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Acidic leucine-rich nuclear phosphoprotein 32 family member B (ANP32B) contributes to retinoic acid-induced differentiation of leukemic cells

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ABSTRACT

The acidic leucine-rich nuclear phosphoprotein 32B (ANP32B) is a member of a conserved superfamily of nuclear proteins whose functions are largely unknown. In our previous work, ANP32B was identified as a novel direct substrate for caspase-3 and acted as a negative regulator for leukemic cell apoptosis. In this work, we provided the first demonstration that ANP32B expression was down-regulated during differentiation induction of leukemic cells by all-*trans* retinoic acid (ATRA). Knockdown of ANP32B expression by specific shRNA enhanced ATRA-induced leukemic cell differentiation, while ectopic expression of ANP32B attenuated it, indicating an inhibitory role of ANP32B against leukemic cell differentiation. Furthermore, luciferase reporter assay revealed that ANP32B might exert this role through inhibiting the ATRA dependent transcriptional activity of retinoic acid receptor (RARα). These data will shed new insights into understanding the biological functions of ANP32B protein.

1. Introduction

Acute myeloid leukemia (AML), a class of prevalent hematopoietic malignancies, is characterized by complete or partial blockage at different stages of differentiation of myeloid progenitor cells. Accordingly, differentiation induction is a potentially attractive strategy for AML therapy [1]. An outstanding example for this is the success in differentiation induction of leukemic cells by all-*trans* retinoic acid (ATRA) in patients who suffer from acute promyelocytic leukemia (APL), a unique subtype of acute myeloid leukemia (AML), which harbors the chromosome translocation t(15;17) and expresses the fusion protein PML-RARa (for *pro*myelocytic *l*eukemia-*r*etinoic *a*cid *r*eceptor a). This ability of APL cells to respond to ATRA with terminal differentiation is likely due to the presence of RARα gene translocation and expression of the RARα chimeric proteins. Pharmacological doses of ATRA trigger the dissociation of nuclear receptor co-repressors from such "dominant-negative" RARα fusion proteins, restoring the regulation of target genes [2]. Hence, it has been attracting great attentions to understand the mechanisms by which ATRA and other agents induce leukemic cell differentiation. Using fast developing molecular genetic, proteomic and functional genomic approaches, many ATRA target genes have been discovered in the past decade [3,4].

ANP32B is a member of the highly conserved acidic leucine-rich nuclear phosphoprotein 32 (ANP32) family, whose members including ANP32A, B, C, D, E, F, G and H are characterized by a C-terminal acidic tail and a N-terminal leucine-rich repeats (LRR) [5,6]. To date, most of the reports have focused on ANP32A, the founding member of the ANP32 family, which has been implicated in a variety of cellular functions. As a phosphatase 2A (PP2A) inhibitor, ANP32A was involved in cell differentiation or proliferation by the regulation of MAP kinases [7], protein kinase C (PKC) [8], or Wnt signaling pathway [9]. ANP32A may regulate neuronal differentiation through the inhibition of acetyl transferases complex [10]. Additionally, ANP32A reduction induces differentiation of prostate carcinoma cells [11]. However, so far there is poor understanding on the function of other members of the family including ANP32B. In our previous subcellular proteomic analysis of apoptotic acute myeloid leukemia (AML) cells induced by NSC606985, a camptothecin ester derivative, ANP32B was found to be down-regulated during apoptosis [12]. Further study revealed it as a novel direct substrate for caspase-3 and acted as a negative regulator for leukemic cell apoptosis [13]. Induction of apoptosis and differentiation are two main strategies for therapeutic treatment of leukemia. In order to find out whether down-regulation of ANP32B also benefits leukemic cell differentiation, here we investigated the effect of ANP32B on AML cell differentiation induced by ATRA.

2. Materials and methods

2.1. Cell lines and differentiation assay

Human leukemic cell lines NB4 and U937, human embryonal kidney cell line 293 T were grown in RPMI-1640 or Dulbecco's

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Modified Eagle's Medium (Sigma–Aldrich, St Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco BRL, Gaithersburg, ML) in 5% CO_2/95% air humidified atmosphere at 37 °C. For morphological characterization, cells were collected onto slides by cytospinning (Shandon,Runcorn, UK), stained with Wright's stain and examined by light microscopy (Olympus, BX-51, Tokyo, Japan). The differentiation antigens CD11b, CD11c and CD14 were measured by flow cytometry (Beckman–Coulter, Miami, FL, USA). The nitroblue tetrazolium (NBT) reduction test was performed as previously described [14].

2.2. Plasmids, siRNA designs and transfections

cDNA of human ANP32B was cloned and inserted into pMSCVpuro Vector (Clontech). The human RARβ promoter was subcloned into pGL3-Basic (Promega, Madison, WI) respectively. Two pairs of complementary siRNA oligonucleotides against ANP32B and a pair of scrambled negative control siRNA were synthesized by Invitrogen (CA), annealed and ligated into pSIREN-RetroQ vector (Clontech). The target sequence for ANP32B were 5′-TGACTACCGAGAGAGTGTC-3′ for siRNA-A3, 5′-GCGAAATAAACAGTTACTC-3′ for siRNA-A4. The sequence for scrambled negative control siRNA was 5′-ACTACCGTTGTTATAGGTG-3′. Retrovirus was generated by transient transfection of the 293T cell line with FuGENE6 transfection reagent (Roche). Forty-eight hours after transfection, the viral supernatant was harvested and used for infection of target cells. Stable retroviral transduction was achieved by infection for 48 h, after which selection with either puromycin (1 g/ml) was initiated. Selection was stopped as soon as the non-infected control cell died off, and the media were replaced with normal-growing media.

2.3. Quantitative RT-PCR

For quantitative real-time RT-PCR assay, total cellular mRNAs from cell lines were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA) and were treated with RNase-free DNase (Promega, Madison, WI). Then, RT was performed by TaKaRa RNA PCR kit (Takara, Dalian, China) following manufacturer's protocol. Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) on the ABI PRISM 7900 system (Perkin-Elmer, Torrance, CA). The following specific oligonucleotide primers were used respectively for ANP32B (forward primer: 5′-ACTTGGATGGCTATGACCGAG-3′ and reverse primer: 5′-TCCTCACTGACTTCATCGTCG-3′), with β-actin (forward primer: 5′-CATC CTCACCCTGAAGTACCC-3′; and reverse primer: 5′-AGCCTGGATAGCAACGTACATG-3′) as internal control. Real-time RT-PCR was performed and data were analyzed according to the previous report [15].

2.4. Luciferase reporter assays

For luciferase assay, 293 T cells were seeded in a 12-well plate (Becton Dickinson, Franklin lakes, NJ). After overnight incubation, cells were transfected either with 100 ng of firefly luciferase reporter plasmid and 4 ng of pSV-*Renilla* plasmid, or further cotransfected with expression vectors. The total amount of DNA was held constant by addition of empty vector. Twenty-four hours after transfection, cells were lysed and analyzed by the Dual-Luciferase Assay system (Promega) according to the manufacturer's instructions. For each experiment, at least three independent transfections in triplicate were performed.

2.5. Western blots

The protein lysates were equally loaded on a 10–12% SDS–PAGE, and subsequently, transferred to an NC membrane (Bio-Rad). The membranes were blocked with 5% nonfat dry milk solution in TBS for 1 h at room temperature, and then incubated in primary antibody dissolved in block solution at 4 °C overnight. The protein was probed by antibodies against ANP32B (Proteintech Group, Chicago, IL), ANP32A (Santa Cruz, CA) and β-actin (Oncogene, San Diego, CA, USA). After washing, the blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Dako Cytomation, Denmark) corresponding to the primary antibody in blocking buffer for 1 h at room temperature, and proteins were detected using a luminol detection reagent (Santa Cruz), and developed onto Kodak X-ray films.

2.6. Statistical analysis

The difference between two different groups was compared for statistical significance by the nonparametric Wilcoxon rank sum test. A value of $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. ATRA decreased ANP32B mRNA and protein level in leukemic cells

To investigate whether ANP32B protein could be modulated during the differentiation induction of leukemic cells, acute myeloid leukemic NB4 cells were treated with appropriate concentrations of the differentiation-inducing agents ATRA (10^{-6} M), PMA (20 nM) and VD_3 (10^{-7} M) for 3 days. As described previously [14], these treatments significantly induced cell differentiation as evaluated by morphologic examination and flow cytometry assay for CD11b and CD14 antigens (data not shown). To our interest, among the three agents, only ATRA significantly decreased ANP32B protein expression (Fig. 1A). Time-course analysis with ATRA revealed that the decrease of ANP32B protein and mRNA level began as early as 24 h, and continued after 72 h (Fig. 1B).

3.2. Suppression of ANP32B expression by shRNA significantly enhanced ATRA-induced differentiation of myeloid leukemic cells

The above observation suggested that decreased ANP32B protein expression was an early event during ATRA-induced differentiation. To investigate whether the down-regulation of ANP32B expression was associated with leukemic cell differentiation, NB4 cells were transfected with two shRNAs specifically targeting ANP32B (A3 and A4) as well as a negative control shRNA (NC). As shown in Fig. 2A, A3 and A4 significantly eliminated ANP32B protein without affecting ANP32A expression. Intriguingly, in contrast to NC transfected cells, A3 and A4 cells gained a morphological change upon ATRA treatment characterized by condensed chromatin and decreased nucleus/cytoplasm ratio, with smaller and distorted nuclei (Fig. 2B). Furthermore, knock-down of ANP32B remarkably increased NBT reduction (Fig. 2C) and CD11b expression (Fig. 2D). These results indicated that suppression of ANP32B enhances ATRA-induced differentiation of myeloid leukemic cells.

3.3. ANP32B induction antagonized ATRA-induced differentiation of myeloid leukemic cells

To further validate the role of ANP32B in myeloid cell differentiation, full-length ANP32B or an empty vector (EV) were respectively transfected into leukemic U937 cells by retrovirus infection. It should be pointed out that U937 cells were used here instead of NB4 cells because over-expression is difficult to achieve in NB4 cells. Compared with $U937^{EV}$, $U937^{ANP32B}$ cells which were enforced to express higher level of ANP32B protein (Fig. 3A) remarkably inhibited ATRA-induced differentiation, as assessed

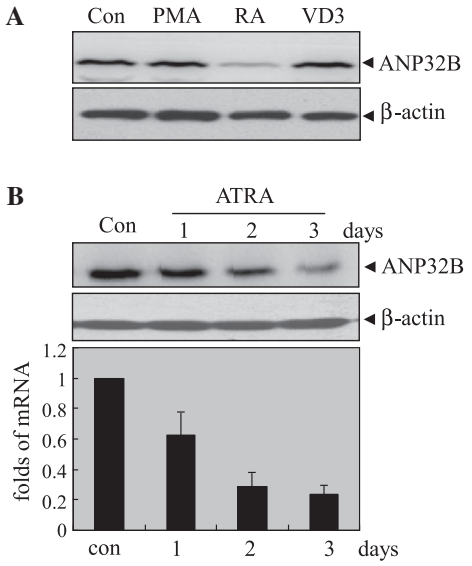

Fig. 1. Effects of three differentiation-inducing agents on ANP32B expression in leukemic NB4 cells. Leukemic NB4 cells were treated with 10^{-6} M ATRA, 20 nM PMA and 10^{-7} M VD_3 for 3 days (A) or 10^{-6} M ATRA for days as indicated (B). ANP32B protein and mRNA were detected. All experiments were repeated at least three times with the same results. The values for mRNA represent mean ± S.D. from triplicate samples for change folds against the untreated cells.

by morphology (Fig. 3B) and decreased CD11b, CD11c expression for indicated times (Fig. 3C).

Next, we evaluated whether the inhibitory effect of ANP32B on differentiation was specific to ATRA treatment or a general response to most differentiation inducers. Toward this end, $U937^{EV}$ and $U937^{ANP32B}$ cells were treated with the other two commonly used differentiation inducers PMA and VD_3. As evidenced by flow cytometry analysis of CD11b, CD11c and CD14 expression, ANP32B over-expression failed to inhibit PMA and VD3-induced differentiation (Fig. 4A). All the above results suggested that ANP32B is a negative regulator specific for ATRA induced myeloid leukemic cells differentiation.

3.4. ANP32B blocked retinoic acid receptor signaling

As mentioned above, ATRA induces leukemic cell differentiation through binding their nuclear receptors, RA receptors (RARs) and retinoid X receptors (RXRs), which in turn switch on the transcription program [16]. As ANP32B was recently shown to participate in transcription regulation as a histone chaprone [5], we examined whether ANP32B could regulate the ATRA dependent transcription activity of the RARα gene, which plays a crucial role in leukemic cell differentiation [17]. For this purpose, a DNA fragment carrying the RARE from the promoter of RARβ, a target gene of RARα, was subcloned, into the luciferase reporter plasmid pGL3-basic, which was transfected into cells together with the internal control Renilla. Twelve hours later, these cells were treated with ATRA for additional 24 h. As depicted in Fig. 4B, in the presence of ANP32B, ATRA activated transcriptional activity of RARα was inhibited. These results suggested a role of ANP32B in regulating retinoic acid receptor signaling.

4. Discussion

Induction of apoptosis and differentiation are two most important strategies for therapeutic treatment of leukemia. In our previous report, we found that down-regulation of ANP32B dramatically enhanced apoptosis induction in leukemic cells. In order to find out whether down-regulation of ANP32B also benefits differentiation induction in leukemic cell, we have investigated the regulation and role of ANP32B during leukemic cell differentiation in this

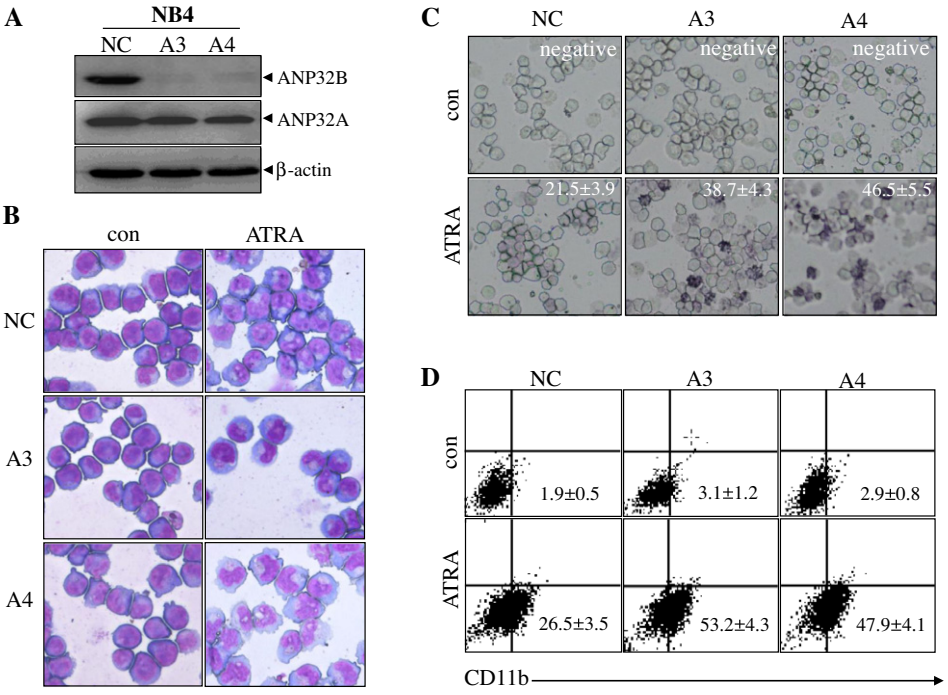

Fig. 2. Silencing of ANP32B expression enhances ATRA-induced differentiation of leukemic NB4 cells. NB4 cells were stably transfected with shRNA-A3, A4 against ANP32B or negative control vector (NC), and ANP32B protein was detected by Western blot with β-actin as a loading control (A). NB4 cells stably transfected with siRNA-A3, A4 or NC were treated with or without 10^{-7} M ATRA for 48 h (B–D). For morphological characterization, cells were collected onto slides by cytospin, stained by Wright's staining and observed under a microscope (100×/1.30 Oil objective lens) (B). NBT reduction test was performed (C) and CD11b positive cells were measured by flow cytometry (D). The values represent mean ± S.D. of triplicate in an independent experiment, which were repeated more than three times with the same results.

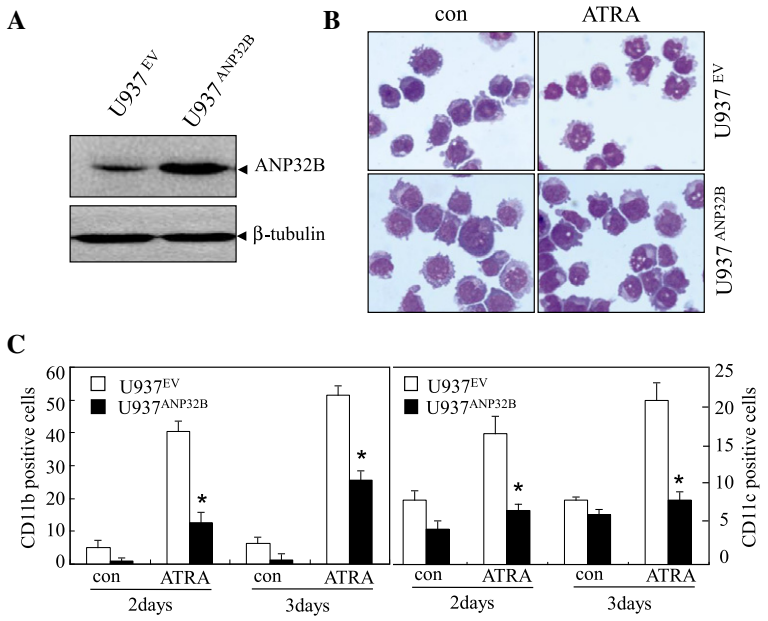

Fig. 3. ANP32B over-expression inhibits ATRA-induced differentiation of leukemic U937 cells. U937 cells were infected with pMSCV-puro-ANP32B or empty vector. The ANP32B protein expression was examined by Western blot (A). For morphological characterization, $U937^{EV}$ and $U937^{ANP32B}$ cells treated with or without 10^{-6} M ATRA for 48 h were collected onto slides by cytospin, stained by Wright's staining and observed under a microscope (100×/1.30 Oil objective lens) (B). $U937^{EV}$ and $U937^{ANP32B}$ cells treated with or without 10^{-6} M ATRA for the indicated hours, CD11b and CD11c positive cells percentage were determined on flow cytometry (C). All values represent mean ± S.D. of triplicate in an independent experiment, which was repeated for more than three times. The symbol * represents $p < 0.05$ compare with $U937^{EV}$ cells with the corresponding treatment.

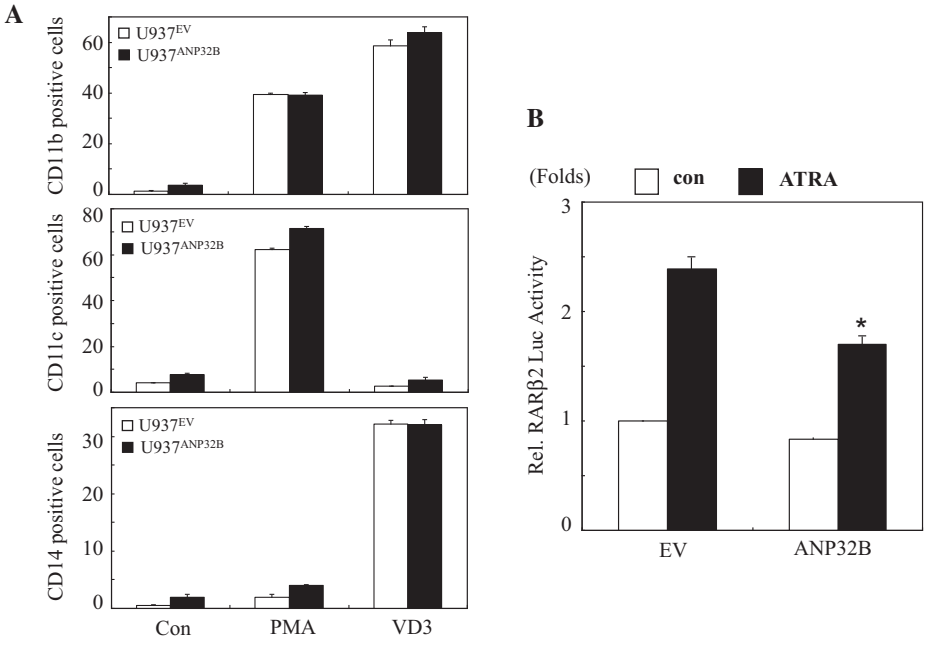

Fig. 4. ANP32B inhibits ATRA dependent transcriptional activity of RARα. $U937^{EV}$ and $U937^{ANP32B}$ cells treated with or without 10^{-6} M PMA and 10^{-7} M VD3 for 3 days, CD11b, CD11c and CD14 positive cells were measured by flow cytometry (A). RARβ2 promoter-driven luciferase reporter was cotransfected with EV or vector encoding ANP32B into 293T cells. Then 293T cells were treated by ATRA for 24 h. The luciferase activities of RARβ2 promoter were normalized by pSV40-Renilla and the relative luciferase activities were shown as folds against that of empty vector transfected cells (B). All values represent mean ± S.D. of triplicate in an independent experiment, which was repeated for more than three times. The symbol *represents $p < 0.05$ compare with $293T^{EV}$ cells with the corresponding treatment.

work. First, we treated leukemic NB4 cells with three differentiation-inducing agents, ATRA, PMA and VD_3. Interestingly, only ATRA decreased ANP32B mRNA and protein levels during differentiation induction. As the down-regulation of ANP32B appeared to be an early event, we next attempted to investigate whether endogenous ANP32B was related to ATRA-induced leukemic cell differentiation. For this purpose, endogenous ANP32B was knocked down in leukemic NB4 cells by specific shRNAs. The results showed that suppression of ANP32B strongly enhanced ATRA-induced leukemic cell differentiation, as evidenced by morphologic feature, NBT reduction and increased CD11b expression. Furthermore, over-expression of ANP32B rendered leukemic U937 cells with low sensitivity

to differentiation, which also appeared to be ATRA specific. All these data strongly suggested that ANP32B contributes to ATRA-induced leukemic cell differentiation.

It is well known that ATRA signaling via RARα is required for myeloid cell differentiation [18,19], during which RARα binds to retinoc acid response element (RARE) located in the promoter of many genes such as RARβ [20] and RARγ [21] and stimulates gene transcription responsible for ATRA-induced differentiation. More recently, ANP32B was identified as a novel histone chaperone that specifically bound to the transcription factor KLF5, leading to transcriptional repression of KLF5-downstream genes through inhibition of promoter histone acetylation [22]. Based on these findings, we proposed that RARα was another transcription factor modulated by ANP32B. Therefore, we sought to examine whether ANP32B could regulate the transcription activity of RARα and found that ANP32B over-expression significantly inhibited ATRA dependent activation of RARβ promoter by RARα as detected by luciferase reporter assay. It still remains to be investigated how ANP32B inhibits ATRA activated transcriptional activity of RARα.

Thus far, we have demonstrated that down-regulation of ANP32B not only enhanced leukemic cell apoptosis in our last work, but also contributed to ATRA-induced leukemic cell differentiation in the present work. Therefore, ANP32B may serve as a potential therapeutic target for leukemia treatment.

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