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Minor expression of fascin-1 gene (FSCN1) in NTera2 cells depleted of CREB-binding protein

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Abstract

CREB-binding protein (CBP) is a transcriptional coactivator whose mutations may cause a generalized perturbation of gene expression. We silenced the CBP gene in NT2 neuronal precursor cells by RNA interference. Hybridization experiments on 1.2 K human cDNA microarrays showed that the FSCN1 gene, encoding for fascin-1 protein, was clearly less expressed in CBP-depleted cells than in controls. This reduction was confirmed by Real Time PCR and Western blotting assays. We also analyzed FSCN1 expression profile during NT2 neuronal differentiation induced by retinoic acid (RA), showing that FSCN1 was up-regulated during neurogenesis. This mRNA increasing suggests the importance of fascin-1 in the formation of mature neurons, in accordance with its actin-bundling function and its localization in neurites and neuronal growth cones. The lower amount of FSCN1 transcript in the absence of the CBP factor was also established in RA-treated cells. In conclusion, this research supports FSCN1 as a novel marker of NT2 neuronal differentiation and the possible role of CBP in its regulation.

Keywords: CBP transcriptional coactivator; NT2 cells; Small interfering RNA; Microarray analysis; Real Time PCR; Fascin-1 protein

CREB-binding protein (CBP) is a coactivator that promotes gene transcription by providing a scaffold for numerous DNA-binding transcription factors and the basic transcription machinery. Furthermore, CBP contains an intrinsic histone acetyltransferase (HAT) activity and may modulate gene expression through acetylation of nucleosomal proteins and various regulatory factors [10,13]. CBP and the closely related p300 protein are involved in multiple physiological processes and several studies indicate that they have similar functions: chromosomal translocations affecting p300 and CBP genes cause hematological malignancies, and point-mutations or heterozygosity of these genes have been found in various kinds of tumors [13,28]. Despite their extensive similarities, CBP and p300 have unique and different functions. Retinoic acid-induced differentiation of F9 embryonal carcinoma cells was inhibited by a ribozyme directed against the p300 mRNA but not by a CBP ribozyme [15]. In a murine model, a full dose of CBP is crucial for hematopoietic stem cell self-renewal; on the contrary, only p300 is essential for a correct hematopoietic differentiation [25]. In addition, CBP haplo-insufficience is found in Rubinstein–Taybi syndrome (RTS) that is characterized by growth and mental retardation, multiple congenital malformations and increased tumor risk [6,22]. No mutations of the p300 cofactor have been described in human congenital diseases. The presence of a particular spectrum of affected tissues in RTS indicates that CBP mutations modify the expression of a restricted class of genes, many of which are as yet unidentified.

In this work, we propose an in vitro cellular system to dissect the biological CBP function in human NTera2/cloneD1 (NT2) neuronal precursor cells [18,23]. In particular, we suppressed CBP expression by RNA interference (RNAi) using a specific small interfering RNA (siRNA), and subsequently we looked for genes that presented modified transcriptional profiles. Microarray, Real Time PCR and Western blotting assays revealed a significant down-expression of the gene encoding the actin-bundling protein fascin-1 (FSCN1) in CBP-depleted cells. Furthermore, we excluded that the reduced amount of FSCN1 gene was attributable to an unspecific effect of type 1 interferon (IFN) activation.

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NT2 cells were maintained in proliferating conditions or differentiated into mature neurons by treatment with retinoic acid (RA) for 14 days and plating on a poly-D-lysin/murine laminin (PDL/LAM) matrix, as previously described [18].

CBP sense (5′-CGGCACAGCCUCUCAGUCAdTdT-3′) and antisense (5′-UGACUGAGAGGCUGUGCCGdTdT-3′) RNA oligos were designed against the human CBP transcript (GeneBank accession no. U85962) according to the guidelines available at http://www.dharmacon.it website. GL2 siRNA, directed against *Photinus pyralis* luciferase transcript, was used as control [9]. Sense and antisense RNAs, chemically synthesized by Dharmacon Research Inc., were annealed in order to create the duplex short (21-mer) interfering RNA (siRNA). Approximately 5×10^4 NT2 cells were plated in 24-well plates and, the following day, transfected with 100 nM siRNA and 3 μl of oligofectamine reagent (Invitrogen) according to datasheet instructions. Cells were harvested and lysed before and at different hours after transfection to obtain total RNA and/or proteins.

One microgram of total RNA, extracted with TRIzol reagent (Invitrogen), was reverse transcribed by GeneAmp RT-PCR kit following the manufacturer's instructions (Applied Biosystems). CBP forward (5′-TGTTTTCGCGAGCAGGTG-3′), and reverse (5′-AGCCTGATTAATTAAGCTATG-3′) primers, designed to specifically detect human CBP mRNA, were synthesized by MWG-Biotech. Two microliters of each cDNA template were amplified in a 50 μl reaction volume at the following conditions: denaturing step at 95 °C for 5 min, 30 cycles at 95 °C/1 min, 56 °C/1 min, 72 °C/2 min and a final extension at 72 °C for 10 min. The endogenous control glyceraldehyde-3-phosphate dehydrogenase (GAPDH; GeneBank accession no. NM002046) was amplified using specific primers (forward: 5′-ACCACAGTCCATGCCATCAC-3′ and reverse: 5′-TCCACCACCCTGTTGCTGTA-3′; 60 °C annealing, 28 cycles). PCR products were detected in a 1.5% agarose/ethidium bromide gel.

For Western blotting analysis, protein extracts and hybridization conditions were as previously reported [18] using the following primary antibodies: 1:400 anti-CBP (C-20/sc-583, Santa Cruz Biotechnology), 1:1000 anti-fascin (55K-2/sc-21743, Santa Cruz Biotechnology), 1:500 anti-p300 (clone RW128, Upstate Biotechnology) and 1:6500 anti-β-actin (clone AC-15, Sigma). Blots were incubated with the appropriate peroxidase-conjugated secondary antibodies (Jackson Immunoresearch Laboratory) and the specific bands were detected by enhanced chemiluminescence (ECL, Amersham).

In immunofluorescence experiments, NT2 cells were plated on glass coverslips in a 24-well plate and fixed, the following day, with 4% paraformaldehyde for 20 min at RT. After a permeabilization with 0.1% TritonX-100 for 10 min at RT, cells were incubated with 1:20 mouse monoclonal anti-CBP antibody (C-1/sc-7300, Santa Cruz Biotechnology) and with 1:100 secondary Texas Red-conjugated antibody (Jackson Immunoresearch Laboratory). Cells were washed in 1× PBS and visualized under a Leica fluorescence microscope. Staining without primary antibody was used as a negative control (data not shown).

Microarray analysis was performed with Atlas cDNA Expression Arrays 1.2 III, including 1176 human cDNA spotted on positive nylon membranes (BD Biosciences, Clontech). Total RNA was isolated from NT2 cells, 72 h after transfection with CBP or GL2 siRNA, using TRIzol reagent. One hundred micrograms of RNA were treated with 10 U DNase I (Sigma) for 30 min at 37 °C and subsequently extracted with phenol:chloroform:isoamyl alcohol (25:24:1), precipitated with 100% ethanol and washed twice with cold 70% ethanol. cDNA probes, from CBP− (CBP siRNA-transfected cells) and CBP+ (GL2 siRNA-transfected cells) samples, were generated from 10 to 20 μg of RNA samples using [α-^{32}P]dATP (3000 Ci/mmol, 10 μCi/μl; Amersham Biosciences), CDS Primer Mix (for Atlas Human 1.2 Array III) and the reagents included in the Atlas Pure Total RNA Labeling System (BD Biosciences, Clontech) according to the user manual. Prehybridization, hybridization and washings were performed as reported in Atlas protocol. Membranes were exposed to a phosphor screen (Eastman Kodak Company, Rochester, NY) at RT for 1–3 days. Phosphorimager scanning was performed to obtained signals from CBP+ and CBP− hybridizations that were processed using AtlasImage v2.7 software (BD Biosciences, Clontech). The set of housekeeping genes was used to normalize mRNA levels and changes in expression were considered significant when the ratio of the signals from CBP− array to CBP+ array had a value $\geq$1.7.

For Real Time PCR assays, 1.5 μg of total RNA from CBP+ and CBP− cells were converted into cDNA with TaqMan Reverse Transcription Reagents Kit (Applied Biosystems) using 20 U RNase Inhibitor, 62.5 U Reverse Transcriptase and 2.5 μM Oligo(dT)$_{16}$ in a 50 μl reaction volume at the following conditions: 25 °C/10 min, 48 °C/30 min, 95 °C/5 min. Unlabelled primers and TaqMan MGB probe (6-FAM dye-labeled) were supplied as Assay-on-Demand Gene Expression product for human FSCN1 transcript (Hs00602051_mH; Applied Biosystems) and as PDAR (Pre-Developed TaqMan Assay Reagents) product for human GAPDH mRNA (4333764T; Applied Biosystems), used as control. Real Time PCR experiments were performed in a ABI PRISM 7000 Sequence Detection System (Applied Biosystems) utilizing 1.5 μl cDNA, 1× TaqMan Universal PCR Master Mix/No AmpErase UNG (Applied Biosystems) and 1× Assay-on-Demand or PDAR in a final volume of 25 μl at the following cycler parameters: 95 °C/10 min followed by 40 cycles at 95 °C/15 s and 60 °C/1 min. All templates were run in triplicate and the ABI Prism 7000 SDS Software (Applied Biosystems) was used to determine the cycle, called threshold cycle or C_t, in which fluorescence significantly increased above background. Standard curves for FSCN1 and GAPDH mRNAs were generated with a dilution series of cDNA template (1–10–40–100–300 ng) and the amplification efficiencies were calculated by the slop of the regression line (Efficiency = $[10^{-1/\text{slope}}] - 1$). The quantifica-