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SATB2 expression increased anchorage-independent growth and cell migration in human bronchial epithelial cells

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ABSTRACT

The special AT-rich sequence-binding protein 2 (SATB2) is a protein that binds to the nuclear matrix attachment region of the cell and regulates gene expression by altering chromatin structure. In our previous study, we reported that *SATB2* gene expression was induced in human bronchial epithelial BEAS-2B cells transformed by arsenic, chromium, nickel and vanadium. In this study, we show that ectopic expression of SATB2 in the normal human bronchial epithelial cell-line BEAS-2B increased anchorage-independent growth and cell migration, meanwhile, shRNA-mediated knockdown of SATB2 significantly decreased anchorage-independent growth in Ni transformed BEAS-2B cells. RNA sequencing analyses of SATB2 regulated genes revealed the enrichment of those involved in cytoskeleton, cell adhesion and cell-movement pathways. Our evidence supports the hypothesis that SATB2 plays an important role in BEAS-2B cell transformation.

1. Introduction

The special AT-rich sequence-binding protein 2 (*SATB2*) gene is located on chromosome 2, spans 191 kilobases, and encodes a 82.5 kDa protein. First reported in 2003, SATB2 is a member of the SATB transcription factor family that binds to AT-rich sequences in the nuclear matrix (Dobreva et al., 2003) and regulates gene expression by orchestrating chromatin organization and remodeling. Subsequent findings showed that SATB2 is involved in skeletogenesis (Dobreva et al., 2006) and neuronal development (Gyorgy et al., 2008). The expression of SATB2 in adult tissues is restricted to the brain and lower gastrointestinal tract (Lindskog et al., 2014). However, the role SATB2 plays in cancer development and prognosis seems to be tumor-specific. In a laryngeal squamous cell carcinoma study, lower expression of SATB2 was correlated with a more advanced tumor grade and higher tumor recurrence rate, and overexpression of SATB2 reduced the tumorigenicity of HEp2 cells in vitro and in vivo (Liu et al., 2012). In colorectal cancer, high expression of SATB2 is associated with a favorable prognosis and increased sensitivity to radiation and chemotherapy (Brocato & Costa, 2015), and overexpression of SATB2 in DLD-1 cells reduced anchorage-independent growth and tumor size when injected to nude mice (Mansour et al., 2015), indicating a tumor suppressor role for SATB2. On the other hand, high SATB2 expression was observed in osteoscarcoma tumors cells, and migration and invasion was decreased by SATB2 knockdown (Mansour et al., 2015; Seong et al., 2015). Moreover, in a breast cancer study, SATB2 mRNA expression was associated with increased tumor grade and poor overall survival (Patani et al., 2009) indicating a tumor promoting activity.

In our previous study (Clancy et al., 2012), we analyzed transformation of the immortalized normal human bronchial epithelial cell-line BEAS-2B by 4 metals, including nickel (Ni), hexavalent chromium (Cr), arsenic (As) and vanadium (V). Among these metals, Ni, As and Cr are known carcinogens associated with many types of cancer in humans (Salnikow & Zhitkovich, 2008; Martinez et al., 2011), and V can function as a tumor promoter of mice lung cancer (Rondini et al., 2010). While each of these metals has their own unique gene expression signature in transformed BEAS-2B cells, the expression of SATB2 is uniformly increased in every metal transformed clones (Clancy et al., 2012). Given the gaps in our understanding of metals carcinogenesis, investigating the role that SATB2 plays in the cellular transformation could elucidate the mechanisms involved in this process.

2. Materials and methods

2.1. Cell culture

The BEAS-2B immortalized human bronchial epithelial cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium (DMEM,

Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; G418, Geneticin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2 H-tetrazolium); SATB2, special AT-rich sequence-binding protein 2; shRNA, short hairpin RNA; TBS-T, Tris Buffered Saline with Tween® 20; Ni-BEAS-2B, nickel transformed BEAS-2B cells; Ni-BEAS-2B -shSATB2-B, Ni-BEAS-2B transfected with SATB2 shRNA-B; Ni-BEAS-2B-shSATB2-C, Ni-BEAS-2B transfected with SATB2 shRNA-C.

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Invitrogen, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and 1% of penicillin/streptomycin (GIBCO, Grand Island, NY). The cells were routinely cultured at 37 °C with 5% CO_2.

2.2. Stable transfection of SATB2

The full-length human SATB2 cDNA cloned into the pcDNA3.1 vector was kindly provided by Dr. Rudolf Grosschedl (Max Planck Institute of Immunobiology and Epigenetics). BEAS-2B cells were transfected with pcDNA3.1 vector or pcDNA3.1-SATB2 DNA using Lipofectamine® LTX Reagent with PLUS™ Reagent (Life technologies, New York, NY) according to manufacturer's protocol. Briefly, when cells reached 80–90% confluency in a 6-well plate, transfection was carried out. For each transfection well, 2.5 μg of plasmid DNA combined with 2.5 μl of PLUS reagent in 150 μl of serum-free media. This was then combined with a mixture of 10 μl Lipofectamine LTX in 150 μl serum free media. This final mixture was then incubated for 5 min before being added to the cells. Forty-eight hour after transfection, cells were harvested and plated in two 10 cm^2 tissue culture dishes with fresh medium containing G418 (500 μg/ml, GIBCO BRL, Gaithersburg, MD). Colonies were picked and expanded after two weeks of selection.

2.3. Small Interfering RNA (shRNA) transfection

Ni transformed BEAS-2B cells (Ni-BEAS-2B) were cultured in Dulbeco's modified Eagle medium (DMEM) with 10% FBS and 1% penicillin/streptomycin. Four SATB2 shRNAs (TG301833A, B, C and D) and scramble control shRNA plasmid (TR30013) were purchased from OriGene (Rockville, MD). The sequences of these four construct were as follows: shSATB2-A: 5′-TCCGCAATGCCTTAAAGGAACTGCTCAAA-3′; shSATB2-B: 5′-GTTCAAAGTTGGAAGACTTGCCTGCGGAG-3′; shSATB2-C: 5′-TGAACCAGAGCACATTAGCCAAAGAATGC-3′; shSATB2-D: 5′-AATGTGTCAGCAACCAAGTGCCAGGAGTT-3′.

The knockdown transfection was performed using PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories, Toronto, Ontario, Canada) following the manufacturer's protocol. The cells were placed under selection with 0.5 μg/ml puromycin for one week and harvested for Western blot and real-time qPCR analysis.

2.4. Soft agar assay

Anchorage-independent growth was tested by the ability of cells to grow in soft agar. In brief, a bottom layer of 0.5% 2-hydroxyethylagarose (Type VII low gelling temperature, Sigma-Aldrich, St Louis, MO) and a top layer of 5000 cells in 0.35% 2-hydroxyethylagarose was placed in a 6-well non-treated polystyrene plate. After three weeks, individual colonies were picked from the agar for growth or the wells were stained with 0.005% crystal violet solution in PBS containing 10% methanol. Images of each stained well were scanned using a Bio-Rad Molecular Imager Gel-Doc XR^+ documentation system and Image Lab software (Biorad, Hercules, CA), and colony numbers were estimated using Image J software with defined particle size of 20-infinity pixel unit and circularity of 0.30–1.00. In addition, when seeding cells into the soft agar plate, 200 cells were simultaneously seeded into a 10 cm^2 dish in order to determine the plating efficiency in monolayer culture — defined as the ratio of the number of colonies (formed in cell culture dish) versus the number of cells seeded. After two weeks of incubation, plates were fixed with methanol and stained with 0.05% crystal violet solution, cell colony numbers were determined and plating efficiencies were calculated.

2.5. Real-time PCR analysis

Total RNA was isolated with TRIzol Reagent (Life Technologies, Gaithersburg, MD). Reverse transcription was performed using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). Real time-PCR was performed by using SYBR Select Master Mix (Applied Biosystems, Foster City, CA) on a 7900HT Fast Real-time PCR System (Applied Biosystems). Each sample was run in triplicate. The relative mRNA expression levels were normalized by using *GAPDH* as the endogenous control. The sequences of primers used to amplify each gene were forward: 5′-TCTCCCCAAACACACCATCA-3′/reverse: 5′-GCAGCTCCTCGTCCTTATATTCA-3′ for *SATB2*, and forward: 5′-TGCACCACCAACTGCTTAGC-3′/reverse: 5′-GGCATGGACTGTGGTCATGA-3′ for *GAPDH*. The results were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

2.6. Western blotting analysis

Cells were lysed with cell extraction buffer (Invitrogen, Camarillo, CA) supplemented with proteinase inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Fifty μg of each protein lysate was loaded and electrophoresed in a 12% SDS-polyacrylamide gel, then transferred to a PVDF membrane. After blocking in 5% skim milk in TBS-T for 2 h at room temperature, the membrane was incubated with SATB2 mouse monoclonal antibody (Abcam, Cambridge, MA., ab51140)502, 1:100) overnight at 4 °C, and then probed with HRP labeled goat anti mouse secondary antibody (1:2000) for 1 h at room temperature before the visualization by the chemiluminescene. Quantification of immunodetected proteins was performed using Image J software.

2.7. Scratch test

Cells (1×10^6) were plated into 35 mm culture dishes with 2×2 mm grids (Nunc) on the bottom. Upon reaching confluency, a single scratch was made across the monolayer using a 200 μL pipette tip held perpendicular to the plate bottom. The plate was then gently washed with medium to remove detached cells, and fresh DMEM media containing 2.5% of FBS were added back into the plates. Images were acquired at 0, 5, and 10 h after the scratch using a Nikon digital DS-Fi1-U3 camera unit controlled by NIS-Elements F3.2 software on an Nikon Eclipse TS100 microscope (Nikon Instruments Inc., Tempe, AZ).

2.8. MTS assay

Cell proliferation was assessed using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI) to measure the absorbance of formazan, the bioreduced product tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium (MTS), which is directly proportional to the number of viable cells in culture. Briefly, 1×10^4 cells were seeded into each well of 96-well microplates overnight to allow cells to attach and grow for 24 h. Subsequently, 20 μl of the combined MTS/PMS solution was added directly into each well of the 96-well assay plate containing 100 μl of cells in culture medium. The plate was incubated at 37 °C for additional 2 h and absorbance at 490 nm was measured using the SpectraMax®M2 Microplate Reader (Molecular Devices®, Sunnyvale, CA). This assay was performed in triplicate.

2.9. RNA sequencing

Total RNA samples from two vector transfected (vector-4 and vector- 5) and two SATB2 transfected (SATB2-3 and SATB2-7, see Fig. 1) BEAS-2B clones were converted into cDNA libraries using a Truseq RNA Sample Preparation v2 Kit (Illumina, San Diego, CA). Reads were aligned to Ensembl gene model (Homo_sapiens.GRCh37.71.gtf) (Morgan et al., 2009) using HTseq (0.6.1.p.1) (Robinson et al., 2010). For the statistical analysis, DESeq2 R/Bioconductor package was used and the raw reads counts were normalized by using trimmed mean of M-values normalization method (Oshlack et al., 2010). The common dispersion and statistical significance for genes cross sample groups