

Y-box Binding Protein-1 Enhances Oncogenic Transforming Growth Factor β Signaling in Breast Cancer Cells *via* Triggering Phospho-Activation of Smad2

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Abstract. *Background/Aim:* Transforming growth factor β (TGF β) plays a role in diverse oncogenic pathways including cell proliferation and cell motility and is regulated by the pleiotropic factor Y-box binding protein-1 (YB-1). In breast cancer, Smad/Mad related protein 2 (Smad2) represents the most common downstream transducer in TGF β signaling. *Materials and Methods:* Here, YB-1's impact on Smad2 phospho-activation was characterized by incubation of the breast cancer cell line MCF-7 with or without TGF β 1 in the absence or presence of overexpressed YB-1 protein. The phospho-status of Smad2 was assessed via western blotting. *Results:* Analysis of MCF-7 cells revealed no induction of total Smad2 neither in the presence of TGF β 1, nor during YB-1 overexpression. In contrast, incubation with TGF β 1 led to an increase of phosphorylated Smad2 forms which was significantly amplified by simultaneously overexpressed YB-1 (2.8 \pm 0.2-fold). *Conclusion:* Oncogenic YB-1 indirectly enhances TGF β signaling cascades via Smad2 phospho-activation and may represent a promising factor for future diagnosis and therapy of breast cancer.

Breast cancer is the most common disease in women and the second leading cause of cancer death among female individuals (1). Many efforts have been undertaken to stratify breast cancer

entities into sub-classes regarding the receptor and oncogene status, mutation frequency, as well as histological properties (2). Additionally, the oncogenic machinery of breast cancer is frequently modulated by anticancer treatment, thus leading to a highly heterogeneous malignant landscape. For instance, anti-estrogens like tamoxifen and fulvestrant are successfully used in the endocrine therapy of estrogen receptor positive breast cancer and, moreover, have been shown to be potent inducers of transforming growth factor β (TGF β) signaling cascades (3, 4). TGF β itself plays a role in diverse oncogenic pathways in breast cancer including cell growth, cell motility, and immune suppression (5). Current research projects of our group have identified the DNA and RNA binding factor Y-box binding protein-1 (YB-1) as a pivotal modulator of TGF β properties (6). In breast cancer, the pleiotropic cold-shock protein YB-1 induces proliferation and tumor growth, controls cell cycle machinery, and is generally correlated with poor prognosis and higher aggressiveness (6-10). YB-1 mediated induction of TGF β activation has been previously shown by TGF β -specific reporter gene assays and by induction of the direct TGF β target plasminogen activator inhibitor-1 (PAI-1) and the TGF β receptor 2 (T β R2) mRNA (6). Missing a proof of TGF β activation on the protein level, we aimed to examine the phospho-activation of the TGF β down-stream signaling protein Smad/Mad related protein 2 (Smad2) which belongs to the main pathway of the TGF β signaling network.

Materials and Methods

Cell culture experiments. Breast cancer cells MCF-7 (NCI, Bethesda, MD, USA) were cultivated in Dulbecco's Modified Eagle's Medium (DMEM)/F12 supplemented with 4.5 g/l glucose (Invitrogen, Karlsruhe, Germany), 5% fetal bovine serum (Sigma-Aldrich, Deisenhofen, Germany), 1 mM sodium pyruvate

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(Invitrogen, Karlsruhe, Germany), and 50 µg/ml gentamycin (Invitrogen, Karlsruhe, Germany).

For transfection experiments, 1×10^5 cells/3.5-cm dish were incubated for 24 h and transfected applying Effectene Transfection Reagent (Qiagen, Hilden, Germany). Each transfection was performed with a total amount of 500 ng DNA/dish using the YB-1 overexpression vector pCMV6-YB1 (OriGene Technologies, Rockville, MD, USA) and the control vector pCMV6 (AMS Biotechnology, Abingdon, UK). Activation of TGFβ pathways was done by incubation with 10^{-10} M recombinant human TGFβ1 (R&D, Wiesbaden, Germany).

Protein analysis. For analysis of YB-1 expression, MCF-7 cells were lysed and equal amounts of total protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a nitrocellulose membrane (Whatman, Dassel, Germany) and blocked with 5% non-fat dry milk. Proteins were detected by antibodies directed against Smad2 (BD Biosciences, Heidelberg, Germany), phospho-Smad2 (p-Smad2; Cell Signaling Technology, Danvers, MA, USA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Biodesign, Asbach, Germany). Signals were visualized by peroxidase-conjugated secondary antibodies and LumiGLO Reagent (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instructions.

Data analysis. Specific protein signals were standardized to GAPDH loading control. P-Smad2 signals were subsequently normalized to total Smad2 protein. Data are given as the mean ± the standard deviation (SD). Statistical comparison was performed using the unpaired Student's *t*-test. $p \leq 0.01$ was considered as significant.

Results

Western blot analysis of MCF-7 cells revealed no induction of Smad2 (Figure 1A, upper row) in the presence of TGFβ1 when compared to control samples and normalized to GAPDH loading control (Figure 1A, lower row). Similarly, the overexpression of YB-1 by pCMV6-YB1 vector transfection was not followed by any alterations of TGFβ1 protein level.

In contrast to Smad2 protein's alterations, the incubation with TGFβ1 led to an increase of phosphorylated Smad2 forms (Figure 1A, middle row) indicating that posttranscriptional modifications, such as protein phosphorylation and the change of oligomerization state, may be crucial mechanisms for Smad2 activation and control.

The impact of TGFβ1 and YB-1 on Smad-2 phosphorylation was evaluated by Western blot analysis, using antibodies directed against p-Smad2. Notably, induction of YB-1 simultaneously to TGFβ1 overexpression resulted in an amplified phosphorylation of Smad2. Quantification of total Smad2 and phosphorylated Smad2 forms demonstrated a shift in Smad2 phospho-status (Figure 1B). When related to total protein (p-Smad2/Smad2) the phosphorylation of Smad2 was significantly enhanced. The unique stimulation with TGFβ1 caused a 1.6 ± 0.1 -fold increase in relative p-Smad2 compared with baseline activity. When simultaneously overexpressing YB-1, however, the relative p-Smad2 level increased to 2.8 ± 0.2 -fold ($p \leq 0.01$).

Discussion

A regulatory impact of YB-1 on cellular TGFβ properties has been described previously (6, 11, 12), whereas involvement of TGFβ's primary downstream signaling factors, the members of the Smad protein family, is poorly characterized. In human fibroblasts, YB-1 antagonizes TGFβ-stimulated expression of collagen type Ia2 (COL1A2) via Smad3 (12). In breast cancer cells, however, Smad2 is the most common downstream transducer in TGFβ signaling (13). Thus, we examined Smad2's phospho-activation by YB-1.

In this study, we showed that MCF-7 breast cancer cells revealed no induction of Smad2 neither in the presence of recombinant TGFβ1 nor during YB-1 overexpression (Figure 1A). These observations are in accordance with the commonly accepted model in which Smad proteins including Smad2 are controlled by post-transcriptional modifications, namely protein phosphorylation and oligomerization, and not by transcriptional regulation. More precisely, Smad2 activation is initiated through TGFβ receptor phosphorylation of the protein followed by association of p-Smad2 with regulatory Smad4. After translocation into the nucleus and binding to accessory co-factors, expression of TGFβ-dependent genes occurs (14, 15).

In contrast to total Smad2 levels, the incubation with TGFβ1 led to an increase of phosphorylated Smad2 forms (Figure 1A, middle row), additionally amplified when overexpressing YB-1 (Figure 1B). Within TGFβ signaling cascades, Smad phosphorylation primarily occurs by serine/threonine kinase activity of the ligand-activated TGFβ receptor complex (15). Besides this main pathway, further kinases have been identified catalyzing Smad protein phosphorylation (16). YB-1 itself exhibits versatile molecular functions including DNA degradation by exonuclease activity (7, 17), however, kinase activity has not been identified yet and thus Smad2 phosphorylation directly catalyzed by YB-1 appears unlikely.

Recently, certain cellular factors have been characterized which indirectly provoke TGFβ signaling and Smad2 phosphorylation. High-mobility group box 1 (HMGB1) for instance is a transcription-like factor in inflammatory processes, inducing Smad2 phosphorylation and subsequently triggering TGF-dependent epithelial-to-mesenchymal transition of epithelial cells (18). The transmembrane glycoprotein mucin 1 (MUC1) has been shown to be significantly involved in the regulation of several regulatory kinases including C-terminal Src kinase (c-Src), phosphatidylinositol-3-kinase (PI3K), protein kinase B (AKT), glycogen synthase kinase 3β (GSK3β), and c-Jun N-terminal kinase (JNK). Although the kinase taking part in Smad2 phosphorylation is still unknown, MUC1 also mediates the phosphorylation of Smad2 in hepatocellular carcinoma cells (19). Interestingly, MUC1 induced Smad2 phosphorylation

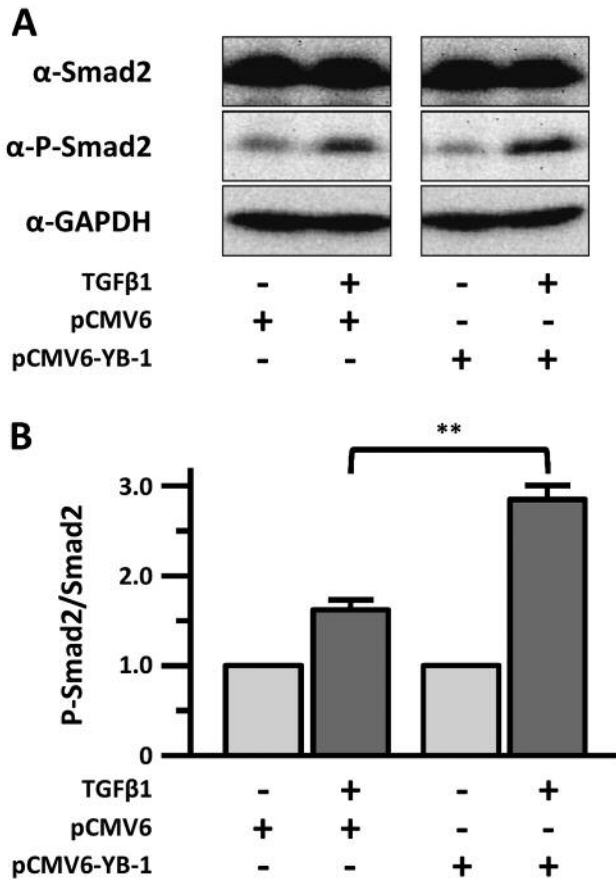


Figure 1. Western blot analysis of p-Smad2 in the absence and presence of TGFβ1 (–/+TGFβ1) and overexpression of YB-1 (pCMV6-YB-1) compared to control transfection (pCMV6). (A) Western blot analysis of total Smad2 (α-Smad2), phosphorylated Smad2 (α-p-Smad2), and GAPDH (α-GAPDH) as loading control. (B) Relative quantification of p-Smad2 standardized to GAPDH signals and normalized to total Smad2 signals. Statistical comparison was performed using the unpaired Student's *t*-test. Columns, mean; bars, \pm SD; ** $p \leq 0.01$.

does not only occur at the C-terminal region of Smad2 (position Ser₄₅₆ and Ser₄₆₇), but also at the linker region (position Ser₂₄₅, Ser₂₅₀, and Ser₂₅₅) (19, 20). Since the antibody used in this study is specific for Smad2 phosphorylation at sites Ser₄₅₆/Ser₄₆₇ only, regulatory effects of alternative phospho-sites may play an important role in alternative Smad2 phosphorylation and should be subject of further investigation.

In conclusion, it can be assumed that YB-1 enhances Smad2 phospho-activation indirectly by general properties in the gene expression machinery. According to the pleiotropic function in breast cancerogenesis, oncogenic YB-1 may represent a promising factor for future diagnosis and therapy of breast cancer.

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