Quantitative Nuclear Proteomics Identifies that miR-137-mediated EZH2 Reduction Regulates Resveratrol-induced Apoptosis of Neuroblastoma Cells*

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Neuroblastoma is the most common pediatric extracranial solid tumor with a broad spectrum of clinical behavior and poor prognosis. Despite intensive multimodal therapy, ongoing clinical trials, and basic science investigations, neuroblastoma remains a complex medical challenge with a long-term survival rate less than 40%. In our study, we found that resveratrol (3, 5, 4′-trihydroxystilbene, RSV), a naturally occurring phytoalexin, possesses an anticancer activity through blocking cell growth and inducing apoptosis in neuroblastoma cell line Neuro-2a (N-2a) cells. Using stable isotope labeling with amino acids in cell culture (SILAC) and quantitative proteomic analysis, we found that 395 proteins were up-regulated and 302 proteins were down-regulated in the nucleus of N-2a cells treated with RSV. Among these, the polycomb protein histone methyltransferase EZH2 was reduced significantly, which is aberrantly overexpressed in neuroblastoma and crucial to maintain the malignant phenotype of neuroblastoma by epigenetic repression of multiple tumor suppressor genes. EZH2 reduction further led to decreased H3K27me3 level and reactivation of neuroblastoma tumor suppressor genes CLU and NGFR. Disruption EZH2 expression by RNA interference-mediated knockdown or pharmacologic inhibition with DZNep triggered cellular apoptosis in N-2a cells. We found that the up-regulation of miR-137 level was responsible for reduced EZH2 level in tumor suppression induced by RSV. Inhibition of miR-137 expression rescued the cellular apoptosis phenotypes, EZH2 reduction, and CLU and NGFR reactivation, associated with RSV treatment. Taken together, our findings present for the first time, an epigenetic mechanism involving miR-137-mediated EZH2 repression in RSV-induced apoptosis and tumor suppression of neuroblastoma, which would provide a key potential therapeutic target in neuroblastoma treatment. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.041905, 316–328, 2015.

Neuroblastoma is a tumor derived from primitive cells of the sympathetic nervous system and is the most common solid tumor in childhood, accounting for 15% of pediatric cancer mortality (1, 2). A subset of neuroblastoma will undergo complete regression or differentiation, whereas others often end fatally despite recent intensive multimodal therapy. Around 50% of patients are currently classified as high-risk for disease relapse. The long-term survival rate of neuroblastoma patients is less than 40% (3, 4). Many features of neuroblastoma have been found to be associated with its high-risk clinical outcome, such as MYCN oncogene amplification (5), allelic loss of chromosome 1p or 11q (6), DNA ploidy (7), and overexpression of receptor tyrosine kinases TrkA and EPHB6 (8, 9). Although more and more evidences have been shown to elucidate the neuroblastoma pathogenesis, the targeted and effective treatments are still in development.

Heritable epigenetic mechanisms, including DNA methylation, histone modifications, nucleosome remodeling, and non-coding RNAs, play an essential role in the regulation of the mammalian genome complexity. Recent advances have shown that global epigenetic abnormalities occur in human cancer cells. Polycomb protein histone methyltransferase enhancer of zeste homolog 2 (EZH2)†, which is aberrantly over-

† The abbreviations used are: EZH2, enhancer of zeste homolog 2; RSV, resveratrol; N-2a, Neuro-2a; DMEM, Dulbecco’s Modified Eagle Medium; FBS, fetal bovine serum; PI, propidium iodide; IAA, iodoacetamide; SILAC, stable isotope labeling with amino acids in cell culture;
expressed in multiple types of human tumors, including neuroblastoma, specifically catalyzes trimethylation of histone 3 on Lys 27 (H3K27me3), a well-known histone mark associated with gene silencing (10). In neuroblastoma, EZH2 represses tumor suppressors CLU, NGFR, RUNX3, and CASZ1, which contribute to the genesis and maintenance of the high-risk phenotype of neuroblastoma (11). MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at a post-transcriptional level by binding to the 3′-UTR of mRNAs to repress translation or induce degradation of mRNAs. Dysregulation of miRNAs expression plays oncogenic or tumor-suppressive role in a wide variety of cancers including neuroblastoma (12). In neuroblastoma, miR-137 is low expressed and it functions as a tumor suppressor by decreasing LSD1 (13). MiR-137 post-transcriptionally represses the expression of EZH2 in stem cells, modulating the proliferation and differentiation (14). Targeting these epigenetic regulators may represent a promising therapeutic strategy for neuroblastoma.

Resveratrol (3, 5, 4′-trihydroxystilbene, RSV) is a naturally occurring phytoalexin present in grapes, peanuts, berries, and Chinese herbs (15). It has received tremendous attention over the past couple of decades because of its chemopreventive and chemoprotective roles in cancers (16), aging (17), ischemic injuries (18), and neurodegenerative diseases (19). In 1997, Jang et al. firstly reported that RSV exerted potent chemopreventive activity in the initiation, promotion, and progression of carcinogenesis (20). RSV has been assessed in phase I clinical trials for human colorectal cancers (15). Previous studies have shown that RSV can inhibit cell proliferation, induce apoptosis (21, 22), and disrupt cell cycle transition at the G1-S phase (21) through inhibiting a number of key regulators of cell survival pathways, such as AP-2 (22), NF-κB (23), PI3K/Akt (24), and MAPK, and activating tumor suppressor genes such as p53 (25) and phosphatase and tensin homolog (PTEN) (24). However, the molecular mechanisms underlying RSV-mediated tumor-suppressing activities are not yet completely elucidated. In order to apply RSV into future clinical trials for cancer therapy, we examined the signaling pathway involved in RSV-mediated tumor suppression in neuroblastoma cells.

In this study, we used stable isotope labeling with amino acids in cell culture (SILAC), a highly reproducible and accurate technology for relative protein quantitation at a global proteome scale, to identify changes in the expression of nuclear proteins in RSV-treated neuroblastoma cells (26). We found that 395 proteins were up-regulated and 302 proteins were down-regulated in the nucleus of neuroblastoma cell line Neuro-2a (N-2a) cells treated with RSV. Epigenetic repressor EZH2 decreased significantly and led to decreased H3K27me3 level. As a result, tumor suppressors of CLU and NGFR silenced by EZH2 were reactivated after RSV treatment, which were involved in the apoptosis induction and tumor suppression. Importantly, we found that EZH2 expression was inhibited by miR-137, which was up-regulated after RSV treatment. Inhibition of miR-137 rescued the RSV-induced EZH2 reduction and cellular apoptosis. Our findings revealed an epigenetic regulatory mechanism involving miR-137-mediated EZH2 reduction in RSV-induced apoptosis of neuroblastoma cells, which would be a key therapeutic target in neuroblastoma treatment.

**EXPERIMENTAL PROCEDURES**

**Cell Culture** — The mouse neuroblastoma cell line Neuro-2a (N-2a) and human neuroblastoma cell line SH-SYSY were obtained from Cell Resource of Peking Union Medical College Hospital. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Hyclone, Los Angeles, CA) containing 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), penicillin (100 U/ml), and streptomycin sulfate (100 mg/ml) at 37 °C in a humidified atmosphere with 5% CO2.

**Cell Viability Assay** — The effect of RSV (≥99% pure) (Sigma Chemical Co., St. Louis, MO) on the viability of N-2a cells was evaluated by MTT assay. Cells were seeded in 96 wells and treated with RSV at different concentrations (DMSO, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 80 μM, 100 μM, 120 μM, and 150 μM) for 24 h. We set nine determinations for each concentration. Then we added 20 μL 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) (Sigma Chemical Co.) to each well and incubated the plate at 37 °C for 4 h. The formazan crystal forming in viable cells was dissolved in 150 μL DMSO. After slight vortex, the absorbance was measured at 490 nm by Microplate Reader (Bio-Rad, Hercules, CA). The cell viability was normalized by the DMSO group.

**Cell Morphology Observation** — Cell Morphology was Observed by Optical Microscopy (Olympus IX71, Japan).

**Apoptosis Assay** — Cell apoptosis was detected by Hoechst 33258 staining, Western blotting, and annexin V/PI staining with flow cytometry. For Hoechst 33258 staining, cells were fixed with 4% paraformaldehyde (pH 7.4) for 10 min at room temperature and then stained by Hoechst 33258 (5 μg/ml) for 30 min at 37 °C in dark. Then cells were washed twice with phosphate-buffered saline buffer (PBS). The blue fluorescence was observed under a Live Cell Imaging System (Olympus, Japan) using a 350 nm excitation and a 460 nm emission-fluorescent filter. We used Western blotting to detect the cleavage of caspase 3 and its substrate protein PARP cleavage. To determine the percentage of early apoptosis, annexin V/PI staining was performed by Annexin V-FITC Apoptosis Detection Kit (Beyotime Institute of Biotechnology, Hangzhou, China). Then the apoptotic cells were detected by FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ). The results were analyzed by WinMDI 2.9 software.

**Preparation of Nuclei and Sample Processing** — N-2a cells for SILAC analysis were cultured in two different SILAC medium containing either light isotopes of 12C6 L-lysine and 12C6 14N4 L-arginine (Lys0/Arg0) or heavy isotopes of 13C6 L-lysine and 13C6 15N4 L-arginine (Lys6/Arg10) (ThermoFisher Scientific, Rockford, IL) for five rounds of cell division. Then the heavy-labeled cells were treated with 80 μM RSV and light-labeled cells were treated with the same volume of DMSO as the control. Nuclei were isolated as following (27). Briefly, cells were harvested in the hypotonic lysis buffer consisting of 5 mM Tris, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 1 mM CaCl2, 250 mM sucrose, 1 mM DTT, 0.6% Nonidet P-40, 1% (v/v) protease inhibitor mixture (Sigma Chemical Co.), and incubated for 5 min on ice. The cells burst and nuclei released. The nuclei were collected by centri-
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fugation at 960 × g and 4 °C for 5 min and the nuclei were washed twice by the hypotonic lysis buffer. Nuclei were lysed in the buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM dithiothreitol (DTT), 1% (v/v) protease inhibitor mixture (Sigma Chemical Co.), and 10% volume of the mixture of Deoxyribonuclease I (1 mg/ml) and Ribonuclease A (0.25 mg/ml) at 4 °C for 30 min. The lysates were dissolved with repeated vortex and ultra-sonication, and centrifuged at 15,000 × g and 4 °C for 30 min to remove insoluble substances.

The protein concentration was measured by a 2-D Quant Kit (GE Healthcare, Pittsburgh, PA). Nuclei lysates of “heavy” and “light” cells were mixed at 1:1 and protein samples containing 100 μg of protein were prepared. The protein mixture was resolved by one-dimensional 12.5% SDS-PAGE gel and visualized by Coomassie Brilliant Blue R-250 staining. Then the gel was excised into 24 slices, cut into 1 mm² particles and destained in 50% acetoneitrile with 25 mM ammonium bicarbonate. The in-gel proteins were reduced by incubation with 10 mM DTT for 40 min at 56 °C and alkylated with 55 mM iodoacetamide (IAA) for 80 min in dark. After being washed and dehydrated, the proteins were digested by sequencing grade trypsin (Promega, Fitchburg, WI) at a 1: 100 ratio (enzyme: substrate) at 37 °C overnight. The peptides were extracted from gel particles twice with 0.1% trifluoroacetic acid in 50% acetoniitrile for 2 h each time, and dried in a vacuum centrifuge (ThermoFisher Scientific).

LC-MS/MS Analysis—Peptides were analyzed with Easy-nLC1000 nanoflow UHPLC (Micro-Techn Scientific, Vista, CA) coupled to an LTQ Orbitrap XL mass spectrometer (ThermoFisher Scientific). Peptides were separated on a 100 μm × 10 cm in-house made column packed with a reversed-phase ReproSil-Pur C18-AQ resin (3 μm, 120 Å, Dr. Maisch GmbH, Ammerbuch, Germany). Separations were performed using linear gradients of 5–32% acetonitrile in 0.1% formic acid at a flow rate of 300 nl/min for 100 min. An electrospray voltage of 2.0 kV was applied. The instrument control was performed with Tune 2.6.0 and Xcalibur 2.2 (ThermoFisher Scientific). Full scan MS spectra (m/z 300–1650) were acquired in the Orbitrap analyzer with resolution 60,000 at 400 m/z after accumulation to a target value of 1,000,000. The five most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation Q, 0.250; and activation time, 30 ms) with a target value of 3000 ions. We set the maximal filling times at 500 ms for the full scans and 150 ms for the MS/MS scans. We enabled the precursor ion charge state screening, and rejected all ions below 10 μm DTT for 40 min at 56 °C and alkylated with 55 mM iodoacetamide (IAA) for 80 min in dark. After being washed and dehydrated, the proteins were digested by sequencing grade trypsin (Promega, Fitchburg, WI) at a 1: 100 ratio (enzyme: substrate) at 37 °C overnight. The peptides were extracted from gel particles twice with 0.1% trifluoroacetic acid in 50% acetoniitrile for 2 h each time, and dried in a vacuum centrifuge (ThermoFisher Scientific).

Bioinformatic Analysis—The classification of up- and down-regulated proteins for Gene Ontology biological process (GOBP) was performed by GeneCodis 3 system (http://genecodis.cnb.csic.es/webservices) (31), in which the hypergeometric test was employed for enrichment. The categories with a p < 0.05 were filtered for hierarchical clustering.

Quantitative Real-time PCR—Cells were harvested and washed three times with ice-cold PBS, then lysed in Trizol reagent (Invitrogen, Carlsbad, CA) to recover the total RNA according to the manufacturer’s protocol. The total RNA was reversely transcribed to cDNA using a HiFi-MMLV cDNA first strand synthesis Kit (CW Bio, Beijing, China). Quantitative real-time PCR was performed using GoTaq® qPCR Master Mix (Promega, Madison, WI) on the CFX96™ Real-Time System (Bio-Rad). GAPDH was amplified in parallel as an internal control. Primer sequences for the mRNA quantitative real-time PCR were listed in supplemental Table S3.

After total RNA extraction, for analysis of mature miRNA expression, polyadenylation, and reverse transcription were carried out using NCode™ VILO™ miRNA cDNA Synthesis Kit (A11193-050, Invitrogen) and quantitative real-time PCR was performed by NCode™ EXPRESS SYBR GreenER™ miRNA qRT-PCR Kit Universal (A11193-051, Invitrogen) on the CFX96™ Real-Time System (Bio-Rad) along with miRNA specific primers. U6 snRNA was used as an endogenous control. Specific primer sequences for the miRNA quantitative real-time PCR were listed in supplemental Table S3.

Western Blotting—Cells were harvested in the buffer consisting of 7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT, 1% (v/v) protease inhibitor mixture (Sigma Chemical Co.), and 10% (v/v) the mixture of DNase I (1 mg/ml) and RNase A (0.25 mg/ml), and incubated at 4 °C for 30 min. Insoluble substances were removed by centrifugation at 15,000 × g and 4 °C for 30 min. Protein homogenates of about 10 μg protein sample were separated by SDS-PAGE and transferred to PVDF membranes using a semi-dry blotting apparatus (Bio-Rad). The membranes were blocked with 5% nonfat milk in PBS with 0.1% Tween-20 for 1 h at room temperature, incubated with the indicated primary antibodies overnight at 4 °C and then the HRP-conjugated secondary antibodies for 1 h at room temperature. Antibodies against caspase 3, PARP, EZH2, SUZ12, and RING1B were from Cell Signaling Technology (Danvers, MA). Antibodies against EED, histone 3, and GAPDH were from Abcam Inc. (Cambridge, MA). Antibodies against BMI-1 and H3K27me3 were from Merck Millipore (Billerica, MA). The bands of protein were visualized by chemiluminescence. The protein content was normalized to GAPDH expression level. Bands were quantified using Image J software.

RNA Interference—N-2a cells seeded in 6-well plates were transfected with siRNA against EZH2 (sc-156000, Santa Cruz Biotechnology, Santa Cruz, CA) or control siRNA (sc-37007, Santa Cruz Biotechnology, Santa Cruz, CA) with Lipofectamine RNAiMAX (Invitrogen). At 48 h post-transfection, cells were harvested and apoptosis assay, then Western blotting and quantitative real-time PCR were performed as described above.

Pharmacologic Inhibition of EZH2—N-2a cells seeded in 6-well plates were treated with 5 μM DZNep (~99% pure) (Sigma Chemical Co.) for 24 h and 48 h. Cells were harvested and apoptosis assay, then Western blotting and quantitative real-time PCR were performed as described above.

Luciferase Reporter Assay—HEK293 cells were plated in a 24-well plate and co-transfected with 50 nm of either miR-137 mimics or miR-137 mimics control (RiboBio, Guangzhou, China), and 200 ng of either psiCHECK-EZH2–3’-UTR-WT plasmid or psiCHECK-EZH2–3’- UTR-Deletion plasmid (psiCHECK vector from Pro. Liang in Peking University), using Lipofectamine LTX (Invitrogen). After 24 h post-
transfection, luciferase reporter assays were performed using dual luciferase reporter assay system (Promega, Fitchburg, WI). Renilla luciferase activity was standardized to Firefly luciferase control. Cells transfected with miR-137 mimics control and psiCHECK-EZH2-3’-UTR-Deletion plasmid were set as negative control.

Inhibition of Endogenous MiR-137—Cells seeded in 6-well plates were transfected with anti-miR-137 inhibitor or anti-miR-137 inhibitor negative control (AM17010, Ambion, Carlsbad, CA) with Lipofectamine RNAiMAX. At 4 h post-transfection, cells were treated with 80 μM RSV for 24 h. Then cells were harvested and apoptosis assay, then Western blotting and quantitative real-time PCR were performed as described above.

Overexpression of MiR-137—N-2a cells grown in 6-well plates were transfected with miR-137 mimics or miR-137 mimics control with Lipofectamine RNAiMAX. At 48 h post-transfection, cells were harvested and apoptosis assay, then Western blotting and quantitative real-time PCR were performed as described above.

Statistics—All functional experiments were repeated at least three times. Data were analyzed by GraphPad Prism 5.0 software and shown by the means ± standard deviation. Multiple sets of data were analyzed by one-way ANOVA, and Student’s t test was used to analyze two sets of data, considering statistically significant when p < 0.05.

RESULTS

RSV Inhibits Cell Growth and Induces Apoptosis in N-2a Cells—RSV inhibits a variety of cancer cell growth and induces cellular apoptosis in vivo and in vitro in a dose range of 1–150 μM (16, 32, 33), so we firstly examined the cell viability and cellular apoptosis in N-2a neuroblastoma cells exposed to a series of RSV concentrations of 0, 10 μM, 20 μM, 30 μM, 40 μM, 50 μM, 80 μM, 100 μM, 120 μM, and 150 μM. To determine the effect of RSV on N-2a cells growth, we used MTT assay to examine the cell viability. As shown in supplemental Fig. S1B, the growth of N-2a cells was markedly inhibited by RSV. Moreover, the inhibition effect of RSV was in a dose-dependent manner and an 80 μM dose caused nearly 50% reduction in cell viability. Light microscopic observation showed that N-2a cells became rounded and lost adherent characteristics after RSV treatment (supplemental Fig. S1C). These results indicated that RSV inhibited N-2a cell growth in a dose-dependent manner.

Apoptosis is characterized with morphological and biochemical changes, including nuclear chromatin compaction and fragmentation, cytoplasm shrinkage, membrane asymmetry loss, and caspase activation (34). The nuclear chromatin aggregates into uniformly dense masses, which abut on the nuclear membrane, is an early event during apoptosis. The morphology of apoptotic nuclei was judged by DNA staining with Hoechst 33258 after RSV treatment. Fig. 1A showed the typical apoptotic nuclei morphology of N-2a cells after being treated with RSV at 80 μM. The nucleus was smaller than normal nucleus and chromatin condensed and aggregated at the nuclear membrane. To detect the loss of membrane asymmetry of apoptosis in N-2a cells after RSV treatment, we used annexin V/PI staining for flow cytometry analysis. The apoptotic cells could be stained by annexin V but not by PI. The percentage of apoptotic cells increased dramatically after RSV treatment (Fig. 1B). Treatment with 80 μM RSV for 24 h caused almost 40% apoptosis in N-2a cells. To assess the mitochondria-dependent apoptosis, we measured the active caspase 3 and its substrate PARP cleavage by Western blotting. As shown in Fig. 1C, 80 μM RSV induced markedly caspase 3 activation and PARP cleavage. These data showed that treatment with RSV resulted in typical apoptosis characterized by chromatin compaction and fragmentation, membrane asymmetry loss, and caspase 3 activation in N-2a cells.

SILAC Identifies Nuclear Proteins Perturbed by RSV in N-2a Cells—Given that RSV inhibited N-2a cell growth and induced apoptosis significantly, we undertook SILAC analysis to measure the relative changes in nuclear protein abundance. SILAC analysis work flow was shown in Fig. 2A. Firstly, N-2a cells were labeled in two different SILAC mediums containing either light isotopes of L-lysine and L-arginine (Lys0/Arg0) or heavy isotopes of L-13C6-lysine and L-15N2-13C6-arginine (Lys6/Arg10). The heavy labeled cells were treated with 80 μM RSV for 24 h and the light labeled cells were treated with DMSO as control. Identification and quantification of nuclear proteins of the RSV-treated group and DMSO-treated group were performed by LC-MS/MS. All identification and quantification from SILAC experiment were based on four independent biological experiments, and 3833 proteins were identified in this study using four independent biological replicates and 3322 proteins (87%) were identified in at least three replicates (supplemental Table S1 and supplemental Fig. S2A). By calculating the correlation between the four separate biological replicates, we obtained a mean correlation between measurements of 0.92 (supplemental Fig. S2B). Only proteins with a significant (p < 0.001) fold change >2 were considered to be differentially expressed. 395 proteins were up-regulated and 302 proteins were down-regulated after RSV treatment. As shown in Fig. 2B, the red plots represented for up-regulated proteins and the blue for down-regulated proteins. A list of all differentially regulated proteins could be found in supplemental Table S1.

Bioinformatic Analysis of Key Nuclear Proteins Regulated by RSV in N-2a Cells—After we analyzed the expression profiles of all nuclear proteins of RSV-treated N-2a cells, we manually annotated the proteins into several functional categories. The differentially expressed proteins were categorized using GO biological process (GOBP) enrichment analysis (supplemental Table S2). As shown in Fig. 2C, the resulting GOBP groups of up-regulated proteins were in red columns and down-regulated proteins in blue columns. GOBP results indicated that the up-regulated proteins were enriched in carboxylic acid metabolic process, fatty acid metabolic process, regulation of apoptotic process, and so on. Down-regulated proteins were...
predominantly involved in DNA repair, DNA replication, regulation of apoptotic process, histone modification, and interphase of mitotic cell cycle. The GOBP category “histone modification” is of great importance in cancer because epigenetic modifications are regarded as ideal targets for cancer therapy (35). In the category “histone modification,” the core catalytic components of PRC2, EZH2 was significantly downregulated with a H/L ratio of 0.31 (p = 0.00018) after RSV treatment.

Validation of EZH2 Protein Level Reduction in a Dose- and Time-dependent Manner after RSV Treatment in N-2a Cells—Quantitative proteomics analysis showed that the core catalytic components of epigenetic modification complex PRC2, EZH2 was decreased responding to RSV treatment. This was the first time that we found PRC complexes may be involved in the cellular apoptosis triggered by RSV in neuroblastoma. In order to validate the quantitative proteomics results, the mRNA levels of the PRC2 complex components (EZH2, EED, and SUZ12) and PRC1 complex components (RING1B and BMI-1) were measured using qPCR and protein levels were measured by Western blotting in N-2a cells treated with 80 µM RSV for 24 h. Not only EZH2, but also the other components of PRC2 and PRC1 protein levels were all dramatically down-regulated after RSV treatment (Fig. 3B), whereas the mRNA levels were not significantly changed (Fig. 3A). Consequently, the level of H3K27me3 was reduced markedly, whereas H3 expression was not changed (Fig. 3B).

EZH2, as the core component of the PRC2 complex, is a histone methyltransferase responsible for H3K27me3, which is a critical epigenetic mark for tumor suppressor silencing (10). Therefore, we chose EZH2 for detailed studies. Firstly, we measured EZH2 protein level in N-2a cells after treatment with increasing concentrations of RSV (0, 20 µM, 50 µM, 80 µM, 100 µM, and 150 µM) by Western blotting. The result showed that RSV reduced EZH2 protein level in a dose-dependent manner and EZH2 was significantly down-regulated after treatment with only 20 µM RSV (Fig. 3C). Then we detected the EZH2 protein level in N-2a cells treated with 80 µM RSV for 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h. In line with expectations, EZH2 was decreased in a time-dependent manner and as early as 2 h, EZH2 was reduced significantly (Fig. 3D). In neuroblastoma, EZH2-mediated H3K27me3 silenced a series of tumor suppressors including CASZ1, CLU, RUNX3, and NGFR (11). To determine whether RSV-induced EZH2
reduction reactivated these tumor suppressor genes, we detected the mRNA levels of these genes by qPCR. As shown in Fig. 3E, CLU and NGFR mRNA levels were up-regulated after RSV treatment, whereas CASZ1 and RUNX3 mRNA levels did not change (data not shown), which might be involved in tumor suppression.

Both Knockdown and Pharmacological Disruption of EZH2 Induce Apoptosis in N-2a Cells—To further establish that EZH2 was involved in apoptosis induction, siRNA-mediated knockdown and pharmacological disruption of EZH2 were performed by transfection of EZH2-specific siRNA or DZNep treatment. EZH2-specific siRNA was transfected into N-2a cells. After 48 h transfection, the mRNA level and protein level decreased to 30% and 6% of that in the control (Fig. 4A and B). H3K27me3 level was also down-regulated after EZH2 knockdown. To determine mitochondria-dependent apoptosis, we detected caspase 3 activation and PARP cleavage. As shown in Fig. 4B, caspase 3 was activated and PARP was cleaved. Then we used a specific EZH2 inhibitor, DZNep, to further examine whether the inhibition of EZH2 could induce apoptosis. DZNep treatment did not decrease the mRNA level of EZH2 but reduced EZH2 protein level and the associated H3K27me3 level significantly in a time-dependent manner (Fig. 4C and D). Activation of caspase 3 and PARP cleavage
Fig. 3. Validation of PRC2 and PRC1 components in N-2a cell after treated with RSV. A, N-2a cells were treated with 80 \( \mu \text{M} \) RSV for 24 h. The PRC2 and PRC1 components mRNA levels were detected by qPCR. GAPDH was used as the control. All mRNA levels were not changed after RSV treatment. B, N-2a cells were treated with 80 \( \mu \text{M} \) RSV for 24 h. The PRC2 and PRC1 components protein levels and H3K27me3 level were detected by Western blotting. H3 and GAPDH were used as control. All components protein levels and H3K27me3 level were down-regulated after RSV treatment. C, N-2a cells were treated with RSV of different concentrations (0, 20 \( \mu \text{M} \), 50 \( \mu \text{M} \), 80 \( \mu \text{M} \), 100 \( \mu \text{M} \), and 150 \( \mu \text{M} \)) for 24 h. EZH2 level was detected by Western blotting. H3 and GAPDH were used as loading control. EZH2 was decreased in a dose-dependent manner. D, N-2a cells were treated with 80 \( \mu \text{M} \) RSV for a series of times (0 h, 2 h, 4 h, 6 h, 12 h, and 24 h). EZH2 level was detected by Western blotting. H3 and GAPDH were used as loading control. EZH2 was decreased in a time-dependent manner. E, Cells were treated as in A. The mRNA level of CLU and NGFR were detected by qPCR analysis. GAPDH was used as the control. The bands of Western blotting were analyzed by Image J, and the relative gray-scale was presented by the column. Each column was the average ratio of three independent trials and presented as mean \( \pm \) standard deviation (*** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \); two-tailed t test. compared with DMSO).
were detected using Western blotting after DZNep treatment (Fig. 4D). These results indicated that both knockdown and pharmacological disruption of EZH2 induced apoptosis in N-2a cells.

miR-137 Participates in RSV-induced Apoptosis through Inhibiting EZH2 mRNA Translation—In N-2a cells, EZH2 mRNA was not down-regulated, but the protein level dramatically decreased after RSV treatment, indicating that EZH2 protein level was reduced through a post-transcriptional mechanism (Fig. 3A). A series of miRNAs, including miR-101, miR-26a, and miR-137, were reported to repress EZH2 expression in cancer cells and stem cells (36–39). To examine the post-transcriptionally regulation mechanism of EZH2 reduction, we determined the levels of these miRNAs (miR-101,
miR-26a, and miR-137) in mouse neuroblastoma N-2a cells and human neuroblastoma SH-SY5Y cells after RSV treatment. As shown in Fig. 5A, miR-137 was significantly upregulated after RSV treatment in both N-2a cells and SH-SY5Y cells. To determine whether miR-137 was participated in RSV-induced apoptosis through inhibiting EZH2 mRNA translation, we then performed functional rescue experiments. Anti-miR-137 inhibitor was transfected to N-2a cells and SH-SY5Y cells before RSV treatment. Anti-miR-137 inhibitor did not change EZH2 mRNA level but rescued the reduction of

**Fig. 5.** **MiR-137 regulates the EZH2-mediated apoptosis after RSV treatment in both N-2a cells and SH-SY5Y cells.** A, Quantitation of miRNAs targeting EZH2 after RSV treatment. N-2a cells were treated with 80 μM RSV for 24 h and SH-SY5Y cells with 50 μM RSV for 24 h. Three miRNAs (miR-137, miR-26a, and miR-101) were examined by qPCR. U6 snRNA was an endogenous control. Each column was the average percentage of three independent trials and presented as mean ± standard deviation (**p < 0.01; comparison against DMSO, two-tailed t test). B, Cells were transfected with anti-miR-137 inhibitor before RSV treatment. Cell morphology was observed by optical microscopy. C, Cells were treated as in B. Apoptotic cell percentage was detected by annexin V/PI staining with flow cytometry analysis. Apoptotic cells (positive for annexin V and negative for PI) were distributed in the lower and right panels. Each column was the average percentage of three independent trials and presented as mean ± standard deviation. (⁎⁎p < 0.05; **p < 0.01; comparison against RSV, two-tailed t test.) D, Cells were treated as in B. The levels of EZH2, H3K27me3, cleaved caspase 3, and cleaved PARP were determined by Western blotting. GAPDH was used as the loading control. The bands of Western blotting were analyzed by Image J, and the relative gray-scale was presented by the column. Each column was the average ratio of three independent trials and presented as mean ± standard deviation. (**⁎⁎p < 0.001, **⁎⁎p < 0.01, *p < 0.05; compared with RSV, two-tailed t test.). E, Cells were treated as in B. The mRNA levels of CLU and NGFR were detected by qPCR. GAPDH was used as the control. Each column was the average ratio of three independent trials and presented as mean ± standard deviation (**⁎⁎p < 0.001, **⁎⁎p < 0.01, *p < 0.05; compared with RSV, two-tailed t test.).
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Fig. 6. **miR-137 induces apoptosis through inhibiting EZH2 expression in both N-2a cells and SH-SY5Y cells.** A, EZH2 3’-UTR dependent luciferase assays were performed by transfection of miR-137 mimics or miR-137 mimics negative control. Dual luciferase assays were performed 48 h after miR-137 mimics and luciferase reporter vectors co-transfection. Renilla luciferase activity was standardized to Firefly luciferase control. EZH2 3’-UTR-dependent activity of a luciferase reporter gene was suppressed by miR-137 mimics in HEK293 cells. Each
miR-137-EZH2 Regulates RSV-induced Apoptosis in Cancer Cells

EZH2 protein level and H3K27me3 level associated with RSV treatment (supplemental Fig. S3 and Fig. 5D). Inhibition of miR-137 rescued the cell morphology change (Fig. 5B), membrane asymmetry loss (Fig. 5C), caspase 3 activation (Fig. 5D), and the activation of tumor suppressor CLU and NGFR (Fig. 5E). In summary, in both N-2a cells and SH-SY5Y cells, anti-miR-137 inhibitor rescued the cellular apoptosis phenotypes associated with RSV treatment. These results showed that miR-137 participated in cellular apoptosis through inhibiting EZH2 mRNA translation.

Transfecting N-2a cells with anti-miR-137 inhibitor or anti-miR-137 inhibitor control, EZH2 mRNA level was not changed but EZH2 level and H3K27me3 level were up-regulated (supplemental Fig. S4C and D). There was no apparent change in cell morphology after cell-miR-137 inhibition (supplemental Fig. S4A) and no membrane asymmetry loss was detected by annexin V/PI staining (supplemental Fig. S4B).

**miR-137 Induces Apoptosis through Inhibiting EZH2 Expression in both N-2a Cells and SH-SY5Y Cells**—MiRNA can target to the 3'-UTR of the target gene mRNA to induce mRNA degradation or translation repression. To validate that miR-137 could indeed directly target to EZH2 3'-UTR, we constructed luciferase reporter vector containing 3'-UTR region of EZH2. Then we measured the activity of luciferase as dependent on EZH2 3'-UTR in response to miR-137. A series of reporter assays showed that overexpression of miR-137 markedly suppressed activity of EZH2 3'-UTR luciferase reporter gene in HEK293 cells, but deletion of the EZH2 3'-UTR seed sequence targeted by miR-137 in the luciferase reporter gene promoter led to complete abrogation of the suppressive effect of miR-137 (Fig. 6A).

Next we examined the effect of miR-137 on endogenous EZH2 expression. As shown in Fig. 6C, overexpression of miR-137 reduced EZH2 protein level to 40% in N-2a cells and 53% in SH-SY5Y. But the mRNA levels were not changed (Fig. 6B), which indicated the degradation of EZH2 mRNA by miR-137. MiR-137 mimics also induced caspase 3 activation and PARP cleavage in both N-2a cells and SH-SY5Y cells (Fig. 6C). The results suggested that miR-137 was a potent epigenetic target for cancer therapy.

EZH2 is high expressed while miR-137 is low expressed in a series of neuroblastoma cells, which may be associated with a poor prognosis of neuroblastoma. To investigate this possibility, we used R2 microarray analysis and visualization platform (http://hgservver1.aml.nl/cgi-bin/r2/main.cgi) to generate Kaplan-Meier plot of overall survival based on EZH2 and miR-137 expression (Fig. 6D). The results showed that higher expression of EZH2 and lower expression of miR-137 were associated with a poor prognosis with p values of 3.7e-04 and 7.6e-07, separately. Then we used Oncogenomics Section Data Center database (http://pob.abcc.ncifcrf.gov/cgi-bin/JK) to extract the median-centered log2 values for EZH2 and miR-137 expression, which were clustered and color-coded using ClIminer software (Fig. 6E). Among these samples, higher EZH2 expression samples were associated with lower miR-137 expression samples, whereas vice versa. The correlation between EZH2 and miR-137 expression was statistically significant (r = −0.243, p = 0.02).

**DISCUSSION**

RSV, a naturally occurring phytoalexin found in grapes, has appealed to the eyes because of its potent tumor-suppressive activity. It blocks proliferation and induces apoptosis in types of tumor cells, including neuroblastoma, the most common extracranial solid cancer in children. However, much less is known about the detailed mechanisms underlying RSV anti-tumor effect. To illustrate the signaling pathway involved in RSV-mediated antitumor effect, we applied an unbiased SILAC quantitative proteomics approach to examine changes in nuclear proteins in RSV-treated neuroblastoma N-2a cells. We identified 395 up-regulated proteins and 302 down-regulated proteins. Several of these proteins were closely associated with histone modification. Among these, the histone methyltransferase EZH2 protein level was decreased in a dose- and time-dependent manner but RNA level was not changed after RSV treatment. The silenced tumor suppressors CLU and NGFR were activated responding to EZH2 reduction, which were involved in the tumor suppression of RSV. Furthermore, both EZH2 knockdown by specific-siRNA and pharmacological disruption by DZNep induced apoptosis.
in N-2a cells. Interestingly, EZH2 expression inhibition was mediated by miR-137, which was increased after RSV treatment. Inhibition of miR-137 rescued the EZH2 reduction, EZH2 target gene CLU and NGFR activation, and apoptosis phenotype triggered by RSV treatment.

Extensive reprogramming of every component of the epigenetic machinery occurred in cancer including DNA methylation, histone modifications, chromatin remodeling, and noncoding RNAs, specifically microRNA expression. The abnormalities of epigenetic regulation are associated with tumor initiation and progression along with genetic alterations. Targeting these reversible epigenetic regulation abnormalities may represent a promising strategy for cancer therapeutic intervention.

The polycomb group transcriptional repressor EZH2, possessing an intrinsic activity in methylating histone H3K27, is frequently overexpressed in human malignancies (40, 41). EZH2 is widely involved in tumor aggressiveness and poor prognosis through controlling NF-κB, Wnt/β-catenin, BMP, and Notch signaling pathways in cancer cells (42–46). Recently, EZH2 was shown to be highly expressed in neuroblastoma and was crucial to the genesis and maintenance of poor prognostic status of neuroblastoma by epigenetic repression of multiple tumor suppressor genes of CASZ1, CLU, RUNX3, and NGFR (10, 11). Targeting EZH2 activity has been suggested as an attractive strategy for killing neuroblastoma cells (10, 40, 47). In the current study, we found that RSV decreased EZH2 expression, which lead to reduced H3K27me3 level. And the repressed tumor suppressor CLU and NGFR by EZH2 were increased at mRNA level after RSV treatment. Our findings show, for the first time, that EZH2 is a functionally important target for the antitumor effects of RSV in neuroblastoma cells.

Dysregulation of miRNA expression plays oncogenic or tumor-suppressive role in virtually all forms of cancers. MiRNAs are involved in tumor initiation and progression through regulating the expression of oncogenes and tumor suppressor genes. Several recent studies reported that EZH2 expression was negatively regulated by miR-101 and miR-26a in cancer cells (48, 49). Interestingly, miR-137-mediated EZH2 down-regulation is involved in adult neurogenesis (14). Althoff et al. reported that low levels of miR-137 expression correlated with a poor prognosis of neuroblastoma patients (13). This study demonstrated that EZH2 expression was modulated by miR-137 in neuroblastoma cells, which would contribute to tumor progression.

In summary, our SILAC quantitative proteomics approach identified EZH2 as a functional regulator in the RSV-induced apoptosis of neuroblastoma cells. We provided evidence that the decrease of EZH2 expression participated in tumor suppression of RSV. Importantly, we showed that up-regulation of miR-137 was responsible for the repression of EZH2 mRNA translation. Collectively, our study presents a novel pathway involving miR-137 elevation, EZH2 reduction and tumor suppressor CLU and NGFR activation, which contributes to the cellular apoptosis of neuroblastoma cells triggered by RSV. These findings extend our understanding of the RSV-antitumor mechanism and therapeutic application possibility of RSV in neuroblastoma therapy.

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REFERENCES

1. Park, J. R., Eggert, A., and Caron, H. (2010) Neuroblastoma: biology, prognosis, and treatment. Hematol. Oncol. Clin. North Am. 24, 65–86

2. Goldsby, R. E., and Matthay, K. K. (2004) Neuroblastoma: evolving therapies for a disease with many faces. Paediatr. drugs 6, 107–122

3. De Bernardi, B., Nicolas, B., Boni, L., Indolfi, P., Carli, M., Cordero Di Montezemolo, L., Donfrancesco, A., Pession, A., Provenz, M., di Cataldo, A., Rizzo, A., Tonini, G. P., Dalloro, S., Conte, M., Gambini, C., Garaventa, A., Bonetti, F., Zanazzo, A., D’Angelo, P., Bruzzi, P., and Italian Co-Operative Group for, N. (2003) Disseminated neuroblastoma in children older than one year at diagnosis: comparable results with three consecutive high-dose protocols adopted by the Italian Co-Operative Group for Neuroblastoma. J. Clin. Oncol. 21, 1592–1601

4. Yang, G. (2004) Treatment of high-risk neuroblastoma with intensive chemotherapy, autologous peripheral blood stem cell transplantation, and 13-cis-retinoic acid. Zhonghua er ke za zhi 42, 486–489

5. Huang, M., and Weiss, W. A. (2013) Neuroblastoma and MYCN. Cold Spring Harb. Perspect. Med. 3, a014415

6. Maris, J. M., White, P. S., Beltinger, C. P., Sulman, E. P., Castlebeyrn, R. P., Shuster, J. J., Look, A. T., and Brodeur, G. M. (1995) Significance of chromosome 1p loss of heterozygosity in neuroblastoma. Cancer Res. 55, 4664–4669

7. Look, A. T., Hayes, F. A., Shuster, J. J., Douglass, E. C., Castlebeyrn, R. P., Bowman, L. C., Smith, E. I., and Brodeur, G. M. (1991) Clinical relevance of tumor cell ploidy and N-myc gene amplification in childhood neuroblastoma: a Pediatric Oncology Group study. J. Clin. Oncol. 9, 581–591

8. Nakagawara, A., Arima-Nakagawara, M., Scavarda, N. J., Azar, C. G., Cantor, A. B., and Brodeur, G. M. (1993) Association between high levels of expression of the TRK gene and favorable outcome in human neuroblastoma. N. Engl. J. Med. 328, 847–854

9. Teng, X., Zhao, H., Robinson, M. E., Cohen, B., Cnaan, A., London, W., Cohn, S. L., Cheung, N. K., Brodeur, G. M., Evans, A. E., and Ikegaki, N. (2000) Implications of EPHB6, EFNB2, and EFNB3 expressions in human neuroblastoma. Proc. Natl. Acad. Sci. U.S.A. 97, 10936–10941

10. Alimova, I., Venkataraman, S., Harris, P., Marquez, V. E., Northcott, P. A., Dubuc, A., Taylor, M. D., Foreman, N. K., and Vibhakar, R. (2012) Targeting the enhancer of zeste homolog 2 in medulloblastoma. Int. J. Cancer 131, 1800–1809

11. Wang, C., Liu, Z., Wu, C. W., Li, Z., Wang, L., Wei, J. S., Marquez, V. E., Bates, S. E., Jin, Q., Khan, J., Ge, K., and Thiele, C. J. (2012) EZH2 Mediates epigenetic silencing of neuroblastoma suppressor genes CASZ1, CLU, RUNX3, and NGFR. Cancer Res. 72, 315–324

12. Zhi, F., Wang, R., Wang, Q., Xue, L., Deng, D., Wang, S., and Yang, Y. (2014) MicroRNAs in Neuroblastoma: small-sized players with a large impact. Neurochem. Res. 39, 613–623

13. Althoff, K., Beckers, A., Odersky, A., Mestdagh, P., Koster, J., Bray, I. M.,
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Bryan, K., Vandesompele, J., Speleman, F., Stallings, R. L., Schramm, A., Eggert, A., Sprussel, A., and Schulte, J. H. (2013) MiR-137 functions as a tumor suppressor in neuroblastoma by downregulating KDM1A. Int. J. Cancer 133, 1064–1073.

Suzulch, K. E., Li, X., Smrt, R. D., Li, Y., Luo, Y., Li, L., Santisivatan, N. J., Li, W., Zhao, X., and Jin, P. (2010) Cross talk between microRNA and epigenetic regulation in adult neurogenesis. J. Cell Biol. 189, 127–141.

Baur, J. A., and Sinclair, D. A. (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat. Rev. Drug Discov. 5, 493–506.

Cox, J., and Mann, M. (2012) 1D and 2D annotation enrichment: a statistical approach for resveratrol-induced colon cancer cell apoptosis: caspase-6-mediated cleavage of lamin A is a major signaling loop. Proteomics 6, 2386–2394.

Allen, R. T., Hunter, W. J., 3rd, and Agramov, D. K. (1997) Morphological and biochemical characterization and analysis of apoptosis. J. Pharmacol. Toxicol. Methods 37, 215–228.

Tan, J., Yang, X., Zhuang, L., Jiang, X., Chen, W., Lee, P. L., Karuturi, R. K., Tan, P. B., Liu, E. T., and Yu, Q. (2007) Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev. 21, 1050–1063.

Gidich, J., Jiang, J., Tan, W., Xia, Y., Cao, H., Meng, Y., Da, Z., Liu, H., and Cheng, C. (2013) p53/p21 Pathway involved in mediating cellular senescence of bone marrow-derived mesenchymal stem cells from systemic lupus erythematosus patients. Clin. Dev. Immunol. 2013:134243.

Courval, J., Jagelska, E. B., Liao, J. C., and Brazda, V. (2013) Preferential binding of p53 tumor suppressor to p21 promoter sites that contain inverted repeats capable of forming cruciform structure. Biochem. Biophys. Res. Commun. 441, 83–88.

Nashir Udden, S. M., Morita-Fujimura, Y., Satake, M., and Ikawa, S. (2013) c-ABL tyrosine kinase modulates p53-dependent p21 induction and ensuing cell fate decision in response to DNA damage. Cell. Signal. 26, 444–452.

Nashir Udden, S. M., Morita-Fujimura, Y., Satake, M., and Ikawa, S. (2013) c-ABL tyrosine kinase modulates p53-dependent p21 induction and ensuing cell fate decision in response to DNA damage. Cell. Signal. 26, 444–452.

Fujii, S., Tokita, K., Wada, N., Ito, K., Yamacho, C., Ito, Y., and Ochiai, A. (2011) MEK-ERK pathway regulates EZH2 overexpression in association with aggressive breast cancer subtypes. Oncogene 30, 3118–3126.

Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanda, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., and Chinnaiyan, A. M. (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419, 624–629.

Tsang, D. P., and Cheng, A. S. (2011) Epigenetic regulation of signaling pathways in cancer: role of the histone methyltransferase EZH2. J. Hepatol. 55, 16–27.

Selwers, W. R., and Loda, M. (2002) The EZH2 polycomb transcriptional repressor—a marker of metastatic prostate cancer? Cancer Cell 1, 349–350.

Lee, S. T., Li, Z., Wu, Z., Aou, M., Guan, P., Karuturi, R. K., Liou, Y. C., and Yu, Q. (2011) Context-specific regulation of NF-kappaB target gene expression by EZH2 in breast cancers. Mol. Cell 43, 798–810.

Yamagishi, M., Nakano, K., Miyake, A., Yamochi, T., Kagiym, Y., Tsutsumi, A., Matsuda, Y., Sato-Otsubo, A., Muto, S., Utsunomiya, A., Yamaguchi, K., Uchimaru, K., Ogawa, S., and Watanabe, T. (2012) Polycomb-mediated loss of miR-31 activates NKD-dependent NF-kappaB pathway in adult T cell leukemia and other cancers. Cancer Cell 21, 121–135.

Zhou, J., Bi, C., Cheong, L. L., Mahara, J., Liu, S. C., Liou, Y. C., and Chung, W. J. (2011) The histone methyltransferase inhibitor, DZNep, up-regulates TNFIP3, increases ROS production, and targets leukemia cells in AML. Blood 118, 2830–2839.