Rap1GAP alters leukemia cell differentiation, apoptosis and invasion *in vitro*

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Abstract. Rap1GAP which regulates the GTP-GDP form switch of Rap1 is a member of the GTPase-activating protein (GAP) family and has recently received substantial attention. Rap1GAP is thought of as a putative tumor suppressor gene and plays an important role in human tumor progression including pancreatic cancer, thyroid cancer and melanoma. In the current study, we found that the expression of Rap1GAP was lower in acute myeloid leukemia (AML) patients compared to non-malignant blood disease patients. The expression of Rap1GAP was also low in HL-60, NB4, U937 and SHI-1 myeloid leukemia cell lines. Upregulated Rap1GAP in NB4 and HL-60 cells promoted cell differentiation induced by ATRA or TPA compared to the empty vector control cells. Furthermore, Rap1GAP-transfected cells also showed a higher rate of apoptosis in response to arsenic trioxide compared to the control counterpart cells. In addition, we found that increased expression of Rap1GAP promoted leukemia cell invasion may be due to matrix metalloproteinase 9 (MMP9). In conclusion, these results demonstrated that Rap1GAP promoted leukemia cell differentiation and apoptosis, but increased leukemia cell invasion *in vitro*.

Introduction

Ras mutation has been found in many types of human malignancies including hematologic malignancies with 15% point mutation in acute myeloid leukemia (AML). Ras-associated protein 1 (Rap1), a close member of Ras-family, has attracted increased attention in tumorigenesis, progression and metastasis (1,2). Rap1 regulates two important cellular processes including B-RAF/MEK/ERK activation and integrin-mediated cell adhesion (3,4). Like other Ras family proteins, Rap1 functions as a molecular switch between the active form binding GTP and the inactive form binding GDP. The GDP-GTP cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) (5). GEFs promote the release of GDP, rendering Rap1 to rebind a new GTP to stimulate the downstream signal cascade. On the contrary, GAPs stimulate the low intrinsic GTPase activity of Rap1 to hydrolyze GTP resulting in the inactivation of Rap1.

Rap1GAP gene locating at chromosome 1p36.1-p35 is a member of GAPs family which also including SPA-1, E6TP1 (E6-targeted protein 1) and SPAR (spine-associated Rap1GAP) (6). Little is known about the function of Rap1GAP in tumorigenesis although the gene is receiving increased attention. Loss of heterozygosity (LOH) of Rap1GAP was found in pancreatic cancer and promoter hypermethylation was recently reported in melanoma cells (7,8). Decreased expression of Rap1GAP was also reported in papillary thyroid cancer (9,10). Also upregulated expression of Rap1GAP exhibited proliferation suppression *in vitro* as well as *in vivo* models. These data led to the proposal that Rap1GAP may act as a putative tumor suppressor gene in these tumors.

Previous data of our laboratory showed that Rap1GAP may play important functions in myelodysplastic syndromes (MDS) (11,12). Here, we investigated whether Rap1GAP plays a role, and how, in the pathogenesis and progression of hematologic malignancies.

Materials and methods

Cell culture. Human 293T cells as the packaging cell for lentivirus was kindly provided by Dr Yun Zhao (Cyrus Tang Hematology Research Center, Soochow University). HL-60, NB-4, U937 and SHI-1 human leukemic cell lines were cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco) with 10% fetal bovine serum (FBS) (Gibco) in 5% CO_2 humidified atmosphere at 37˚C.

Patients and sample preparation. After approved by Hospital Ethics Committee and signing the informed consent, bone marrow mononuclear cells (BMNCs) were isolated from patients including 8 cases of M2, 7 cases of M3, 6 cases of M5 (according to FAB classification). Eight cases of non-malignant blood disease patients (including 5 iron deficiency anemia,

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2 hypercellular anemia and 1 idiopathic thrombocytopenia) were chosen as controls. Non-parametric tests in SPSS were used for statistical analyses.

Rap1GAP-expressing lentiviral vector and virus production. A human full-length of Rap1GAP was amplified by PCR from pcDNA3.1-Rap1GAP (generously offered by Dr P. Stork, Oregon Health Sciences University, Portland, OR), using Pfu polymerase with forward primer 5'-GCGGGATCCATGAT TGAGAAGATGCAG-3' and reverse primer 5'-GCGGGATC CCTAACAGCCCAGCTGG-3'. To the subcloned lentivirus pRRL-Venus vector (gifted from Dr Yun Zhao), *Bam*HI digestion sites were added. All plasmids were conformed by DNA sequencing.

After packaged according to the protocol of literature (13), the virus in the supernatant of transfected cells was centrifuged and kept in -80˚C for later use.

Transduction of human leukemia cells and FACS sorting. Human leukemic cells HL-60 and NB4 were transfected with lentivirus. Briefly, $1x10^6$ cells were seeded into 6-well dishes within 2 ml IMDM medium with 10% FBS, then 100 μl Rap1GAP-virus or control virus was added to each well. After 8-10 h of incubation, cells were washed twice with sterile phosphate-buffered saline (PBS) and transferred into 45 cm^2 flask for a 3-4 day expansion. Both the Rap1GAP-transducted cells and control cells were sorted by YFP signal using FACS (BD Biosciences) and expanded for one more week. The stable monoclonal cells transfected with Rap1GAP were then obtained by limited dilution.

Western blot analysis. For western blot analysis, cell pellets were lysed in 100 μl lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% Nonidet P-40, 1% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin) for 60 min on ice. After centrifugation for 15 min at 12,000 g (4˚C), the supernatants were collected for western blot analysis. Primary antibody concentrations were as follows: rabbit anti-Rap1GAP monoclonal antibody (Santa Cruz Biotechnology), 1:1,000; rabbit anti-Rap1 monoclonal antibody (Pierce), 1:500; mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody, 1:1,000; rabbit anti-ERK monoclonal antibody 1:1,000 and rabbit anti-p-ERK monoclonal antibody 1:500 (Cell Signaling).

Rap1 activity assay. Active Rap1 was detected by glutathione S-transferase (GST)-tagged RalGDS according to the protocol (Pierce). Briefly, $1x10^7$ cells were collected and lysed by lysis/binding/washing buffer in which protease inhibitors was previously added. GST-RalGDS-RBD (20 μg) was added to a spin cup containing 100-μl glutathione resin within a collection tube. Immediately, 700 μl cell lysate containing about 500 μg proteins was transferred into the spin cup. For the prevention of leakage, the spin cup was closed and sealed with laboratory film. The reaction mixture was incubated at 4˚C for 1 h with gentle rocking. After incubation, the spin cup was transferred into a new collection tube and washed by 400 μl lysis/binding/washing buffer thrice. Finally, 50 μl 2X reducing sample buffer was added to the resin and centrifuged at 6,000 g for 2 min following a 2-min incubation at room temperature. The eluted samples containing active Rap1 were heated for 5 min at 95-100˚C and stored at -20˚C for later immunoblot assay.

Reverse transcriptase PCR and quantitative real-time PCR. Total RNA was extracted from aliquots of cells with TRIzol reagent according to the manufacturer's procedure, cDNA was synthesized using MMLV reverse transcriptase (Gibco). Expression level was measured by PCR or qRT-PCR. Primers were as follows: Human Rap1GAP: Forward 5'-CA GAGGAGGACTACATTCCATACCCG-3' and reverse 5'-CCAACTTTGCCATCTGGACAACATT-A-3'; β-actin: forward 5'-AGGCCGGCTTCGCGGGCGAC-3' and reverse 5'-CTCGGGAGCCACACGCAGCTC-3'; p21: forward 5'-AT GTCAGAACCGGCTGGGGAT-3' and reverse 5'-GGGC TTCCTCTTGGAGAAGAT-3'.

For qRT-PCR, all reactions were performed in 96-well MJ white optical plates. β-actin was used as an endogenous control. The primers and Taqman probes were designed by the software Primer Express 2.0. Human Rap1GAP: forward 5'-CAAGAACAGAGCGGAGACC-3', reverse 5'-GCCACGT GCTATAGATGAAG-3', probe 5'-AGCAGAGGCGCTCAA GGACTTCTCC-3'; Human β-actin: forward 5'-TCACCC ACACTGTGCCCATCTACGA-3', reverse 5'-CAGCGGAAC CGCTCA TTGCCAATGG-3', probe 5'-ATGCCCTCCCCCA TGCCATCCTGCGT-3'; Human MMP9: forward 5'-GCTGG GCTTAGATCATTCCTCA-3', reverse 5'-AGGGCGAGGA CCATAGAGGT-3', probe 5'-CCCGGCCTTCTGTTCCTGA TAAACCC-3'.

Each reaction with a final volume of 25 μl was composed of the sample cDNA, TaqMan Universal PCR Mastermix (Applied Biosystems), primers and probe. Amplification was performed by a denaturation step at 95 ˚C for 10 min; followed by 40 cycles of 95˚C for 30 sec, 50˚C for 30 sec, and 72˚C for 30 sec. The relative levels of Rap1GAP and MMP9 were evaluated by comparative CT value against β-actin and expressed as $2^{-\Delta CT}$ and $2^{-\Delta\Delta CT}$. All samples were measured in triplicate.

Cell differentiation determination. Cells ($5x10^5$/ml) were seeded in 6-well plates and incubated with ATRA (final concentration 10^{-6} M) or TPA (final concentration 20 nM) for 24, 48 and 72 h. Cells were collected then washed with PBS for flow cytometry analysis, PE-conjugated CD11b was used. Nitroblue tetrazolium test (NBT) was done at the same time.

Detection of apoptosis by fluorescence microscope. After induced by arsenic trioxide (final concentration HL-60 cells $5x10^{-6}$ M, NB4 cells $2x10^{-6}$ M) for 24 h, cells were collected and washed twice with binding buffer before the next step. Because the lentiviral vector contained YFP protein, resuspended cells were stained with PE-conjugated Annexin V to visualize the apoptosis cells with red fluorescence.

Cell invasion assay. *In vitro* cell invasion were measured by the costar transwell chamber system. The membrane filters were coated with 60 μl Matrigel (BD Biosciences) which was reconstituted with IMDM at a dilution of 1:10, then dried at 37˚C under sterile conditions overnight. HL-60 cells were suspended in 200 μl IMDM with 10% FBS at the final density of $2.0x10^5$ cells/well and seeded into the inserts. The outside

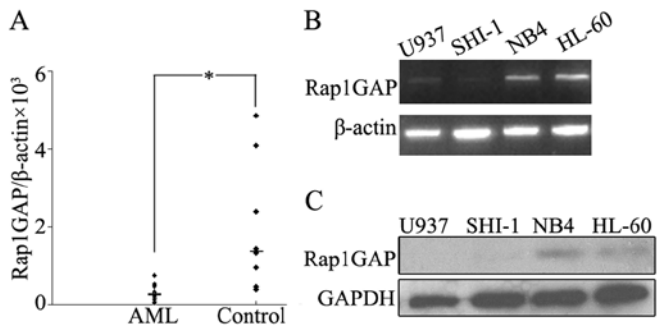

Figure 1. Rap1GAP1 is downregulated in acute myeloid leukemia. (A) Total RNA was collected from 21 cases of acute myeloid leukemia (8 cases of M2, 7 cases of M3, 6 cases of M5) and 8 cases of non-malignant blood diseases as control, and 100 ng RNA was subjected to qRT-PCR. Experiments were repeated in triplicate. Compare with control *P<0.05. (B) qRT-PCR analysis of Rap1GAP gene transcription in HL-60, NB4, U937 and SHI-1 cells. β-actin was used as an internal control. Experiments were repeated thrice with essentially the similar results. (C) Western blot detection of Rap1GAP in HL-60, NB4, U937 and SHI-1 cells lysates. Aliquots (10 μl) standardized protein samples were separated by SDS-PAGE and blotted with the specific antibodies. GAPDH was used as control.

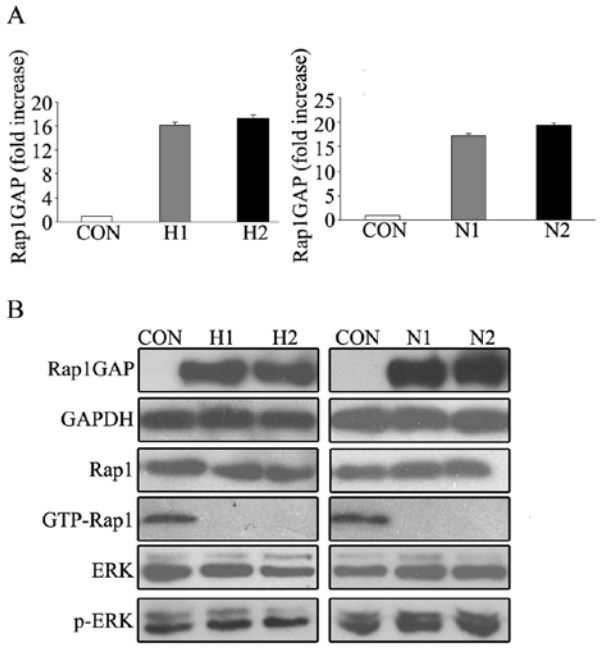

Figure 2. Upregulated expression of Rap1GAP inhibits the activation of Rap1. (A) qRT-PCR analysis of Rap1GAP gene transcription in HL-60 and NB4 stable transfected cell lines. NB4 and HL-60 cells were transfected with lentivirus containing Rap1GAP, empty lentivirus vector as control (CON). β-actin mRNA was used as an internal control. (B) Western blot analysis of the levels of Rap1GAP in Rap1GAP-transfected cells (H1, H2 or N1, N2). Thirty microliters of Ral GDS-RBD agarose were used in 0.5-ml cell extract to pull down the activated form of Rap1 (GTP-Rap1). The total Rap1 and GTP-Rap1 were detected by Rap1 antibody. The total ERK and p-ERK were observed by specific antibodies. Empty-vector as control (CON).

of the insert was filled with 500 μl IMDM with 10% FBS to form the lower chamber. After 24-48 h of incubation, the cells that had migrated into the lower chamber were collected and counted. The invasion rate was calculated by the ratio of the number of cells in the lower chamber to the total number of leukemic cells. Each invasion experiment was performed in triplicate.

Gelatin zymography. Cells were suspended in 100 μl serum-free medium at the final density of $2.0x10^5$ cells/well and seeded into 96-well plates. After 24 h of incubation, the culture supernatants were harvested and centrifuged to remove cellular debris for zymography. Briefly, 10-15 μl supernatants mixed with loading buffer were subjected to SDS-PAGE using 10% acrylamide gels with 0.1% gelatin. The gels were washed three times for 30 min in 2.5% Triton X-100, then incubated overnight in activation buffer (50 mM Tris-Cl pH 7.5, 10 mM $CaCl_2$, 150 mM NaCl, 1 mM $ZnCl_2$, 0.02% NaN_3) at 37˚C. The gelatinolytic activity was observed as transparent bands after the gels were stained with 0.1% Coomassie Blue R-250 and destained in 10% acetic acid and 30% alcohol.

Results

Rap1GAP1 is downregulated in acute myeloid leukemia. We detected the expression level of Rap1GAP in BMNCs from patients with acute myeloid leukemia and patients with non-malignant blood diseases by using qRT-PCR. The median levels of Rap1GAP transcripts in patients with AML and non-malignant blood diseases were 0.0002 (ranging from 0 to 0.0025) and 0.0014 (ranging from 0.0004 to 0.005), respectively. The Rap1GAP expression was significantly lower in AML than in control groups (P<0.05) (Fig. 1A). In leukemic cell lines (HL-60, NB4, U937 and SHI-1) Rap1GAP expression was very low, both in RNA and protein level (Fig. 1B and C). Collectively, decreased Rap1GAP expression was observed in BMNCs of AML patients as well as in leukemic cell lines (i.e. HL-60, NB4, U937 and SHI-1).

Upregulated Rap1GAP blocks Rap1 activation in leukemic cells. To explore the function of Rap1GAP in hematologic malignancies, two stable Rap1GAP-transfected subclones of both HL-60 and NB4 cells were identified by qRT-PCR and western blot analysis. In the results of qRT-PCR, the mRNA level of Rap1GAP increased about 16.2, 17.3, 17.5 and 19.3 fold in HL-60 (H1 and H2), NB4 (N1 and N2) stable cell lines, respectively (Fig. 2A). Western blot analysis showed similar results. As expected, Rap1 activity was almost undetectable in these Rap1GAP-overexpressed cells. In contrast, high basal Rap1 activity was observed in control cells (Fig. 2B). These results suggested that increased expression of Rap1GAP in leukemic cells lead to the abolishment of Rap1 activity.

Upregulated Rap1GAP do not affect myeloid leukemic cell growth. A large number of studies have reported that the expression of Rap1GAP was downregulated in a variety of human malignancies and upregulated Rap1GAP inhibited the proliferation of malignancies *in vivo* and *in vitro* models. But in hematologic malignancies, increased expression of Rap1GAP in AML cell lines including HL-60 and NB4 does not influence the proliferation capability compared to empty control or wild-type cells (data not shown). Neither was the difference of p-ERK or total ERK levels between Rap1GAP-transfected cells and their counterparts observed (Fig. 2B).

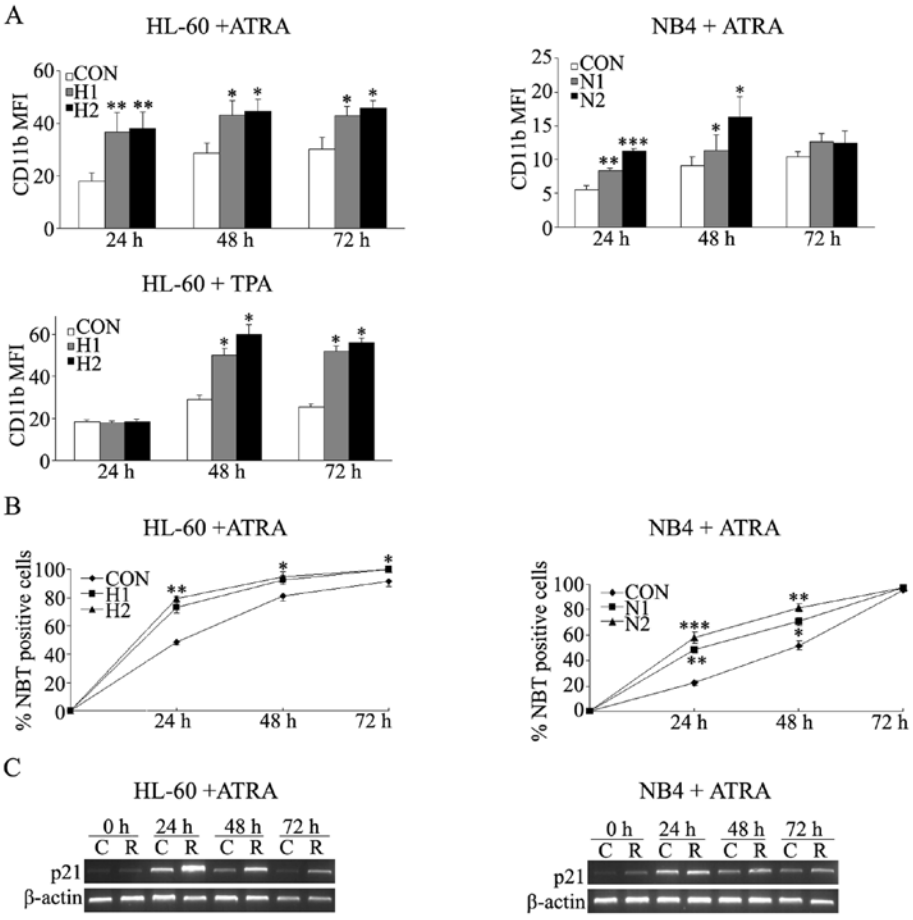

Figure 3. Upregulated expression of Rap1GAP promotes differentiation of HL-60 and NB4 cells induced by ATRA or TPA. (A) The CD11b MFI values of leukemic cells (HL-60 and NB4 cells) induced by ATRA or TPA at 24, 48 and 72 h measured by flow cytometry. The summary results of CD11b from three separate experiments are presented. Data represent the mean ± SD of triplicate measurements representative for three induced experiments. (B) NBT analysis of empty-vector control cells (CON) and Rap1GAP-transfected cells (H1, H2 and N1, N2). Compared with control $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (C) Different mRNA levels of p21 of Rap1GAP-transfected cells (R) compared to the counterparts (C). β-actin mRNA was used as an internal control.

Upregulated Rap1GAP promotes the differentiation of NB4 and HL-60 cells induced by ATRA or TPA. HL-60 cells, previously considered as a promyelocytic leukemia cells, can be induced to differentiate towards more mature stage by some inducers. All-trans-retinoic acid (ATRA) induces HL-60 differentiate into granulocyte and phorbol 12-myristate 13-acetate (TPA) stimulates cells to differentiate into a macrophage-like phenotype. Here, two stable Rap1GAP-transfected subclones were examined. During ATRA induction, the CD11b mean fluorescence index (MFI) of HL-60 cells increased for 1.4- to 2.1-fold in two transfected monoclones than empty control each day ($P<0.05$) (Fig. 3A). NBT assay also demonstrated a similar result (Fig. 3B). As shown in Fig. 3A, TPA-induction also resulted in much higher CD11b MFI of HL-60 cells in Rap1GAP-transfected monoclones than the counterpart cells at 48 and 72 h ($P<0.05$), but the difference was not observed at 24 h. Moreover, TPA-induced morphological changes of HL-60 cells were observed more obviously in Rap1GAP-transfected cells than in the empty control cells (data not shown).

ATRA can also induce NB4 cells to differentiate into mature granulocyte. The CD11b MFI increased for 1.2- to 2.0-fold in two transfected monoclones at 24 and 48 h, but the difference was not observed at 72 h (Fig. 3A). NBT assay was consistent with the CD11b results (Fig. 3B). During induction, the mRNA level of p21 of both HL-60 and NB4 cells were measured by RT-PCR (30 cycles) at each time point. The expression of p21 showed higher level in the Rap1GAP-transfected cells compared to the control cells (Fig. 3C).

Upregulated Rap1GAP promotes HL-60 and NB4 cells apoptosis. To determine the effect of Rap1GAP on leukemic cell apoptosis, we use arsenic trioxide to induce HL-60 and NB4 cells to undergo apoptosis. HL-60 and NB4 cells were transfected with lentivirus vector carrying YFP protein whose emitting wave interfere with other fluorescence and can not be analyzed by flow cytometry without light filter, so the apoptosis cells were observed directly through fluorescence microscope. For both HL-60 cells in Fig. 4A and NB4 cells shown in Fig. 4B, we have observed that the number of cells stained with Annexin V-PE were much greater in Rap1GAP-transfected cells than that in the counterparts. These results may indicate that increased expression of Rap1GAP render leukemic cells to undergo apoptosis.

Increased expression of Rap1GAP promotes leukemic cell invasion in vitro. Cell invasion ability was measured by the transwell system. After 24-48 h of incubation, the cells migrated into the lower chamber were collected and counted.

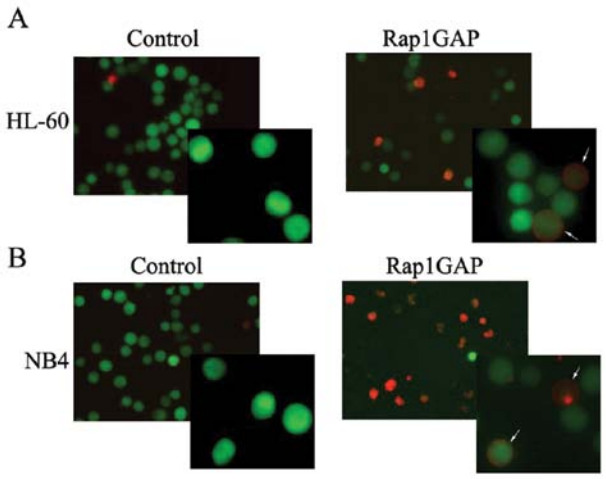

Figure 4. Increased expression of Rap1GAP promotes leukemic cell apoptosis. (A) Rap1GAP-transfected HL-60 cells (Rap1GAP) and the control cells (CON) were treated by arsenic trioxide and detected as apoptotic cells by fluorescence microscopy. (B) Rap1GAP-transfected NB4 cells (Rap1GAP) and the corresponding control cells (CON) were treated by arsenic trioxide and stained with Annexin V-PE to detect apoptotic cells by fluorescence microscopy. The apoptotic cells were detected using Annexin V-PE (red).

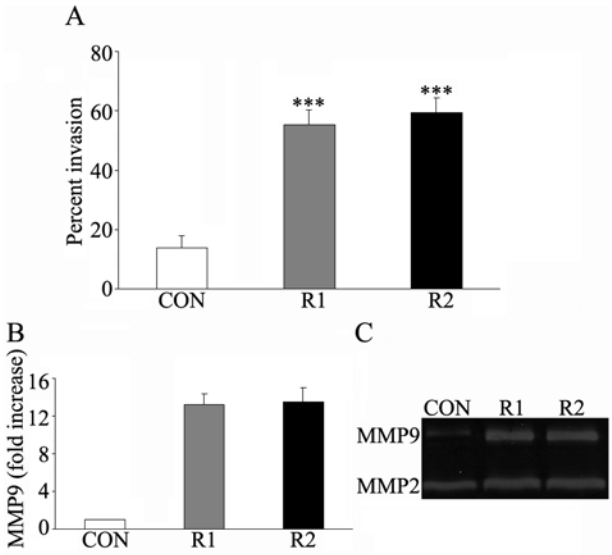

Figure 5. Rap1GAP increases the invasion capability of HL-60 cells *in vitro*. (A) Rap1GAP-transfected HL-60 cells (R1 and R2) exhibited higher invasion rate than the control cells (CON). Compare with control ***P<0.001. (B) The mRNA level of MMP9 was higher in Rap1GAP-transfected cells compared with the counterpart cells. (C) Gelatin zymogram showed that more functional MMP9 was secreted in Rap1GAP-transfected HL-60 cells than in the control cells. All experiments were repeated in triplicate.

The invasion rate of Rap1GAP-transfected HL-60 cells was about 4-fold that of the empty control cells (Fig. 5A). The filter of the upper chamber was coated with Matrigel which was composed of laminin, collagen type IV, and proteoglycans to imitate human basement membranes. To invade into the lower chamber, leukemic cells secreted some matrix metalloproteinases (MMPs) to resolve the barrier. We therefore investigated the MMP secretion, mainly MMP9 and MMP2 in control- and Rap1GAP-transfected cells. As shown in the gelatin zymogram, Rap1GAP-transfected cells showed higher MMP9 secretion than the control cells, but changes in MMP2 secretion was not obvious (Fig. 5C). qRT-PCR was also performed to determine the mRNA level of MMP9 in Rap1GAP-transfected cells and the control cells. The mRNA level of MMP9 was upregulated in Rap1GAP-transfected HL-60 cells (about 12-fold increase) in comparison with the corresponding control cells (Fig. 5B). These results suggested that upregulated expression of Rap1GAP enhance the invasion ability of the leukemic cells probably due to the expression and secretion of MMP9.

Discussion

In the present study, we demonstrate that Rap1GAP expression is downregulated in acute myeloid leukemia compared with non-malignant blood diseases. Upregulated Rap1GAP is associated with increased differentiation and apoptosis of the leukemic cells. We also show that increased expression of Rap1GAP promotes leukemic cell invasion *in vitro* and increased secretion of MMP9.

The earliest report of Rap1 by Kitayama *et al* identified Rap1 as a protein which could counteract the activation of Ras (14). However, later studies found that in different cell lines Rap1 activation may exert contradictory functions not just a counteracting molecule of Ras (2,3,15). Although it was unknown whether Rap1 played a 'good role' or was a 'bad actor' in cancer development, Rap1 was reported to regulate a variety of cellular processes such as the maturation of megakaryocytes (16), neuronal differentiation (17) and T-cell anergy (18). Some other molecules which regulate the Rap1 activation were also reported. Tuberin which shares structural similarity with Rap1GAP was subject to loss in tuberous sclerosis (19) and E6TP1 was believed to involved in the development of cervical cancer (20,21). More recently, Rap1GAP was reported as a tumor suppressor gene in pancreatic cancer (7), thyroid cancer (9,10), melanoma (1,8) and oropharyngeal squamous cell carcinoma (22). According to the SPA-1 knock-out B6 murine model, which presented a highly activated Rap1 and ultimately developed myeloid leukemia, SPA-1 was thought of as an important GAP in hematologic malignancies (23). However, little was known about the function and effect of Rap1GAP in hematologic malignancies. To verify if Rap1GAP also acts as a tumor suppressor gene in hematologic malignancies, we detected the expression of Rap1GAP in AML and leukemic cell lines. Based on the results that the expression of Rap1GAP was downregulated in AML as well as in leukemic cell lines, we thus upregulated the expression of Rap1GAP in HL-60 and NB4 cells to study the function of Rap1GAP *in vitro*.

Rap1GAP regulates GTP-GDP cycles to influence the B-RAF/MEK/ERK signaling pathway which is involved in cell proliferation, differentiation and apoptosis (24). Some studies reported that the upregulated expression of Rap1GAP reduced the level of both Rap1-GTP and p-ERK resulting in the suppression of cell proliferation (8-10), while others reported that upregulated Rap1GAP suppressed the cell proliferation with lower Rap1-GTP but without change of p-ERK (7). In our study, increased Rap1GAP can abolish Rap1-GTP, but we did not find suppressed growth or the

change of p-ERK. We do not exactly know why Rap1GAP has no effect on leukemic cell proliferation and we postulate that it may due to different cell types. Rap1GAP is mainly expressed in linage marker-positive BMCs according to a study on B6 mice (23). We believe that Rap1GAP may play an important role in hematologic cell differentiation and apoptosis. Interestingly, our results proved this consideration. We found that increased expression of Rap1GAP promoted the differentiation of HL-60 and NB4 cells induced by TPA or ATRA and render the cells to apoptosis in response to arsenic trioxide.

During ATRA treatment, we found the induced expression of p21 in both NB4 and HL-60 cells by semi-quantitative RT-PCR. p21 is a cdk (cyclin dependent kinase) inhibitor which suppresses Rb protein phosphorylation to inhibit cell division. Enhanced p21 expression may play a critical role in cell cycle arrest and differentiation (25,26). In fact, we have observed a much higher expression of p21 in the Rap1GAP-transfected cells than that in the control cells especially at 24 h. Upregulated Rap1GAP may shut off the GTP-GDP energy cycles of Rap1 via p21 to influence the cell differentiation and the exact process must be more complicated. In addition to the traditional pathway, Rap1GAP also interacted with the Gz member of trimeric G and may exert different cell activities (27,28).

Tumor invasion and metastasis involved several events including tumor cell adhesion, extracellular matrix (ECM) proteolysis and cell migration (29). In some reports, increased expression of Rap1GAP reduced tumor invasion by altering cell adhesion or migration (7,22). Zheng *et al* demonstrated that Rap1GAP suppressed two integrin-dependent events, focal adhesion formation and filamentous actin (F-actin) assembly, which resulting in the inhibition of melanoma cell migration (8). However, Mitra *et al* reported that Rap1GAP promoted invasion of squamous cell carcinomas via induction of MMP9 secretion and was associated with poor survival, which was the first report regarding the link between Rap1GAP and MMP9 (30). MMPs, particularly MMP9 and MMP2, play an important part in the degradation of ECM to render the spreading of tumor cells. Here, we also observed the increased invasion rate of Rap1GAP-transfected HL-60 cells as well as the higher expression and secretion of MMP9 compared to the control cells. The secretion and activation of MMPs were a complicated process which involved several genes including tissue inhibitor of metalloproteinase 2 (TIMP-2), membrane type 1 MMP (MT1-MMP) and other genes. Presently, we do not know why an increased expression of Rap1GAP promotes invasion of leukemic cells and whether it affects the prognosis of AML. This was the first report on the relationship between Rap1GAP and MMPs in hematopoietic cells.

In conclusion, our results demonstrated for the first time that the expression of Rap1GAP was downregulated in AML compared with the control group. Upregulated expression of Rap1GAP promoted leukemic cells (i.e. HL-60 or NB4 cells) differentiation and apoptosis while also promoted cell invasion. This study provides initial results on the function of Rap1GAP in leukemia cell lines *in vitro*. However, the exact mechanism of the effect of Rap1GAP in leukemogenesis and the relationship between Rap1GAP and MMP9 needs further exploration.

Acknowledgements

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