

Inhibition of Long Noncoding RNA CRNDE Increases Chemosensitivity of Medulloblastoma Cells by Targeting miR-29c-3p

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Long noncoding RNA CRNDE (CRNDE) recently emerged as a carcinogenic promoter in various cancers including medulloblastoma. However, the functions and molecular mechanisms of CRNDE to the acquired drug resistance of medulloblastoma are still unclear. The transcript levels of CRNDE were examined in four medulloblastoma cell lines exposed to cisplatin treatment, and IC_{50} values were calculated. Effects of CRNDE knockdown or miR-29c-3p overexpression on cell viability, colony formation, apoptosis, migration, and invasion were assessed using the CCK-8, colony formation assay, flow cytometry, and Transwell assays, respectively. RNA pulldown and RNA-binding protein immunoprecipitation (RIP) were performed to confirm the molecular interactions between CRNDE and miR-29c-3p involved in medulloblastoma cells. The *in vivo* role of CRNDE knockdown or miR-29c-3p overexpression on tumor growth and apoptosis was evaluated in a xenograft mouse model of human medulloblastoma. The transcript levels of lncRNA CRNDE were significantly higher in cisplatin-treated tumor cells with higher IC_{50} values. Depletion of CRNDE inhibited tumor cell proliferation and colony formation, induced cell apoptosis, and suppressed migration and invasion in medulloblastoma cells. Moreover, overexpression of miR-29c-3p inhibited tumor cell proliferation and colony formation, migration, and invasion, and enhanced apoptosis and chemosensitivity to cisplatin. In addition, CRNDE was found to act as a miR-29c-3p sponge. Furthermore, *in vivo* experiments showed the CRNDE/miR-29c-3p interactions involved in medulloblastoma. Our study demonstrates that CRNDE acts as a critical mediator of proliferation, apoptosis, migration, invasion, and resistance to chemotherapeutics via binding to and negatively regulating miR-29c-3p in medulloblastoma cells. These results provide novel molecular targets for treatment of medulloblastoma.

Key words: Medulloblastoma; Long noncoding RNA (lncRNA); CRNDE; miR-29c-3p

INTRODUCTION

Medulloblastoma is the most frequent malignant brain tumor in children. To date, therapeutic options for medulloblastoma include surgery, radiotherapy, and chemotherapy¹. Although there have been advances in multimodal treatments such as surgery, radiotherapy, and chemotherapy, overall survival of most patients with medulloblastoma remains dismal². Thus, further molecular mechanism and novel therapies with more specificity are urgently needed.

Long noncoding RNAs (lncRNAs) are nonprotein-coding transcripts more than 200 nucleotides (nt) in length. Mounting evidence has revealed that lncRNAs are involved in a wide range of biological behaviors, such as cell growth, apoptosis, and differentiation^{3,4}.

Recently, many studies have suggested that lncRNAs exhibit extensive roles in the initiation and progression of cancer^{5,6}. Colorectal neoplasia differentially expressed (CRNDE) was originally discovered to be upregulated in colorectal carcinomas⁷. CRNDE is located on chromosome 16 and identified as an lncRNA. It is reported to be aberrantly expressed in several malignancies, including glioma, colorectal cancer, and medulloblastoma^{8,9}. The pivotal role of lncRNAs in anticancer drug resistance has been confirmed in many cancers¹⁰. Our previous study identified elevated transcript levels of CRNDE in clinical medulloblastoma tissues compared to the adjacent noncancerous tissues. Moreover, our data revealed that CRNDE promotes tumor growth in medulloblastoma¹¹. In this study, we aimed to explore the role of CRNDE

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in the drug resistance of medulloblastoma cells and its underlying mechanism.

MATERIALS AND METHODS

Cell Culture

Medulloblastoma cell lines D283, Daoy, D425, and D341 were obtained from the Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (CAS, Shanghai, P.R. China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco), 100 µg/ml streptomycin, and 100 µg/ml penicillin (Hyclone, Logan, UT, USA). Cells were cultured at 37°C in a 5% CO₂ humidity-controlled incubator.

Cell Viability Assay

For cell viability determination, CCK-8 assay was performed based on the manufacturer's protocol. In brief, cells were seeded onto 96-well plates and transfected with negative control groups (shRNA group, miR-NC) or target gene-manipulated groups (shCRNDE, miR-29c-3p). The cell viability in each well was determined by adding 10 µl of CCK-8 solution. After further incubation at 37°C for 2 h, absorbance was measured using an ELISA reader at a wavelength of 450 nm.

Real-Time PCR

Total RNA was extracted from cultured cells using TRIzol reagent (TaKaRa, Dalian, P.R. China), and RNA was subjected to reverse transcription into cDNA using a PrimeScript RT Master Mix Perfect Real Time (TaKaRa) kit. Real-time PCR was performed using an ABI 7900 system. The transcript levels of CRNDE and miR-29c-3p were determined using the equation 2^{-Ct} . The assay was performed in triplicate for each case.

Colony Formation Assay

Cells pretransfected with the specific shRNA against CRNDE or miR-29c-3p were seeded on a 60-mm dish and cultured. After 14 days of culture, colonies were stained with crystal violet (0.1%). The number of surviving colonies was counted.

Cell Apoptosis

Cell apoptosis was analyzed using Annexin-V/PI apoptosis kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. In brief, cells were seeded in a six-well plate and transfected with the specific shRNA against CRNDE or miR-29c-3p. The cells from each group were washed with ice-cold PBS and were resuspended in 100 µl of binding buffer. Annexin V-FITC (5 µl) and PI (5 µl) were added to the cell suspension and incubated for 15 min in the dark at room temperature. Samples were then analyzed by flow cytometry.

RNA Immunoprecipitation (RIP)

According to the manufacturer's protocol, RNA immunoprecipitation was performed in glioma cells 48 h after transfection with either the miR-29c-3p overexpression or miR-NC vectors using the Magna RIP™ RNA Binding Protein Immunoprecipitation kit (Millipore, Bedford, MA, USA). A total of 1×10^7 cells were lysed in RNA lysis buffer. The cell lysate was introduced to a magnetic bead solution conjugated to either human anti-Argonaute 2 (AGO2) antibodies (Millipore) or control mouse IgG molecules (Millipore). The RIP immunoprecipitation buffer was incubated together, while the samples were incubated with proteinase K (Gibco). Following incubation, IP RNA was isolated from samples and examined by reverse transcription PCR to investigate the enrichment of CRNDE.

Luciferase Reporter Assay

HEK-293T cells were cotransfected with luciferase reporter plasmids and miR-29c-3p mimics or miR-NC plasmids using Lipofectamine 2000. Then firefly and *Renilla* luciferase activities were measured by the Dual-Luciferase Reporter Assay System kit (Promega, Madison, WI, USA) to analyze the interaction between CRNDE and miR-29c-3p.

Transwell Cell Invasion and Migration Assay

The QCM™ 24-well Fluorimetric Cell Invasion assay kit and the Migration kit (Chemicon, Billerica, MA, USA) were used to determine migration and invasion. Cells (2×10^4) were suspended in 200 µl of DMEM containing 1% FBS and added to the upper chamber. DMEM (700 µl) containing 10% FBS was added to the lower chamber. After incubation, cells on the upper surface of the membrane were removed with a cotton swab. Following 24 h of incubation, the cells that had not migrated were pipetted out from the upper surface of the insets, and the cells that had migrated to the lower filters were detached using the cell detachment solution. Cells were fixed with 4% paraformaldehyde for 15 min and stained with 0.2% crystal violet for 10 min. The numbers of migrated and invaded cells were calculated.

Xenograft Mouse

Six-week-old male athymic BALB/c nude mice were maintained in special pathogen-free (SPF) conditions. Prior to the experiment, both cell lines were transfected with specific shRNA against CRNDE or miR-29c-3p. The cells (5×10^6) were injected subcutaneously into the right flank in each group of mice. Tumor dimensions [(length (*L*) and width (*W*)] were measured, and tumor volumes were then calculated as $TV = (L \times W^2) / 2$. All procedures were approved by the Animal Ethical and Welfare Committee of Qingdao Women and Children's Hospital.

Statistical Analysis

Data are expressed as mean \pm standard deviation (SD). Comparison between groups was performed using the Student's *t*-test or one-way ANOVA. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Elevated Expression of lncRNA CRNDE Is Associated With Drug Resistance in Medulloblastoma Cells

First, four medulloblastoma cell lines (D283, Daoy, D425, and D341) were exposed to cisplatin at different doses. After 24 h of treatment, CCK-8 assay was conducted to evaluate the cell viability of medulloblastoma cells, and the IC_{50} of each cell line was determined (Fig. 1A and B). We found that the IC_{50} values of D341 and Daoy cells exposed to cisplatin were significantly higher than those in the D283 and D425 cells. Moreover, the transcript levels and cellular location of lncRNA CRNDE were measured in D283, Daoy, D425, and D341 cells exposed to cisplatin at 10 $\mu\text{g}/\text{ml}$ for 24 h using real-time PCR and FISH. Consequently, we found that the transcript levels of lncRNA CRNDE in D341 and Daoy cells were significantly higher than those in D283 and D425 cells (Fig. 1C and D). Moreover, the expression of lncRNA CRNDE in D341 and Daoy cells exposed to cisplatin was also elevated compared to those in D283 and D425 cells (Fig. 1E and F). These data suggested that the expression of lncRNA CRNDE may be associated with drug resistance in medulloblastoma cells.

Inhibition of CRNDE Exhibits Antitumor Property in Medulloblastoma Cells

Next we explored whether cell viability *in vitro* was influenced by CRNDE knockdown. Real-time PCR validated the downregulation of CRNDE in D283 and D425 cells (Fig. 2A). Consequently, CCK-8 assay showed that D341 and Daoy cells depleted of CRNDE exhibited slower proliferative rate (Fig. 2B and C). Colony formation assay showed that colony number was less in the CRNDE-shRNA-transfected group than those in the shNC group in Daoy and D341 cells (Fig. 2F and G). In addition, flow cytometric and Transwell analysis showed that depletion of CRNDE induced apoptosis (Fig. 2D and E), and inhibited migration and invasion (Fig. 2H and I) in Daoy and D341 cells. Taken together, these data validated that depletion of CRNDE exhibited antitumor property in medulloblastoma cells.

CRNDE Binds to miR-29c-3p and Negatively Regulates its Expression

To test the hypothesis that CRNDE acts as a ceRNA, we first searched for potential CRNDE/miRNA interactions using bioinformatics analysis and found that miR-29c-3p can bind the lncRNA product of the CRNDE gene (Fig. 3A). To verify this prediction, we generated wild-type (wt) CRNDE luciferase plasmids containing potential miR-29c-3p binding sites, as well as mutant variants of each site. After cotransfection, luciferase assays showed that luciferase activity was lower in the

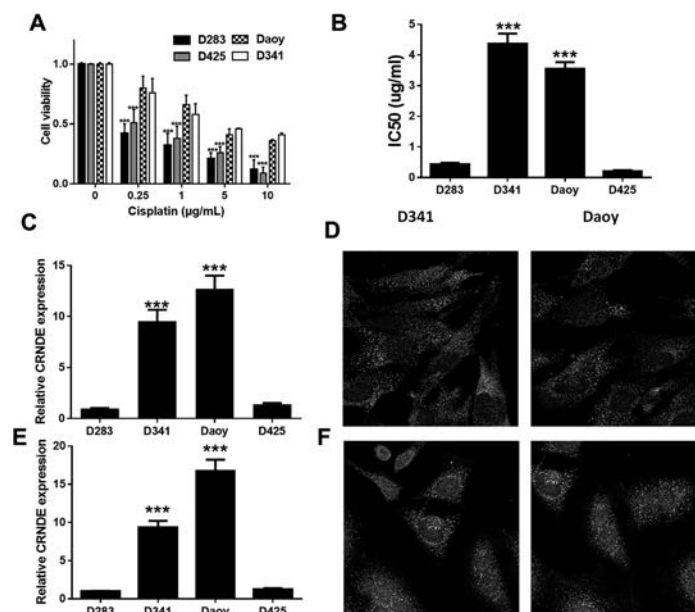


Figure 1. Elevated expression of long-noncoding RNA (lncRNA) CRNDE is associated with drug resistance in medulloblastoma cells. CCK-8 assay was conducted to evaluate the cell viability of medulloblastoma cell lines (D283, Daoy, D425, and D341), and the IC_{50} of each cell line was determined (A, B). Moreover, the transcript levels and cellular location of lncRNA CRNDE was measured using real-time PCR and FISH without cisplatin (C, D) or with cisplatin treatment (E, F). *** $p < 0.001$.

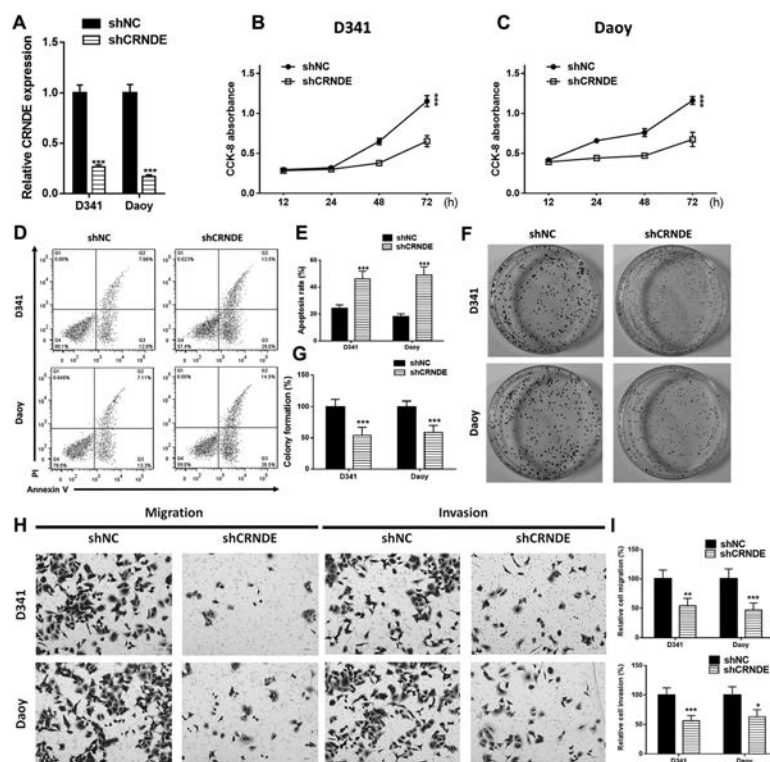


Figure 2. Inhibition of CRNDE exhibits antitumor property in medulloblastoma cells. Real-time PCR validated the downregulation of CRNDE in D283 and D425 cells (A). Thereafter, proliferation and colony formation of CRNDE-depleted D341 and Daoy cells were assessed using CCK-8 and colony formation assay (B, C, F, G). Flow cytometric and Transwell analysis was conducted to measure the apoptosis, migration, and invasion in cells with depletion of CRNDE (D, E, H, I). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

CRNDE wt+miR-29c-3p group than in the miR-NC group, while it was comparable in the CRNDE-mut+miR-29c-3p group and the miR-NC group, in both Daoy and D341 cells (Fig. 3B). Moreover, real-time PCR showed that miR-29c-3p expression was upregulated after transfection with shCRNDE and downregulated after transfection with CRNDE in Daoy and D341

cells (Fig. 3C). Furthermore, RNA pulldown and RNA-binding protein immunoprecipitation (RIP) assay confirmed the molecular interactions of CRNDE and miR-29c-3p involved in medulloblastoma cells (Fig. 3D). Taken together, these data suggest that CRNDE targeted miR-29c-3p and negatively regulates its expression in medulloblastoma cells.

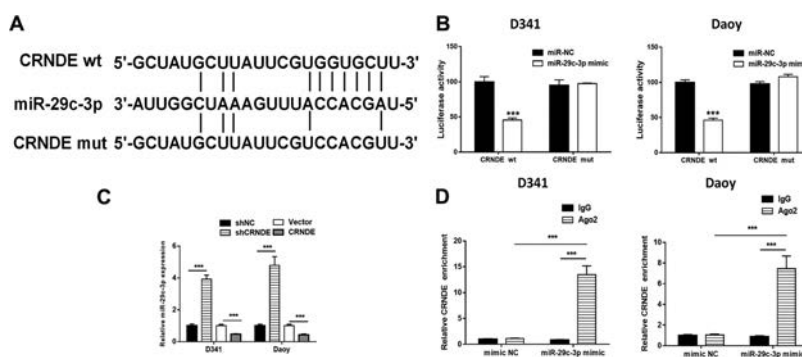


Figure 3. CRNDE binds to miR-29c-3p and negatively regulates its expression. Bioinformatics analysis of potential CRNDE/miRNA interactions (A). A dual-luciferase reporter plasmid containing CRNDE-wt or CRNDE-mut was cotransfected into HEK293T cells along with miR-29c-3p mimics or miR-NC, and luciferase activities were determined (B). Determination of miR-29c-3p expression by real-time PCR in CRNDE-depleted or CRNDE-overexpressed cells (C). Validation of molecular interactions of CRNDE and miR-29c-3p by RNA pulldown and RNA-binding protein immunoprecipitation (RIP) assay (D). *** $p < 0.001$.

miR-29c-3p Overexpression Sensitizes Medulloblastoma Cells to Cisplatin

Furthermore, we explored whether cell viability was affected by miR-29c-3p overexpression. Real-time PCR validated the upregulation of miR-29c-3p in D341 and

Daoy cells (Fig. 4A). As a result, CCK-8 assay indicated that D341 and Daoy cells ectopically expressed miR-29c-3p showed a slower growth rate and exhibited increased chemosensitivity, which were reversed after cotransfection with CRNDE (Fig. 4B and C). Colony

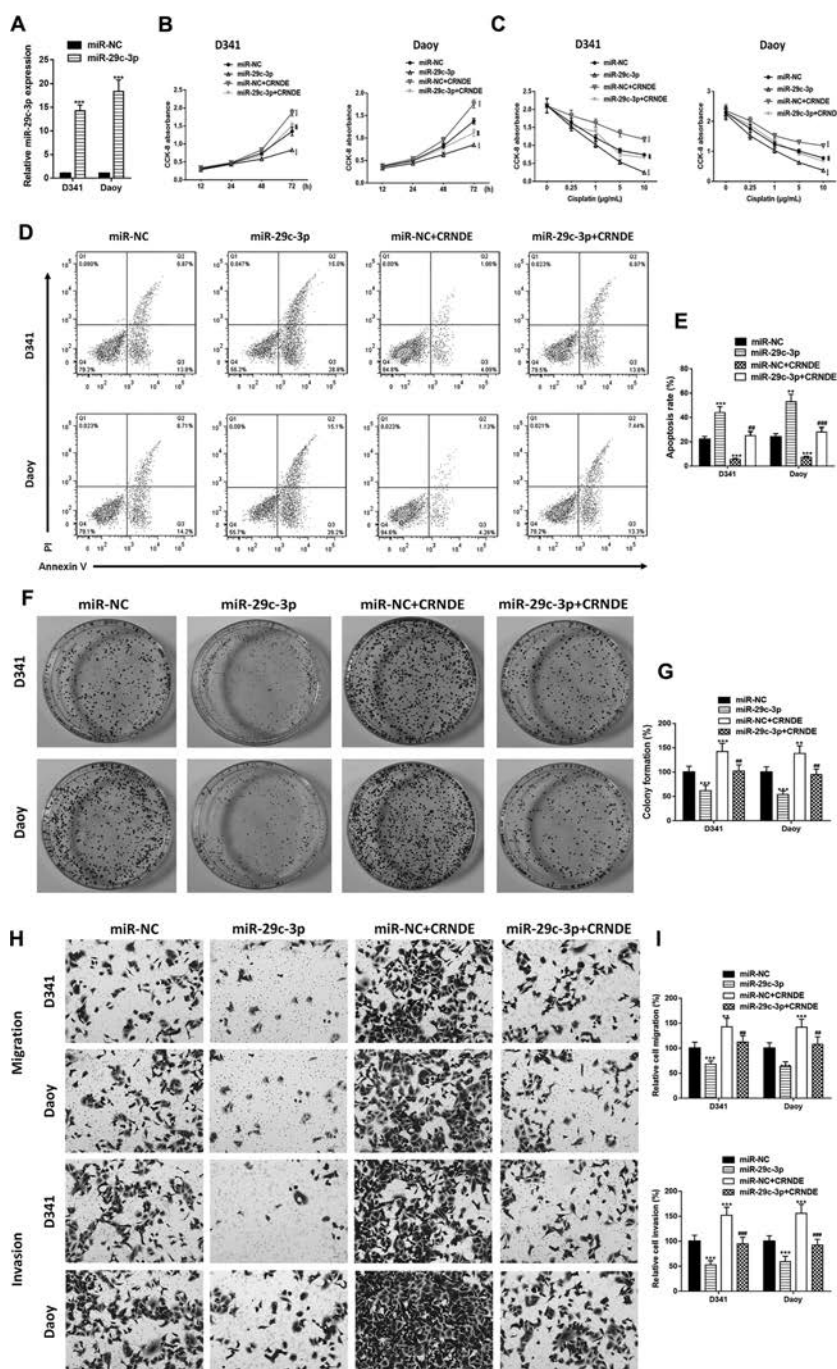


Figure 4. miR-29c-3p overexpression sensitizes medulloblastoma cell to cisplatin. Real-time PCR validated the upregulation of miR-29c-3p in D283 and D425 cells (A). Thereafter, proliferation and colony formation of miR-29c-3p-overexpressed D341 and Daoy cells were assessed using CCK-8 and colony formation assay (B, C, F, G). Flow cytometric and Transwell analysis were conducted to measure the apoptosis, migration, and invasion in cells with depletion or overexpression of miR-29c-3p (D, E, H, I). ** $p < 0.01$, *** $p < 0.001$, compared to miR-NC; ## $p < 0.01$, ### $p < 0.001$, compared to miR-NC+CRNDE.

formation assay showed that colony number was less in the miR-29c-3p transfected group, but was elevated after cotransfection with CRNDE in Daoy and D341 cells (Fig. 4F and G). In addition, flow cytometric and Transwell analysis showed that overexpression of miR-29c-3p induced apoptosis (Fig. 4D and E), inhibited migration and invasion (Fig. 4H and I), which were reversed by cotransfection with CRNDE in Daoy and D341 cells. Taken together, these data suggested that miR-29c-3p exerted a tumor suppressor role in medulloblastoma cells.

In Vivo Role of miR-29c-3p Overexpression on Tumor Growth in Medulloblastoma

To evaluate the *in vivo* role of miR-29c-3p and CRNDE on tumor growth, a mouse model bearing medulloblastoma was established. Starting from week 2, tumor volumes in the CRNDE-overexpressed group of mice were observed to be significantly larger than those in the control mice, which were obviously decreased after cotransfection with miR-29c-3p. Moreover, tumor volumes were even diminished in transfection with miR-29c-3p alone (Fig. 5A and B). In addition, we evaluated the *in vivo* role of miR-29c-3p and CRNDE on tumor cell apoptosis. TUNEL assay showed that overexpression of CRNDE inhibited apoptosis, which was reversed after cotransfection with miR-29c-3p. Moreover, transfection with miR-29c-3p alone dramatically induced apoptosis in both Daoy and D341 cell transplanted tumor in nude

mice (Fig. 5C). Taken together, these data revealed the *in vivo* inhibitive role of miR-29c-3p overexpression on tumor growth in medulloblastoma.

DISCUSSION

Medulloblastoma is the most common malignant brain tumor during childhood, which is the leading cause of central nervous system tumor-related deaths. Moreover, the early diagnosis and treatment for medulloblastoma remain difficult medical problems¹². Recent studies have revealed that some lncRNAs play critical roles in medulloblastoma pathogenesis, progression, and acquired resistance to chemotherapy, representing novel biomarkers and therapeutic targets for medulloblastoma diagnosis and treatment¹³.

Some clues regarding the roles of CRNDE on the pathogenesis and progression of central nervous system-related malignancies, such as glioma and medulloblastoma, have recently been reported^{11,14,15}. Li et al. analyzed microarray data of glioma clinical samples and identified a 30-fold increase in the expression of CRNDE compared to normal brain tissues¹⁶. Further mechanical investigation revealed the essential roles for the CRNDE-miR-136-5p-Bcl-2/Wnt2 regulatory pathway in the malignant progression of glioma. Our previous study for the first time found that CRNDE, a lncRNA, acted as a critical mediator of tumor growth in medulloblastoma both *in vitro* and *in vivo*¹¹. Resistance to chemotherapy and molecularly targeted therapies is a major

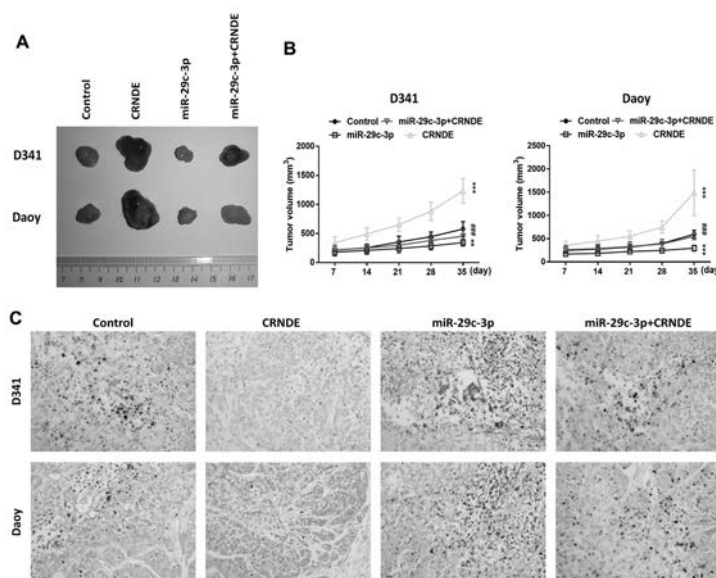


Figure 5. *In vivo* role of miR-29c-3p overexpression on tumor growth in medulloblastoma. A mouse model bearing medulloblastoma was established using Daoy cells and D341 cells. The average tumor volume in the different groups was determined (A, B). Tumor tissues from each group were further subjected to TUNEL assay (C). ** $p < 0.01$, *** $p < 0.001$ compared to control; ### $p < 0.001$ compared to CRNDE.

problem facing current cancer research¹⁷. Thus, we further explored the role of CRNDE in drug resistance and the underlying mechanisms contributing to the oncogenic phenotype of CRNDE, focusing on ceRNA/miRNA interaction, in medulloblastoma.

Actually, many previous studies have reported the pivotal role of CRNDE/miRNA interactions involved in tumor initiation and progression, including gliomas, pancreatic cancer, and colorectal cancer^{14,16,18}. In our study, we for the first time found that the transcript levels of lncRNA CRNDE were significantly higher in cisplatin-treated tumor cells with higher IC₅₀ values, suggesting a possible link between CRNDE and drug resistance in medulloblastoma cells. Moreover, both in vitro and in vivo data suggested that depletion of CRNDE could inhibit tumor cell proliferation and colony formation, induce cell apoptosis, and suppress migration and invasion in medulloblastoma cells.

Furthermore, we searched for potential binding sites of CRNDE to miRNAs based on bioinformatics databases. Consequently, a potential interaction between miR-29c-3p and CRNDE was identified and validated by dual luciferase activity and RNA pulldown and RIP assays, suggesting that CRNDE acts as miR-29c-3p sponge to mediate the tumorigenicity. As is well known, many miRNAs act as a tumor suppressor gene or oncogene, and regulate tumor invasion, metastasis, and drug resistance. It has been found that miR-29c act as a tumor suppressor miRNA in several malignancies, including breast, lung, and stomach cancer^{19–21}. However, it has been unknown whether miR-29c plays a role in the drug resistance of medulloblastoma cells. Our study showed that overexpression of miR-29c-3p inhibited tumor cell proliferation and colony formation, migration, and invasion, and enhanced apoptosis and chemosensitivity to cisplatin, implying that miR-29c-3p exerts a tumor suppressive function in medulloblastoma cells.

In addition, our in vivo experiments showed that tumor volumes were decreased and apoptosis rates were elevated in mice transfection with miR-29c-3p, which was reversed after overexpression of CRNDE, confirming the in vivo role of CRNDE/miR-29c-3p interactions in Daoy and D341 cell transplanted tumor in nude mice.

In summary, our study demonstrates that CRNDE, an lncRNA, acts as a critical mediator of tumor proliferation, apoptosis, migration, invasion, and resistance to chemotherapeutics using in vitro and in vivo assays. Mechanically, CRNDE functions as a ceRNA that directly binds to and negatively regulates miR-29c-3p, thereby mediating medulloblastoma growth and drug resistance. Our study provides novel molecular targets for targeted treatment of medulloblastoma.

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