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BAMBI is overexpressed in ovarian cancer and co-translocates with Smads into the nucleus upon TGF-ß treatment

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ABSTRACT

Objective. Transforming growth factor beta (TGF-ß) signaling via Smads plays a central role in carcinogenesis. Bmp and activin membrane-bound inhibitor (*BAMBI*) was initially described as a pseudoreceptor antagonizing TGF-ß receptor activation, thus impairing signaling. Here we wanted to estimate the role of BAMBI in ovarian cancer.

Methods. The function of BAMBI was studied using a cell line model and intracellular localization experiments. The impact of BAMBI expression on patient outcome was estimated by real-time PCR and immunohistochemistry.

Results. We demonstrate for the first time a nuclear co-translocation of BAMBI with Smad2/3 upon TGF-ß treatment. Moreover, overexpression of *BAMBI* in an *in vitro* model led to significantly increased proliferation (doubling time −37.0%, $P=0.010$), migration (+581.2%, $P=0.004$) and resistance to TGF-ß-mediated apoptosis (decrease of apoptosis from 30% in the control cells to 7% in the BAMBI-overexpressing cells). Although—*prima facie*—this fits to the thesis of BAMBI as a pseudoreceptor, it may also be explained by modulation of TGF-ß signaling in the nucleus, leading to the observed pro-oncogenic properties. The tumor promoting impact of *BAMBI* mRNA overexpression *in vitro* could not be confirmed in primary tumor samples, and while nearly all tumor samples showed up-regulation of BAMBI (37.3% 1+, 39.2% 2+, and 16.7% 3+, respectively) compared to undetectable BAMBI in healthy pre- and post-menopausal ovarian epithelia, no impact of BAMBI expression on recurrence free and overall survival could be observed.

Conclusion. These findings provide new insights into the Smad-mediated pathway by inferring that BAMBI is a novel modulator of TGF-ß signaling.

Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy afflicting roughly 6% of women and has become the fourth most frequent cause of cancer-related death of women in western countries. Estimates indicate that one of 70 women will develop ovarian cancer in her lifetime with a median survival rate of approximately 4.5 years. Recent cancer statistics estimated 22,430 new cases and 15,280 deaths per year in the United States alone [1].

Loss of accurate response to growth inhibiting signals, such as transforming growth factor (TGF)-ß occurs frequently during carcinogenesis. TGF-ß affects various cellular processes including regulation of differentiation, migration, apoptosis, and proliferation of epithelial cells [2,3]. The three TGF-ß isoforms (TGF-ß1, TGF-ß2 and TGF-ß3) recognize type I, II, and III TGF receptors (TGF-ßRI, TGF-ßRII and betaglycan or endoglin, respectively), which when bound initiate signaling via the activation of transcriptional co-regulators named Smads [3]. In detail, activation of the TGF-ßRI triggers Smad2 and Smad3 phosphorylation which subsequently translocate from the cytoplasm to the nucleus as a complex with Smad4. Through interaction with specific DNA-binding sites they induce or repress the transcription of corresponding target genes [4].

The functional TGF-ßRII/TGF-ßRI heteromeric signaling complex acts as a tumor promoter via SMAD-dependent and alternative pathways [5–8]. Smad-independent TGF-regulated networks include the signaling molecules RHOA, cell division cycle 42 (CDC42), RAC1, Ras, phosphatidylinositol 3-kinase (PI3K), protein phosphatase 2A (PP2A),

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mitogen-activated protein kinase kinase kinase 1 (MAP3K1, also known as MEKK1), and TGF-activated kinase 1 (TAK1) [3].

Resistance against antiproliferative effects of TGF-ß signaling contributes to the development of many epithelial cancers [2,9], including ovarian cancer [10–13]. Although accumulating knowledge concerning TGF-ß resistance is available, the mechanisms are poorly understood in ovarian cancer. Recently, Sunde et al. found a number of genes involved in TGF-ß signaling which are differentially expressed in ovarian cancer specimens compared to normal ovarian surface epithelium. In particular, genes that inhibit TGF-ß signaling were up-regulated in advanced-stage ovarian cancers while genes that enhance TGF-ß signaling were down-regulated [14].

The pseudoreceptor BAMBI (*B*MP and *a*ctivin *m*embrane-*b*ound *i*nhibitor), formerly also called NMA, is known as an inhibitor of the TGF-ß signaling pathway [15–18]. BAMBI exhibits structural homology to TGF-ßRI but lacks an intracellular kinase domain [16].

Expression of *BAMBI* has been shown to be aberrantly elevated in most colorectal and hepatocellular carcinomas, caused by ß-catenin signaling [15]. In HepG2 hepatoma cells, transcription of *BAMBI* is regulated via a feedback loop which confers binding of Smad3 and Smad4 to the Smad-responsive elements of the *BAMBI* promoter [18].

The significance of TGF-ß signaling raised the question of the function of BAMBI in ovarian cancer. Thus, we characterised the role of BAMBI in an ovarian cancer cell line and evaluated the impact of *BAMBI* expression on ovarian cancer patient outcome.

Materials and methods

Cell lines, BAMBI cloning, and transfection of SKOV3

The human ovarian carcinoma cell lines MDAH 2774 and H134 were cultured in RPMI medium with 10% FCS (fetal calf serum), 50 U ml^{-1} penicillin G, and 50 µg ml^{-1} streptomycin sulfate at 37 °C in a humidified atmosphere of 95% air with 5% CO_2. SKOV3, SKOV3 Co, and SKOV3 BAMBI cells were cultured in McCoy's medium supplemented with 10% FCS, 50 U ml^{-1} penicillin G, and 50 µg ml^{-1} streptomycin sulfate. Selection of stable transfectants was achieved by growth in the presence of 200 µg ml^{-1} G418 and subculturing with 100 µg ml^{-1} G418. Growth medium was supplemented with 1 or 2.5 ng ml^{-1} TGF-ß for at least 1 week with medium change every second day. For *BAMBI* overexpression, the full length ORF of *BAMBI* (acc. no. BC019252) was cloned from the vector pOTB7 (Sma I, Xho I) into the pLP-IRES neo vector (Eco RV). This construct and pLP-IRESneo as a control were transfected into SKOV3 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Stable clones were selectively harvested and subcultured.

Patient material

For mRNA expression analysis, ovarian tumor samples from primary tumors and corresponding clinicopathologic parameters were obtained from 69 patients who underwent optimal debulking surgery at the Charité Hospital in Berlin, Germany. For immunohistochemical studies using paraffin-embedded tissues, 51 ovarian tumor samples were obtained from ovarian tumor patients at primary diagnosis who underwent optimal debulking surgery, and four normal ovaries were obtained from patients without malignant disease, both surgically removed at the Medical University of Vienna (AKH). Relevant clinical information was collected, and tissue samples and clinical data anonymized. Informed consent for the scientific use of biological material was obtained from all patients in accordance to the requirements of the ethics committee of the institutions involved.

Phenotypical characterization of cell lines

Expression of *BAMBI* was measured by quantitative real-time RT-PCR essentially as described previously [19] using the TaqMan® Gene Expression Assays Hs00180818_m1 for BAMBI, and Hs99999909_m1 for HPRT1 or Hs99999907_m1 for beta-2-microglobulin as housekeeping controls (Applied Biosystems, Foster City, CA, USA). Level of expression is represented in arbitrary units. For the BAMBI Western blot analysis, 30 µg of total protein prepared with RIPA^{+}-buffer was separated by 12% SDS-PAGE and blotted on PVDF membranes. The antibodies used were: monoclonal mouse anti-BAMBI (Abnova Corp., Taipei, Taiwan) in a 1:400 dilution, polyclonal goat anti-mouse HRP-conjugate (Calbiochem, Darmstadt, Germany) in a 1:10,000 dilution, and rabbit anti-actin HRP-conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in a 1:500 dilution.

Proliferation (doubling time) was measured in triplicates by subsequent cultivation and cell counting with a CASY cell counter (Innovatis AG, Bielefeld, Germany). Doubling time (t_d) was calculated as follows: $\mu = (\ln(x_t) - \ln(x_0)) / t$, $t_d = \ln(2) / \mu$(x_t, cell number at time t; x_0, cell number at start of experiment; t, duration of experiment).

Migration and invasion assays were performed in a 24-well plate trans-well system (BD Biosciences, San Jose, CA, USA). Therefore 2.5×10^4 cells were seeded in triplicate on 8.0 µm pore size control chambers for migration and the BD Matrigel invasion chambers (pore size: 8.0 µm) for invasion. 30% FCS was used as chemoattractant. After 24 h incubation cells from both sides of the membrane were trypsinised, harvested and reseeded in 96-well plates in appropriate densities. Cells were allowed to settle for 4 h after which cell count was estimated using the Cell Titer-Blue® cell viability assay (Promega, Madison, WI, USA). Results were given in percent calculated as follows: after subtraction of blank values, the fluorescence ($560_{Ex}/595_{Em}$ nm) in cells from the lower side of the membrane (corresponding to the migrated/invaded cells) was divided by the sum of fluorescence values in cells from both sides of the filter (corresponding to all seeded cells) and multiplied with 100. Apoptosis was analysed as follows: upon treatment of the cells the medium was aspirated, cells were trypsinised, harvested and washed twice with ice cold PBS. Then the cells were resuspended in Binding Buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells ml^{-1}. Aliquots of 500 µl were prepared for each probe in FACS tubes and complemented with Annexin-V-FITC and PI (propidium iodide), respectively, or in combination for gating and analysis of the cells. After an incubation period of 15 min in the dark apoptosis was determined by flow cytometry.

Immunocytochemical and immunohistochemical staining

For immunocytochemical staining cells were grown overnight on sterile four-well Lab-Tek™ Chamber Slides™ (Nagle Nunc International, Rochester, NY, USA). After washing with PBS, cells were fixed in 3% formaldehyde/PBS for 20 min at room temperature and permeabilized with 0.5% Triton X-100/PBS for 5 min. Endogenous peroxidase activity was blocked by a 10 min incubation with 3% H_2O_2/PBS. Subsequently, cells were blocked for 30 min with 0.2% fish gelatine/PBS and incubated with the primary antibody diluted in 0.2% fish gelatine/PBS for 60 min at room temperature. Primary antibodies were as follows: anti-BAMBI, 1:200; anti-Smad2/3, 1:100 (BD Biosciences, San Jose, CA, USA). After washing with PBS, biotin-conjugated secondary antibody goat anti-mouse, 1:200 in 0.2% fish gelatine/PBS (BA9200, Vector Laboratories, Burlingame, CA, USA) was applied for 30 min followed by incubation with the streptavidin ABComplex–HRP (ABC-Kit from Dako, Glostrup, Denmark) for 45 min and DAB + (Dako) according to the manufacturer's instruction. Finally cells were stained with hematoxylin/eosin and mounted in Eukitt (O. Kindler GmbH, Freiburg, Germany). Microscopy was performed on an Olympus BX50 upright light microscope (Olympus Europe, Hamburg, Germany) equipped with the Soft Imaging system CC12.

For immunohistochemical staining [20], 5 µm paraffin tissue sections were deparaffinized with xylene and rehydrated by incubation in serial dilutions of ethanol. Subsequently, antigen retrieval was