INVOLVEMENT OF β-CATENIN IN TRANSCRIPTIONAL REGULATION OF REST IN NEUROBLASTOMA CELLS

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Summary

The repressor element-1 (RE-1) silencing transcription factor (REST) plays a crucial role in neural development. REST is one of 50 target genes of the Wnt signaling pathway whose activation allows β-catenin to move into the nucleus where, together with the factor TCF, it binds LEF-responsive elements and activates expression of target genes involved in oncogenesis. This study found that in neuroblastoma cells differentiated with phorbol 12-myristate 13-acetate (PMA), REST is regulated in a time-dependent manner and β-catenin is involved in its regulation. Reporter gene assays showed that, in neuroblastoma cells, PMA could lead to β-catenin movement in the nucleus where it regulates the transcription of REST gene by binding to LEF sites within a region of its promoter, here called 'CTRL'.

Keywords: transcription factors, neuroblastoma, Wnt signaling pathway, phorbol 12-myristate 13-acetate, RE1 silencing transcription factor.

Introduction

During the organism life, gene expression leads to cellular processes such as proliferation, differentiation and programmed cell death. Structural and functional identity adopted by cells depends on activation or inhibition of specific gene combinations (1). Previous studies dealt with functions and mechanisms that regulate the expression of an important transcription factor called REST (repressor element-1 silencing transcription factor) involved in oncogenesis. Although REST function was initially associated to neuronal gene repression in non neural cells (2), later, a more complex tissue-dependent expression was observed (3) (4). REST was seen to regulate the expression of specific genes involved in neurogenesis: they need to be silenced in the first phases of neural differentiation and then to be induced in terminal differentiation. REST influence in maintenance of correct gene expression confirms his key role in neural cells. In fact, REST loss (5) (6) and over-expression (7) were associated to different processes that are neural terminal differentiation and neoplastic progression (8).

REST is one of 50 target genes of Wnt proteins whose signaling is known to regulate REST expression directly by TCF (T Cell Factor)/β-catenin protein complex (9). Wnt signaling activation stabilizes β-catenin in the cell cytoplasm. Then, it moves into the nucleus and activates gene expression together with TCF/LEF factors (10). β-catenin over-expression was associated to several kinds of cancer such as colon and breast cancer (11). This regulation could aid to neoplastic malignancy by increasing or maintaining REST expression.

REST gene has a very complex structure (Fig. 1) with seven exons and six introns. The first transcription codon (ATG) is collocated into exon 4. The exons 1-2-3 are in REST regulatory region and codify for no protein motif (12). Bioinformatics analysis of REST gene promoter identified a responsive site in its exon 1 for TCF transcription factor (14).

The aim of this work was to investigate putative regulation mechanisms of REST transcription factor by studying Wnt/β-catenin involvement in neuronal differentiation and transcriptional regulation of a specific REST promoter region that it never was previously analyzed.

Figure 1

Figure 1. Genomic structure of human REST gene (11).

Methods

Cell culture

Human neuroblastoma SH-SY5Y cells (American Type Culture Collection: Human neuroblastoma. Cancer Res, 1978, J. Nat. Cancer institute, 1983) (Fig. 2) were grown as monolayers in MEM and Ham's F12 (1:1) medium supplemented with 10% (v/v) fetal calf serum, L-glutamine (2 mM), pyruvic acid (1 mM), 1x non-essential amino acids and 1x antibiotic–antimycotic solution in a humidified environment containing 5% CO2 and 95% air.

Figure 2

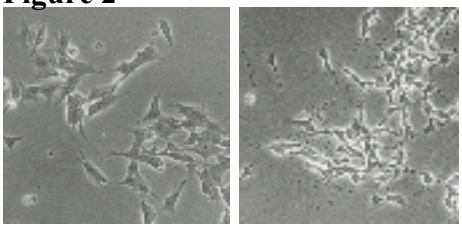


Figure 2. Microphotography of neuroblastoma cell line SH-SY5Y; in the left there are undifferentiated cells; at the right there are differentiated cells with PMA (16nM).

PCR Analysis

For PCR experiments, SH-SY5Y cells were collected from tissue culture flasks, centrifuged (500 x g for 5 min) and rinsed with phosphate-buffered saline. Genomic cellular DNA was extracted using DNAzol reagent (Invitrogen) according to the manufacturer's instructions. Forward and reverse primers used in this work are reported in Table 1; amplification conditions are reported in Table 2.

Table 1

ZONE	PRIMER	PRIMER SEQUENCE	Tm (°C)
FOXO	***FOXO FOR*** Zone	TTGGGATCCATCTTCCTACTGGCAA	61.5
	FOXO REV Zone	AAAAGTTCCCCCCAGCCCTGG	61.9
CTRL	***CTRL FOR*** Zone	AAGGGTATCTTGGAGCGCTTTAGACTTG	61.3
	CTRL REV Zone	ACTTACCGGCCTTCCTCGGTGAG	61.4
FOXO-CTRL	***FOXO FOR*** Zone	TTGGGATCCATCTTCCTACTGGCAA	61.5
	CTRL REV Zone	ACTTACCGGCCTTCCTCGGTGAG	61.4

Table 1. Primer sequences and Melting temperature (Tm) used to amplify the three REST promoter regions FOXO, CTRL and FOXO-CTRL.

Table 2

Amplification Phases	FOXO zone	CTRL zone	FOXO-CTRL zone
Denaturation	95°C; 10 min		
Denaturation	95°C; 1,5 min		
Annealing *35 cicles*	50°C; 45 sec		
Elongation	72°C; 2 min	72°C; 1,45 min	72°C; 2,52 min
Final elongation	72°C; 3 min		

Table 2. Description of working conditions used to amplify the three REST promoter regions, FOXO, CTRL and FOXO-CTRL.

Western blotting analysis

Cells were scraped off in cold phosphate-buffered saline, centrifuged and the pellet resuspended in 100 µl of CER I buffer (NE-PERTM Extraction Reagent; Pierce, Rockford, IL, USA). After 10 min incubation on ice, 5.5 µl of CER II buffer was added and the suspension was resuspended by a vortex, incubated on ice for 1 min then resuspended again. The cytoplasmic fraction was separated by centrifugation at 16,000 x g for 5 min. To obtain the nuclear extract, the cell pellet was resuspended in 50 µl of NER buffer and incubated on ice for 40 min. The soluble proteins in the lysate were separated by centrifugation at 16,000 x g for 10 min at 4°C. The protein content was quantified using a BCA protein assay (Pierce). Nuclear or cytoplasmic extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cruz marker (sc-2035; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was also loaded on the gel as molecular weight standard. Proteins were then transferred to HybondTM ECLTM nitrocellulose membranes (Amersham Biotech, Milan, Italy), which were blocked in a solution of 5% non-fat milk TBS (10 mmTris–HCl, pH 8, containing 150 mm NaCl) with 0.1% Tween 20 for 1.5 h at room temperature (25°C). Blots were probed with the same solution and anti-REST antibody for 3 h, or anti-histone H1 antibody at room temperature. Dilutions were 1:50 for REST antibody, 1:1000 for β-catenin antibody, 1:1000 for histone H1 antibody and 1:5000 for β-actin antibody. Membranes were incubated with peroxidase-conjugated anti-mouse secondary antibodies (Santa Cruz

Biotechnology) at room temperature for 1.5 h and the blots were finally developed with SuperSignal West Pico chemiluminescent substrate according to the manufacturer's protocol (Pierce). Blot images were digitally acquired by LAS3000 (Fujifilm Corporation Image). AIDA (Raytest Isotopenmessgeräte) was used for semiquantitative analysis of protein expression.

Small interfering RNA

A pre-designed and validated duplex small interfering RNA (siRNA) for β-catenin was used. A Stealth™ RNAi negative control duplex with a GC content similar to that of siRNA and a scrambled siRNA duplex were used as negative controls. Duplex siRNAs were transfected into SH-SY5Y cells using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The transfection efficiency of each duplex siRNA was confirmed using the Block-IT™ fluorescent oligo (Invitrogen). About 80–90% of cells were transfected, as also confirmed by flow cytometry (data not shown).

Plasmid constructs, cell transfection and reporter gene assays

All reporter plasmids are based on the pGL3Basic vector system (Promega, Madison, WI, USA) containing the luciferase reporter gene. Briefly, three REST promoter fragments were amplified by PCR, cloned into pCRII Blunt TOPO (Invitrogen), sequenced (BMR Genomics, Padua, Italy) and inserted into pGL3Basic (Promega) plasmid between the *KpnI* and *XhoI* restriction sites. SH-SY5Y cells were plated in 24-well dishes and at 50–60% confluence were transiently transfected with each REST promoter/luciferase reporter plasmid (1.5 mg/well) and pSV-bGal (0.5 mg/well; Promega) using EXGEN 500 Transfection Reagent (Fermentas, Hanover, MD, USA); 24 h after transfection cells were exposed to PMA 16nM for 24 h-4d and β-catenin siRNA 50nM for 48 h, or left untreated. Finally, transfected cells were lysed in Reporter Lysis Buffer (Promega) and samples collected. Luciferase and β-galactosidase activities of each lysate were measured with the Bright-Glo™ Luciferase Assay System and Beta-Glo™ Assay System (Promega) respectively, according to the manufacturer's instructions.

Data analysis

To quantify the intensity of the bands, membranes were scanned and analysed by an imaging densitometer (GS-700; Bio-Rad, Hercules, CA, USA). The bands were quantified with the Molecular Analyst™ image analysis software (Bio-Rad). Statistical significance was determined by Newman-Keuls test after ANOVA using GraphPad Prism (version 3.0; GraphPad Software, Inc., San Diego, CA, USA). P-values<0.05 were considered to be significant.

Results

PMA effect on β-catenin and REST transcription factor protein levels

In order to investigate a putative regulatory role of β-catenin on REST transcription during neuronal differentiation, SH-SY5Y cell line was exposed to PMA for time-course going from 24 hours (h) to 4 days (d) in complete culture medium. SH-SY5Y cells exposed to PMA show neurite extension and specific marker expression such as neuro-specific enolase (NSE) and GAP-43 protein (14).

Western blot results, using the specific β-catenin monoclonal antibody, show that β-catenin nuclear and cytoplasmic levels increase at 24 h up to 4 d of exposition to PMA (Fig. 3).

As showed in western blot analysis using anti-REST monoclonal antibody (Fig. 4), this protein levels have a time-dependent trend; the reduction at the last treatment phase to PMA is in accordance to a marked neuronal differentiation (15).

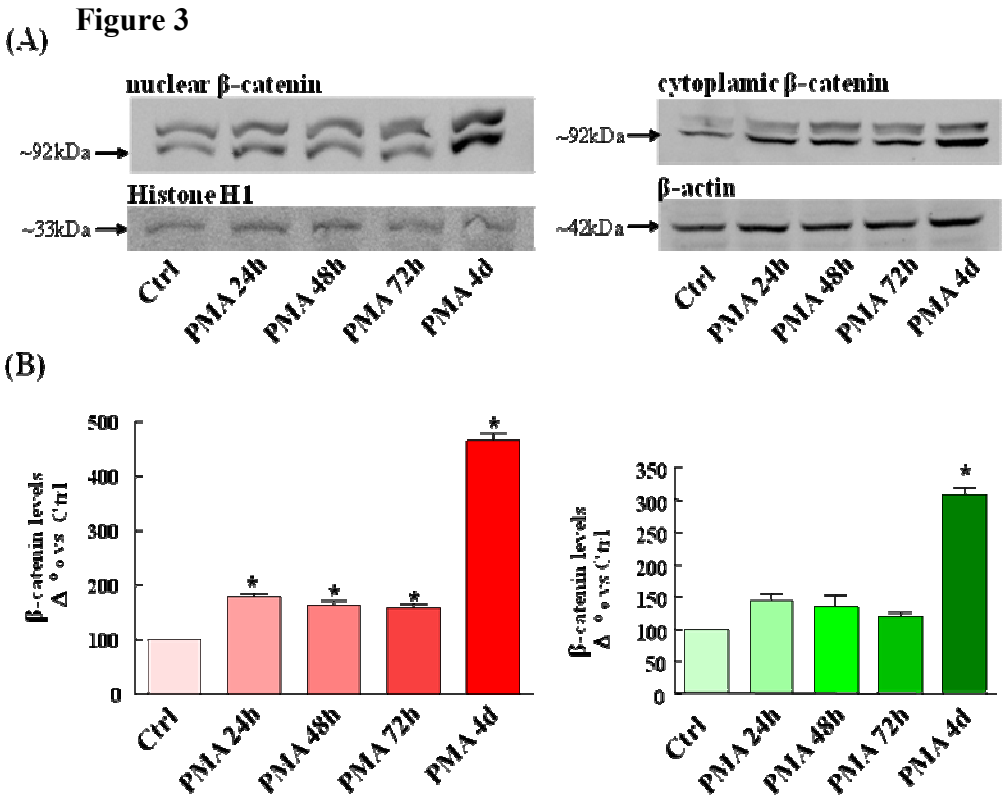


Figure 3. (A) Representative western blot experiment showing β-catenin regulation in nuclear and cytoplasmic extracts of SH-SY5Y cells exposed to PMA 16 nM for 24 h-4 d or left untreated (CTRL). (B) Densitometric analysis of the bands. Histone H1 and β-actin were evaluated in the same samples and used for loading control. *p<0.001 compared with CTRL.

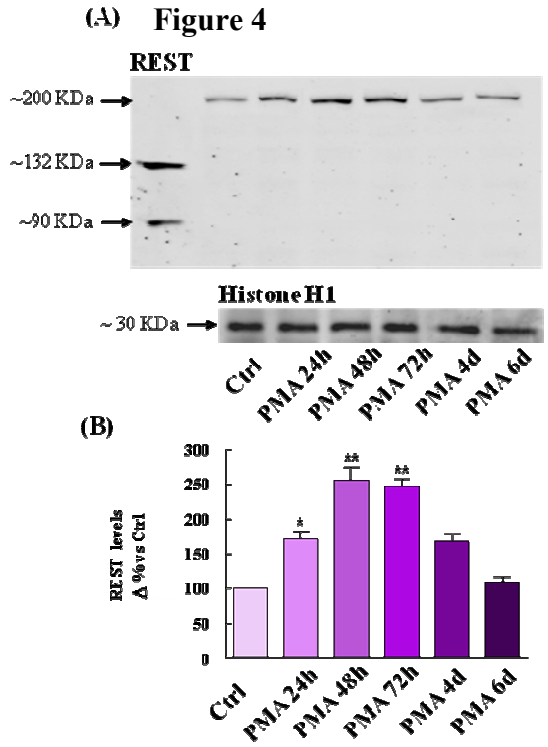


Figure 4. REST regulation in nuclear cell extracts of SH-SY5Y cells exposed to PMA 16 nM for 24 h-6 d or left untreated (CTRL). (A) Representative western blot analysis. (B) Densitometric analysis of the bands. Histone H1 was evaluated in the same samples and used for loading control. *p<0.05 compared with CTRL, **p<0.01 compared with CTRL.

Analysis of β-catenin protein expression in SH-SY5Y cells exposed to β-catenin siRNA

In order to evaluate β-catenin role in REST regulation, SH-SY5Y cells were exposed to PMA and siRNA specific for β-catenin mRNA. Previous studies reported that the treatment for 48 hours with siRNA 50 nM is needed to reduce the levels of this protein in a significant manner. Western blot analysis of β-catenin protein (Fig. 5) shows that the exposition of SH-SY5Y cells to both siRNA and PMA is able to reduce β-catenin levels than to only PMA one.

Figure 5

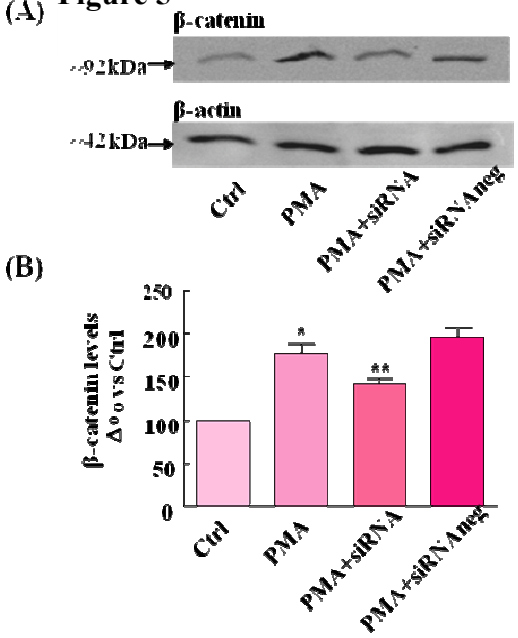


Figure 5. (A) Reduction of β-catenin protein levels in cell extracts of SH-SY5Y cells exposed to PMA 16 nM and β-catenin siRNA (50nM) for 48 h. (B) Densitometric analysis of the bands. β-actin was evaluated in the same samples and used for loading control. *p<0.001 compared with CTRL,*p < 0.01 compared with PMA.

Effect of PMA and siRNA specific for β-catenin on REST expression in SH-SY5Y cells

In order to investigate a putative involvement of β-catenin in REST regulation during neuronal differentiation inducted by PMA, neuroblastoma cells were exposed to β-catenin siRNA and to PMA. REST nuclear level achieved by western blot (Fig. 6) is significantly reduced and its effect observed with only PMA is turned off. These results get stronger the hypothesis that β-catenin could have a regulatory role on REST transcription factor.

Figure 6

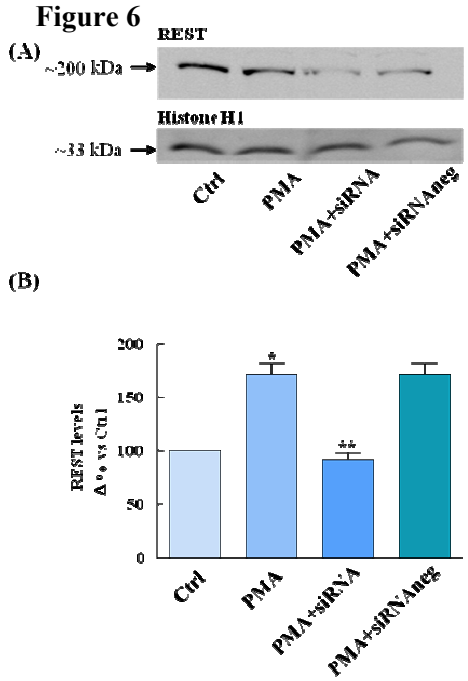


Figure 6. (A) Reduction of REST nuclear levels in SH-SY5Y cells exposed to PMA (16nM) and β-catenin siRNA (50nM) for 48 h. (B) Densitometric analysis of the bands. Histone H1 was evaluated in the same samples and used for loading control. *p<0.001 compared with CTRL, **p<0.01 compared with PMA.

Analysis of REST transcription factor in SH-SY5Y cells transiently transfected with a plasmid that contains β-catenin cDNA sequence

Since in exon 1 of REST gene there is the responsive element for TCF/β-catenin transcription factor, SH-SY5Y cells were transiently transfected with the recombinant plasmid pcDNA3.1^{+}+β-catenin (Fig. 7) that codifies β-catenin murine gene, in order to go into more depth β-catenin effect on REST. Previous studied reported that β-catenin interacts with REST forming a complex that is able to bind DNA in specific manner and to induce the expression of several genes involved in Wnt signaling (11). 48 hours after transfection, nuclear and cytoplasmic proteins were extracted and β-catenin expression was evaluated by western blot (Fig. 8). Both nuclear and cytoplasmic levels of this protein are increased by β-catenin transfection. Then, REST expression was evaluated with western blot and, as Figure 9 shows, nuclear and cytoplasmic levels of this protein are increased.

Figure 7

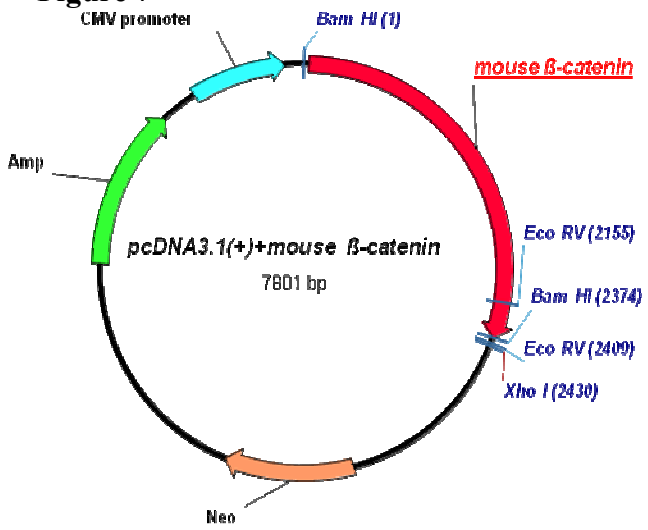
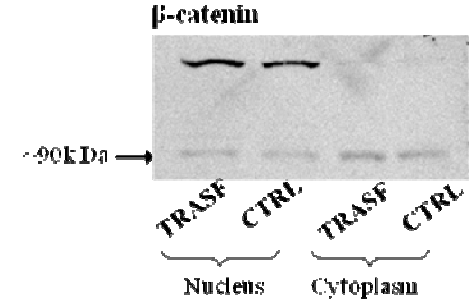


Figure 7. Map of recombinant pcDNA3.1(+)+mouseβ-catenin achieved by cloning mouse β-catenin gene in pCS+mutMMBC6*Myc plasmid. *BamHI* restriction sites used are indicated in blue color.

A Figure 8



B

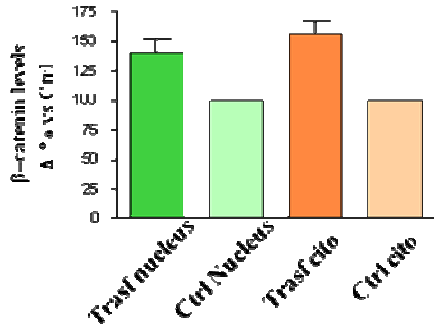


Figure 8. Over-expression of nuclear and cytoplasmic β-catenin protein in SH-SY5Y cells transiently transfected with pcDNA3.1(+)+ mouseβ-catenin. (A) Representative western blot analysis. (B) Densitometric analysis of the bands.

Figure 9

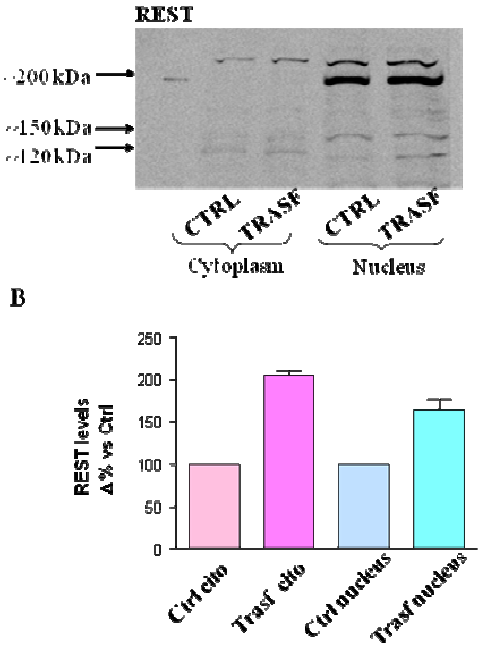


Figure 9. Over-expression of nuclear and cytoplasmic REST protein in SH-SY5Y cells transientlly transfected with pcDNA3.1(+)+mouseβ-catenin. (A) Representative western blot analysis. (B) Densitometric analysis of the bands.

Preparation of reporter vectors that contain different REST gene promoter fragments

In order to verify the presence of responsive elements for several transcription factors on REST gene promoter, a specific 5' regulatory region was analyzed with PROMO 3.0 software and different binding sites were predicted by Vector NTI (Informax).

Three zones called FOXO (831 bp), CTRL (727 bp) and FOXO-CTRL (1427 bp) of a specific REST promoter region that spans -3960 base pairs (bp) to -2531 (bp) position (considering the start codon adenine as +1) (Fig. 10A) were amplified from SH-SY5Y cells genomic DNA by PCR. The three amplificated regions were cloned in pCRII blunt-TOPO cloning vectors and then sub-cloned in pGL3basic reporter vectors, upstream to Luciferase gene. The prediction analysis of these regions shows the presence of many transcription factor binding sites. In FOXO (fig. 10B) there are five binding sites for retinoic acid, two for FoxO3a and two for TCF4/β-catenin. In CTRL (fig. 10C) there are three binding sites for retinoic acid, two for FoxO3a and two for REST itself (RE1). These sequence predictions were verified with enzymatic techniques by using appropriate restriction enzymes.

Figure 10

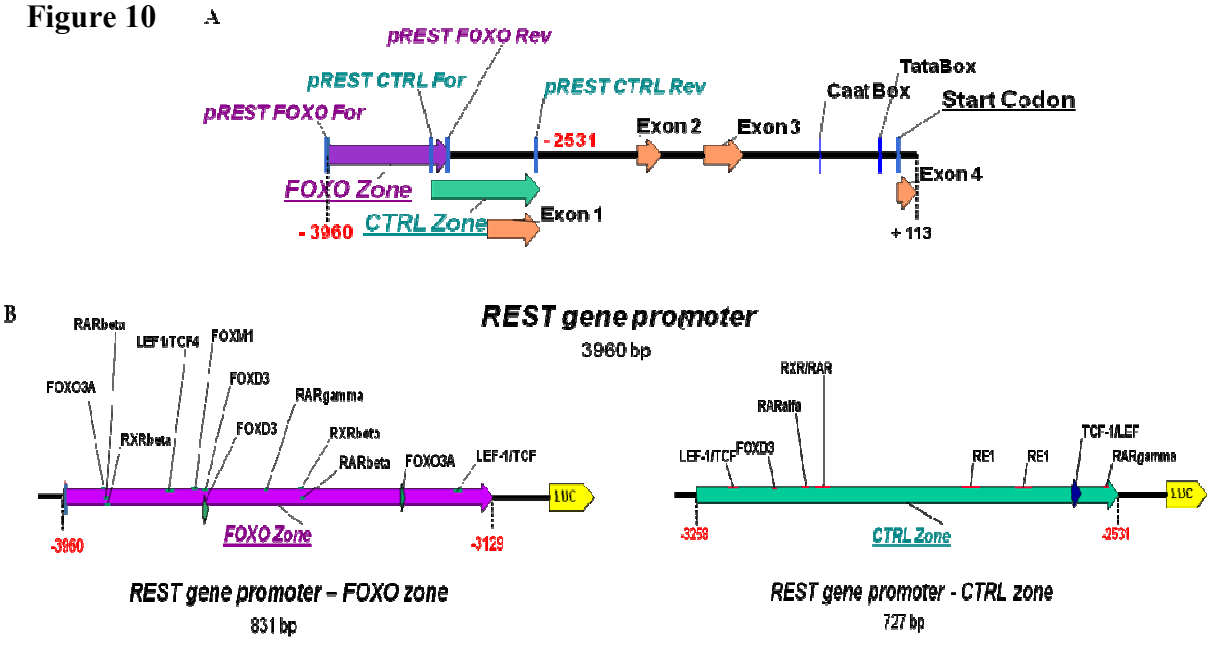


Figure 10. (A) REST promoter region from -3960 to +113 position, considering the start codon adenine as +1. The exons and the three constructs amplified are indicated (B) FOXO zone of REST promoter region (from -3960 to -3129 position). (C) CTRL zone of REST promoter region (from -3258 to -2531 position). Different transcription factor binding sites predicted by Vector NTI software are indicated.

Modulation of REST gene expression by reporter vectors that contain different fragments of REST promoter in SH-SY5Y cells

From results obtained up to now β-catenin seems to have a positive regulatory role on REST protein levels and this modulation could be mediated by TCF/β-catenin interaction with specific responsive sites on REST promoter.

In order to confirm this hypothesis and to study how β-catenin could regulate REST gene promoter, SH-SY5Y cells were transfected with each reporter plasmid pGL3basic containing FOXO, CTRL and FOXO-CTRL regions and then were exposed to PMA (16 nM for 48 h). Moreover, a β-catenin siRNA (50nM) was used. Finally SH-SY5Y cells were lysed and Luciferase reporter gene expression was evaluated. Luciferase activity is directly proportional to transcriptional activity of specific promoter fragment. As Figure 11 shows, PMA is able to increase Luciferase expression and therefore transcriptional activity of CTRL and FOXO-CTRL promoter upstream to reporter gene. PMA and siRNA together reduce activity of CTRL and FOXO-CTRL zones. FOXO activity shows a different profile that could be due to a different effect of transcription factors on this region. However it is interesting to note that REST promoter activity is in accordance to REST protein expression evaluated with Western blot in the same treatment conditions.

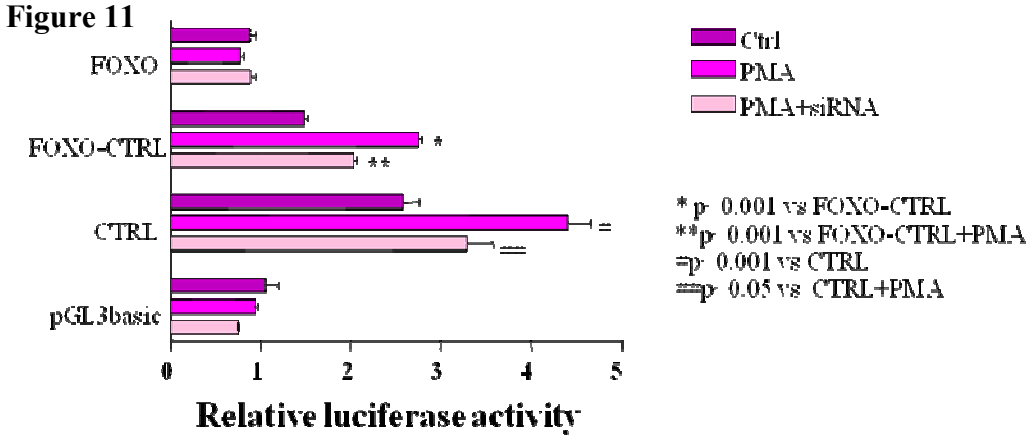


Figure 11. PMA and β-catenin siRNA effect on transcriptional activity of REST promoter different fragments in SH-SY5Y cells. Cells were transiently transfected with REST reporter gene constructs inserted into the pGL3-basic, promoter-less luciferase plasmid vector. Alternatively, cells were transfected with the empty pGL3 plasmid or not transfected (data not shown). After 24 h, cells were left untreated (control) or exposed either to PMA (16nM) alone for 48 h or to β-catenin siRNA (50nM) alone or to both of them; thereafter, Luciferase activity was measured in cell lysates. The transcriptional activity of each construct is expressed as luciferase activity relative to the activity of not transfected cells, which had assigned a value of 1.0. Transfection efficiencies are normalized to β-galactosidase activity by co-transfection of the internal control plasmid, pSV-βGal. This data show that PMA induce a relative luciferase activity increase in cells transfected with both CTRL and FOXO-CTRL gene constructs.

Discussion

In this work we observed that PMA is able to modify REST and β-catenin protein levels. In fact, during neural differentiation with PMA, REST expression seems to behave in a time-dependent manner. In the first phases of differentiation, REST increase could be correlated with its gene silencer role. Instead, in terminal differentiation, REST could be silenced to allow the expression of specific neural genes.
In order to investigate β-catenin involvement in REST regulation, β-catenin was silenced and over-expressed (reproducing Wnt signalling activation) in SH-SY5Y cells exposed to PMA. In the first case, REST expression was reduced and, in the second case, it was increased, demonstrating that β-catenin can influence REST expression. This observation is in accordance to previous studies where REST was demonstrated to be one of several target genes involved in Wnt signaling and to have TCF/β-catenin binding site (13).
Hence, putative mechanisms of REST transcription regulation were investigated by analysing its gene promoter. Gene reporter assays show that in SH-SY5Y cells exposed to PMA the transcriptional activity of CTRL and FOXO-CTRL zones, which have putative binding sites for TCF/β-catenin, was increased. In the presence of β-catenin siRNA, their transcriptional activity was reduced. This reduction was not observed in FOXO zone that seems to be regulated in a completely different way. Therefore, β-catenin regulatory action on REST expression could occur in CTRL zone. Nevertheless, further studies are necessary to better understand this regulatory role. In fact, REST expression is based on a very complex balance: its gene promoter presents a lot of consensus sites for different transcription factors that act as transcriptional repressors and enhancers.

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