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Action of HMGB1 on miR-221/222 cluster in neuroblastoma cell lines

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Abstract. microRNA (miR/miRNA) are small non-coding RNAs that control gene expression at the post-transcriptional level by targeting mRNAs. Aberrant expression of miRNAs is often observed in different types of cancer. Specific miRNAs function as tumor suppressors or oncogenes and interfere with various aspects of carcinogenesis, including differentiation, proliferation and invasion. Upregulation of miRNAs 221 and 222 has been shown to induce a malignant phenotype in numerous human cancers via inhibition of phosphatase and tensin homolog (PTEN) expression. Neuroblastoma is the most common extracranial solid malignancy in children, which is characterized by cellular heterogeneity that corresponds to different clinical outcomes. The different cellular phenotypes are associated with different gene mutations and miRs that control genetic and epigenetic factors. For this reason miRs are considered a potential therapeutic target in neuroblastoma. The aim of the present study was to investigate the mechanisms by which extracellular high mobility group box 1 (HMGB1) promotes cell growth in neuroblastoma. SK-N-BE(2) and SH-SY5Y neuroblastoma derived cell lines were transfected with the antisense oligonucleotides, anti-miR-221 and -222, followed by treatment with HMGB1 to investigate the expression of the oncosuppressor PTEN. In this study, it was demonstrated that HMGB1, which is released by damaged cells and tumor cells, upregulates miR-221/222 oncogenic clusters in the two human neuroblastoma derived cell lines. The results revealed that the oncogenic cluster miRs 221/222 were more highly expressed by the most undifferentiated cell line [SK-N-BE(2)] compared with the the less tumorigenic cell line (SH-SY5Y) and that exogenous HMGB1 increases this expression. In addition, HMGB1 modulates PTEN expression via miR-221/222, as demonstrated by transiently blocking miR-221/222 with anti-sense oligonucleotides. These results may lead to the development of novel therapeutic strategies for neuroblastoma.

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Introduction

Neuroblastoma arises from neural crest precursors that do not differentiate into terminal neurons. The hallmark of these tumors are the numerous different clinical variables, ranging from highly metastatic with rapid progression and resistance to therapy to spontaneous regression or development into benign ganglioneuromas (1). Neuroblastoma accounts for >7% of malignancies in pediatric patients (<15 years) (1). Approximately 65% of primary tumors occur in the adrenal glands. Tumors also arise in the neck, chest and pelvis. The symptoms of neuroblastoma are dependent on tumor location, as well as the presence or absence of metastasis and paraneoplastic syndromes (1). At diagnosis, 40% of cases are classified as high-risk according to age of onset (<18 months) and the presence of genetic mutations (2). Despite advances in chemotherapy, 60% of high-risk neuroblastomas recur and thus mortality rates are extremely high (3). For low-risk patients, surgery alone is curative in the majority of cases, while intermediate-risk patients (without MYCN amplification) are administered low doses of cyclophosphamide and vincristine until resection is possible (4). Treatments for high-risk patients with stage III and IV neuroblastoma include intensification of conventional chemotherapy agents with the addition of 13-cis-retinoic acid. Clinical trials with viral agents able to target tumor cells or immune therapies with exposure of neuroblastoma cells to interferon γ, prior to conventional chemotherapy, are returning encouraging results (5).

The different clinical variables correspond to genetic mutations in crucial genes, including oncogenes, oncosuppressor genes and transcription factors. Furthermore, scientists are concentrating their efforts on determining the network of regulatory microRNAs (miRs/miRNAs) and genes in neuroblastoma (6,7). A possible therapeutic approach would be to enhance the expression of miRNAs that induce differentiation while suppressing those associated with tumor progression. As well as regulating the expression of oncogenes and transcription factors, miRNAs are associated with epigenetic

mechanisms in neuroblastoma, such as DNA methylation and the induction of autophagy (8-10).

The key prognostic factor in neuroblastoma is MYCN amplification (11), which occurs in ~20% of cases and is associated with poor outcome. MYCN amplification is correlated to the transcription of proteins that regulate differentiation, such as nestin (12), or HMG proteins, which suppress apoptosis, induce autophagy and amplify AKT signaling (13,14). A recent study demonstrated that high mobility group box 1 (HMGB1) is upregulated in neuroblastoma (15), inducing cell proliferation and autophagy in Schwann cells. In our recent study, the HMGB1/receptor for advanced glycation end products (RAGE)/miR-221/222 signaling pathway, which is associated with cellular proliferation and phosphatase and tensin homolog (PTEN) inhibition, was identified in a model of thyroid cancer (16). *In vitro* models of neuroblastoma derive from different tumors, and maintain their heterogeneous genetic variances and, thus, tumorigenic properties. It is known that each neuroblastoma and its deriving cell line has at least three different phenotypic patterns that include stem, neuroblastic and non-neuronal cells. The different phenotypes show different tumorigenicity. Tumorigenicity in neuroblastoma is directly associated with MYCN amplification and differently expressed miRNAs (17). Furthermore, numerous miRNAs induce neuroblastoma cell differentiation and interact with endogenous substances. The understanding of these interactions may provide novel strategies for the treatment of neuroblastoma.

The present study reports the role of HMGB1 in an MYCN-amplified neuroblastoma cell line [SK-N-BE(2)] and a neuroblastoma cell line without MYCN amplification (SH-SY5Y). The results show that HMGB1 exerts different effects on the two cell lines and that its interaction with miR-221/222 influences important pathways associated with cell growth.

Materials and methods

Reagents. Anti-human PTEN monoclonal antibody (cat. no. M3627; 1:1,000 dilution) was obtained from Dako (Carpinteria, CA, USA). Monoclonal anti-mouse IgG horseradish peroxidase conjugate (cat. no. NXA931; 1:2,000 dilution) was purchased from GE Healthcare (Little Chalfont, UK). Monoclonal anti-human HMGB1 (cat. no. H9537; 1:2,000) and β-actin (cat. no. A5316; 1:10,000) antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human recombinant HMGB-1 expressed in *Escherichia coli* was from Sigma-Aldrich (cat no. H4652; St. Louis, MO, USA). miRIDIAN Hairpin Inhibitor hsa-miRNA-221 (cat. no. IH300578-07-005) and hsa-miRNA-222 (cat. no. IH301176-02 -005) and miRIDIAN microRNA Hairpin Inhibitor Transfection Control with Dy547 (cat. no. FE5IP0045000105) (control oligonucleotide) and DharmaFECT 1 Transfection Reagent (cat. no. FE5T200103) were purchased from GE Healthcare Dharmacon Inc. (Lafayette, CO, USA). The mature miRNA-221 sequence was 5'-AGCUACAUUGUCUGCUGG GUUUC-3' and the mature miRNA-222 sequence was 5'-CUC AGUAGCCAGUGUAGAUCCU-3'. Reverse transcription (RT) primers and TaqMan probes were obtained from TaqMan miRNA assays (Applied Biosystems, Foster City, CA, USA).

Cell lines. SK-N-BE(2) (DSMZ no. ACC632) and SH-SY5Y (DSMZ no. ACC209) cells were obtained from DSMZ (Braunschweig, Germany) in October 2012 and maintained in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.), supplemented with heat-inactivated 10% fetal calf serum (FCS) containing 2 mM L-glutamine. Where required, 10 nM HMGB1 was added to the cultures for 24, 48 or 72 h. Cell viability was determined by a trypan blue exclusion test. Cells were frozen in nitrogen liquid tanks ($2x10^6$ cells/ml FCS, 10% dimethylsulfoxide) until use between the 4th and 8th passage after revival.

Cell viability. Cell viability was determined using the Trypan blue exclusion test. SK-N-BE(2) or SH-SY5Y cells ($5x10^4$/ml) were suspended in a 10-μl suspension containing trypan blue (0.4% w/v; Sigma-Aldrich) in phosphate-buffered saline (PBS) and incubated at room temperature for 5 min, followed by examination under a light microscope to determine the percentage of cells with clear (viable cells) and blue cytoplasm (non-viable cells).

Knockdown of miRNA. AntagomiRs against miR-221 and -222, and control oligonucleotide with Dy547 were transiently transfected into SK-N-BE(2) and SH-SY5Y cells using DharmaFECT 1 Transfection Reagent, prior to treatment with HMGB1. Briefly, cells were seeded in 12-well plates at a density of $2x10^5$ cells/well and incubated at 37˚C for 18 h prior to transfection. Subsequently, culture medium was replaced with 1 ml antibiotic-free medium containing DharmaFECT 1 Transfection Reagent (2 μl/ml) and antagomiRs at a final concentration of 25 nM for 24 h, according to manufacturer's instructions. Controls with Dy547 and DharmaFECT 1 Transfection Reagent alone were included in the experiments as indicators of transfection efficiency and as negative controls, respectively. Transfection efficiency was determined by cytofluorimetric readings in the red light spectrum (575 nm; Cytofluorimetric Coulter Epics XL; Beckman Coulter, Brea, CA, USA) and reached 69-76% in Dy547-positive cells.

Relative quantification of miRNA by RT-quantitative polymerase chain reaction (qPCR). Total RNA was extracted from SK-N-BE(2) and SH-SY5Y cells with TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Briefly, 1 ml TRIzol and 0.2 ml chloroform (Sigma-Aldrich) were added to pellets and centrifuged at 12,000 x g at 4˚C for 15 min. For the detection of mature miR-221 and -222, 50 ng total RNA was reverse transcribed using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed using an miRNA-specific TaqMan assay (cat. no. PN4427975; Applied Biosystems; Thermo Fisher Scientific, Inc.) and hsa-miR221 and -222 and an ABI Prism 7900HT Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR conditions were as follows: 50˚C for 2 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. Amplifications were performed in triplicate and repeated twice. The ubiquitously expressed U6b small nuclear RNA was used for normalization. An experimental negative control (no cDNA) was used, which confirmed the absence of amplification.

Relative quantification of miRNA expression was performed using the ΔΔCq method (18).

Western blot analysis. Briefly, whole cell lysates were heat denatured for 5 min, loaded on a standard Tris-HCl 12.5% SDS-PAGE gel, and run on ice at 40 V for the stacking gel and 80 V for the running gel to separate the proteins, as previously described (12). Proteins were transferred onto a previously activated PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Membranes were then placed in Tris-buffered saline (Sigma-Aldrich) with Tween 20 and 5% bovine serum albumin (Sigma-Aldrich) for 1 h, and probed overnight with the specific antibody at 4˚C. At the end of incubation time, membranes were washed with Tris-buffered saline and incubated with anti-mouse IgG peroxidase conjugated secondary antibody (1:2,000 dilution) for 1 h at room temperature. Membranes were stripped with stripping buffer (β-mercaptoethanol, SDS and Tris-HCl) and incubated with β-actin monoclonal antibody as a loading control. Signals were detected by autoradiography (Kodak Biomax; Kodak, Rochester, NY, USA) using a chemiluminescent peroxidase substrate kit (Sigma-Aldrich), then quantified by densitometric analysis using Quantity-One software (version 4.6.5; Bio-Rad Laboratories, Inc.).

Cytofluorimetric determination of cell cycle. Parental control cells and transfectant cells (10^5) were seeded into a 6-well plate with and without 10 nM HMGB1. After 24 h, cells were fixed with cold 70% ethanol in PBS for 1 h at 4˚C. After centrifugation at 200 x g for 10 min at 4˚C, cells were washed once in PBS. The pellet was resuspended in a solution of 0.5 ml propidium iodide (PI; 0.1 mg/1 ml in PBS; Sigma-Aldrich) and 50 μl RNAse (Type I-A; 10 mg/ml in PBS; Sigma-Aldrich). PI-stained nuclei were incubated in the dark at room temperature for 15 min and maintained at 4˚C until the nuclear DNA content of the cells was evaluated by flow cytometry (Cytofluorimetric Coulter Epics XL; Beckman Coulter).

Statistical analysis. All statistical analyses were performed using KaleidaGraph version 4.5.1 (Synergy Software Inc., Reading, PA, USA). All determinations were performed three times. Data are expressed as means + standard deviation. The differences between cell populations [SK-N-BE(2) cells and SH-SY5Y cells, untransfected or transfected with antagomiRs; untreated or treated with HMGB1] were analyzed for miR-221/222, HMGB1 and PTEN expression, using Student's t-test. P-values were evaluated in the differently treated cell populations. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of HMGB1 on miR-221 and -222 expression in SK-N-BE(2) and SH-SY5Y cells. Fig. 1 shows that the two cell lines express different amounts of miR-221 and -222. SK-N-BE(2) cells express markedly higher levels of miR-221 (100-fold; P=0.004) and miR-222 (80-fold; P=0.007) than SH-SY5Y cells.

Treatment with 10 nM HMGB1 for 24 h increased expression of miR-221 and -222 in both SK-N-BE(2) and SH-SY5Y cells. By contrast, transfection of SK-N-BE2 cells with miR-221 and -222 antagomiRs reduced their expression by 49% each compared with the controls. In transfected SH-SY5Y cells, expression was reduced by 41% for miR-221 and 40% for miR-222, compared with the controls.

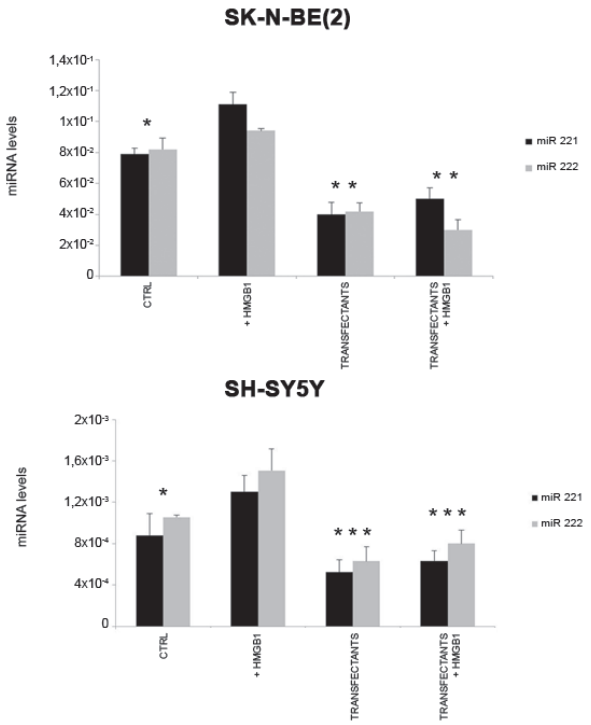

Figure 1. Effect of HMGB1 on miR-221 and -222 expression in SK-N-BE(2) and SH-SY5Y cells. SK-N-BE(2) (upper panel) and SH-SY5Y cells (lower panel) were cultured for 24 h with or without 10 nM HMGB1. Expression levels of miRNAs were detected by reverse transcription-quantitative polymerase chain reaction. miRNA expression was quantified using the ΔΔCq method and normalized to U6b small nuclear RNA expression. Histograms represent the mean ± standard deviation of 3 independent experiments. *P<0.05, SK-N-BE(2) vs. SH-SY5Y Ctrl. **P<0.05, SK-N-BE(2) transfectants vs. SK-N-BE(2) transfectants + HMGB1. ***P<0.05, SH-SY5Y transfectants vs. SH-SY5Y transfectants + HMGB1. miR/miRNA, microRNA; Ctrl, control; HMGB1, high mobility group box 1; Ctrl, control.

The treatment of transfected cells (transfectants) with HMGB1 for 24 h did not markedly increase or decrease the expression of miR-221/222 in either cell line.

Expression of HMGB1 in parental control and transfectant SK-N-BE(2) and SH-SY5Y cells. Endogenous HMGB1 protein was expressed in both cell lines (Fig. 2). While endogenous HMGB1 levels decreased in SK-N-BE(2) transfectants compared with the controls (P=0.04), the opposite effect was observed in SH-SY5Y transfectants, which expressed markedly higher levels of HMGB1 than the control SH-SY5Y cells (P=0.003).

Expression of PTEN in miR-221 and -222 in parental control and transfectant SK-N-BE(2) and SH-SY5Y cells. To show that the action of exogenous HMGB1 on miRNAs is functional, the expression of PTEN, a known target of miR-221/222, was analyzed in control and transfectant cells. Fig. 3 shows that PTEN expression is markedly decreased in SK-N-BE(2) transfectants (miR-221/222) compared with controls, while the addition of HMGB1 increases PTEN expression to the same

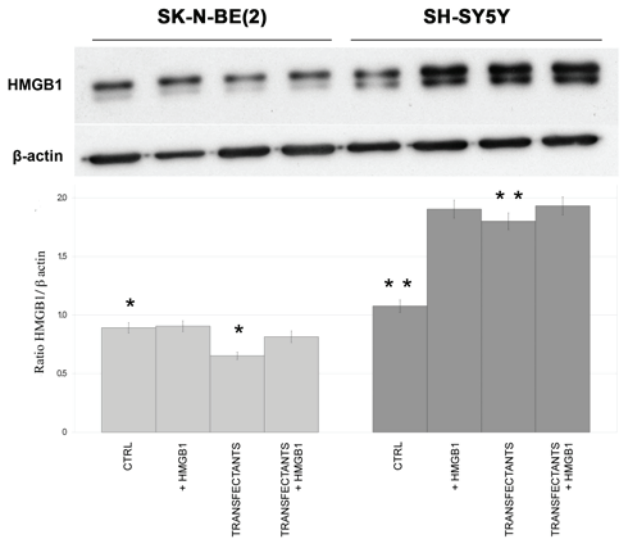

Figure 2. HMGB1 expression in parental ctrl and transfectant SK-N-BE(2) and SH-SY5Y cells. SK-N-BE(2) and SH-SY5Y cells were cultured for 24 h with and without 10 nM HMGB1. The expression level of HMGB1 protein (28-30 kDa) was detected by western blotting in whole cell lysates. Densitometry was performed on the western blot bands and each plot is shown underneath each band. Results are representative of 3 different western blot experiments. *P<0.05, SK-N-BE(2) Ctrl vs. transfectants. **P<0.05, SH-SY5Y Ctrl vs. transfectants. HMGB1, high mobility group box 1; Ctrl, control.

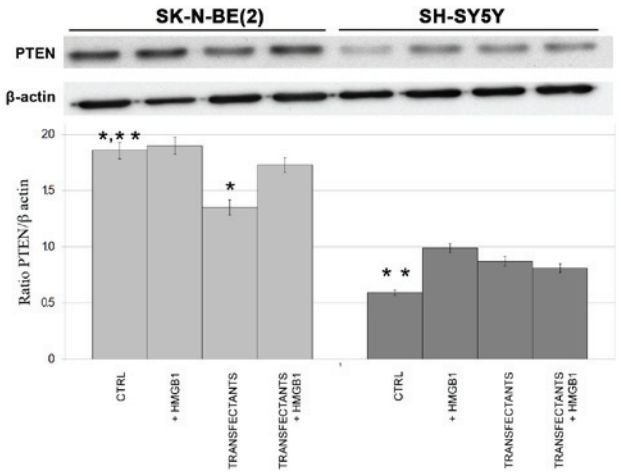

Figure 3. PTEN expression in parental ctrl and microRNA-221/222 SK-N-BE(2) and SH-SY5Y cells. SK-N-BE(2) and SH-SY5Y cells were cultured for 24 h with and without 10 nM HMGB1. Protein expression level of PTEN protein (55 kDa) was detected by western blot analysis in whole cell lysates. Densitometry was performed on western blot bands and each plot is shown underneath each band. Results are representative of 3 different western blot experiments. *P<0.05, SK-N-BE(2) vs. transfectants. **P<0.05, SK-N-BE(2) Ctrl vs. SH-5H5Y Ctrl. PTEN, phosphatase and tensin homolog; ctrl, control; HMGB1, high mobility group box 1.

levels as the controls (P=0.008). The decreased PTEN expression in SK-N-BE(2) transfectant cells may be explained by the different roles of miRNAs in neuroblastoma, as the same miRNAs may induce proliferation or survival of certain cells and concurrent differentiation of others.

In SH-SY5Y cells, HMGB1 appeared to increase PTEN expression compared with the control cells, while it did not show any effect in the transfectant cells. It is of note that PTEN expression in control SH-SY5Y cells was markedly lower than in control SK-N-BE(2) cells (P=0.001), in agreement with a previous study (19).

HMGB1 has opposite effects on the growth of SK-N-BE(2) and SH-SY5Y cells. Fig. 4 shows growth curves of parental control and transfectant SK-N-BE(2) and SH-SY5Y cells. The addition of 10 nM HMGB1 to SK-N-BE(2) cells for 24, 48 and 72 h induced an increase in growth of 56, 284 and 125%. Notably, the opposite result was obtained in SH-SY5Y cells cultured in the presence of HMGB1, with growth increasing to 28 and 75% at 24 and 48 h, but decreasing to 0.9% at 72 h. SK-N-BE(2) cells transfected with miR-221 and -222 antagomiRs demonstrated a decrease in growth compared with control cells. HMGB1 added to transfectant SK-N-BE(2) and SH-SY5Y cells further reduced growth compared with untreated transfectants.

Analysis of DNA content in parental control cells and transfectants of both cell lines treated with or without HMGB1 for 24 h confirmed the growth curves results. As shown in Fig. 5, a high tetraploid peak was observed in SK-N-BE(2) cells following treatment with HMGB1, while the tetraploid peak was reduced in transfectant cells (A3) and there was a high hypodiploid peak in SK-N-BE(2) transfectant cells (A4), indicating cellular apoptosis. Regarding SH-SY5Y cells, the reduction in growth following treatment with HMGB1 was confirmed by the high hypodiploid peak (B2) and low tetraploidy (B4) peaks observed in both transfectant and control cells following treatment with HMGB1.

Discussion

Transcription of miR-221 is induced by N-Myc transcription factor (20) in MYCN-amplified SK-N-BE(2) cells. The present study demonstrated that miR-221 and -222 are more highly expressed in SK-N-BE(2) cells than in SH-SY5Y cells. It is known that the miR-221/222 cluster targets PTEN (21), an oncosuppressor gene that is considered to be one of the primary regulators of AKT-activating pathways. The PTEN gene is frequently lost on chromosome 10q2317 in various types of human cancer. Notably, aberrant methylation of the PTEN gene has been observed in neuroblastoma tissues, resulting in PTEN inactivation and AKT hyperactivation. Our previous study reported that HMGB1 decreases PTEN expression in thyroid cancer cell lines by cooperating with the miR-221/222 cluster. Similarly, the present study reports that HMGB1 has an effect on neuroblastoma cell lines by enhancing miR-221 and -222 expression in both MYCN-amplified and non-amplified cell lines. The growth increase induced by HMGB1 in SK-N-BE(2) cells may be explained by the known association between N-Myc transcription factor and HMGB1, which acts on AKT-activating pathways (22). Furthermore, the present study showed that HMGB1 enhances miR-221/222 expression and induces growth in SK-N-BE(2) cells. When miR-221/222 expression was reduced by antisense oligonucleotides, PTEN expression also decreased; however, the addition of HMGB1 for 24 h was able to re-establish PTEN function and, consequently, reduce the cell growth rate. We hypothesize that HMGB1 exerts its action on miR-221/222 and PTEN expression as a consequence of their interaction. Furthermore, the

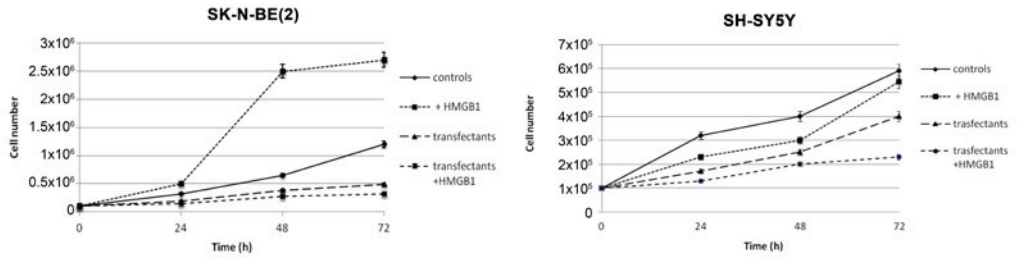

Figure 4. Effect of HMGB1 on the proliferation rate of parental control and transfected cells. Parental control cells and transfectant cells (10^5) were seeded into a 6-well plate at time 0 h with and without 10 nM HMGB1. At the indicated time points, cell number and cell viability of each well were counted with a hemocytometer. Data represent the mean ± standard deviation of 3 independent experiments. HMGB1, high mobility group box 1.

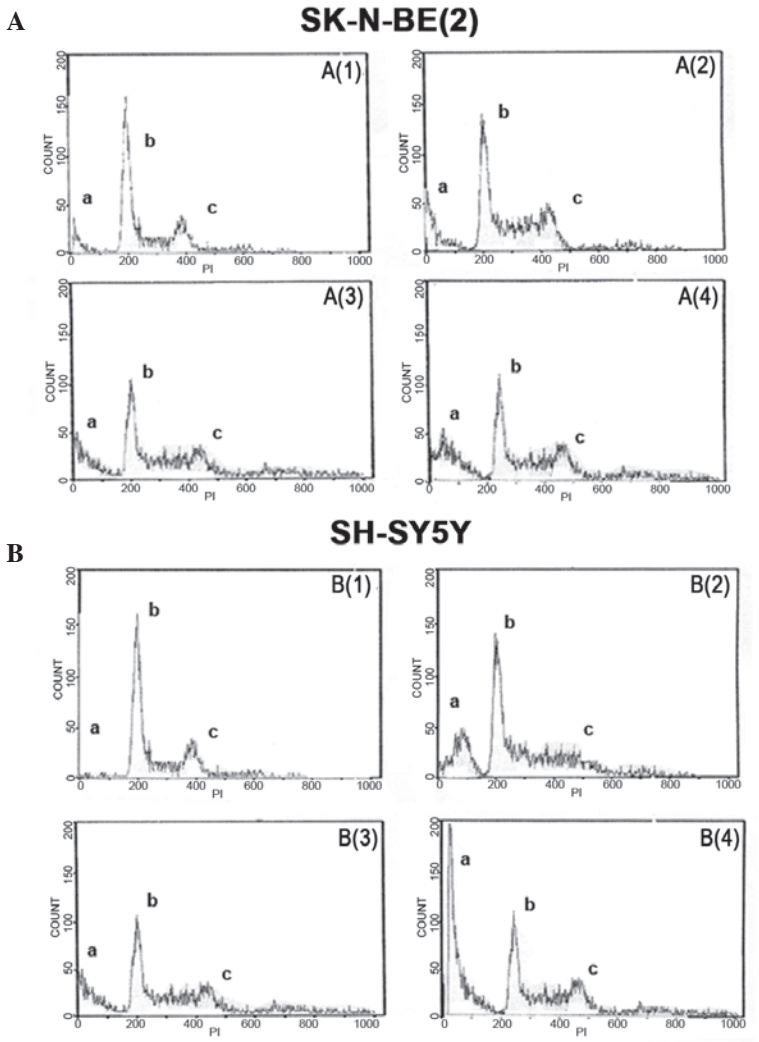

Figure 5. Cell cycle of control parental and transfected (A) SK-N-BE(2) and (B) SH-SY5Y cells. Parental control cells and transfectant cells (10^5) were seeded into a 6-well plate at 0 h with and without 10 nM high mobility group box 1 (HMGB1). After 24 h, cells were fixed in 70% ethanol at 4˚C for 1 h and then resuspended in PI solution until DNA content was determined on a cytofluorimeter. Photograms (A) and (B) both show control parental cells (top left), transfected cells (top right) control parental cells treated with HMGB1 (bottom left) and transfected cells treated with HMGB1 (bottom right). Peaks: a indicates hypodiploid peaks, b indicates diploid peaks and c indicates tetraploid peaks. Data are representative of 3 independent experiments. PI, propidium iodide.

decreased PTEN expression observed in SK-N-BE(2) cells may be explained by the different roles of miRNAs in neuroblastoma, as the same miRNAs may induce both inhibition and potentiation of certain mRNAs in the different neuroblastoma lines. It is known that exogenous HMGB1 binds RAGE, which in turn activates intracellular pathways that lead to AKT activation. The present study identified a possible pathway through which HMGB1 increases the growth of SK-N-BE(2) cells, triggered by the action of miR-221/222 on PTEN. This is supported by the observation that when miR-221 and -222 function are reduced by antagomiRs, HMGB1 increases PTEN expression. In SH-SY5Y cells,

which express markedly less miR-221 and -222 than SK-N-BE(2) cells, HMGB1 reduces growth. The differential expression of miR-221/222 in the two cell lines reported in the present study is consistent with a previous finding that expression of miR-21, -221 and -335 are associated with non-tumorigenic and neuroblastoma cell differentiation (23). By contrast, RAGE (24), which interacts with HMGB1, has a prominent role in neuritic extension in neurons (25). During early development, neurons undergoing differentiation express higher levels of RAGE and HMGB1 (26). A previous study (27) reported that the interaction between RAGE and HMGB1 potentiates retinoic acid-induced differentiation of SH-SY5Y cells. Furthermore, in thyroid cancer, in which the miR-221/222 cluster has an oncogenic effect, it has been shown that blocking RAGE prevents the action HMGB1 on miRNAs. Therefore, it is hypothesized that binding of RAGE with HMGB1 can regulate the actions of miR-221/222, even in neuroblastoma cells (unpublished data).

In conclusion, HMGB1 acts on PTEN through the enhancement of miR-221/222 in SK-N-BE(2) and SH-SY5Y cells. In cells transfected with miR-221/222 antagomiRs, HMGB1 was able to enhance PTEN expression and consequently reduce cell growth. The present study indicates that HMGB1 contributes to neuroblastoma growth and differentiation by cooperating with miRNAs that in turn function as oncogenes, oncosuppressor genes or differentiating factors. The present study indicates that HMGB1 signaling bridges the extracellular microenvironment of neuroblastoma and its tumorigenicity via the enhancement of miR-221/222 that in turn suppress the oncosuppressor PTEN. Accordingly, targeting HMGB1 and miR-221/222 may provide novel strategies for therapies in neuroblastoma as extracellular HMGB1 is an inflammatory molecule that is implicated in the pro-tumorigenic microenvironment and increased expression of oncogenic miRs in neuroblastoma.

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