

Suppression of RND3 activity by AES downregulation promotes cancer cell proliferation and invasion

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Abstract. Amino-terminal enhancer of split (AES) is a member of the Groucho/TLE family. Although it has no DNA-binding site, AES can regulate transcriptional activity by interacting with transcriptional factors. Emerging evidence indicates that AES may play an important role in tumor metastasis, but the molecular mechanism is still poorly understood. In this study, we found that knockdown of AES by RNA interference (RNAi) downregulated RND3 expression at the mRNA and protein levels in MDA-MB-231 and HepG2, two cancer cell lines. Furthermore, luciferase assays showed that overexpression of AES significantly enhanced RND3 promoter activity. Moreover, inhibition of AES both in MDA-MB-231 and HepG2 cells by RNAi significantly promoted cell proliferation, cell cycle progression and invasion, consistent with the effects of RNAi-mediated RND3 knockdown in these cells. For the first time, data are presented showing that alteration of the malignant behavior of cancer cells by AES is related to RND3 regulation, and these findings also provide new insights into the mechanism of AES action in regulating tumor malignancy.

Introduction

The majority of patients with malignant carcinoma succumb to metastasis (1), and inhibition of cancer cell metastasis is an effective method to prolong the survival time of patients. Yet, the mechanisms by which tumor cells become metastatic are poorly understood (2). Thus, to explore the molecular mechanisms and molecular targets of metastasis is of vital importance in the targeted therapy of cancer (3).

Amino-terminal enhancer of split (AES), which is known as Groucho (Gro) or transducin-like enhancer of split (TLE), is a member of the transcriptional co-repressor family (4). The Gro/TLE protein family consists of five members and is highly conserved within different species (5). These proteins have no DNA-binding region but can interact with DNA-binding transcription factors (TFs) and mediate transcription activity of target genes (6,7). AES, as well as its mouse homolog GRG5, is the shortest member of the Gro/TLE family (4,8). It was considered that AES may act as a negative regulator in the process of transcription. Moreover, a series of previous research described that AES recruits various TFs such as NF- κ B and gp130 to gene promoters, and functions as a transcription repressor (9,10). Most of the previous studies concerning AES, as well as other members of the Gro/TLE family, have mainly focused on development, including bone growth, hematopoiesis and eye development (11-14), while there are only a few studies concerning the function of AES in cancer. AES also forms complexes with the mitochondrial protein Bit1 and induces cell death with characteristics of caspase-independent apoptosis (15). Recently, AES was reported as a metastatic repressor in colorectal cancer (CRC) by inhibiting the NOTCH signaling pathway, and composite depletion of AES in Apc intestinal polyposis mice caused marked tumor invasion and intravasation (16,17). Thus, loss of AES may be an important factor for inducing tumor invasion. Although AES is known to regulate the activity of the Notch intercellular domain (NICD), other pathways involved in AES regulation during the process of tumor metastasis must be explored.

The Rho GTPase family is a group of small GTPase proteins that are implicated in several cellular functions such as actin cytoskeleton organization, microtubule dynamics and cell cycle progression. Thus, they play an important role in

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Abbreviations: AES, amino-terminal enhancer of split; TLE, transducin-like enhancer of split; TFs, transcription factors; RNAi, RNA interference; CRC, colorectal cancer; NICD, Notch intercellular domain; RhoGAP, GTPase-activating protein for Rho; ROCK, Rho kinase-I; HCC, hepatocellular carcinoma; ESCC, esophageal squamous cell carcinoma

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cancer progression (18). Most Rho GTPase members function as molecular switches, cycling between an active GTP-bound and an inactive GDP-bound conformation (19). RND3, also known as RhoE, is an atypical member of the Rho GTPase family in that it binds, but does not hydrolyze GTP. The best-characterized function of RND3 is inhibition of RhoA/ROCK signaling by binding to p190 GTPase-activating protein for Rho (RhoGAP), thus reducing the RhoA-GTP levels, as well as directly regulating the RhoA effector, Rho kinase-I (ROCK-I) (20). Recent studies have indicated that RND3 could act as a tumor-suppressor gene in cancer progression (21,22). However, the molecular mechanism that regulates the expression of RND3 is poorly understood.

Herein, the results revealed that reduced AES expression downregulates RND3 expression at the mRNA and protein levels, while enhanced AES expression significantly increases the activity of the RND3 promoter. Further studies have shown that AES regulates tumor cell proliferation, cell cycle progression and invasion, and the process is related to RND3 regulation. For the first time, we demonstrated that AES regulates the expression of RND3, and the results further elucidate the mechanisms of AES in regulating tumor malignancies.

Materials and methods

Cell culture. The human hepatocellular carcinoma cell line HepG2 and the breast cancer cell line MDA-MB-231 were maintained in RPMI-1640 medium (Invitrogen, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Gaithersburg, MD, USA), 100 U/ml of penicillin G sodium, and 100 µg/ml streptomycin sulfate (Sigma, St. Louis, MO, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

Plasmid construct. The human AES gene (NM_001130, 197 amino acids) was PCR amplified from human HEK293 cDNA. To construct HA-AES, the AES coding sequence was amplified by sense primer 5'-GCCCCGAATTCCGATTGACATGA-3' and anti-sense primer 5'-GAACGGTACCCCCTGCTAATCCGACTTCTCGCCAT-3' and then cloned into the *Eco*R1 and *Kpn*I sites of the pCMV-HA vector. RND3 promoter was cloned into the pGL3-basic vector to generate the pGL3-RND3-promoter as previous described (23).

Small interfering RNA transfection. The siRNAs against AES were designed and synthesized by Ribobio (Guangzhou, China). The sequence of AES siRNA was 5'-CCUACGG CUUGAACAUUCGAdTdT-3' and the sequence of the RND3 siRNA strand was 5'-AACAGATTGGAGCAGCTACdTdT-3'. siRNAs against AES and RND3 were transfected into MDA-MB-231 and HepG2 cells respectively using Lipofectamine 2000 (Invitrogen).

Luciferase reporter assay. The pGL3-RND3-promoter (0.4 µg) and pRL-TK (0.005 µg) were co-transfected into HeLa cells in each well of 24-well plates together with pCMV-HA or pCMV-HA-AES. Firefly and *Renilla* luciferase activities were measured 48 h after transfection using the Dual-Luciferase reporter assay system (Promega) according to the manufacturer's protocol.

Values are shown as relative *Renilla* and Firefly luciferase activity, and data values are reported as means ± SEM.

RT-PCR. Total RNAs were extracted from transfected cells by TRIzol (Life Technologies) using the manufacturer's protocol and reconstituted in 1.0 µg/µl with nuclease-free water. For semi-quantitative reverse transcription-PCR, cDNA was synthesized from total RNA using Olig-dT primer. The primers used for specific RND3 PCR reactions were: forward 5'-AAGATA GTTGTGGTGGGAGA-3' and reverse 5'-CATAGTAAGGAGA ACCCGAA-3'. The specific AES PCR reaction primers used were: forward 5'-CACCAGGAGGATGATGGCGAG-3' and reverse 5'-GGCGTGGAGGTGTCTGGAAC-3'. The primers used for specific GAPDH PCR reactions were: forward 5'-CAA GGCCAACCGCGAGAA-3' and reverse 5'-CCCTCGTAGAT GGGCACAGT-3'.

Western blotting. Transfected cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 50 mM Tris-HCl pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 mM deoxycholic acid and 1 mM EDTA) containing a cocktail of protease inhibitors and phosphatase inhibitors (Calbiochem, Darmstadt, Germany). Equal amounts of the protein sample (30-50 µg) were separated by 12% SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA) using the Bio-Rad semi-dry transfer system. The following antibodies were used for western blotting: anti-AES (Sigma), anti-α-tubulin (Biostar, Wuhan, China), anti-RND3 (Millipore).

Cell proliferation assay. MDA-MB-231 and HepG2 cells were seeded in 96-well plates, and after 24 h, cells were transfected with two types of siRNAs or the negative control, respectively. Relative cell growth was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

Matrigel invasion assay. After 24 h of transfection, cells were collected and suspended in serum-free medium. Cells (4×10⁴) in 0.2 ml serum-free medium were plated in the top chamber with a Matrigel-coated membrane (24-well insert; pore size, 8 mm; Becton Dickinson), with 10% FBS as a attractant. The cells were incubated for 48 h. The cells that did not invade through the pores were removed, and the filter was stained with hematoxylin and eosin (H&E) for visualization and counting.

Cell cycle analysis by flow cytometry. Cells were transfected with siRNA against AES or RND3 for 48 h. The cells were then digested by trypsin, collected by centrifugation, washed with PBS and fixed overnight at 4°C by 70% ethanol. The cells were then washed with PBS and stained by PI at 4°C for 30 min using the Cell Cycle Detection kit (KeyGen, Nanjing, China). The cells were then analyzed using a flow cytometer (BD FACSCalibur).

Statistical analysis. The data are presented as the means ± SEM. The Student's t-test was used for comparisons. *p* < 0.05 was considered to indicate a statistically significant result.

Results

siRNA-mediated AES knockdown downregulates RND3 expression at the mRNA and protein levels. Previous studies

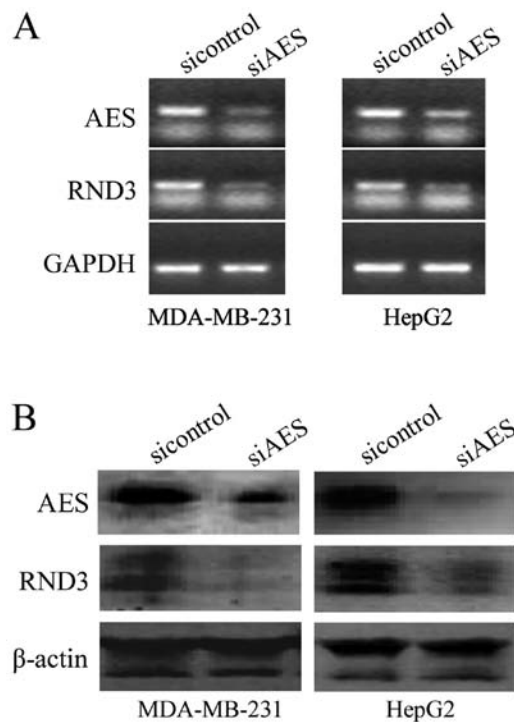


Figure 1. AES regulates the expression of RND3. (A) The mRNA expression level of AES and RND3 in MDA-MB-231 and HepG2 was analyzed by RT-PCR. GAPDH was used as a loading control. (B) Immunoblot analysis of AES and RND3 expression in MDA-MB-231 and HepG2 cells transfected with sicontrol or siAES. β -actin was used as a loading control.

reported that AES acted as a potential tumor-metastasis suppressor (16,17). However, the molecular mechanisms remain unclear (17). RND3, a typical member of the Rho family, was demonstrated as a tumor-suppressor in several studies (14,24). Recent studies indicate that both AES and RND3 are involved in tumor metastasis. Thus, we hypothesized that AES could regulate RND3 expression levels in cancer cells. Breast cancer cell line MDA-MB-231 and hepatocellular carcinoma cell (HCC) line HepG2 were transfected with AES siRNA. RT-PCR results revealed that knockdown of AES by RNA interference (RNAi) downregulated RND3 expression at the mRNA level (Fig. 1A). Western blot analysis showed that downregulation of AES also inhibited RND3 expression at the protein level in both the MDA-MB-231 and HepG2 cell lines (Fig. 1B). These results reveal that AES regulates RND3 at the mRNA and protein levels.

Enhanced AES expression increases RND3 promoter activity. The relationship between AES and RND3 was further investigated. To investigate whether AES regulates RND3 promoter activity, the promoter sequence of RND3 was subcloned into the pGL3-basic vector. Then, the pGL3-RND3-promoter was co-transfected with pCMV-HA-AES or pCMV-HA, respectively, into HeLa cells. Dual-luciferase reporter assay revealed that forced AES expression increased the luciferase activity of the pGL3-RND3-promoter by 5.6-fold compared with the control pCMV-HA group. Meanwhile, AES siRNA abolished the activation of luciferase activity induced by enhanced AES expression (Fig. 2). In summary, these data indicate that AES functions as a positive regulator of the RND3 promoter.

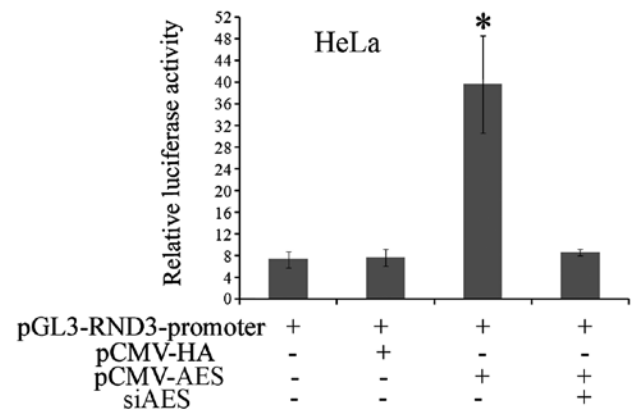


Figure 2. AES regulates RND3 promoter activity. pGL3-RND3-promoter luciferase constructs and the pRL-TK reporter plasmid were cotransfected into HeLa cells with pCMV-HA, pCMV-AES or RND3 siRNA, respectively. Luciferase activity was determined 48 h after transfection. The pRL-TK reporter plasmid was used as the transfection control. The ratio of normalized sensor to control luciferase activity is shown. Data are shown as the means \pm SD and were obtained from three independent experiments performed in triplicate. * $p < 0.05$, a significant difference from the control transfected cells.

Downregulation of AES and RND3 expression enhances the proliferation of cancer cells. Previous studies have shown that RND3 functions as a tumor-suppressor gene in esophageal squamous cell carcinogenesis, and is involved in the regulation of tumor cell proliferation, invasion and cell cycle progression (25-27). Recent research also indicates that AES may have a similar function with RND3 (16). Our results showed that AES regulates the expression of RND3 in breast cancer and HCC cells. Thus, whether AES and RND3 function similarly in these two cancer cell lines needs to be further investigated. To determine the influence of AES and RND3 on tumor proliferation, specific siRNAs of AES and RND3 were separately transfected into MDA-MB-231 and HepG2 cells. After transfection, the viability of both HepG2 and MDA-MB-231 cells was markedly increased at each time point similar to the effect of RND3 knockdown (Fig. 3). This result demonstrated that downregulation of AES promotes cancer cell proliferation.

siRNA-mediated AES and RND3 downregulation induces cell cycle progression of cancer cells. The cell cycle progression of cancer cells with different transfection was assayed by flow cytometry. The percentage of G1 phase cells transfected with AES siRNA was decreased significantly compared with the controls (Fig. 4). Downregulation of AES in both MDA-MB-231 and HepG2 cells promoted S-G2-M phase progression, which was in correspondence with the effect of RND3 knockdown by siRNA (Fig. 4). These results indicate that AES also contributed to cell cycle progression and this function involved RND3 regulation.

Knockdown of AES and RND3 increases the invasive activity of cancer cells. We performed a Matrigel invasion assay to investigate the invasive activity of cancer cell lines. MDA-MB-231 and HepG2 cells were transfected with control, AES or RND3 siRNAs separately. Knockdown of both AES and RND3 expression resulted in a significant increase in the

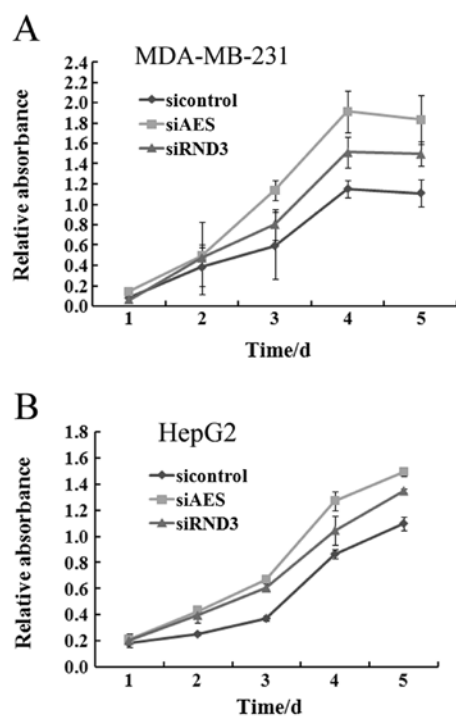


Figure 3. AES influences proliferation potential of cancer cells. Proliferation potential of (A) MDA-MB-231 and (B) HepG2 cells 24 h after transfection with sicontrol, siAES or siRND3, was determined by the CCK-8 assay. The data represent means \pm SD from 3 independent experiments performed in triplicate.

number of invasive cells compared with the control groups (Fig. 5). This result suggests that downregulation of AES enhances breast cancer and HCC cell invasion by mimicking the inhibition of RND3 expression.

Discussion

AES, a member of the Gro/TLE family, regulates gene expression at the transcription level by interacting with various transcriptional factors (TFs) (28-31). In the past few years, studies concerning AES as well as other members of the TLE/GRG family have mainly focused on various developmental and pathological processes (32-34). Recently, Sonoshita *et al* reported that AES/GRG5 prevents metastasis of colorectal cancer cells by inactivating Notch signaling and may function as a metastasis-suppressor gene, which highlights an innovative therapy for anti-metastasis (16). However, the regulatory mechanism of AES in tumorigenesis and cancer progression remains to be further determined. Our present study suggests that AES-mediated RND3 regulation plays an important role in the process of cell proliferation, cell cycle progression and invasion.

In the present study, small specific siRNA was used to knockdown AES expression in MDA-MB-231 and HepG2 cells, and the results showed that RND3 expression was decreased accompanied by AES downregulation. These data indicate that AES regulates RND3 directly or indirectly. A dual-luciferase reporter assay was used to determine whether AES influences RND3 promoter activity. Forced AES expression significantly activated RND3 promoter activity in HeLa cells. However,

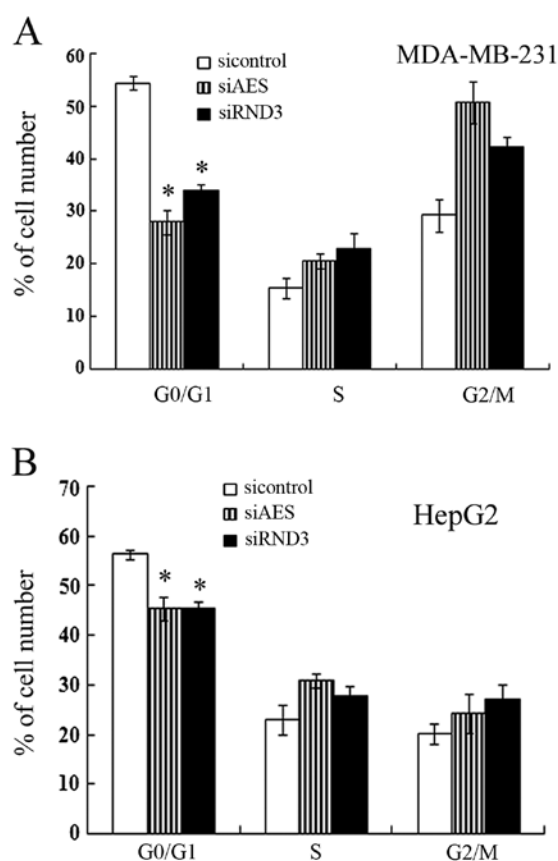


Figure 4. Downregulation of both AES and RND3 promotes cell cycle progression. Cell cycle progression of (A) MDA-MB-231 and (B) HepG2 cells 48 h after transfection with sicontrol, siAES or siRND3, was determined by FACS analysis. Data are representative of three independent experiments. * $p < 0.05$, a significant difference from oligo-transfected control cells.

whether AES interacts with the RND3 promoter directly still requires further investigation.

At the transcriptional level, RND3 may be directly regulated by many TFs, including P53, HIF-1 α and Foxd3 (35-37). Moreover, DNA damage-inducing stimuli, including chemotherapeutic agents and ultraviolet (UV) irradiation, could upregulate the RND3 gene expression at both the mRNA and protein levels (38). According to the characteristics of the Gro/TLE family members, we hypothesized that AES interacts with various TFs and promotes RND3 expression at the transcription level. Therefore, it will be interesting to explore the accurate molecular mechanisms by which AES interacts with TFs and affects RND3 transcription.

RND3 is an atypical member of the Rho family, and studies concerning this molecule are relatively fewer compared with studies of members of the Rho family (39). Previous studies showed that RND3 regulates a diverse set of biological activities including actin organization, cell motility, cell-cycle progression and apoptosis (24,27).

Recent research revealed that elevated RND3 expression markedly increased the expression levels of PTEN and p27, while decreasing pAkt expression, thus inhibiting cell cycle progression at the G1 phase (26,40). RND3 also blocks cell cycle progression at the G2/M phase. A study using a prostate cancer cell line showed that forced RND3 overexpression inhibits the expression levels of CDC2 and cyclin B1 which are essential

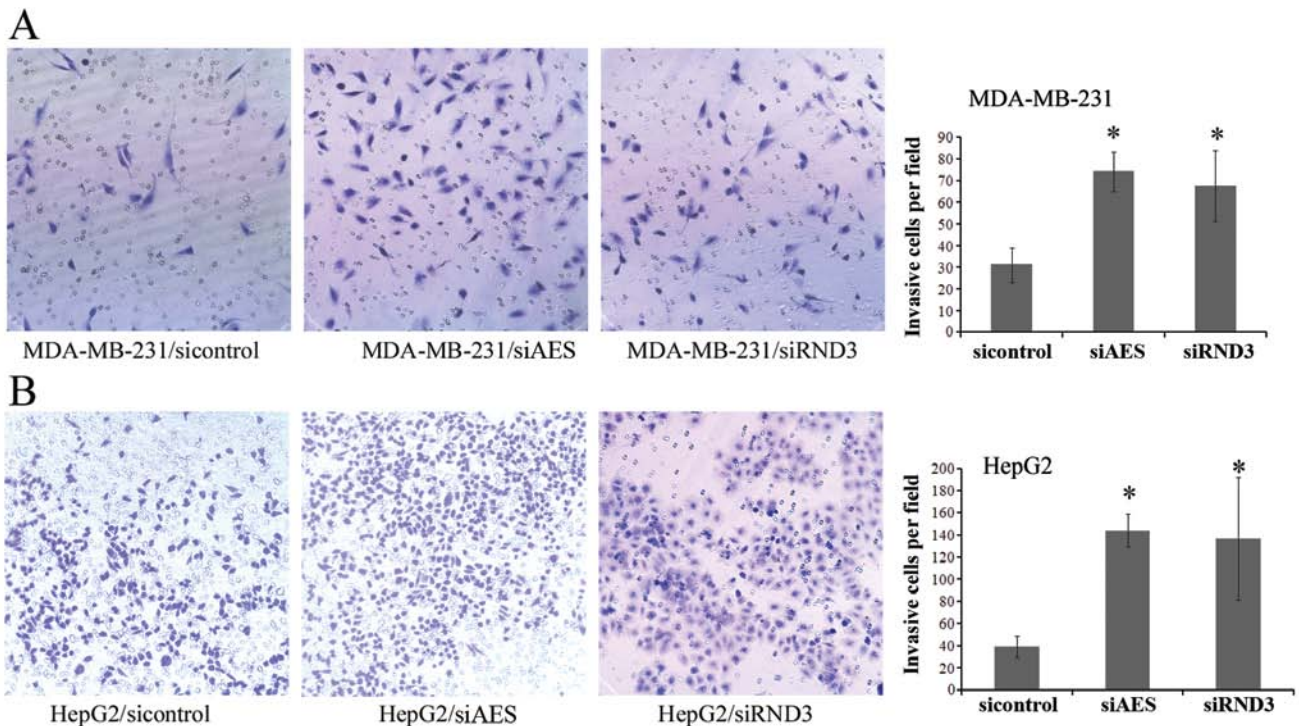


Figure 5. AES influences cancer cell invasion. The invasive ability of (A) MDA-MB-231 and (B) HepG2 cells 48 h after transfected with sicontrol, siAES or siRND3, was assayed using a Matrigel-coated Transwell. The cells that successfully invaded into the Matrigel were quantified 48 h after plating. Data are represented as the means \pm SD for three independent experiments. * $p < 0.05$, a significant difference from the control oligo-transfected cells.

for G2/M transition, and induces G2/M phase arrest (41). Moreover, downregulation of RND3 in ESCC cells promoted cell proliferation, cell cycle progression, as well as cell invasion *in vitro* (26).

Our results revealed that siRNA-mediated AES down-regulation decreased the expression of RND3; therefore, we hypothesized that AES executes its function through RND3. Further functional experiments were carried out using these two genes. Rapid proliferation was induced by AES-specific siRNA transfection in both MDA-MB-231 and HepG2 cells. Knockdown of AES in these two cell lines also induced cell cycle progression and promoted cell invasion. These effects were also noted when cells were transfected with RND3-specific siRNA and were consistent with the effects of AES-specific siRNA transfection. The above results indicate that AES regulates RND3 expression and suggest that down-regulation of AES promotes tumor cell proliferation, cell cycle progression and invasion which involves RND3 expression. We demonstrated, for the first time, that there is a connection between AES and RND3, a typical member of the Rho family. Our results also elucidate the mechanisms of AES regulation and offer new insights into the molecular mechanisms of how AES executes its tumor repressor functions.

Acknowledgements

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