of Smad3
Smad3 nuclear translocation was tested in TGF-β responsive L6 myoblasts (panels A-F) and in PC12 cells (panels G-L) both stably expressing the GFP-Smad3 fusion protein. Cells were plated on collagen-coated dishes, starved and stimulated for 24h with either 200pM TGF-β1 (panels D-F), 2nM NGF (panels J-L) or were left untreated (panels A-C and G-I). Cells were fixed, nuclei stained with Hoechst 33258 as described in 3.4.5 and cells were used for fluorescent microscopy.

Fig. 4.23 shows that in L6 cells, TGF-β1 is capable of triggering nuclear translocation of GFP-Smad3 (compare panel B and E). In PC12 cells, NGF treatment equally induces nuclear accumulation of the GFP-Smad3 fusion protein (panel K) which was distributed throughout the cell in absence of ligand (panel H).

4.6.2 Investigation of Smad4 dependence

To investigate whether NGF-induced Smad signaling shows analogy to the TGF-β/Smad cascade in that it requires Smad4 as a common mediator, the effects of a Smad4 mutant were observed in a reporter gene assay. The Smad4 variant that was used represents a deletion mutant lacking the Smad activation domain (SAD), a proline rich domain that is located in the C-terminal part of the linker region and provides transcriptional capability of the Smad4 protein [134, 287]. The Smad4(ΔSAD) construct was kindly provided by M. de Caestecker (Bethesda, USA).

As a functional control for the Smad4 deletion mutant, its inhibiting effect was first verified in TGF-β responsive L6 cells using the p3TP-luc(+) reporter.

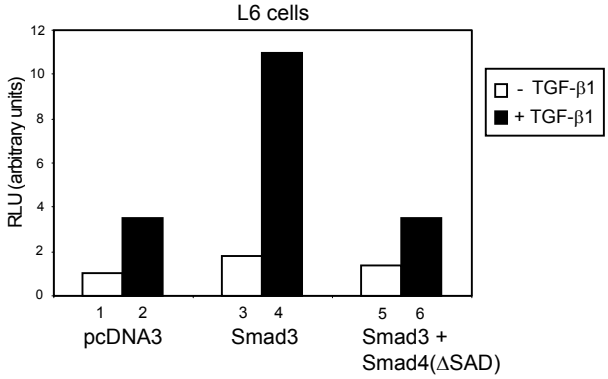


Fig. 4.24a Confirmation of the inhibiting effect of the Smad4(ΔSAD) mutant in L6 cells
L6 cells were transfected with p3TP-luc(+), pRL-TK and the indicated Smad constructs using lipofectamine™. The cells were starved and stimulated with 200pM TGF-β1 for 24h before reporter activity was analyzed.

As depicted in Fig. 4.24a, the Smad4(ΔSAD) mutant effectively impairs TGF-β-mediated reporter activation in L6 myoblasts. Although Smad4(ΔSAD) does not provoke a dominant-negative effect (Fig. 4.24a, compare bars 2 and 6) it completely abrogates the strong transcriptional activity that is mediated by ectopic expression of Smad3 (compare bars 4 and 6).

In PC12 cells, different combinations of Smad3 and either wild-type or mutant Smad4 were equivalently examined for their ability to induce transcription from the p3TP-luc(+) reporter in response to NGF.

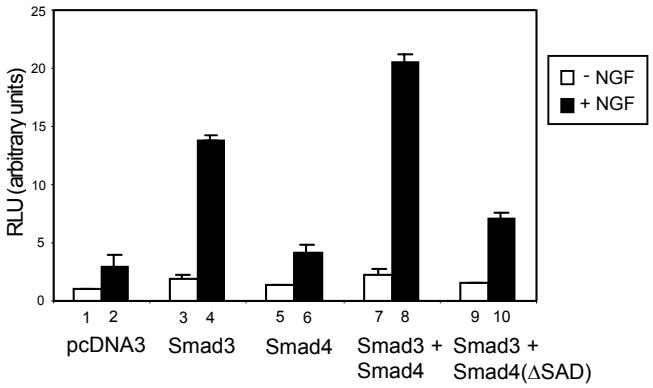


Fig. 4.24b Investigation of the Smad4 dependence of NGF-induced reporter activation
PC12 cells were transiently transfected with p3TP-luc(+), pRL-TK and the indicated Smad constructs. Total amounts of DNA were kept constant by addition of pcDNA3. Following starvation and cultivation in absence or in presence of 2nM NGF for 24h, cells were lysed and analyzed for luciferase activity.

Whereas cells transfected with Smad3 elicit a strong NGF induced reporter signal (Fig. 4.24b, bar 4), ectopic expression of wild-type Smad4 alone shows no effect on luciferase induction from the p3TP-luc(+) reporter (Fig. 4.24b, bar 6). Cotransfection of Smad3 and wild-type Smad4, however, results in an amplification of the signal obtained from cells transfected with Smad3 (compare bars 4 and 8). In contrast, coexpression of Smad3 and the functionally inactive Smad4 variant, Smad4(ΔSAD), largely prevents Smad3-mediated reporter gene activation (compare bars 8 and 10). These results were also confirmed by using the $p(CAGA)_{12}$-luc reporter (data not shown) and suggest that functional Smad4 is required to mediate NGF effects on Smad-dependent reporter constructs.

4.6.3 Effects of the inhibitory Smad7 on NGF-induced transcriptional activation

Besides the R-Smads and the common mediator Smad4, the inhibitory Smad protein, Smad7, plays an important role in TGF-β signal transduction in that it represents an immediate early gene of TGF-β that potently blocks the TGF-β signaling cascade, thereby providing an effective negative feedback mechanism [98, 99].

To address the question whether Smad7 is capable of blocking NGF-initiated Smad signaling, its capacity to prevent transcription was investigated in cells expressing either wild-type Smad7 or a truncated variant of Smad7, Smad7ΔC. Smad7ΔC (AA 1-407) lacks the very C-terminal 19 amino acids which were described to be essential for the interaction with TβRI and thus for preventing R-Smads from binding to and getting phosphorylated by TβRI [288]. Smad7 and Smad7ΔC constructs were obtained from P. ten Dijke (Amsterdam, The Netherlands).

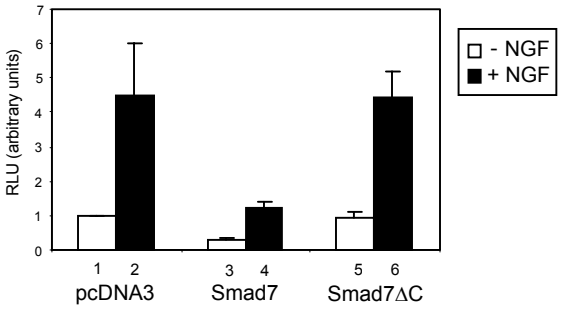


Fig. 4.25 Studies on the effect of Smad7 on NGF-induced Smad signaling
PC12 cells were transiently transfected with pSBE-luc, pRL-TK and the indicated Smad7 variants. Following starvation, cells were left untreated or were stimulated with 2nM NGF for 24h prior to cell lysis and luciferase measurement.

The experiment depicted in Fig. 4.25 demonstrates, that Smad7 is a potent inhibitor of NGF-mediated Smad signaling (Fig. 4.25, compare bars 2 and 4). Ectopic expression of Smad7 even reduces the ligand-independent signal in PC12 cells (bar 3). In contrast to Smad7, the truncated Smad7ΔC mutant does not reveal impairment of Smad signaling (bar 6), indicating that the very C-terminal amino acids are crucial for the inhibitory effect of Smad7.

4.7 DETERMINATION OF THE ROLE OF TRANSMEMBRANE RECEPTORS FOR SIGNAL TRANSDUCTION

In the TGF-β/Smad signaling cascade, signal transducers upstream of the Smad proteins are represented by the transmembrane receptors, TβRI and TβRII. Following TGF-β1 activation, TβRI triggers phosphorylation of R-Smads which serve as direct substrates for TβRI. Based on the observation that - unlike TGF-β1 - NGF does not trigger C-terminal phosphorylation of Smad proteins (Fig. 4.22) it is hypothesized that other transmembrane receptors are involved in mediating Smad activation.

The following experiments were performed to elucidate whether TGF-β and/or NGF receptors contribute to transduction of NGF-induced signals via the Smad pathway.

4.7.1 Participation of the TrkA receptor

To assess whether the NGF receptors, TrkA and $p75^{NTR}$, play a key role, their function in NGF induced Smad activation was investigated. As $p75^{NTR}$ does not contain any known signaling entity in its intracellular part [237], efforts were focused on examination of the TrkA receptor which belongs to the family of tyrosine kinase receptors [224, 278].

PC12 cells were transfected with constructs coding for either wild-type TrkA, kinase-deficient TrkA or wild-type TrkA together with Smad7. Reporter activation was determined from the pSBE-luc reporter in response to NGF stimulation. The kinase-deficient variant of TrkA (TrkA-KA) was kindly provided by M. Chao (New York, USA) and carries a mutation (K538A) resulting in inactivation of the tyrosine kinase activity.

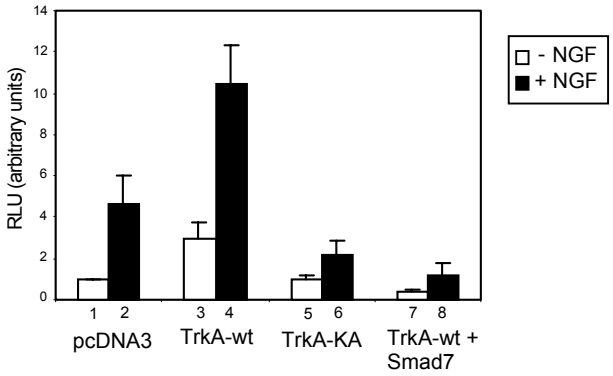


Fig. 4.26 Evaluation of the role of functional TrkA receptors in NGF-induced Smad activation
Constructs for pSBE-luc, pRL-TK-luc as well as wild-type or kinase-deficient TrkA, respectively, were transfected into PC12 cells. TrkA-wt was transfected either alone or together with Smad7. Following starvation, cells were stimulated with 2nM NGF for 24h or were left untreated. Reporter activity from three independent experiments was determined by luminescence measurement.

Even though cells transfected with the wild-type TrkA receptor show an elevated level of luciferase activity already in the absence of ligand, the signal can be potently enhanced by stimulation with NGF (Fig. 4.26, bar 4). However, transfection of the inactive TrkA variant (TrkA-KA) causes a significant reduction of responsiveness (Fig. 4.26, bar 6). An even stronger inhibitory effect can be observed after cotransfection of the wild-type TrkA receptor together with Smad7 (Fig. 4.26, bar 8). These results suggest functional TrkA receptors to be necessary for NGF-mediated activation of Smad dependent reporter genes and demonstrate the inhibitory role of Smad7 on this NGF-mediated effect.

4.7.2 Investigation of the role of the TGF-β receptors in NGF-mediated signaling

To assess the importance of functional TGF-β receptors for NGF effects on Smad-dependent reporter genes, various TGF-β receptor constructs and their respective functionally inactive mutants were investigated.

The TβRI-KR mutant contains a point mutation which causes an exchange of lysine 232 to arginine (K232R). As the lysine at this position is critical for the kinase activity of the receptor, the amino acid substitution results in kinase deficiency of the receptor [29, 289].

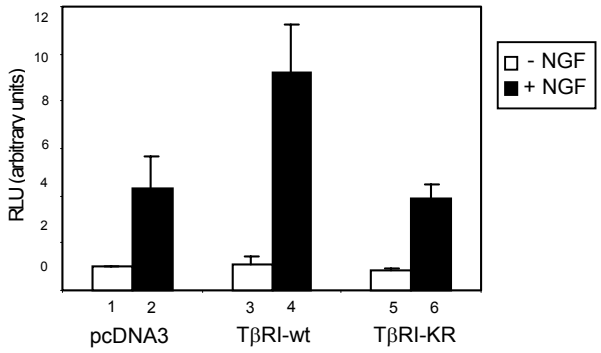


Fig. 4.27 Effects of TβRI variants on NGF-induced signals
PC12 cells were transiently transfected with pSBE-luc, pRL-TK-luc and the indicated TβRI variants. Prior to cell lysis, cells were starved and were either left untreated or were stimulated with 2nM NGF for 24h. Cell lysates were prepared and used for luciferase measurement.

The experiment demonstrates that ectopic expression of wild-type TβRI is able to augment the NGF-triggered reporter signal (Fig. 4.27, compare bars 2 and 4). In contrast, the kinase deficient TβRI-KR does not provoke changes in transcriptional activity compared to the signal obtained from mock transfected cells (Fig. 4.27, compare bars 2 and 6).

Concerning the type II receptors, the consequences of expressing either wild-type or two different functionally inactive TβRII receptors (TβRII-Δcyt and TβRII-KR) was investigated in PC12 cells in response to NGF. TβRII-Δcyt lacks the whole cytoplasmic domain except of the most membrane proximal 9 amino acids (see chapter 4.2.1.3). TβRII-KR contains a point mutation that substitutes the critical lysine of the active center by an arginine (K277R). Both mutated receptors when overexpressed in TGF-β responsive cells potently inhibit TGF-β-induced responses [29, 284].

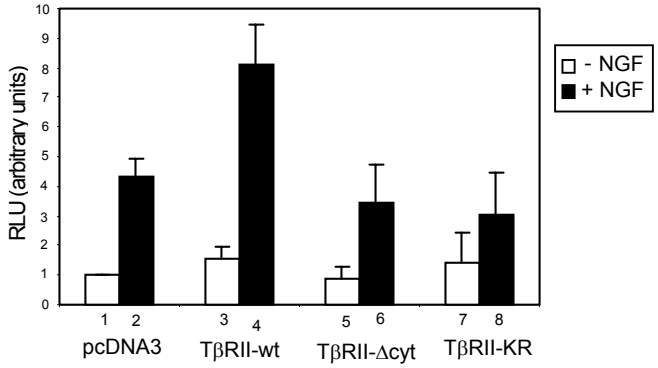


Fig. 4.28 Effects of several TβRII variants on NGF-induced signals
PC12 cells were transfected with pSBE-luc, pRL-TK and either wild-type TβRII or two different mutants (TβRII-Δcyt and TβRII-KR) that are both functionally inactive. Starvation of the cells was followed by NGF stimulation for 24h and cell lysis. Luciferase activity was recorded and error bars are calculated from three independent experiments.

Whereas expression of the wild-type TβRII increased the transcriptional activity induced by NGF (Fig. 4.28, compare bar 2 and bar 4), none of the kinase deficient receptor mutants is capable to produce a signal that exceeds the level of mock transfected cells (Fig. 4.28, bars 6 and 8). However, the TβRII mutants do not show either a significant dominant-negative effect in presence of NGF, suggesting that the TGF-β type II receptor plays a supportive but not a crucial role in NGF-mediated activation of Smad-dependent reporter genes.

4.8 RECEPTOR INTERACTION STUDIES BETWEEN TGF-β AND TrkA RECEPTORS IN COS CELLS

From the previous experiments it can be proposed that TβRII, when overexpressed in PC12 cells, can support NGF-induced Smad activation (Fig. 4.28). To elucidate the underlying mechanism, a potential direct physical interaction of TβRII and TrkA was examined.
Interaction studies were performed either in 293T cells or in COS cells, both of which allow strong overexpression of the respective receptors.

4.8.1 Epitope tagging of the TrkA receptor

For coimmunoprecipitation studies it was necessary to supply the TrkA receptor construct with an epitope tag to achieve efficient immunoprecipitation of the receptor or detection by Western blot, respectively.
Therefor, the sequence encoding the TrkA receptor was first excised from the pCMV5 vector by restriction with *EcoR*I and subsequently subcloned into the pcDNA3 vector backbone which was linearized by digestion with *EcoR*I and dephosphorylated prior to ligation. The resulting pcDNA3/TrkA construct was used as template for PCR mutagenesis which was performed to introduce a Flag epitope downstream of the signal peptide according to the scheme below (Fig. 4.29a)

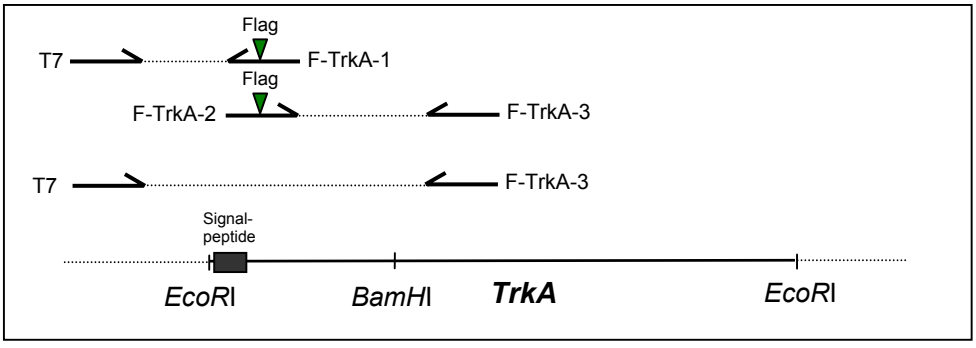


Fig. 4.29a Schematic illustration of PCR mutagenesis performed to generate F-TrkA

The obtained PCR fragment was digested with *EcoR*I and *BamH*I. In parallel, the pcDNA3/TrkA construct was also digested with *EcoR*I and *BamH*I to obtain the vector backbone and the 3'-part of the TrkA receptor. For ligation, the dephosphorylated pcDNA3

vector backbone was incubated with the epitope containing PCR-fragment as well as with the fragment that encodes the 3'-part of the TrkA receptor and ligation was achieved by addition of T4-DNA ligase (see chapter 3.2.13). The resulting construct was verified by sequencing. The composition of the newly generated vector containing the flag-tagged TrkA receptor is shown in Fig. 4.29b.

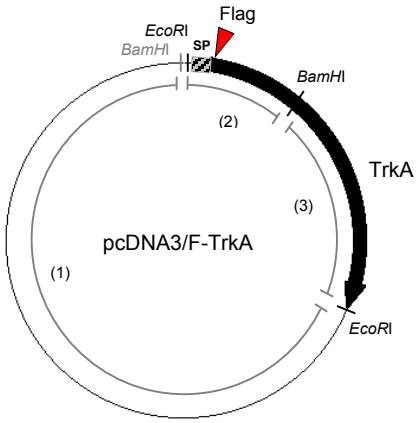


Fig. 4.29b Schematic illustration of the joined components of pcDNA3/F-TrkA
The pcDNA3 vector backbone (1) is ligated with the 5'-terminal fragment of the TrkA receptor which contains a Flag-epitope that was introduced by PCR-mutagenesis right after the signal peptide (SP) (2) and with the 3'-terminal fragment of TrkA that was gained by restriction from the original construct (3).

4.8.2 Verification of direct physical interaction between TβRII and TrkA

4.8.2.1 Coimmunoprecipitation studies with TβRII and TrkA

Direct physical interaction of two proteins can be detected by performing an immunoprecipitation against one protein and subsequent detection of the other protein by immunoblotting. In this way, a signal for the second protein will only appear if both proteins were associated and thus immunoprecipitated as a complex.

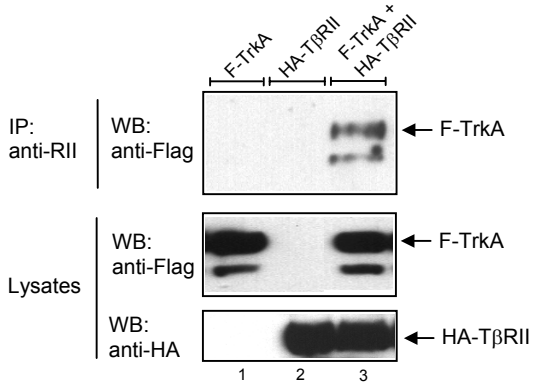


Fig. 4.30 Coimmunoprecipitation of TrkA and TβRII receptors in 293T cells
293T cells were transiently transfected (see chapter 3.3.6.1) with either F-TrkA (lane 1), HA-TβRII (lane 2) or both receptor constructs (lane 3). 48h post transfection, lysates were prepared in lysis buffer containing 0.5% TritonX-100 (see chapter 3.4.1). Immunoprecipitation was carried out using anti-RII. Following separation by SDS-PAGE and immunoblotting, TrkA-associated proteins were detected by an anti-Flag antibody (upper panel). To control the expression level, aliquots of total cell lysates were immunoblotted with the appropriate antibodies (middle and lower panel).

Fig. 4.30 shows that the receptors are expressed in comparable amounts (Fig. 4.30, middle and lower panel) and that only if both receptors are coexpressed, they give rise to a signal following sequential immunoprecipitation and Western blotting (Fig. 4.30, upper panel). Thus, the coimmunoprecipitation reveals that TrkA and TβRII are indeed capable to directly interact in a ligand-independent manner.

4.8.2.2 Binding and cross-linking studies with [^{125}I]-NGF

Further evidence for direct association of TrkA with TβRII but not TβRI is presented by a binding and cross-linking experiment with [^{125}I]-NGF (see chapter 3.4.9.2) shown in Fig. 4.31. COS cells were transfected with either TrkA alone or in combination with TβRI and TβRII. Iodinated NGF was bound and chemically cross-linked to the receptors which were subsequently immunoprecipitated using appropriate antibodies.

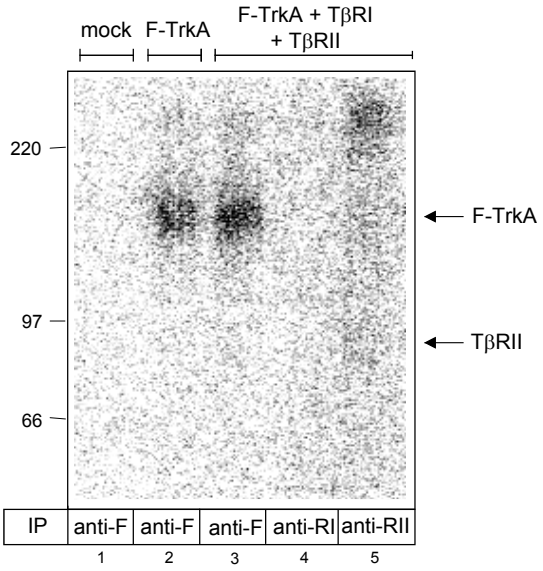


Fig. 4.31 Binding and cross-linking of [^{125}I]-NGF to transmembrane receptors in COS cells
COS cells were transfected with either empty vector (lane 1), F-TrkA (lane 2) or F-TrkA together with TβRI and TβRII (lanes 3-5). 48h post transfection, cells were subjected to binding and cross-linking with 1nM [^{125}I]-NGF. Different antibodies were used to immunoprecipitate the receptors: anti-Flag (anti-F) for F-TrkA (lane 1-3), anti-RI for TβRI (lane 4) and anti-RII to precipitate TβRII (lane 5). Following SDS-PAGE, receptors that were cross-linked to [^{125}I]-NGF were visualized by exposure to a phosphor imager screen.

Precipitation of the TrkA receptor shows that [^{125}I]-NGF strongly binds to TrkA in absence or in presence of TGF-β receptors (Fig. 4.31, lanes 2 and 3). Whereas immunoprecipitation using antiserum against TβRI does not evoke any signal (Fig. 4.31, lane 4), precipitation of TβRII by specific antibodies (anti-RII) gives rise to weak but detectable signals that represent [^{125}I]-NGF-bound TrkA and [^{125}I]-NGF-bound TβRII (Fig. 4.31, lane 5). The strong band at the top of lane 5 reflects high molecular weight complexes of ligand-bound and cross-linked receptors.

4.8.2.3 Competition assay with [^{125}I]-TGF-β1 and cold NGF

The purpose of an additional experiment was to investigate whether NGF can interfere with binding of [^{125}I]-TGF-β1 to TβRII and TβRI in a competitive binding and cross-linking assay. COS7 cells were transfected with the indicated receptors prior to performing binding and cross-linking as described in 3.4.9.1. Deviating from the basic protocol, the binding buffer contained - in addition to the radiolabeled TGF-β1 - the indicated amounts of cold, *i.e.* unlabeled NGF or TGF-β1.

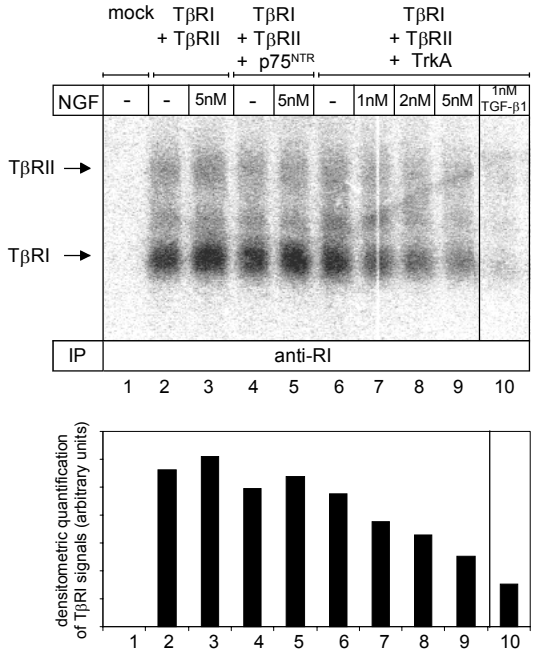


Fig. 4.32 Competition of NGF with [^{125}I]-TGF-β1 for binding to TGF-β receptors
COS7 cells were transfected with the indicated receptor constructs. Binding of 500pM [^{125}I]-TGF-β1 was carried out as described in 3.4.9.1. At the same time, cold NGF (lanes 3, 5, 7, 8 and 9) or cold TGF-β1 as a positive control (lane 10) were added in the amounts indicated above each lane. After cross-linking, receptors were immunoprecipitated from cell lysates with an antibody detecting TβRI (anti-RI) followed by SDS-PAGE and autoradiography. The histogram below shows the quantification of the signal for TβRI.

Fig. 4.32 shows that with increasing amounts of unlabeled NGF, binding of [^{125}I]-TGF-β1 (500pM) to its receptors gets impaired. This is only observed when TβRI and TβRII are coexpressed with the high affinity NGF receptor, TrkA (Fig. 4.32, lanes 6-9). However, transfection of only the TGF-β receptors (TβRI and TβRII) or TGF-β receptors in combination with the low affinity NGF receptor, p75^{NTR}, causes no NGF-mediated decrease in TGF-β1 binding to its receptors (lanes 2-5). As control, an excess of unlabeled TGF-β1 was applied, resulting in an almost complete displacement of the iodinated ligand from the receptor binding sites (lane 10).

4.9 INVESTIGATION OF OTHER PATHWAYS AND MOLECULES THAT ARE POTENTIALLY INVOLVED

In the light of the complex network of signals that mutually regulate each other, it is likely that molecules from other pathways play a crucial role in mediating NGF-induced Smad activation in PC12 cells.

Candidates are the proteins of the MAP kinase cascade signaling through the extracellular signal-regulated kinase-1 and -2 (Erk-1 and -2). This pathway is the most prominent one that originates from activated TrkA receptors (Fig. 4. 33, panel A) [216, 245] and was already described to crosstalk with the TGF-β/Smad pathway [132, 133, 198].

Moreover, TGF-β activated kinase (TAK1) represents an interesting candidate because on the one hand, TAK1 is suggested to play a role in TGF-β-induced gene expression [106, 107, 127] and on the other hand, TAK1 was shown to be involved in mediating BMP-2-induced neurite outgrowth of PC12 cells [187, 290]. Additionally, this BMP-2-induced neurite outgrowth could be inhibited by Smad6 and Smad7, both of which are able to directly interact with TAB1 (Fig. 4.33, panel B).

The MAP kinase pathway and the cascades downstream of TAB1/TAK1 are schematically depicted in Fig. 4.33.

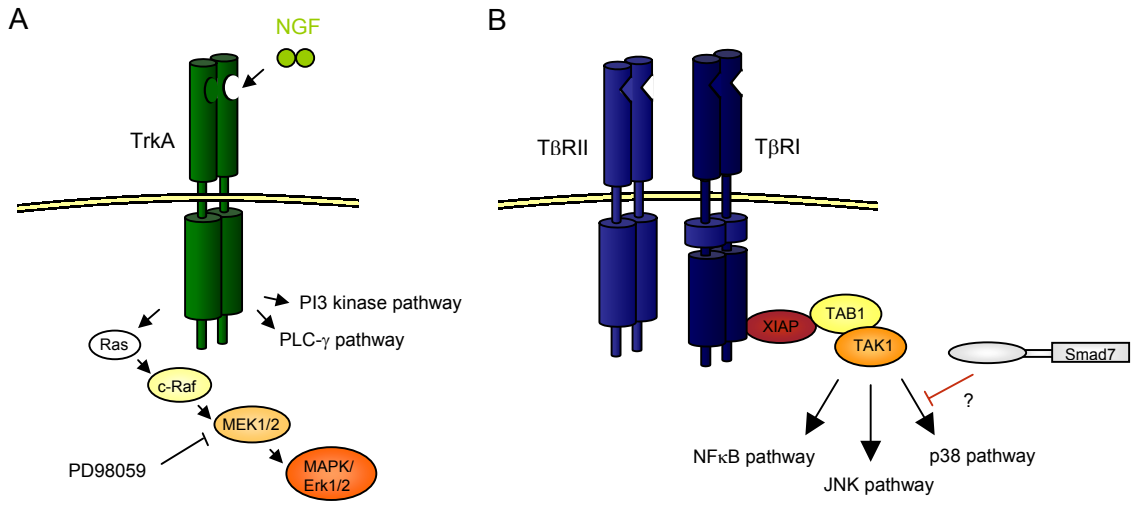


Fig. 4.33 Pathways that are potentially involved in NGF-mediated Smad activation
Schematic illustration of (A) the pathways downstream of the activated TrkA receptor and (B) the cascades that are activated by TAK1. TAK1 becomes activated by the TAK1 binding protein (TAB1) which itself interacts with the X-linked inhibitor of apoptosis (XIAP) protein that links the whole complex to the TGF-β receptors. Direct interaction of TAB1 and Smad7, might lead to abrogation of the p38 MAP kinase pathway (this was so far only shown for BMP-2-induced signals [187] and is therefore indicated with a question-mark).

4.9.1 Contribution of the MAPK/Erk pathway in NGF-mediated Smad activation

To elucidate the role of the aforementioned pathways, the MAP kinase pathway was first blocked by adding the MEK1/2-inhibitor, PD98059. The effects of abrogation of the MAP kinase pathway were investigated by using the pSBE-luc reporter as a readout (Fig. 4.34, panel A). In order to control the functionality of the MEK1/2-inhibitor, PC12 cells were transfected in parallel with a Gal4-luc construct and a second construct that contains a Gal4 binding domain upstream of an Elk-1 promoter (pGal4-BD-Elk-1) which specifically responds to Erk/MAP kinase signaling (Fig. 4.34, panel B). The Gal4-luc and the pGal4-BD-Elk-1 constructs were provided by K. Krieglstein (Homburg) and were previously published [291, 292].

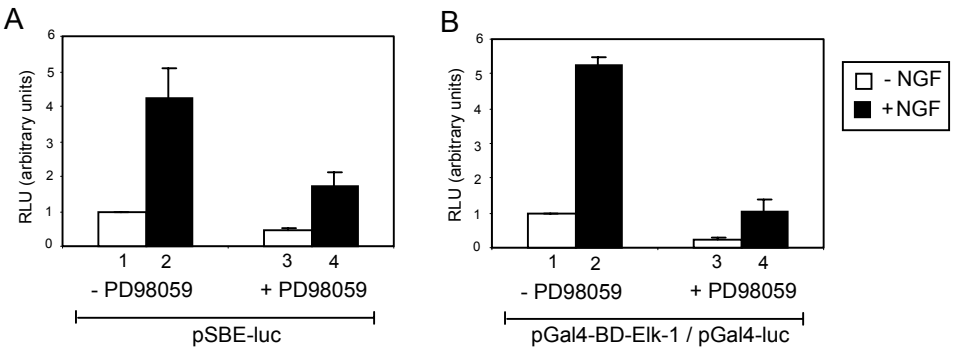


Fig. 4.34 Studies on the contribution of the MAPK/Erk-pathway
PC12 cells were transfected with pRL-TK and either pSBE-luc (panel A) or pGal4-BD-Elk1 and pGal4-luc (panel B). Following starvation, cells were treated with 50µM PD98059 1h prior to stimulation with control medium or medium containing 2nM NGF. Luciferase activity was determined and error bars were calculated from two independent experiments.

In PC12 cells that were not treated with the MEK1/2 inhibitor, NGF stimulation results in a significant activation of the Smad-dependent pSBE-luc reporter (Fig. 4.34, panel A, bar 2). However, if NGF is applied on cells that were pretreated with the MEK1/2 inhibitor PD98059, reporter activation is markedly reduced compared to the results obtained in absence of PD98059 (bar 4), proposing a contribution of the MAPK/Erk pathway. Interestingly, also the ligand-independent signal is decreased by the inhibitor (bar 3).

The potential of the MEK1/2 inhibitor PD98059 to effectively block the MAP kinase cascade was verified in a complementary experiment shown in Fig. 4.34, panel B. The 5-fold induction of the Gal4-Elk-1 reporter construct in response to NGF stimulation (Fig. 4.34, panel B, bar 2) is completely prevented by the addition of PD98059 (bar 4). Again, as observed in the previous experiment (panel A), the ligand-independent signal is also decreased by the inhibitor (bar 3).

4.9.2 Contribution of the TAK1-p38 kinase pathway in NGF-mediated Smad activation

The TAK1-p38 pathway is initiated by TAK1 activation which is mediated by TAB1 and leads to sequential activation of the MAP kinase kinase (MKK6), p38 kinase and finally transcription factors such as the activating transcription factor-2 (ATF-2) [107, 127].

To investigate a potential participation of the TAK1-p38 kinase pathway in mediating NGF-initiated Smad activation, dominant-negative mutants of the signal transducers TAK1 (DN-TAK1) and MKK6 (DN-MKK6) were analyzed. The constructs for DN-TAK1 and DN-MKK6 were kindly provided by A. Hoffmann (Braunschweig) and S. Ludwig (Würzburg), respectively.

In the first experiment, PC12 cells were transfected with dominant-negative MKK6 - either alone or together with the TrkA receptor construct. The effect of the mutant was then determined using the pSBE-luc reporter.

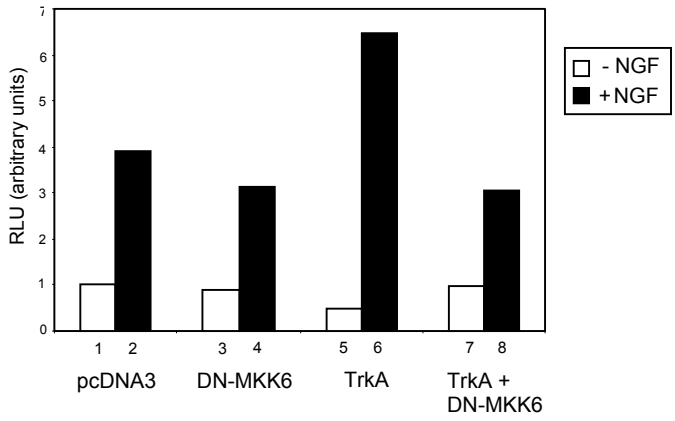


Fig. 4.35 Effects of dominant-negative MKK6 in NGF-induced pSBE-luc reporter activation
PC12 cells were transiently transfected with pSBE-luc, pRL-TK and the indicated constructs. Total amounts of DNA were kept constant by addition of pcDNA3. Cells were starved and subsequently stimulated with 2nM NGF for 24h. Luciferase activity was determined and data are represented from a single experiment.

Whereas the dominant-negative MKK6 variant does not show strong inhibition if expressed alone (Fig. 4.35, bar 4) it is capable to completely abrogate the signal induced by TrkA overexpression (compare bars 6 and 8) meaning that MKK6 seems to act downstream of the TrkA receptor.

In the next experiment, the effects of XIAP and of a dominant-negative mutant of TAK1, both of which are molecules that act upstream of MKK6, were investigated. As depicted in Fig. 4.33, XIAP represents both, a TAB1 binding protein [109] and a protein that can bind to TβRI and several other members of the type I class of the TGF-β receptor superfamily [110]. Thus, XIAP can link the TAB1-TAK1 complex to the type I receptor.

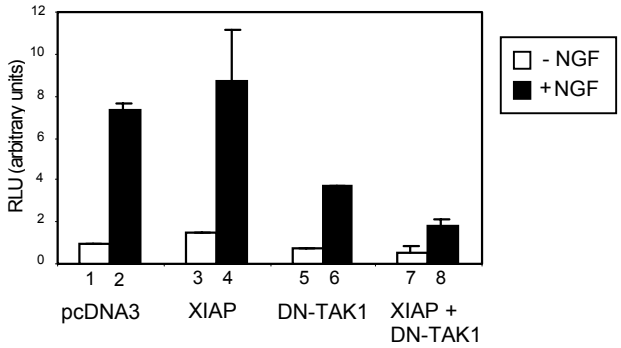


Fig. 4.36 Effects of XIAP and dominant-negative TAK1 in NGF-induced reporter activation
PC12 cells were transiently transfected with p3TP-luc(+), pRL-TK and the indicated constructs. Total amounts of DNA were kept constant by addition of pcDNA3. Following starvation, cells were cultured in medium supplemented with or without 2nM NGF for 24h. Luciferase activity was measured and error bars are calculated from two independent experiments.

Fig. 4.36 shows that overexpression of XIAP alone does not lead to a significant alteration of NGF-induced reporter activation (Fig. 4.36, bar 4). However, introduction of dominant-negative TAK1 leads to a 50-75% reduction of transcriptional activity compared to mock transfected cells (bars 6 and 8).

Finally, the intention was to elucidate whether the dominant-negative variants of TAK1 and MKK6 are also able to interfere with the strong NGF-induced reporter activation that results from Smad3 overexpression.

For that purpose, PC12 cells were transfected either with Smad3 alone or with Smad3 in combination with DN-TAK1 or DN-MKK6 and transcriptional activation of the $p(CAGA)_{12}$-luc reporter was determined in absence or in presence of NGF.

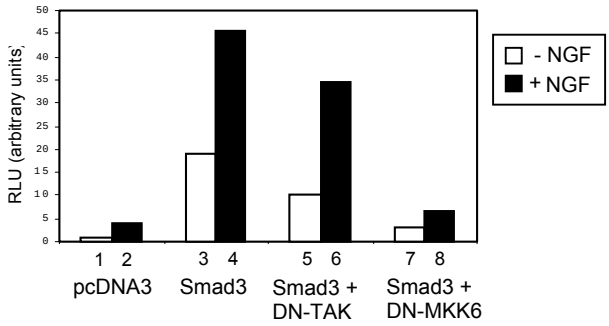


Fig. 4.37 Effects of dominant-negative mutants of TAK1 and MKK6 in NGF-induced reporter activation
PC12 cells were transiently transfected with $p(CAGA)_{12}$-luc, pRL-TK and the indicated constructs. Total amounts of DNA were kept constant by addition of pcDNA3. Following starvation, cells were either left untreated or were stimulated with 2nM NGF. Luciferase activity was measured and data represent the results from a single experiment.

The experiment revealed that both dominant-negative molecules can reduce the signal evoked by Smad3. Whereas, DN-TAK diminishes the Smad3-induced reporter signal only to a limited extent (Fig. 4.37, compare bars 4 and 6), DN-MKK6 completely abrogates the Smad3-triggered luciferase activity such that the luciferase activity even reaches the level of

mock transfected cells. These results strongly suggest a role of MKK6 and probably TAK1 in NGF-induced reporter activation.

4.10 INVESTIGATION OF OTHER CELL LINES

Given that the effects of NGF on the Smad pathway were so far exclusively investigated in PC12 cells, efforts were made to find other cell lines that also initiate transcription from Smad dependent reporter genes in response to NGF.

In the light of the facts that (i) PC12 cells represent a model system for neuronal differentiation and that (ii) NGF acts as a neurotrophin in the nervous system, cells of neuronal origin might show a comparable behavior following NGF treatment.

Moreover, potential clinical relevance is based on the observation that both growth factors, TGF-β1 as well as NGF, are described to play a dual role in tumorigenesis. In normal cells of many different tissues, TGF-β1 is known to act as a tumor suppressor [17, 24]. However, once cells have escaped from TGF-β1-induced antiproliferative effects during tumor development, TGF-β1 starts to elicit its oncogenic activities, resulting in promotion of tumorigenesis and metastasis [23, 25]. Similarly, it is attributed to NGF that it has a crucial function in the initiation and progression of breast tumors. Whereas NGF does not alter proliferation in normal breast epithelial cells, it acts as a mitogenic factor for breast cancer cells [293, 294]. Thus, the search for other cell lines was focused on neuronal cell lines on the one hand and breast cancer cell lines on the other hand.

4.10.1 Receptor repertoire of different cell lines

The first step in identifying an adequate cell line was to characterize the cells in terms of their available receptor repertoire. To this end, the neuroblastoma cell line, Lan-1, as well as two breast cancer cell lines, MCF-7 and MDA-MB 468, were used for RT-PCR analysis to verify the presence of TGF-β and NGF receptors, respectively.

Total RNA was isolated and reverse transcribed as described under 3.2.8. The subsequent RT-PCR reaction (see chapter 3.2.9) was carried out using primer pairs specific for the respective receptor.

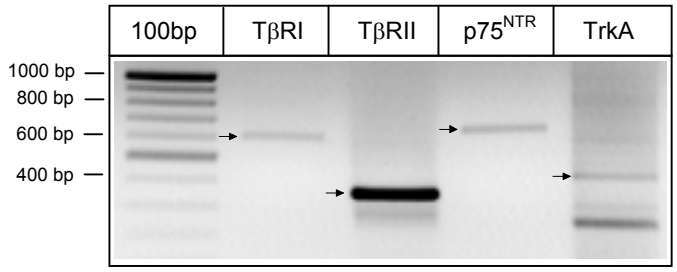


Fig. 4.38a Receptor repertoire of Lan-1 neuroblastoma cells verified by RT-PCR
Total RNA was isolated from Lan-1 cells, reverse transcribed to generate cDNA by random priming and finally, cDNA was amplified by PCR using primers specific for the respective receptor. For TβRI: TR1-4, TR1-9; for TβRII: P9, P11; for $p75^{NTR}$: p75-1, p75-2; for TrkA: F-TrkA2, F-TrkA3) (sequences of the oligonucleotides are listed in the appendix, A.2). PCR-fragments were verified by sequencing.

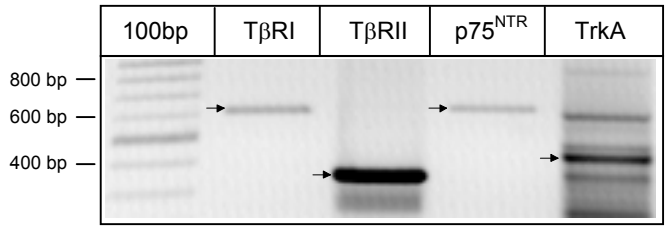


Fig. 4.38b Receptor repertoire of MCF-7 breast cancer cells verified by RT-PCR
Total RNA was isolated from MCF-7 cells and reverse transcription and RT-PCR were performed as described in Fig. 4.38a.

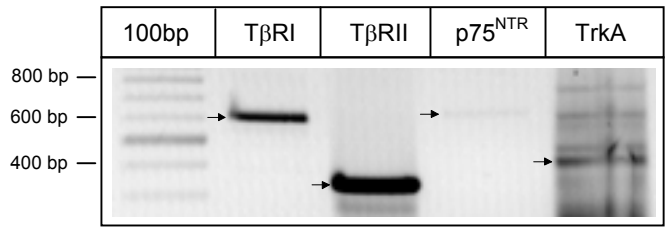


Fig. 4.38c Receptor repertoire of MDA-MB 468 breast cancer cells verified by RT-PCR
Total RNA was isolated from MDA-MB 468 cells and reverse transcription and RT-PCR were performed as described in Fig. 4.38a.

Examination of the different cell lines (Lan-1, MCF-7 and MDA-MB 468) revealed that all of them contain the mRNA for TGF-β as well as NGF receptors (Fig. 4.38a-c). In contrast to PC12 cells, however, the investigated cell lines seem to express higher levels of TβRII because in PC12 cells, two rounds of PCR amplification were necessary to yield detectable amounts of the resulting PCR-fragment whereas in these cell lines already the first PCR amplification produced high amounts of the fragment.

4.10.2 Analysis of NGF-induced Smad-dependent activation of transcription in other cell lines

As the investigated cell lines were shown to express TGF-β and NGF receptors, they were further tested for their capacity to induce transcription from Smad-dependent reporter genes. To this end, cells were transfected with either p3TP-luc(+) or $p(CAGA)_{12}$-luc as reporter constructs and transcriptional activation was determined in response to stimulation with NGF or TGF-β1, respectively.

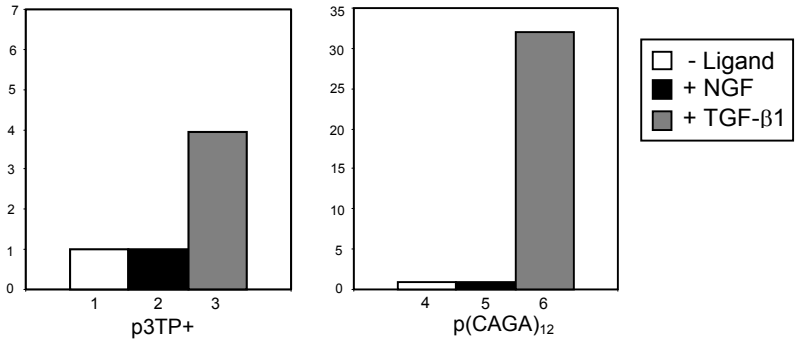


Fig. 4.39a Transcriptional activation of Smad-dependent reporter genes in Lan-1 cells
Lan-1 cells were transfected with pRL-TK and either p3TP-luc(+) or $p(CAGA)_{12}$-luc. Following starvation, cells were treated with 2nM NGF or 200pM TGF-β1 and luciferase activity was measured after 24h of ligand stimulation.

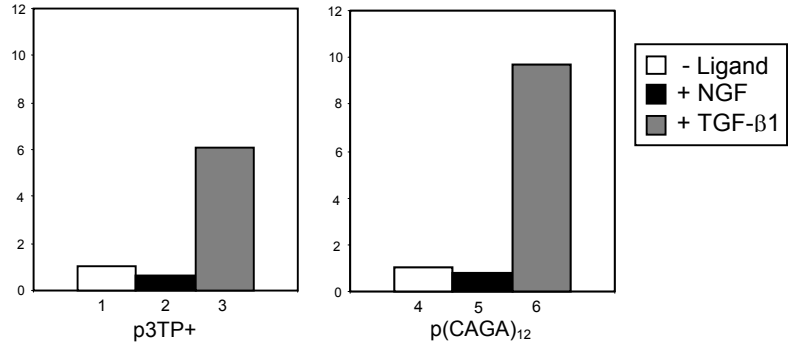


Fig. 4.39b Transcriptional activation of Smad-dependent reporter genes in MCF-7 cells
MCF-7 cells were transfected with pRL-TK and either p3TP-luc(+) or $p(CAGA)_{12}$-luc. Following starvation, cells were treated with 2nM NGF or 200pM TGF-β1 and luciferase activity was measured after 24h of ligand stimulation.

Lan-1 cells as well as MCF-7 cells show transcriptional activation in response to TGF-β1 on both reporter genes that were tested. In contrast, none of the cell lines was sensitive to NGF stimulation (Fig. 4.39a and 4.39b).

A property of the MDA-MB 468 cell line is that it lacks Smad4 expression. For this reason, the following luciferase assays were performed in absence or in presence of ectopic expression of Smad4.

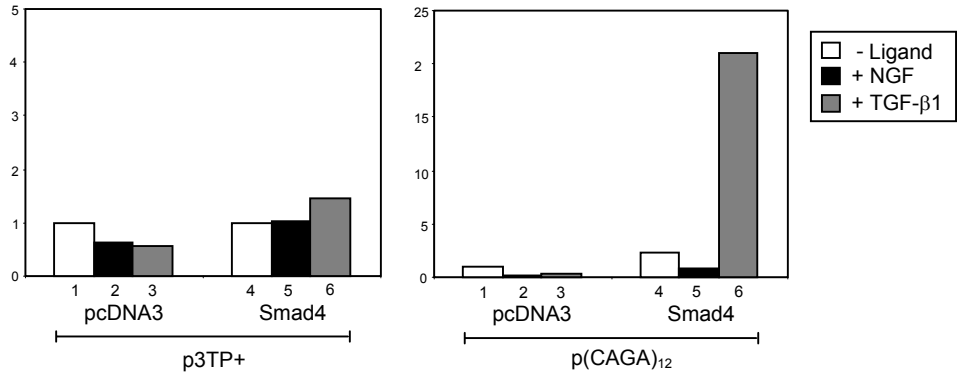


Fig. 4.39c Transcriptional activation of Smad-dependent reporter genes in MDA-MB 468 cells
MDA-MB 468 cells were transfected with, p3TP-luc(+) or $p(CAGA)_{12}$-luc, respectively, pRL-TK and either empty vector or a Smad4 construct. Following starvation, cells were treated with 2nM NGF or 200pM TGF-β1 and luciferase activity was measured after 24h of ligand stimulation.

Experiments using the p3TP-luc(+) reporter do not show significant changes in transcriptional activity (Fig. 4.39c, left panel). However, TGF-β1 evokes a slightly higher signal in Smad4 expressing cells than in mock transfected cells (compare lanes 3 and 6). On the other hand, results obtained from the $p(CAGA)_{12}$-luc reporter demonstrate a clear effect on reporter activation (Fig. 4.39c, right panel). TGF-β1 does not induce transcription in mock transfected cells (bar 3) but ectopic expression of Smad4 restores TGF-β1 responsiveness of MDA-MB 468 cells (bar 6). NGF, however, is not able to induce the Smad-dependent reporter at all – neither in absence nor in presence of Smad4 (bars 2 and 5).

Collectively, these data reveal that the neuroblastoma cell line Lan-1 as well as the breast cancer cell lines MCF-7 and MDA-MB 468 (in presence of Smad4) are all responsive to TGF-β1 but none of them responds to NGF if tested on different Smad-dependent reporter constructs. This is in clear contrast to parental PC12 cells which show opposite results, namely resistance to TGF-β1 but sensitivity to NGF. Consequently, none of the tested cell lines is an adequate cell system to compare and verify the processes observed in PC12 cells.

5 Discussion

Binding of growth factors or cytokines to cell surface receptors initiates signal transduction pathways that finally provoke defined biological responses. However, these specific responses are not caused by a single signaling cascade but rather reflect the result of complex interactions between several pathways that mutually regulate each other. Furthermore, based on the fact that each cell type shows a distinct assortment and expression pattern of signaling molecules, the interplay of signal transducers is often highly cell- and tissue- specific.

Starting-point of the presented investigations on the crosstalk between NGF and the TGF-β/Smad pathway was the observation that NGF treatment results in activation of Smad-dependent reporter genes in PC12 cells.

5.1 PC12 CELLS AS CELL SYSTEM TO INVESTIGATE THE CROSSTALK BETWEEN NGF AND THE TGF-β/SMAD PATHWAY

PC12 cells are known to express both types of NGF receptors, the high-affinity receptor TrkA and the low-affinity receptor $p75^{NTR}$ [277, 278]. Referring to the expression of TGF-β receptors there are inconsistent reports. Kimchi and coworkers could not detect any TGF-β receptors [279] whereas others postulate that PC12 cells express moderate levels of endogenous TβRI [51]. Characterization of PC12 cells in respect of their receptor repertoire confirmed the expression of NGF receptors and revealed that they endogenously express TβRI as well as TβRII (Fig.4.3 and Fig. 4.2). Additionally, binding and cross-linking studies showed that the latter receptors are both expressed at the cell surface (Fig. 4.4). However, detection of TβRII was difficult to achieve by RT-PCR, suggesting that TβRII is expressed only in low amounts in PC12 cells. To elucidate whether PC12 cells respond to TGF-β1 stimulation in biological assays, they were tested for TGF-β1-induced Smad2 phosphorylation by Western blot and for TGF-β1-mediated transcriptional activation by reporter gene assays using a Smad-dependent reporter construct. Phosphorylation analysis demonstrated that TGF-β1 can trigger C-terminal Smad2 phosphorylation only in cells that ectopically express TβRII but not in parental PC12 cells (Fig. 4.5), supporting the hypothesis that TβRII is present in limiting amounts in PC12 cells. These results were further confirmed by a reporter gene assay which showed that expression of TβRI is not sufficient to provoke TGF-β1-induced transcriptional activation but expression of TβRII renders the cells sensitive to TGF-β1 (Fig. 4.6). These data propose that the low amounts of TβRII represent the limiting factor for proper TGF-β1 signaling in PC12 cells.

Considering that PC12 cells were established from a rat pheochromocytoma, low amounts of TβRII are probably a consequence of tumor progression which is frequently accompanied by downregulation or mutation of TGF-β receptors [17, 281, 295].

5.2 GENERATION AND CHARACTERIZATION OF STABLE PC12 CELLS

The purpose of generating stable PC12 cells was first to obtain a high percentage of transduced cells and second to allow ectopic expression of specific genes over a period of at least one week as it is necessary for the performance of neurite outgrowth assays.

Although VSV-G pseudotyped retroviruses show a very broad host range [252] and infection with this type of virus particles usually results in high infection efficiency, PC12 cells could be infected to only 5-10%. Even after sorting for GFP-positive cells (*i.e*. infected cells) the cell pool could not be enriched to a 100% infected population (Fig. 4.10b). This is likely to be due to the fact that PC12 cells tend to aggregate to cell clumps. However, the infection efficiency after sorting (~ 60%) was high enough to yield strong expression of the genes of interest as determined by Western blot analysis (Fig. 4.11a-c).

FACS-analysis (Fig. 4.10c) and Western blotting (Fig. 4.11a) that were performed with the stable PC12 cells demonstrated that expression of the heterologous proteins was induced only in presence of doxycycline, confirming that the inducible promoter does not show leakiness.

From binding and cross-linking experiments (Fig. 4.12) it can be concluded that the TGF-β receptors are properly transported to the cell surface and can bind the ligand, TGF-β1.

5.3 THE ROLE OF TGF-β SIGNALING IN NGF-INDUCED NEURITE OUTGROWTH OF PC12 CELLS

PC12 cells are derived from the rat adrenal gland. Despite their non-neuronal origin, PC12 cells respond to NGF stimulation with initiation of a differentiation program that includes formation of neurite-like elongations, thus changing their morphology towards a neuronal phenotype (Fig. 4.1) [260].

As members of the TGF-β superfamily were previously described to support the neurotrophic effect of neurotrophins [212, 215, 251, 296], it was investigated whether TGF-β signaling is a prerequisite for NGF induced neurite outgrowth of PC12 cells. Abrogation of TGF-β signal transduction was thereby achieved by inducible expression of a truncated TGF-β type II receptor (TβRII-Δcyt) or the inhibitory Smad7 protein in stable cell pools (Fig. 4.13a). Additionally, TGF-β effects were blocked by neutralizing TGF-β in the medium using TGF-β antibodies (Fig. 4.13b). In all cases, inhibition of TGF-β signaling did not prevent neurite formation, indicating that the TGF-β/Smad pathway is dispensable for neuronal

differentiation. However, overexpression of the wild-type type II receptor resulted in slightly enhanced neurite outgrowth (Fig. 4.13a, compare panel A and C). These results propose that the Smad pathway which can be activated by NGF plays a supportive but not a crucial role in neurite formation of PC12 cells and is thus mainly important for other, as yet unidentified, cellular responses.

Interestingly, it was recently published that the inhibitory Smad proteins, Smad6 and Smad7, can impair BMP-2 induced TAK1-mediated neurite outgrowth in PC12 cells by a direct interaction between TAB1 and Smad6 or Smad7, respectively [187]. In this context, it is to note that, in contrast to BMP-2, TGF-β1 is not able to induce neurite outgrowth in absence of NGF. However, the inhibitory function of Smad7 on BMP-induced but not NGF induced neurite formation demonstrates, that NGF and BMP-2 make use of different pathways to provoke neurite outgrowth. This hypothesis is supported by the finding that NGF sequentially induces the MAPK/Erk-pathway and the p38-kinase cascade [297] whereas BMP-2 only activates the TAK1-p38-pathway [290]. Collectively, this demonstrates that although neurite formation of PC12 cells can be induced by NGF as well as BMP-2, each of the growth factors uses a distinct mechanism and none of them is dependent on Smad signaling.

5.4 THE MECHANISM OF NGF-MEDIATED ACTIVATION OF SMAD-DEPENDENT REPORTER GENES

Luciferase assays revealed that treatment of PC12 cells with NGF - but not with TGF-β1 - leads to activation of several distinct Smad-dependent reporter genes (Fig. 4.14), implying that NGF has the potential to trigger Smad activation.

Furthermore, it could be shown that simultaneous treatment with NGF and TGF-β1 did not lead to synergistic effects of both growth factors (Fig. 4.15), rather the additional stimulation with TGF-β1 shows a tendency to reduce the NGF triggered signal. Considering the complex network of signaling cascades in a cell, it is likely that downstream molecules that are activated by TGF-β elicit negative regulation of the NGF triggered pathway. In this context, it is notable that Smad proteins are not the only signal transducers that are activated by TGF-β1. A multitude of publications substantiates that TGF-β1 activates various MAP kinase pathways [107, 197, 298, 299].

The central question, however, was to elucidate the mechanism of NGF-initiated activation of Smad proteins. The results and conclusions from experiments that were performed in order to unravel the signaling events will be discussed in the following.

5.4.1 NGF effects are independent of TGF-β1

Previous reports propose that NGF can upregulate transcription of TGF-β1 in PC12 cells [285]. Consequently, the observed NGF-induced transcriptional responses could be due to secondary effects, namely NGF-triggered secretion of TGF-β and subsequent TGF-β signaling. However, as discussed above, limiting amounts of TβRII in PC12 cells account for the resistance towards stimulation with exogenous TGF-β1. Results from different experimental approaches confirmed that also endogenous TGF-β that acts in an autocrine manner is not capable to initiate transcriptional activation from Smad-dependent reporter genes. Neutralization of all isoforms of TGF-β by using TGF-β antibodies showed that NGF-mediated transcriptional activation is not impaired in absence of TGF-β ligand (Fig. 4.17). Similarly, capturing TGF-β by addition of the extracellular domain of TβRII-B (TβRII-B-ECD) which binds all three TGF-β isoforms [53] does not lead to a significant reduction of NGF-induced transcription from Smad-dependent reporter genes (Fig. 4.18, upper panel, compare bars 2 and 8). From the parallel experiment in which TGF-β- responsive L6 cells were stimulated with the supernatant of PC12 cells that were simultaneously treated with ligand and TβRII-B-ECD, it can be concluded that the TβRII-B-ECD effectively neutralizes TGF-β1 at a 1000-fold molar excess because it strongly impairs TGF-β mediated transcriptional activation (Fig. 4.18, lower panel, compare bars 3 and 9). Moreover, there is no activation of transcription detectable in the L6 cells that were stimulated with the supernatant of NGF-treated PC12 cells (Fig. 4.18, lower panel, bar 2). This confirms that the amount of active TGF-β that is secreted in response to NGF stimulation of PC12 cells, is not high enough to induce Smad-dependent reporter activation in TGF-β-responsive L6 cells.

Although evidence was provided that there is no significant production of TGF-β following NGF treatment, the exact amount of secreted TGF-β was determined. Besides latent (*i.e.* biological inactive) TGF-β, only marginal amounts of active TGF-β could be detected in the supernatant of NGF-treated PC12 cells. The concentration of active TGF-β could be determined to 0,28pM in the absence of NGF and to 1,12pM following stimulation with NGF for 24h. Thus, despite the slight increase of the TGF-β concentration in the medium, the absolute amount of active TGF-β is extremely low – as mentioned before, even too low to stimulate highly responsive L6 cells. Interestingly, the amounts of total TGF-β are clearly higher (18,2pM and ~40pM in the absence and presence of NGF, respectively), indicating that TGF-β is mainly present in the latent form. TGF-β is synthesized as a precursor proprotein that is cleaved during secretion. However, the mature TGF-β remains associated with its propeptide thereby forming a latent complex until activation [35, 38] (see chapter 1.3). As TGF-β activation takes place following secretion into the extracellular compartment, intracellular signaling events that occur prior to TGF-β secretion can be excluded.

Collectively, it became evident that the NGF-triggered effects on Smad-dependent transcriptional activation are independent of TGF-β1.

5.4.2 The mechanism of Smad activation and downstream signal transduction

Originally, Smad proteins were exclusively attributed to pathways activated by TGF-β family members but it becomes increasingly evident, that they are involved in multiple signaling cascades originating from receptor serine/threonine (RSKs) or receptor tyrosine (RTKs) kinases [127, 188, 203, 300-302].

The observation, that NGF stimulation results in activation of different Smad-dependent reporter genes in PC12 cells indicates that the signal that is initiated by NGF binding to the TrkA tyrosine kinase receptor feeds into the TGF-β/Smad pathway at some point. To characterize the point of convergence and the mode of Smad activation, NGF-induced R-Smad activation was evaluated in terms of C-terminal phosphorylation, heteromeric complex formation with Smad4 and nuclear translocation.

To identify which of the R-Smads get activated in response to NGF, several distinct reporter constructs were used that differ in their specificity for either BMP- or TGF-β-activated Smads. Whereas pSBE-luc responds to all R-Smads [274], p3TP-luc(+) shows higher specificity for TGF-β activated R-Smads [28]. However, p3TP-luc was previously described to show some responsiveness to signals mediated by BMP-activated Smads [303] which is likely to be due to the presence of three AP-1 binding sites that are located upstream of the PAI-1 promoter [275]. The most specific reporter is the $p(CAGA)_{12}$-luc which is highly responsive to Smad3-mediated signals [158, 275] (see appendix A.3 for further information on the reporter constructs). Based on the observation that all tested reporter constructs were activated by NGF stimulation (Fig. 4.14) the selection of potentially involved R-Smads could be restricted to TGF-β R-Smads, most likely Smad3. Confirmation was gained from a complementary experiment in which BMP-activated R-Smads (Smad1 and Smad5) as well as TGF-β-activated R-Smads (Smad2 and Smad3) were analyzed (Fig. 4.20). Given that Smad3 clearly showed the most potent effect, further studies were focused on Smad3.

To investigate whether C-terminal phosphorylation contributes to Smad activation as it is known from TGF-β1 signaling [135, 136], the phosphorylation pattern of Smad3 was monitored in response to NGF and TGF-β1, respectively. The experiment demonstrated that whereas in L6 cells TGF-β1 induced C-terminal phosphorylation of Smad3, neither TGF-β1 nor NGF was capable to trigger phosphorylation of the SSXS-motif in PC12 cells (Fig. 4.22). Again, in accordance to previous results (Fig. 4.5), unresponsiveness to TGF-β1 is likely to be due to the limiting amounts of TβRII in parental PC12 cells. Reporter gene assays were performed to elucidate whether the C-terminal SSXS-motif is indeed dispensable for NGF-induced Smad activation. The potential to activate the $p(CAGA)_{12}$-luc reporter in response to

NGF was compared between wild-type Smad3 and a truncated Smad3 protein (Smad3(ΔSSXS)) that lacks the C-terminal SSXS-motif (Fig. 4.21). Interestingly, the Smad3 mutant showed an intermediate response. Despite the fact that the signal was reduced to approximately 30% of the signal obtained with wild-type Smad3, the truncated Smad3 variant could not completely abrogate reporter activation.

Together with the data obtained from phosphorylation experiments, the clear reduction of transcriptional activity in presence of the Smad3(ΔSSXS) mutant indicates that even though NGF does not lead to C-terminal phosphorylation of Smad3, the SSXS-motif seems to play a supportive role in mediating the NGF signals via the Smad pathway. The impact of the SSXS-motif can be explained by structural requirements. It is conceivable that the tail region of the R-Smad protein is needed to achieve the proper conformation to allow heteromeric complex formation with the common mediator Smad4. Recent reports show that phosphorylation of the SSXS-motif plays a crucial role in triggering homo- and hetero-oligomer formation of R-Smads [140, 145]. The C-terminal phosphorylation thereby stabilizes the assembly of three Smad monomers by electrostatic interaction between the L3-loop of one monomer with the phosphorylated tail of the neighboring R-Smad monomer. However, interaction between unphosphorylated Smad3 and Smad4 was also observed to some extent. Given that the NGF initiated processes were shown to be dependent on functional Smad4 proteins (Fig. 4.24b), it is assumed that interaction between R-Smads and Smad4 can occur even if the C-terminal serines are not phosphorylated or that other NGF-initiated modifications cause the same or even a distinct oligomerization pattern of Smads that likewise leads to nuclear translocation (Fig. 4.23) and transcriptional activation of Smad-dependent reporter genes (Fig. 4.14).

Taken together, it became evident that although treatment of PC12 cells with either TGF-β1 or NGF can result in activation of Smad-dependent reporter genes, the preceding signaling events are not identical. While TGF-β signaling requires upregulation or ectopic expression of TβRII in PC12 cells, leading to activation of the Smad pathway by C-terminal phosphorylation of R-Smads, NGF mediates Smad activation through a different mechanism that does not involve C-terminal phosphorylation.

During the past years, an increasing number of publications revealed that Smad proteins are not solely phosphorylated by TβRI but also rank as substrates for a growing number of cellular kinases which trigger modulation of Smad activity by alternative mechanisms that do not require C-terminal phosphorylation (see chapter 1.6.2 and Fig. 1.1).

Proteins of the Erk subfamily of MAP kinases which get activated by receptor tyrosine kinases were described to phosphorylate the linker region of R-Smads, thereby negatively affecting nuclear translocation [132, 133]. Similarly, Cam kinase II was shown to prevent nuclear accumulation by phosphorylating several serine residues within Smad2 [202]. PKC

precludes DNA-binding of Smad3 by phosphorylation of residues located in the MH1 domain [203] and several other proteins such as JNK [197] or MEKK-1 [188] were described to trigger Smad phosphorylation outside the C-terminal SSXS-motif.

These examples support our findings that activation of Smad proteins can occur independent of C-terminal phosphorylation. Furthermore, besides direct phosphorylation events, NGF potentially triggers other modifications of R-Smads or the dissociation of R-Smads from cytoplasmic retention proteins such as SARA [72, 94] or microtubules [138] resulting in enhanced Smad nuclear translocation and transcriptional activation.

In order to further elucidate the mechanism of Smad activation, *in vivo* phosphorylation studies could reveal whether the phosphorylation status of Smad3 changes in response to NGF stimulation. In the case of an increased phosphorylation level following NGF treatment, subsequent phospho-peptide mapping or mutation of potential phosphorylation sites (provided that candidates can be located) could lead to identification of the exact phosphorylation site(s).

5.4.3 Inhibitory functions of Smad7

Smad7 belongs to the class of inhibitory Smads and was originally described to inhibit TGF-β signaling by interacting with activated TβRI, thereby preventing transient association of R-Smads that usually get phosphorylated by TβRI [98, 99]. However, several lines of evidence demonstrate that association with activated TβRI is not the only mechanism by which Smad7 elicits negative regulation of the TGF-β signaling cascade.

During these studies, it could be shown that Smad7 has a clear dominant-negative effect on NGF-induced transcription of Smad-dependent reporter genes (Fig. 4. 25). Even the strong transcriptional activation that is obtained from TrkA overexpressing cells was potently inhibited by Smad7 (Fig. 4.26), suggesting that Smad7 acts downstream of TrkA to impede NGF-triggered Smad activation.

As mentioned before, Smad7 does not necessarily elicit its inhibitory function exclusively by physical interaction with TβRI, rather, there are different scenarios that can account for the signal abrogation by Smad7: First, Smad7 might enhance the turnover of TGF-β receptors as Smad7 was recently described to directly interact with the HECT-domain ubiquitin ligases Smurf1 and Smurf2 which trigger degradation of the Smad7/TGF-β receptor complex [102, 104]. Second, Smad7 interacts with the WD domain containing protein STRAP which elicits synergistic inhibition of TGF-β mediated signals in concert with Smad7 [100]. STRAP was described to stabilize the ternary complex consisting of Smad7, TβRI and STRAP and to be involved in recruiting Smad7 to the TGF-β receptors [103]. However, referring to NGF-initiated effects, both mechanisms would presuppose a central role of the TGF-β receptors in NGF-mediated Smad activation. Given that the activating phosphorylation does not occur at

the C-terminal SSXS-motif (Fig. 4.22), an involvement of the TGF-β receptors would demand that TβRI is able to phosphorylate Smad proteins at residues other than the C-terminal serines, which is rather unlikely. A third scenario can be suggested on the basis of a publication that describes direct interaction between Smad7 and TAB1, leading to abrogation of TAK1 activation and thus prevention of further downstream signaling through the TAK1/p38 pathway [187]. Thus, Smad7 might associate with TAB1/TAK1 or even other – as yet unidentified – proteins that support Smad7 in blocking Smad signaling. Along these lines, an association of Smad7 with cellular kinases was proposed to be involved in inhibition of MEKK-1 induced transcriptional activity of Smad2 [188]. MEKK-1 was shown to enhance Smad2-mediated transcriptional activity whereby Smad2 activation is independent of phosphorylation of the SSXS-motif.

Further studies revealed that the integrity of the C-terminal part of Smad7 is essential to convey the inhibitory function of Smad7 in NGF-mediated Smad activation (Fig. 4.25). As in this experiment, a truncated Smad7 variant that lacks the very C-terminal 19 amino acids did not interfere with NGF-initiated Smad activation, this region is supposed to be critical.

The C-terminal region of Smad7 was reported to enable association with TβRI [98, 288] and with STRAP [103]. The interaction site with TAB1, however, still needs to be mapped [187]. Moreover, it was previously published that the N-terminus of the Smad7 protein interacts with the MH2 domain and that this intramolecular association results in an enhancement of the inhibitory activity of Smad7 in TGF-β1 mediated signaling [186]. Thus, the C-terminal amino acids seem to adopt a critical role in mediating protein-protein interactions of Smad7 with itself and various other proteins.

Taken together, Smad7 acts downstream of TrkA and is a potent inhibitor of NGF-triggered Smad activation. Whether Smad7 interferes with signaling at the receptor level or by association with cellular proteins is not yet completely elucidated. However, due to the facts that (i) the TGF-β receptors seem to play a minor role in mediating NGF-induced signals (see chapter 5.5) and that (ii) the C-terminal part of Smad7 seems to represent a docking site for several proteins, a mechanism that is based on interaction with cellular proteins appears to be more likely.

5.5 INVOLVEMENT OF TRANSMEMBRANE RECEPTORS IN NGF-INDUCED SMAD ACTIVATION

To assess which of the transmembrane receptors are involved in transducing NGF-initiated signals, the effects of wild-type and functionally inactive receptor variants were investigated.

Referring to the NGF receptors, overexpression of wild-type TrkA receptors resulted in a significant increase in NGF-triggered transcriptional activation of Smad-dependent reporter constructs, whereas a kinase deficient variant of TrkA exhibited a dominant-negative effect

(Fig. 4.26). These results imply that the kinase activity of TrkA plays an important role in mediating NGF-induced Smad activation. Interestingly, cotransfection of Smad7 and wild-type TrkA receptors revealed that Smad7 can completely block the strong signal that is evoked by functional TrkA receptors (Fig. 4.26), suggesting that Smad7 acts downstream of the TrkA receptor to abrogate the NGF-induced signals.

Concerning the TGF-β receptors, overexpression of the wild-type receptors likewise leads to an augmentation of the NGF-induced signal. However, kinase inactive variants of the receptors do not provoke dominant-negative effects (Fig. 4.27 and Fig. 4.28). Thus, the TGF-β receptors rather play a supportive but not a crucial role in mediating NGF-triggered Smad activation. This is in accordance with previous findings, demonstrating that NGF treatment does not lead to C-terminal phosphorylation of Smad3 (Fig. 4.22), suggesting that Smad activation by NGF is not mediated by TβRI.

To further elucidate the nature of the supportive effect of the TGF-β receptors, potential direct interactions between the TrkA and the TGF-β receptors were analyzed. First hints for physical interactions between TrkA and TβRII came from a binding and cross-linking assay in which iodinated NGF was applied to cells that were transfected with either TrkA alone or with TrkA together with the TGF-β receptors (Fig. 4.31). In this experiment, weak signals corresponding to NGF-bound TrkA- and TβRII-receptors could be detected following immunoprecipitation with antibodies recognizing TβRII, implying that the interaction between the receptors is strong enough to coimmunoprecipitate TrkA with TβRII. Furthermore, the fact that a band corresponding to TβRII could be detected on the autoradiogram presupposes that ^{125}I-NGF was cross-linked to TβRII. To explain cross-linking of ^{125}I-NGF to TβRII, it must be assumed that either the receptors are in very close proximity or that ^{125}I-NGF itself shows some affinity to TβRII. In contrast to TβRII, the type I receptor does not show any association with TrkA or binding of ^{125}I-NGF.

Direct ligand-independent physical interaction between TrkA and TβRII could be confirmed by coimmunoprecipitation studies (Fig. 4.30). However, it has to be considered that binding and cross-linking assays using radiolabeled NGF as well as coimmunoprecipitation studies were performed under strong overexpressing conditions. Thus, it can not be ruled out that association of the receptors is due to the artificial overexpression in COS or 293T cells which does not represent the physiological situation. Usually, data obtained from overexpressing systems should be confirmed in other cell lines without ectopic expression of the investigated proteins. Unfortunately, due to limiting amounts of receptors present in parental PC12 cells it was not possible to perform coimmunoprecipitation studies in PC12 cells.

Taken together, despite the presented evidences for a direct interaction between TrkA and TβRII (but not TβRI) which potentially allows formation of higher order receptor complexes at

the cell surface, it is questionable whether endogenous TβRII that is expressed in PC12 cells also interacts with TrkA and whether this interaction contributes to mediate NGF-induced Smad activation.

The observation that NGF can impair binding of TGF-β1 to its receptors (Fig. 4.32) and previous data that showed weak cross-linking of ^{125}I-NGF to TβRII (Fig. 4.31) open up the question whether NGF itself displays affinity to the TGF-β receptors. It is important to note that both, NGF and TGF-β1 belong to the homodimeric cystine-knot containing superfamily of growth factors which also includes platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and human chorionic gonadotrophin (HCG) [6-8]. All these growth factors have a similar monomer topology containing a central β-sheet with the cystine-knot on one end. Structural diversity however is based on the distinct modes of dimerization. Whereas members of the TGF-β family form antiparallel dimers, NGF assembles as a parallel dimer [8]. Considering the structural similarity referring the monomer but structural diversity in terms of homodimer formation (which is relevant for receptor-ligand complex formation) it seems rather unlikely that NGF can directly bind to TGF-β receptors.

In conclusion, the initial step in NGF-triggered Smad activation is represented by binding of NGF to TrkA receptors. Subsequently, functional *i.e*. kinase active TrkA receptors are necessary to mediate Smad activation whereas TGF-β receptors seem to play a minor role.

5.6 PARTICIPATION OF OTHER SIGNAL TRANSDUCTION PATHWAYS

As mentioned before (see chapter 4.9), molecules of the MAPK/Erk cascade and the TAK1/p38 pathway represent candidates that are likely to participate in mediating NGF-induced effects on Smad-dependent reporter genes. Experiments in which the MAPK/Erk-pathway was blocked by using the MEK1/2-inhibitor, PD98059, showed a reduction of reporter activation of about 60%, suggesting an involvement of this cascade. The small G protein, Ras, as well as Erk2 were described to directly phosphorylate R-Smads in the linker region, resulting in inhibition of nuclear translocation [132, 133] and kinases downstream of MEK1/2 were proposed to phosphorylated Smad2 at the C-terminal SSXS-motif and two additional sites, leading to activation of Smad2 [300]. These examples demonstrate, that members of the MAPK/Erk pathway are able to directly modulate Smad activity by several distinct phosphorylation events which are highly dependent on the present cellular environment. Moreover, Ras was shown to participate in activation of the p38-pathway upstream of MKK3/6 [304] and oncogenic Ras was likewise described to sequentially activate the MAPK/Erk pathway and the MKK3/6-p38 pathway in human fibroblasts [305]. Activation of MKK3 and MKK6 is regulated through phosphorylation by MAP kinase kinase kinases such as TAK1 [306]. Given that activation of the p38-pathway by proteins of the MAPK/Erk cascade was proposed to occur upstream of MKK3/6 the TAB1/TAK1 complex is

a potential target of phosphorylation and thus of activation by MAPK/Erk kinases. On the basis of this data, a model can be proposed in which, following NGF stimulation, molecules of the MAPK/Erk cascade induce the activation of TAK1 which in turn leads to sequential phosphorylation of MKK3/6 and further downstream signal transducers and transcription factors. This model would be consistent with the previous assumption that Smad7 blocks signaling by binding to TAB1 and preventing TAK1 activation (see chapter 5.4.3). Furthermore, data obtained from experiments in which the effects of dominant-negative mutants of MKK6 and TAK1 were analyzed could also be explained by this model. The mutant MKK6 (DN-MKK6) showed a slight dominant-negative effect on NGF-induced Smad activation when expressed alone and was able to completely abolish the strong activating capacity of cotransfected TrkA receptors (Fig. 4.35). Along these lines, the dominant-negative TAK1 mutant provokes an efficient reduction of NGF-triggered Smad activation (Fig. 4.36). These results strongly suggest that MKK6 acts downstream of the TrkA receptor which is in accordance with the hypothesis of TAK1/MKK6 being activated by members of the MAPK/Erk pathway which originates from TrkA receptors.

Introduction of the XIAP protein which is known to link the TAB1/TAK1 complex to TβRI did not mediate a significant alteration of transcriptional activation in response to NGF, supporting the secondary role of the TGF-β receptors.

Finally, both dominant-negative mutants (DN-TAK1 and DN-MKK6) are capable of interfering with the strong activating capacity of Smad3 in presence of NGF (Fig. 4.37). In particular DN-MKK6 completely blocks the Smad3-triggered effects, demonstrating that functional MKK6 plays a crucial role. As the exact mechanism of Smad3 activation is not yet elucidated, MKK6 or downstream kinases can be considered as potential kinases for Smad3. MKK6 represents a dual specificity kinase which is known to be an upstream activator of p38 kinase. Thus, it remains to be elucidated whether Smad3 can also serve as a substrate for MKK6.

It is worth mentioning that there are also several lines of evidence for crosstalk at the transcriptional level. Activating transcription factor-2 (ATF-2) was described to be a common nuclear target of Smad and TAK1 cascades [107, 127] and nuclear crosstalk between Smad, MAPK/Erk and p38 pathways was implicated in TGF-β induced aggrecan synthesis in chondrogenic cells [299]. However, in the case of NGF-induced activation of Smad-dependent transcription in PC12 cells, nuclear crosstalk is unlikely as the pSBE-luc and the $p(CAGA)_{12}$-luc reporter which were mostly used in luciferase assays are specific for R-Smads and do not contain other binding motifs such as a cAMP response element (CRE) which represents the binding site for ATF-2.

Taken together, NGF-induced Smad activation is dependent on both, the MAPK/Erk pathway and the TAK1/MKK6 cascade. From the present state of investigations it is likely that

MAPK/Erk and TAK1/MKK6 become sequentially activated following NGF binding to the TrkA receptor and that their activation leads to modulation of Smad3 activity.

5.7 EVALUATION OF OTHER CELL SYSTEMS

With intent to find cell lines that can be used to reproduce and confirm the NGF-induced Smad activation that was observed in PC12 cells, different cell lines were characterized in respect of their receptor composition and their potential to mediate Smad activation in response to NGF. The neuroblastoma cell line Lan-1 and the breast cancer cell lines, MCF-7 and MDA-MB 468, were found to endogenously express both, NGF- and TGF-β signaling receptors (Fig. 4.38a-c). Nevertheless, they demonstrated a completely different behavior referring to Smad activation. Whereas Lan-1, MCF-7 as well as Smad4-transfected MDA-MB 468 cells showed TGF-β1 responsiveness, none of them induced transcription from Smad-dependent reporter genes following NGF stimulation (Fig. 4.39a-c). In order to explain these opposite characteristics, it is to note that there is a significant difference in the amount of TβRII expression in PC12 cells on the one hand and the neuroblastoma and breast cancer cells on the other hand. As mentioned before, the low amount of TβRII in PC12 cells conveys resistance to TGF-β1 which can be restored by ectopic expression of TβRII (Fig. 4.5 and 4.6). Consequently, it is not astonishing that the tested cell lines that express both types of TGF-β receptors in sufficient amounts, are sensitive to TGF-β1. However, the distinct level of TβRII expression might be critical for the sensitivity towards NGF: in presence of high amounts of TβRII, the TGF-β signaling cascade is favored whereas in presence of low amounts of TβRII, cells become TGF-β resistant but instead, NGF is able to trigger Smad activation under these circumstances.

Furthermore, it can not be excluded that PC12 cells contain molecules like accessory proteins or signal transducers which are not present in the other tested cells but are necessary for mediating NGF-induced activation of Smad-dependent reporter genes.

5.8 SUMMARIZING DISCUSSION AND CURRENT MODEL

PC12 cells endogenously express both, NGF receptors as well as TGF-β receptors. TGF-β signaling, however, requires ectopic expression of TβRII because low amounts of TβRII represent the limiting factor for proper TGF-β signaling in parental PC12 cells.

In contrast, stimulation of PC12 cells with NGF results in activation of the Smad pathway independent of TGF-β1. Binding of NGF to its high-affinity receptor TrkA induces activation of Smad3 but unlike in TGF-β1 signaling, this process does not include phosphorylation of the C-terminal SSXS-motif of the R-Smad. Thus, other kinases are likely to be involved in activating the R-Smad protein by using phosphorylation sites outside the SSXS-motif. The

activated R-Smad is transferred into the nucleus following heteromeric complex formation with Smad4 and finally activates transcription from Smad-dependent promoter elements.

Smad7 was found to be a potent inhibitor of this NGF-initiated cascade. As the TGF-β receptors are likely to play a minor role, it is assumed that Smad7 does not bind to TβRI but rather to other cellular proteins (*e.g.* TAB1) thereby preventing R-Smad activation and blocking the downstream cascade.

Considering the involvement of both, the MAPK/Erk-kinase pathway and the TAK1/p38-pathway, a lot of complex interactions and co-dependences between molecules of different pathways might be involved in mediating the observed NGF effects. However, based on the current state of investigations, the following hypothetical model of NGF-induced Smad activation can be proposed (Fig. 5.1):

Binding of NGF to the NGF receptor TrkA activates - among others - the MAPK/Erk-pathway. Signal transducers of this cascade such as Ras or MEK1/2 kinases can lead to activation of TAK1 which in turn activates the MAP kinase kinase MKK6. MKK6 itself or other downstream molecules are candidates that mediate phosphorylation of Smad3 outside the SSXS-motif. The activated Smad3 protein can then hetero-oligomerize with Smad4, translocate to the nucleus and activate transcription from Smad-dependent reporter genes.

The TGF-β receptors do not represent key molecules in this cascade but they might contribute to TAK1 activation as the XIAP/TAB1/TAK1 complex is known to be directly associated with the TGF-β receptors.

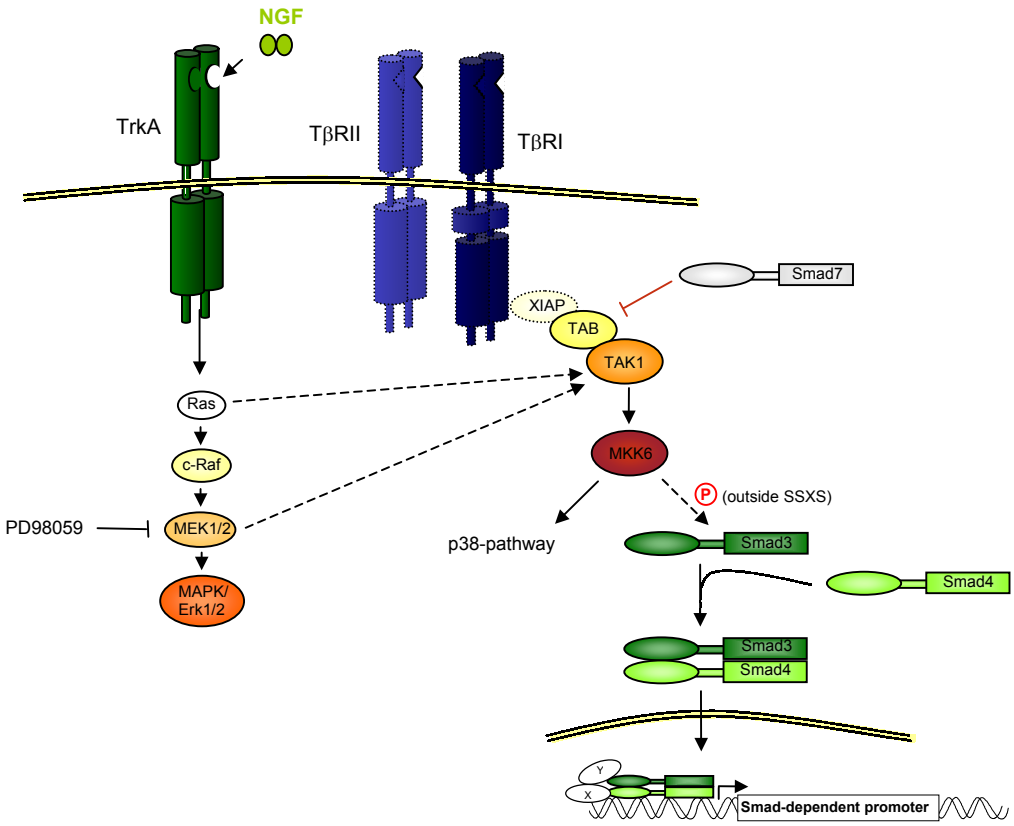


Fig. 5.1 Hypothetical model of NGF-induced transcriptional activation from Smad-dependent reporter genes

Smad7 is likely to abrogate NGF-initiated signals by association with TAB1/TAK1 because direct interaction between TAB1 and Smad7 was recently described. Furthermore, this model of Smad7 inhibition is in accordance with the finding that Smad7 acts downstream of TrkA.

In the light of the enormous variety of interactions between different cascades, in particular the different MAP kinase pathways, the NGF-induced signaling events leading to activation of Smad-dependent genes are likely to be even more complex.

This mechanism of NGF mediated Smad activation which is independent of TGF-β1 might be of special importance in regulating expression of genes that are essential for the development and function of neuronal cells or other NGF sensitive cells – in particular those that are TGF-β-resistant.

6 Summary

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that is engaged in regulating versatile cellular processes that are pivotal for development and homeostasis of most tissues in multicellular organisms.

TGF-β signal transduction is initially propagated by binding of TGF-β to transmembrane serine/threonine kinase receptors, designated TβRI and TβRII. Upon activation, the receptors phosphorylate Smad proteins which serve as downstream mediators that enter the nucleus and finally trigger transcriptional responses of specific genes.

During the past years, it became evident that signaling cascades do not proceed in a linear fashion but rather represent a complex network of numerous pathways that mutually influence each other. Along these lines, members of the TGF-β superfamily are attributed to synergize with neurotrophins. Together, they mediate neurotrophic effects in different populations of the nervous system, suggesting that an interdependence exists between TGF-βs on the one hand and neurotrophins on the other.

In the present work, the crosstalk of NGF and TGF-β/Smad signaling pathways is characterized in rat pheochromocytoma cells (PC12) which are frequently used as a model system for neuronal differentiation.

PC12 cells were found to be unresponsive to TGF-β due to limiting levels of TβRII. However, stimulation with NGF results in initiation of Smad-mediated transcription independent of TGF-β. Binding of NGF to functional TrkA receptors triggers activation of Smad3. This NGF-dependent Smad activation occurs by a mechanism which is different from being induced by TGF-β receptors in that it provokes a different phosphorylation pattern of R-Smads. Together with an inferior role of TβRI, Smad3 is proposed to serve as a substrate for cellular kinases other than TβRI. Based on the presented involvement of components of both, the MAPK/Erk and the TAK1/MKK6 cascade, signal mediators of these pathways rank as candidates to mediate direct activation of Smad3. Smad3 is subsequently translocated to the nucleus and activates transcription in a Smad4-dependent manner.

Negative regulation is provided by Smad7 which was found to act as a potent inhibitor of Smad signaling not only in TGF-β- but also in NGF-mediated cascades.

The potential of NGF to activate the Smad pathway independent of TGF-β might be of special importance in regulating expression of genes that are essential for the development and function of neuronal cells or of other NGF-sensitive cells, in particular those which are TGF-β-resistant.

6 Zusammenfassung

Das multifunktionelle Zytokin TGF-β ist an der Regulation vielfältiger zellulärer Prozesse beteiligt. Diese sind für die Entwicklung und die Homöostase der meisten Gewebe vielzelliger Organismen essenziell.

Die TGF-β Signaltransduktionskaskade wird durch die Bindung des Zytokins an spezifische transmembrane Serin/Threonin-Kinase Rezeptoren (TβRI und TβRII) initiiert. Eine solche Rezeptoraktivierung führt zur Phosphorylierung von Smad Proteinen. Diese dienen der Signalweiterleitung, indem sie anschließend in den Zellkern translozieren und dort die Transkription spezifischer Zielgene modulieren.

In den letzten Jahren wurde deutlich, dass Signalkaskaden nicht nur linear weitergeleitet werden, sondern dass vielmehr ein komplexes Netzwerk aus zahlreichen, sich gegenseitig regulierenden, Signalwegen existiert. In diesem Zusammenhang wird auch den Mitgliedern der TGF-β Superfamilie zugeschrieben, dass sie mit Neurotrophinen kooperieren und somit deren Effekte in unterschiedlichen neuronalen Zellpopulationen unterstützen.

In der vorliegenden Arbeit wurde der "crosstalk" von NGF- und TGF-β/Smad-Signalwegen charakterisiert. Als Zellsystem dienten dazu Ratten Pheochromocytoma Zellen (PC12), die weithin als Modellsystem für neuronale Differenzierung verwendet werden.

Basierend auf der Expression limitierender Mengen an TβRII, zeigen PC12 Zellen keine Responsivität gegenüber TGF-β. Stimulation mit NGF hingegen resultiert - unabhängig von TGF-β - in der Initiation von Smad-vermittelter Transkription. Die initiale Bindung von NGF an TrkA Rezeptoren führt zur Aktivierung von Smad3. Diese NGF-induzierte Smad-Aktivierung unterscheidet sich von der durch TGF-β-Rezeptoren initiierten Aktivierung hinsichtlich des Phosphorylierungsmusters der R-Smads. Da weiterhin gezeigt werden konnte, dass die TGF-β Rezeptoren für NGF-induzierte Ereignisse eine untergeordnete Rolle spielen, wird angenommen, dass Smad3 ein Substrat für andere zelluläre Kinasen als TβRI ist. Die hier nachgewiesene Beteiligung der MAPK/Erk Kaskade sowie des TAK1/MKK6 Signalwegs an der Weiterleitung des NGF-Signals machen deren Signalmoleküle zu potenziellen Kinasen für die direkte Aktivierung von Smad3. Im Anschluß daran erfolgt die nukleäre Translokation des Smad3 und spezifische Promotoraktivierungen unter Beteiligung von Smad4.

Abschließend konnte gezeigt werden, dass das Smad7 Protein, nicht nur nach TGF-β- sondern auch nach NGF-Stimulation als effektiver Inhibitor der Smad Signalkaskade wirkt.

Die bislang unbekannte Fähigkeit, den Smad-Signaltransduktionsweg unabhängig von TGF-β zu aktivieren, schreibt NGF eine besondere Bedeutung für die Genregulation in neuronalen Zellpopulationen oder anderen NGF-sensitiven - insbesondere TGF-β-resistenten - Zellen zu.

7 References

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A.1 Abbreviations

Chemicals/ Materials

Ab	Antibody
AA	Acrylamid
Amp	Ampicillin
APS	Amoniumpersulfat
BA	Methylenbisacrylamid
β-gal	β-galactosidase
bp	Basepairs
BSA	Bovine serum albumin
DEAE	Diethlyminoethyl
DMEM	Dulbecco's modified essential medium
DMSO	Dimethylsulfoxid
dNTP	2'-Desoxy-nucleosid-5'-triphosphat
Dox	Doxycycline
DSS	*Disuccinimidyl suberate*
E. coli	*Escherichia coli*
EDTA	Ethylendiamintetraacetate
EGFP	Enhanced green fluorescent protein
EMEM	Eagle's minimal essential medium
env	Envelope
EtBr	Ethidiumbromide
FCS	Fetal calf serum
gag	Group specific antigen
GFP	Green fluorescent protein
HA	Haemaglutinin
HEPES	N-2-hydroxyethylpiperazin-2'-ethansulfonic acid
HMS	Highmolecular DNA-standard
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IRES	Intervening ribosomal entry site
LB	Luria broth
LMS	Low molecular DNA-standard
Luminol	3-Aminophthalhydrazide
LTR	Long terminal repeat
MuLV	Murine leukemia virus
PAGE	Polyacrylamid-gelelectrophoresis
PAS	Protein A sepharose
PBS	Phosphate buffered saline
PE	Phycoerythrin
Pen	Penicillin
Pfu	*Pyrococcus furiosus*
PIN	PBS + IFS (inactivated fetal calf serum) + Na-Azide
PMSF	Phenylmethylsulfonylfluorid
pol	Polymerase
pox	Peroxidase
P/S	Penicillin/Streptomycin
RNase A	Ribonuclease A
rtTA	Reverse tetracycline-controlled transactivator
SDS	Sodium-dodecylsulfate
Strep	Streptomycin
Taq	*Thermophilus aquaticus*
TBS	Tris buffered saline
TCA	Trichloricacid
TEMED	N,N,N',N',-tetramethyl-ethylendiamine
TK	Thymidine-kinase
Tris	Tris-(hydroxymethyl)-aminomethan
X-gal	5-Bomo-4-chloro-3-indolyl β-D-galactopyranoside

TERMS

α-	Anti-
AA	Amino acid
ALK	Aktivin like kinase
BMP	Bone morphogenetic protein
BMPRI/II	BMP receptor type I/II
CMV	Cytomegalo virus
dH_2O	Deionized water
DNA	Deoxyribonucleic acid
ds	Double stranded
ECD	Extracellular domain
ECL	Enhanced chemiluminescence
FACS	Fluorescence activated cell sorting
Fig.	Figure
GS-domain	Glycin-serine rich domain
IP	Immunoprecipitation
MAD	Mother against dpp
MAPK	Mitogen-activated protein kinase
MH	MAD-homology
NGF	Nerve growth factor
OD	Optical density
P-	Phospho-
PAGE	Polyacrylamid gel electrophoresis
PCR	Polymerase chain reaction
RSK	Receptor serine/threonine kinase
RTK	Receptor tyrosine kinase
SBE	Smad binding element
ss	Single stranded
TβRI/II	TGF-β receptor type I/II
TGF-β	Transforming growth factor-β
TrkA	Tropomyosin-related kinase A
UV	Ultraviolett
WB	Western-blot
wt	Wild-type

UNITS

bp	Basepairs
Bq	Bequerel
°C	Degree Celsius
Ci	Curie
Da	Dalton
g	Gram
h	hours
k	Kilo-
kDa	Kilodalton
l	Liter
µ	Mcyro-
min	Minutes
mM	Millimolar
mol	Molar
n	Nano-
p	Pico-
rpm	Rounds per minute
RT	Room temperature
sec	Second
U	Unit
V	Volt
Vol	Volume

A.2 Sequences of oligonucleotides

Primers specific for TβRI				
Primer	**Sequence**	**↔**	**Template**	**Function**
TRI-4	5'-AAGGGA**C**CCATGCTCATGAT-3'	←	TβRI	deletion of a *Bam*HI-site
TRI-5	5'-ATCATGAGCATGG**G**TCCCTT-3'	→	TβRI	deletion of a *Bam*HI-site
TRI-9	5'-TAGGCCGTTTGTATGTGCAC-3'	→	TβRI	sequencing

Primers specific for TβRII				
Primer	**Sequence**	**↔**	**Template**	**Function**
P 1	5'-TATGACGAGCAGCGGGGTCT-3'	→	TβRII	sequencing
P 9	5'-CAGAGCAGTTTGAGACAGT-3'	→	TβRII	sequencing
P 11	5'-AGGATATTGGAGCTCTTGAGGTC-3'	←	TβRII	sequencing
PK 40	5'-GATGTCTACTC**G**ATGGCTCTGGT-3'	→	TβRII	deletion of a *Nco*I-site
PK 41	5'-ACCAGAGCCAT**C**GAGTAGACATC-3'	←	TβRII	deletion of a *Nco*I-site

Primers for the construction of TβRII-Δcyt				
Primer	**Sequence**	**↔**	**Template**	**Function**
DC-1	5'-CAGGTT**C**AACTCAGCTTCTGCTG-3'	←	TβRII	introduction of a stop codon
DC-2	5'-CAGCAGAAGCTGAGTT**G**AACCTG-3'	→	TβRII	introduction of a stop codon
DC-3	5'-TTCCAAGAGG**G**AT**C**CTCCTCATAG-3'	←	TβRII	introduction of a *Bam*HI-site

Primers for the construction of the retroviral construct encoding Smad7				
Primer	**Sequence**	**↔**	**Template**	**Function**
S7-1	5'-GGCCGGCCATGGACTACAAGGACGA CG ATGAC-3'	→	Smad7	introduction of a *NcoI*-site
S7-2	5'-TGAAACCCGTCCA**A**GGTTGCTGCAT-3'	←	Smad7	deletion of a *Nco*I-site
S7-3	5'-ATGCAGCAACC**T**TGGACGGGTTTCA-3'	→	Smad7	deletion of a *Nco*I-site
S7-6	5'-CCGGCCGGATCCTCACGATCCGCCCTG TCCTCTT-3'	←	Smad7	introduction of a *Bam*HI-site

Primers specific for Smad7				
Primer	**Sequence**	**↔**	**Template**	**Function**
S7-7	5'-AAGCCTACAGCCTGCA-3'	→	Smad7	sequencing
S7-8	5'-TGAGTTTCTTGAGCA-3'	←	Smad7	sequencing
S7-9	5'-CTGGTGTGCTGCAAC-3'	→	Smad7	sequencing
S7-10	5'-ATCTGGAGTAAGGAGG-3'	←	Smad7	sequencing
S7-11	5'-CTGAGGTAGATCATAG-3'	←	Smad7	sequencing

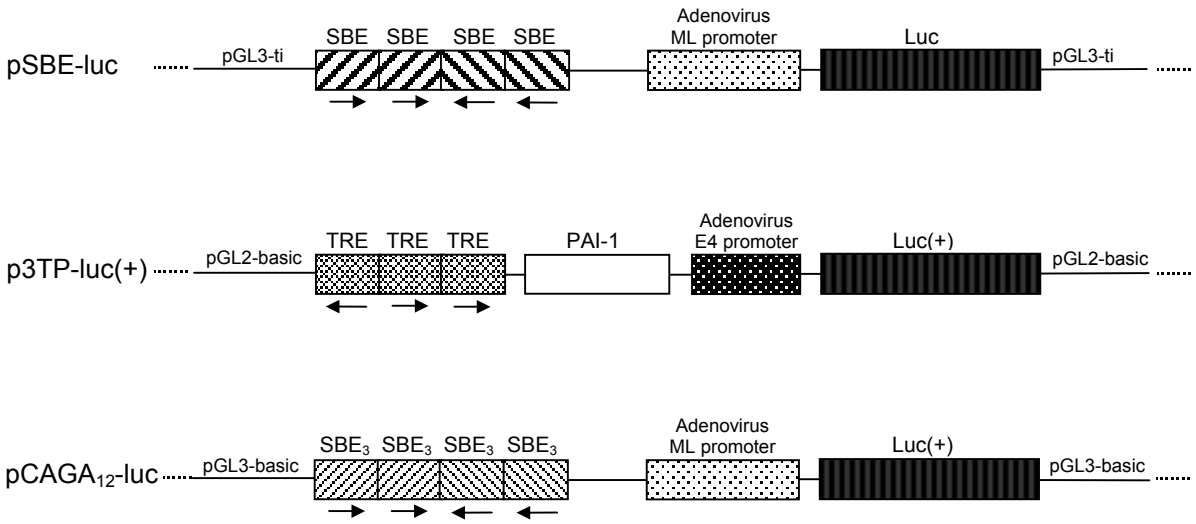
Primers specific for the retroviral vector (pcz CFG EGIRT)				
Primer	**Sequence**	**↔**	**Template**	**Function**
D#685	5'-CATAAAATGAATGCAATTGTTGTT G-3'	→	pczCFG EGIRT	sequencing
D#605	5'-CATAAAATGAATGCAATTGTTGTTG-3'	→	pczCFG EGIRT	sequencing
D#502	5'-TGATATTGTTGAGTCAAAACTAGAGC-3'	←	pczCFG EGIRT	sequencing
GFP3'	5'-GATCACTCTCCTGTACAAGTC-3'	→	GFP (Clontech)	sequencing

Primers for the construction of the epitope tagged TrkA				
Primer	**Sequence**	**↔**	**Template**	**Function**
F-TrkA-1	5'-CTTGTCATCGTCGTCCTTGTAGTCGGG TGCGGCGCCCGCAGATGCCAGTATCA GC-3'	←	TrkA	introduction of a N-terminal Flag-epitope
F-TrkA-2	5'-GACTACAAGGACGACGATGACAAGTGC CCCGATGCCTGCTGCCCCCACGGCTCC -3'	→	TrkA	introduction of a N-terminal Flag-epitope
F-TrkA-3	5'-CAGATTTCATCACCGTGGCTGACTGCT CCA-3'	→	TrkA	introduction of a N-terminal Flag-epitope

Primers specific for TrkA				
Primer	**Sequence**	**↔**	**Template**	**Function**
TrkA-5	5'-CTGGCCACACGCAACTGTCTAGTG-3'	→	TrkA	sequencing
TrkA-7	5'-GTAGAGCTCAGTCAGGTTCTCTG-3'	←	TrkA	sequencing
TrkA-8	5'-CAGATGCCTTCCATTTCACTCCTC-3'	→	TrkA	sequencing
TrkA-9	5'-GAGAGAGACTCCAGAGCGTTGAAG-3'	←	TrkA	sequencing
hTrkA-10	5'-CAGAGAACCTGACTGAGC-3'	→	hu TrkA	sequencing
rTrkA-10	5'-CCGGGAACCTGACGGAGC-3'	→	rat TrkA	sequencing
hTrkA-11	5'-CAGCAGCACGTCGTCCCCC-3'	←	hu TrkA	sequencing
rTrkA-11	5'-CAGAAAAACGTCATCCCCC-3'	←	rat TrkA	sequencing

Primers specific for p75				
Primer	**Sequence**	**↔**	**Template**	**Function**
p75-1	5'-TGTGCGAGGACAC**T**GAGCGCCA-3'	→	p75 (rat, human)	sequencing
p75-2	5'-AGGCCTC**G**TGGGTAAAGGAGTCTAT-3'	→	p75 (rat, human)	sequencing

A.3 Reportergen constructs



The pSBE-reporter contains four repeats of the Smad binding element (SBE) taken from the JunB promoter and is responsive to both, TGF-β and BMP mediated signals [274].

p3TP-luc(+) is derived from p3TP-luc [28] but the luciferase gene was exchanged by an optimized luciferase gene showing enhanced activity [273]. The reporter contains three TPA responsive elements (TRE) taken from the collagenase promoter and part of the plasminogen activator inhibitor-1 (PAI-1) promoter. In contrast to the pSBE-reporter, the p3TP-luc(+) reporter is specific for signals mediated by TGF-β activated Smad proteins, namely Smad2 and Smad3 [28]. In addition, the p3TP-luc reporter contains three AP-1 sites upstream of the PAI-1 promoter [275].

The $p(CAGA)_{12}$-luc reporter is the most specific one in that it is efficiently induced by the TGF-β activated Smad3 but not by other R-Smads. It contains four cassettes composed of three CAGA-boxes, each, which serve as Smad binding elements (SBE) and are oriented in a specific manner [158, 275].

Publikationen

Publikationen

Rotzer, D., Roth, M., **Lutz, M.**, Lindemann, D., Sebald, W. and Knaus, P. (2001). *Type III TGF-β receptor independent signaling of TGF-β2 via TβRII-B, an alternative spliced TGF-β type II receptor*. EMBO J. **20** (3): 480-490.

Lutz, M., Krieglstein, K., ten Dijke, P., Sebald, W. and Knaus, P. (2002). *NGF mediates activation of the Smad pathway in PC12 cells.* Zur Publikation eingereicht.

Lutz, M. and Knaus, P. (2002). (Review) *Integration of the TGF-β pathway into the cellular signalling network.* Cell. Signal. Im Druck.

Veröffentlichte Abstracts

Lutz, M., Farkas, L. M., Lindemann, D., ten Dijke, P., Unsicker, K., Krieglstein, K. and Knaus, P. (2000). *Crosstalk between the NGF and TGF-β signalling pathways.* Signal Transduction ***1***, Supplement 1, 66.

Rotzer, D., Roth, M., **Lutz, M.**, Sebald, W. and Knaus, P. (2000). *Identifizierung und Charakterisierung eines TGF-β2 Rezeptors im Osteoblasten.* Osteologie, ***9***, Suppl. 1, 94.

Rotzer, D., Roth, M., **Lutz, M.**, Lindemann, D., Sebald, W. and Knaus, P. (2000). *Type III TGF-β receptor independent signaling of TGF-β2 via TβRII-B, an alternative spliced TGF-β type II receptor*. Signal Transduction ***1***, Suppl. 1, 89.

Posterpräsentationen

Lutz, M., Farkas, L. M., Heldmann, M., Lindemann, D., ten Dijke, P., Unsicker, K., Krieglstein, K. and Knaus, P. (1999). *TGF-β and NGF act synergistically on the neuronal differentiation of PC12 cells.* NIH-Conference, Bethesda, USA: "TGF-β: Biological Mechanisms and clinical Application".

Farkas, L. M., **Lutz, M.**, Jaszai, J., Goehmans, C., Heumann, R., Knaus, P., Unsicker, K. and Krieglstein, K. (1999). *TGF-β is an essential mediator of NGF-induced neurite outgrowth.* NIH-Conference, Bethesda, USA: "TGF-β: Biological Mechanisms and clinical Application".

Rotzer, D., Roth, M., **Lutz, M.**, Sebald, W. and Knaus, P. (2000). *Identifizierung und Charakterisierung eines TGF-β2 Rezeptors im Osteoblasten.* Conference: "Osteologie 2000, Würzburg".

Lutz, M., ten Dijke, P., Unsicker, K., Krieglstein, K. and Knaus, P. (2000). *Crosstalk between the NGF and TGF-β signalling pathways.* GBM-Symposium on Signaltransduction, Berlin.

Rotzer, D., Roth, M., **Lutz, M.**, Lindemann, D., Sebald, W. and Knaus, P. (2000). *Type III TGF-β receptor independent signaling of TGF-β2 via TβRII-B, an alternative spliced TGF-β type II receptor*. GBM-Symposium on Signaltransduction, Berlin.

Rotzer, D., Roth, M., **Lutz, M.**, Lindemann, D., Sebald, W. and Knaus, P. (2000). *Type III TGF-β receptor independent signaling of TGF-β2 via TβRII-B, an alternative spliced TGF-β type II receptor*. FASEB research conference: "TGF-β: signaling and development", Tucson, Arizona, USA.

Lutz, M., Krieglstein, K., ten Dijke, P., Sebald, W. and Knaus, P. (2001). *NGF mediates activation of the Smad pathway in PC12 cells.* VIIth International Dahlem Symposium on "Cellular Signal Recognition and Transduction", Berlin-Dahlem.

Lebenslauf

Persönliche Daten

Marion Lutz

geboren am 25.02.1973 in Nürnberg

ledig

Schulausbildung

1979 - 1981	Grundschule, Mainz-Finthen
1981 - 1983	Grundschule, Mainz-Gonsenheim
1983 - 1992	Maria-Ward-Gymnasium Mainz
05/1992	Abitur

Hochschulausbildung

10/1992 - 05/1995	Grundstudium an der Gutenberg-Universität Mainz, Fachbereich Biologie
05/1995 - 02/1997	Hauptstudium an der Bayerischen Julius-Maximilians-Universität Würzburg, Fachbereich Biologie Studienschwerpunkte: Biochemie, Humangenetik, Zoologie
02/1997	Diplom-Prüfung
03/1997 - 12/1997	Diplomarbeit am Biozentrum der Universität Würzburg, Lehrstuhl für Physiologische Chemie II, bei Prof. Dr. W. Sebald unter Betreuung von Dr. P. Knaus Thema: „Expression dominant-negativer TGF-β Rezeptoren in cytotoxischen T-Zellen“
seit 02/1998	Promotion am Biozentrum der Universität Würzburg, Lehrstuhl für Physiologische Chemie II, bei Prof. Dr. W. Sebald unter Betreuung von Dr. P. Knaus Thema: „Effects of nerve growth factor on TGF-β/Smad signal transduction in PC12 cells“
01/2001- 06/2001	Stipendiatin des Graduiertenkollegs „Regulation des Zellwachstums“, einer Stiftung der Deutschen Forschungsgemeinschaft (DFG)

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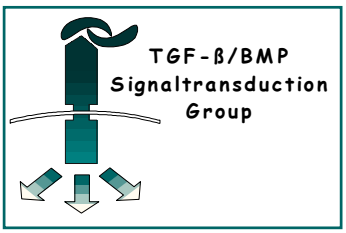

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Erklärung

Hiermit erkläre ich ehrenwörtlich, dass ich die Dissertation „Effects of nerve growth factor on TGF-β/Smad signal transduction in PC12 cells“ selbständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ich erkläre weiterhin, dass diese Dissertation weder in gleicher Weise noch in anderer Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Ich habe außer den mit dem Zulassungsgesuch urkundlich vorgelegten Graden keine weiteren akademischen Grade erworben oder zu erwerben versucht.

Würzburg, den 1. Juli 2002