miR-203, a Tumor Suppressor Frequently Down-regulated by Promoter Hypermethylation in Rhabdomyosarcoma*

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Background: Rhabdomyosarcoma (RMS) is a pediatric tumor that expresses several muscle-specific proteins with poor terminal differentiation.

Results: miR-203 was frequently down-regulated in RMS, and its re-expression in RMS cells inhibited their growth and migration and promoted terminal differentiation.

Conclusion: miR-203 is a tumor suppressor down-regulated in RMS.

Significance: miR-203 can serve as a potential target for therapeutic treatment of RMS.

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma found in children and young adults. It is characterized by the expression of a number of skeletal muscle-specific proteins, including MyoD and muscle α-actin. However, unlike normal myoblasts, RMS cells differentiate poorly both in vivo and in culture. As microRNAs are known to regulate tumorigenesis, intensive efforts have been made to identify microRNAs that are involved in RMS development. In this work, we found that miR-203 was frequently down-regulated by promoter hypermethylation in both RMS cell lines and RMS biopsies and could be reactivated by DNA-demethylating agents. Re-expression of miR-203 in RMS cells inhibited their migration and proliferation and promoted terminal myogenic differentiation. Mechanistically, miR-203 exerts its tumor-suppressive effect by directly targeting p63 and leukemia inhibitory factor receptor in RMS cells, which promotes myogenic differentiation by inhibiting the Notch and the JAK1/STAT1/STAT3 pathways, respectively. Our work reveals that miR-203 functions as a tumor suppressor in RMS development.

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma found in children and young adults (1–4). Based on distinct genetic alterations and cell morphologies, RMS can be classified into two classes: embryonal and alveolar. For both types of RMS cells, a common feature is that they are small and round. For a RMS cell, the size is about 20 μm, and the nuclei contain a dense chromatin pattern and are usually located at the 3’-untranslated region (3’-UTR) of target mRNA transcripts. miRNAs are sequentially processed by the Drosha and Dicer complexes to form double strand miRNAs, which have unknown functions (miRBase, release 19) (12). Initially synthesized as poly(A)-containing single strand primary RNA transcripts, miRNAs are sequentially processed by the Drosha and Dicer complexes to form double strand miRNAs, a class of ~22 nucleotide small noncoding RNAs that also critically regulate tumorigenesis. Since the discovery of the first miRNA (i.e. lin-4) in Caenorhabditis elegans (11), more than 2000 miRNAs have been identified in human, most of which have unknown functions (miRBase, release 19) (12). Initially synthesized as poly(A)-containing single strand primary RNA transcripts, miRNAs are sequentially processed by the Drosha and Dicer complexes to form double strand miRNAs, a class of ~22 nucleotide small noncoding RNAs (13–16). When loaded onto the Argonaute-containing complex, a particular strand of a duplex miRNA, the guide strand, is preferentially selected and incorporated into the miRNA-induced silencing complex and guides the complex to complementary sites (usually located at the 3’-translated region (3’-UTR)) of target mRNAs through imperfect base pairing, which leads to post-transcriptional gene silencing by translational repression and/or deadenylation and decay of target mRNAs. miRNAs have been shown to play important regulatory roles in various biological processes, including cell proliferation, differentiation, apoptosis, and development (17, 18). Aberrant expression of miRNAs is linked to the pathogenesis of many human diseases, including cancers (19, 20). Like their protein counterparts, the miRNAs that are involved in tumorigenesis can also

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3 The abbreviations used are: RMS, rhabdomyosarcoma; qPCR, quantitative PCR; miRNA, microRNA; MS-PCR, methylation-specific PCR; 5-aza-dC, 5-aza-2’-deoxycytidine; LIF, leukemia inhibitory factor; LIFR, LIF receptor; WCE, whole cell extract; PET, polyethylene terephthalate.
be divided into two groups, the oncogenic miRNAs and the tumor-suppressive miRNAs (21–24).

Several miRNAs have been identified that contribute to the development of RMS (25). A group of muscle-specific miRNAs, including miR-1, miR-133, and miR-206, was found to be dysregulated in RMS (26–29). In addition, miR-26a, miR-29, and miR-183 were also shown to be dysregulated in RMS (30–32). miR-203 has been implicated in a number of cancers (33–42). However, its status in RMS was unclear. In this study, we demonstrated that miR-203 was down-regulated mainly by promoter hypermethylation in RMS cell lines and RMS biopsies and could be reactivated by DNA-demethylating agents. Re-expression of miR-203 in RMS cells inhibited cell proliferation and migration and enhanced myogenic differentiation. We showed that miR-203 exerts its tumor-suppressor functions in RMS cells by directly targeting p63 and the leukemia inhibitory factor receptor gene (LIFR), which promotes myogenic differentiation of RMS cells by inhibiting the Notch pathway and the JAK1/STAT1 pathway, respectively.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, DNA Constructs, and Reagents**—RD cells (ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin. RH30 cells were generously provided by Dr. Peter Houghton (The Research Institute at Nationwide Children’s Hospital, Columbus, OH) and maintained in RPMI 1640 medium with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. The growth media above were switched to the differentiation medium (DM) (DMEM with 2% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) to induce differentiation. Human recombinant leukemia inhibitory factor (LIF) was purchased from Chemicon (Billerica, MA). DAPI, 5-aza-2’-deoxycytidine (5-aza-dC), sodium bisulfite, hydroquinone, 6-mercaptopurine, Rose Bengal, and streptomycin were from Sigma. Tripolyphosphate and phenylmethylsulfonyl fluoride were from Roche Applied Science. The sequence of primers is listed as follows: Jagged1 (forward 5’-TTATGCGGGCTTAGACCGAGCACAGAC and reverse 5’-GCCCTGTTTAAACGCTTAGAACCGGCTTAC); fragment containing the miR-203-binding sites 1 and 2, forward 5’-TTATGCCGGGCTTAGACCGAGCACAGAC and reverse 5’-GCCCTGTTTAAACGCTTAGAACCGGCTTAC; fragment without the miR-203-binding sites 1 and 2, forward 5’-TTATGCCGGGCTTAGACCGAGCACAGAC and reverse 5’-GCCCTGTTTAAACGCTTAGAACCGGCTTAC; fragment containing the miR-203-binding sites 3 and 4, forward 5’-TTATGCCGGGCTTAGACCGAGCACAGAC and reverse 5’-GCCCTGTTTAAACGCTTAGAACCGGCTTAC; and fragment without the miR-203-binding sites 3 and 4, forward 5’-TTATGCCGGGCTTAGACCGAGCACAGAC and reverse 5’-GCCCTGTTTAAACGCTTAGAACCGGCTTAC.

**Antibodies and Western Blot Analysis**—Antibodies against myogenin, LIFR, JAK1, STAT1, STAT3, and p63 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-GAPDH was from Ambion. Antibodies against phospho-JAK1 (Tyr-1022/Tyr-1023), phospho-STAT1 (Tyr-705), and phospho-STAT3 (Tyr-705) were from Cell Signaling (Danvers, MA). Anti-Hes1 was from EMD Millipore. Anti-β-tubulin (T4026) was from Sigma. Anti-sarcomeric myosin heavy chain antibody (MF20) was purchased from Developmental Studies Hybridoma Bank (Iowa City, IA). Western blot analysis was carried out according to standard procedures as described previously (43).

**siRNA, miRNA, and Plasmid Transfection**—For siRNA and miRNA transfection, 4 × 10⁴ cells/well of RD or RH30 cells were plated into 12-well plates 3 h before transfection. For each well, 50 nM of each siRNA or miRNA was transfected using the Lipofectamine RNAiMAX (Invitrogen). The following siRNAs and miRNAs (only the guide-strand sequences are shown) were used: enhanced green fluorescent protein (5’-GCCUGAGCCUGAGAUUGUC); p63 (5’-AACACGGAATGCCGATGGA; #2, 5’-AAACGCAAGGTCTCGGACAG); JAK1 (5’-AACCCUGAGAGUGGAGUAC); Hsa-miR-203 (5’-GUGAAGUUGUAGGACACU); The control miRNA that was derived from C. elegans does not match any human mRNA sequence and was purchased from Ribobio Co., Ltd. (Guangzhou, China). For plasmid transfection, 6 × 10⁴ cells/well of RD or RH30 cells were plated into 12-well plates. Cells were transfected with appropriate DNA using the Lipofectamine Plus reagents (Invitrogen) following the manufacturer’s instructions.

**Immunostaining**—Phosphate-buffered saline-washed cells were fixed for 15 min in 4% paraformaldehyde, followed by treatment with 0.2% Triton X-100 for 20 min and 4% bovine serum albumin blocking for 1 h. Cells were then incubated with a primary antibody overnight followed by incubation with a fluorescent dye-conjugated secondary antibody for 1 h. DAPI (100 ng/ml) was added for another 10 min. Fluorescence microscopy was performed with a Nikon Eclipse Ni-U microscope, and the images were captured by a SPOT RT3 color slide digital camera (Diagnostic Instruments Inc., Sterling Heights, MI).

**Quantitative RT-PCR (RT-qPCR)**—Total RNAs from RMS cells were extracted using the TRIZol reagent (Invitrogen). RNAs from formalin-fixed paraffin-embedded RMS biopsies were extracted using the RecoverAll total nucleic acid isolation kit (Ambion) following the manufacturer’s instructions. The expression levels of miR-203 were determined using a miR-203-specific TaqMan probe (Applied Biosystems). U6 was used as an internal control for normalization. All RT-qPCRs were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems). The primer sets were described as previously (44, 45) with modifications by
Kantlehner (46). Briefly, the genomic DNA was isolated from RD or RH30 cells. All buffers used in the reactions were degassed by vacuum. 1 μg of purified genomic DNA was sonicated to generate shorter fragments, heated at 95 °C for 3 min, and chilled on ice, followed by mixing with 100 μl of freshly prepared bisulfite solution (3 M sodium bisulfite, 8 mM hydroquinone, 0.5 mg/ml Triloxy, 1 mM tetraethylammonium chloride, 0.1 M tetraethylpentamine pentahydrochloride, 0.3 M guanidine hydrochloride, adjusted to pH 5.0 with 3 M NaOH). The mixture was heated in a PCR machine under the following conditions: one cycle of 20 s at 95 °C, followed by 20 min at 58 °C, and three cycles of 10 s at 95 °C followed by 20 min at 58 °C. The DNA was then recovered from the mixture using the DNA extract kit (Viogen), followed by denaturation with 0.3 M NaOH at 42 °C for 20 min. The solution was neutralized by 0.25 volume of 5 M ammonium acetate, pH 8.0. The DNA was ethanol-precipitated, resuspended in water, and analyzed by PCR using primers based on methylated CpG sites or unmethylated CpG sites in the human miR-203 promoter (33).

Cell Proliferation Assays—The cell proliferation assays (WST-1 and BrdU labeling experiments) were performed as described previously (47). Briefly, the Premix WST-1 cell proliferation assay system (TaKaRa Bio Inc., Otsu, Japan) was used to examine cell proliferation. RMS cells treated with 5-aza-dC or transfected with various miRNAs were plated into 96-well plates at 3000 cells/well. At different time points, the WST-1 reagent was added into the culture medium (1:10 dilution). After incubation for 1 h, the absorbance was measured at A450 nm. For the BrdU labeling experiments, the in situ cell proliferation kit, FLUOS (Roche Applied Science), was used. Briefly, 30 h after transfection, 10 μM of BrdU was added into the culture medium for 1 h. After the labeling, cells were fixed, permeabilized, and stained with an anti-BrdU antibody to reveal BrdU + (i.e. proliferating) cells. The nuclei were counterstained with DAPI.

Transwell Assays—The cell migration and invasion properties were assayed using 12-well Transwell chambers (8 μm pores, BD Biosciences, catalog no. 353182). For the invasion assays, the PET membrane of the insert wells was pre-coated with 4 mg/ml Matrigel (BD Biosciences). Cells were transfected with a control miRNA or miR-203 for 24 h, followed by trypsinization and then resuspension in DMEM with 10% FBS. A total of 1 × 105 or 4 × 105 cells was plated into the upper well for the migration and invasion assays, respectively. The lower chambers were filled with DMEM containing LIF (100 ng/ml) as a chemoattractant. After 16 h, the insert wells were removed, and the cells on the bottom side of the PET membrane were scraped off with cotton wool. Cells that had passed through the PET membrane were stained by DAPI, and the images were captured using a fluorescent microscope (Olympus IX70) linked to a SPOT RT digital camera. To get the quantitative results, duplicate wells were used for each sample. The cells were counted from five randomly selected microscopic fields (under a ×10 objective lens) of the bottom side of the PET membrane per chamber. The data were presented as mean ± S.D.

 Luciferase Reporter Assays—The luciferase reporter assays were conducted as described previously (43). Briefly, in each well of a 12-well plate, cells were co-transfected with 0.5 μg of a luciferase reporter plasmid together with 100 nM of a control miRNA or miR-203 by Lipofectamine 2000. All the samples were prepared in triplicate. The luciferase activity was assayed by an LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). 10 μl of WCE was added to 150 μl of freshly prepared luciferase buffer (0.4 μM luciferin, 13.3 mM ATP, 0.1 M Tris-HCl, pH 7.8, 1 mM EDTA, pH 8.0, 10 mM MgAC2). Luciferase units were normalized against the total protein concentration determined by the protein assay reagent from Bio-Rad.

RMS Biopsies—Normal human skeletal muscle tissues and formalin-fixed paraffin-embedded RMS biopsies were collected from the Shenzhen- Peking University Hospital (Shenzhen, Guangdong Province) and the School of Stomatology of the Fourth Military Medical University (Xi’an, Shaanxi Province) in China. Approval was obtained from the Medical Ethics Committee in the Shenzhen-Peking University Hospital.

In Vivo Tumorigenesis Assays—4–5-Week-old male nude/ nude mice were used for xenograft studies as described previously (30). RD cells (1 × 107) were injected subcutaneously into the flanks of nude mice. Tumor size was measured with a Vernier caliper, and tumor volume was calculated using the following formula: (p/6) × Dl × Ds2, where Dl is the largest diameter, and Ds is the smallest diameter. miRNA oligonucleotides (5 μM) were incubated with Lipofectamine RNAiMAX (Invitrogen) in a final volume of 60 μl of Opti-MEM for 15 min prior to injection. After incubation, 30 μl of the miRNA/Lipofectamine mixture was injected into each tumor. The injection was performed every 3 days. For the 5-aza treatment, 10 μl of DMSO or 5-aza-dC (10 mM) was injected intratumorally once every 3 days. All animal handling procedures were approved by the Animal Ethics Committee of Hong Kong University of Science and Technology.

Statistics—The Student’s t test was used for all statistical analyses. p value was calculated and presented in the figures (*, p < 0.05; **, p < 0.01).

RESULTS

miR-203 Was Down-regulated in RMS Cell Lines and RMS Biopsies Mainly by Promoter Hypermethylation—To examine the expression status of miR-203 in RMS, we first focused on RD and RH30 cells, two RMS-derived cell lines that were derived from embryonal and alveolar RMS, respectively (48). By RT-qPCR, we found that RD and RH30 cells barely expressed miR-203 compared with normal human skeletal muscle tissues (Fig. 1A). To check whether this was unique to RMS, we also examined the miR-203 level in a panel of other human tumor cell lines. Although cells derived from hepatocarcinoma (i.e. HepG2 and SMMC-7721) and prostate cancers (i.e. PC3 and DU145) also expressed a very low level of miR-203, cells derived from lung cancer (i.e. A549 and H1299) and colon cancer (i.e. SW480, Lovo, and HCT116) expressed miR-203 at a higher level compared with normal human muscle tissues (Fig. 1A), which was consistent with published reports (36–40). Our results suggested that the expression status of miR-203 in different types of tumors was tissue-specific. As miR-203 was reported to be down-regulated by promoter hypermethylation...
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FIGURE 1. miR-203 was down-regulated in RMS-derived cells and RMS biopsies by promoter hypermethylation. A, total RNA from normal human skeletal muscle (50 nM) (n = 4), RMS cells (RD and RH30), hepatocarcinoma cells (HepG2, SMMC-7721), prostate cancer cells (PC3 and DU145), lung cancer cells (A549, H1299), and colon cancer cells (SW480, LoVo, and HCT116) were subjected to RT-qPCR analysis to measure the relative levels of miR-203. Fold change was calculated as the ratio of the relative miR-203 level in various cancer cells over that in normal skeletal muscle. B–D, RD and RH30 cells were treated with either DMSO or 10 μM 5-aza-2’-deoxycytidine (5AZA) for various times. B, after 48 h of pretreatment, cells were trypsinized and re-plated into 96-well plates at 3000 cells/well. Six duplicates were used for each sample at each time point. At various time points, the number of live cells in each well was indirectly quantified by the WST-1 assays. The experiment was repeated three times with similar results, and the data from one representative experiment were presented as mean ± S.D. C, 72 h after treatment, the total RNA was harvested and subjected to RT-qPCR analysis to measure miR-203 level. Fold change was calculated as the ratio of the relative miR-203 level in 5-aza-treated cells over that in untreated cells. D, 72 h after treatment, the genomic DNA was isolated and subjected to MS-PCR analysis. U, primers based on unmethylated CpG sites; M, primers based on methylated CpG sites. E, total RNA from normal skeletal muscle (n = 10) and RMS biopsies (n = 22) were subjected to RT-qPCR analysis with TaqMan probes specific for miR-203 and U6, and the result was presented as log2 values.

TABLE 1

| Sex    | Age (year) | Location of tumors                        | Subtypes | miR-203 fold change |
|--------|------------|--------------------------------------------|----------|---------------------|
| M 1    | Left parotideomasseteric region            | ERMS     | 1.92                 |
| F 45   | Right inguinal region                       | PRMS     | 1.12                 |
| F 29   | Left submandibular and floor of mouth       | PRMS     | 1.07                 |
| M 47   | Left pharynx                                | PRMS     | 1.00                 |
| M 19   | Neck                                       | ERMS     | 0.43                 |
| M 52   | Right soft palate                           | PRMS     | 0.39                 |
| M 26   | Left zygomatic region                        | PRMS     | 0.32                 |
| F 66   | Left upper neck                             | PRMS     | 0.32                 |
| M 21   | Right parotideomasseteric region            | ARMS*    | 0.30                 |
| M 13   | Left lower lip                              | ERMS     | 0.30                 |
| M 24   | Right neck                                  | ARMS*    | 0.25                 |
| M 24   | Right lower gingiva                         | ARMS     | 0.18                 |
| M 2    | Left inguinal region                         | ERMS     | 0.17                 |
| F 6    | Right parotideomasseteric region            | ERMS     | 0.17                 |
| F 19   | Left maxillary                              | ARMS     | 0.11                 |
| M 22   | Left cellarche ethnomoides                 | ARMS*    | 0.09                 |
| F 28   | Right upper arm                             | ERMS     | 0.011                |
| F 28   | Right maxillary                             | ARMS     | 0.0044               |
| F 46   | Right inguinal region                        | PRMS     | 0.0016               |
| M 30   | Left maxillary                              | ARMS     | 0.0015               |
| M 22   | Left testis                                 | PRMS     | 0.00060              |
| M 23   | Left parotideomasseteric region             | ARMS     | 0.000043             |

Information on RMS biopsies used in this study. The level of miR-203 in each biopsy was measured by RT-qPCR using a miR-203-specific TaqMan probe. The average level of miR-203 from 10 normal human skeletal muscle samples was set as 1. Fold change was calculated as the ratio of the relative miR-203 level in RMS biopsies over that in normal skeletal muscle. M, male; F, female; *, metastasis; #, recurrent; PRMS, pleomorphic RMS; ERMS, embryonal RMS; ARMS, alveolar RMS.

in some human cancers (33–36), we tested whether this was also the case in RMS-derived cells. Treatment of RD or RH30 with 5-aza-dC indeed slowed down cell growth in culture (Fig. 1B). Importantly, 5-aza-dC treatment re-activated the expression of miR-203 in both RD and RH30 cells (Fig. 1C), suggesting that miR-203 was down-regulated in RMS cell lines by promoter hypermethylation. To further confirm this notion, RD and RH30 cells were either left untreated or treated with 5-aza-dC for 72 h before harvest. The genomic DNA was isolated, treated with bisulfite, and then subjected to methylation-specific PCR (MS-PCR) (45). As shown in Fig. 1D, only the primer sets that were specific for the methylated CpG (M), but not the unmethylated CpG (U), could amplify DNA of the correct size from nonuntreated RD or RH30 cells (33). In contrast, only the primer sets that were specific for the unmethylated CpG could amplify DNA of the correct size from the 5-aza-dC-treated RD or RH30 cells. To further examine the expression status of miR-203 in RMS tumor biopsies, we obtained 10 normal human skeletal muscle biopsies and 22 RMS biopsies (Table 1) and measured the levels of miR-203 by RT-qPCR using a miR-203-specific TaqMan probe. As shown in Fig. 1E, the miR-203 level in the RMS biopsies was much lower than that in normal skeletal muscles. Collectively, our data showed that the expression of miR-203 was down-regulated in both RMS-derived cell lines and RMS biopsies mainly by promoter hypermethylation.

Re-expression of miR-203 in RMS Cells Inhibited Cell Growth and Promoted Myogenic Differentiation—The down-regulation of miR-203 in RMS cells and tumor biopsies suggested that it could function as a tumor suppressor. To test this hypothesis, we re-expressed miR-203 in either RD or RH30 cells by transient transfection and assessed its impact on cell proliferation by either the WST-1 assay, which measures the activity of the mitochondrial succinate-tetrazolium reductase in live cells, or the BrdU incorporation assay, which reveals the number of cells that incorporated BrdU during S phase. Compared with RMS cells transfected with a control miRNA, both RD and RH30 cells transfected with miR-203 showed reduced cell growth as judged by both assays (Fig. 2, A–C). Moreover, like miR-1 and miR-206 (26, 27), we found that re-expression of miR-203 in RMS cells promoted terminal myogenic differentiation, which was manifested by induction of both myogenin and myosin heavy chain (MHC), two well established differentiation markers, as judged by both Western blot and immunostaining analysis (Fig. 2, D–G). When re-expressed alone at an optimal dose (i.e. 50 nM) in RD cells, both miR-203 and miR-206 were less efficient than miR-1 in inducing MHC expression (Fig. 2, F,
When individually re-expressed at a suboptimal level (i.e. 25 nM) together with an equal amount of a control miRNA, none of the three tumor-suppressive miRNAs could efficiently induce MHC expression (Fig. 2, F, lanes 6–8, and G, panels 6–8). However, when two different miRNAs (each at 25 nM) were co-expressed together, they synergistically induced stronger MHC expression than any of the miRNAs alone did (Fig. 2, F, lanes 9–11, and G, panels 9–11). Notably, miR-1 and miR-203 together led to the strongest MHC induction (Fig. 2, F, lane 9, and G, panel 9). Our data above showed that miR-203 could indeed function as a tumor suppressor in RMS cells by inhibiting tumor cell proliferation and promoting myogenic differentiation.

Re-expression of miR-203 Suppressed RMS Cell Migration and Invasion—Many tumor-suppressive proteins or miRNAs exert their effects by inhibiting cell migration and invasion. To examine whether miR-203 has such properties, we first used Transwell®-based cell migration assays. We found that re-expression of miR-203 in either RD or RH30 cells effectively suppressed their migration ability (Fig. 3, A and B). Furthermore, using the cell invasion assays, we showed that re-expression of miR-203 in either RD or RH30 cells also efficiently inhibited the invasion ability of both RMS cells (Fig. 3, C and D). Taken together, these results demonstrated that re-expression of miR-203 in RMS cells suppressed their migration and invasion potentials.

miR-203 Inhibited the Notch Pathway and Promoted Myogenic Differentiation by Directly Targeting p63—To uncover the mechanisms underlying the tumor-suppressive effects of miR-203, we searched for its direct target genes. It has been shown that miR-203 could regulate the “stemness” of mouse skin stem cells by targeting p63, resulting in enhanced differentiation (49, 50). p63 is a transcription factor of the p53 subfamily (51). As the nucleotide sequence of the mature miR-203 and its target site at the 3’-UTR of p63 are fully conserved in both human and mouse genomes (49), it was likely that miR-203 could also target p63 in RMS cells. Indeed, we found that re-expression of miR-203 reduced the expression levels of p63 in both RMS cell...
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In several human cancer cell lines, p63 was reported to be capable of regulating the Notch pathway by inducing the expression of *Jagged1* that encodes a ligand of the Notch pathway (52–54). Consistently, when RD cells were transfected with either miR-203 or a p63 siRNA, we found that the mRNA level of *Jagged1* was indeed reduced (Fig. 4B), whereas that of other Notch ligands remained unaffected. To examine the activation status of the Notch pathway, we turned to Hes1, a well established downstream target of the Notch pathway (54). As a control, we first showed that two nonoverlapping p63 siRNAs indeed reduced the protein level of Hes1 in RD cells (Fig. 4C, left panel). Importantly, re-expression of miR-203 in RD cells was as effective as p63 siRNAs in reducing the Hes1 level (Fig. 4C, right panel). In normal myoblasts, the Notch pathway was known to negatively regulate myogenic differentiation (55–58). In RD cells, we found that inhibition of the Notch pathway with a γ-secretase inhibitor (L-685,458) was also effective in promoting myogenic differentiation as evidenced by the elevated myogenin level (Fig. 4D) (59). Consistently, when p63 was knocked down in either RD or RH30 cells with two different siRNAs, both RMS cells also underwent enhanced differentiation (Fig. 4E). Collectively, our data above showed that re-expression of miR-203 in RMS cells inhibited the Notch pathway by targeting p63, which in turn promoted myogenic differentiation.

**miR-203 Inhibited the JAK1/STAT1/STAT3 Pathway by Targeting LIFR**—An miRNA is known to exert its diverse functions by directly targeting multiple genes. To look for additional target genes of miR-203, we used both TargetScan (60) and miRanda (61). Human *LIFR* gene was predicted to be a target of miR-203 by both programs. When miR-203 was re-expressed in either RD or RH30 cells, we showed that the protein level of LIFR was indeed reduced (Fig. 5A). There are four putative miR-203-binding sites at the 3′-UTR of *LIFR* (Fig. 5B). To prove that miR-203 directly targets the 3′-UTR of *LIFR*, we cloned the 3′-UTR of *LIFR* downstream of a luciferase reporter gene. Because of the large distance (~3.7 kb) between sites 2 and 3, we generated two separate luciferase reporter genes as follows: one containing sites 1 and 2, and the other containing sites 3 and 4. As shown in Fig. 5C, co-expression of miR-203 with either reporter gene resulted in decreased luciferase activities. In contrast, when we deleted the miR-203-binding sites in both reporter genes, miR-203 was much less effective in repressing the activity of the mutant reporter genes. LIFR pairs with gp130 to serve as a receptor for multiple members of the IL-6 family of cytokines, including leukemia inhibitory factor (LIF) and oncostatin M (62–64). In normal myoblasts, we previously showed that both LIF and oncostatin M potently repress myogenic differentiation via the JAK1/STAT1/STAT3 pathway (65, 66). In RD cells, we found that the LIF-induced JAK1 pathway also negatively regulated myogenin expression; whereas the knockdown of *JAK1* enhanced the expression of myogenin (Fig. 5D), LIF treatment further suppressed basal myogenin expression (Fig. 5E). Consistently, we found that the LIF-induced activation of JAK1, STAT1, and STAT3 in either RD or RH30 cells was also reduced by miR-203 (Fig. 5F). Moreover, we showed that re-expression of an exogenous LIFR together with miR-203 in either RD or RH30 cells restored the defective JAK1/STAT1/STAT3 signaling induced by LIF (Fig. 5G), which further supported our claim that LIFR is a key target of miR-203 in RMS cells. Taken together, our data showed that re-expression of

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miR-203 in RMS cells inhibited the JAK1/STAT1/STAT3 pathway by targeting LIFR, which in turn promoted myogenic differentiation.

**Re-expression of miR-203 Inhibited the Tumorigenesis Potential of RMS Cells in Vivo**—To further reveal whether miR-203 exhibited tumor-suppressive functions in vivo, we employed the xenograft model by injecting RD cells into nude mice subcutaneously. After solid tumor appeared about 2 weeks after injection, we repeatedly injected miRNA mimics intratumorally once every 3 days for a period of 3 weeks. As shown in Fig. 6A, injection of miR-203, but not a control miRNA, efficiently reduced tumor growth. Using RT-qPCR, we confirmed that miR-203 levels were indeed higher in tumors treated with miR-203 (Fig. 6B). Moreover, in paired tumor samples injected separately with the control miRNA and miR-203, we found that the miR-203-expressing tumor samples consistently showed reduced expression of p63, LIFR, and Hes1, with a concomitant increase in the level of myogenin (Fig. 6C). Furthermore, consistent with our previous findings that 5-aza-dC effectively restored the expression of miR-203 in RMS cells and reduced their growth in culture (Fig. 1), we found that repeated injection of 5-aza-dC into tumors in nude mice also effectively reduced the tumor size compared with that of DMSO (Fig. 6D). Thus, our data indicated that both miR-203 and 5-aza-dC effectively inhibited RMS growth in vivo.

**DISCUSSION**

**miR-203 Exerts Tumor-suppressive Effects in RMS Cells**—Like many tumor-suppressive proteins, miRNAs are also known to possess such functions (22, 23). Typically, they exert their tumor-suppressive effects by targeting and down-regulating various molecules that promote tumor growth, survival, migration, and angiogenesis (23, 24). In RMS, several tumor-suppressive miRNAs, including miR-1, miR-29, miR-133a, and miR-206, have already been identified (26–28, 30). Compared with their levels in normal skeletal muscles, miR-1, miR-29, miR-133a, and miR-206 were all found to be down-regulated in both RMS biopsies and RMS-derived cell lines (26–28, 30, 67). Among them, miR-29 was shown to be down-regulated by YY1-EZH2-dependent epigenetic mechanism (30). It remains...
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unknown what causes the down-regulation of miR-1, miR-206, and miR-133a. In RMS cells, YY1 was found to be a key target of miR-29, whereas c-Met was shown to be a critical target of both miR-1 and miR-206 (27, 28, 30). In this study, we showed that miR-203 is also a tumor-suppressive miRNA that is down-regulated in RMS mainly by promoter hypermethylation. Aberrant expression of miR-203 is detected in a number of human cancers. In hematopoietic tumors, hepatocellular carcinoma, and oral squamous cell carcinoma, miR-203 was shown to be down-regulated by promoter hypermethylation (33–36). In addition, miR-203 was also found to be down-regulated by unknown mechanisms in prostate cancer (37, 38). Unexpectedly, an elevated expression of miR-203 was found in lung cancer (39), colon adenocarcinoma (40), pancreatic ductal adenocarcinoma (41), and ovarian cancer (42). Our own assays also confirmed these findings (Fig. 1A), suggesting that the role of miR-203 in tumorigenesis is cancer type-dependent. Similar phenomena have also been observed in other tumor-associated miRNAs (e.g. miR-29 and miR-146) (23, 24). A number of cellular targets have been identified for miR-203. p63 was shown to be directly targeted by miR-203 in mouse skin epithelial cells (50). In several types of human cancer cells, ABCE1, Abl1, and E2F3 were shown to be directly targeted by miR-203 (33, 35, 36, 68). In this study, we found that miR-203 also targets p63 in RMS cells (Fig. 4A). p63 is known to be overexpressed in a number of cancers (51). Moreover, p63-mediated activation of the Notch pathway was thought to contribute to tumor growth (53, 69). In RMS biopsies and RMS-derived cell lines, the Notch pathway was also found to be active (70). p63 is known to transcriptionally regulate genes encoding Notch ligands (53, 69). Among the known Notch ligands, we found that only Jagged1 was up-regulated by p63 in RMS cells (Fig. 4B). In addition to p63, our work also identified LIFR as another key target of miR-203 in RMS cells. The LIF/LIFR-initiated signaling pathway was reported to regulate the metastatic property of RMS cells (71).

As cellular proliferation and terminal differentiation programs are mutually exclusive, approaches that promote terminal differentiation are considered an effective way to inhibit tumor growth in cancer therapies (26, 27, 72, 73). Our data show that the tumor-suppressive effect of miR-203 in RMS is partly due to its ability to promote terminal myogenic differentiation. It does so by suppressing p63 and LIFR, which in turn lead to the down-regulation of two potent differentiation-inhibitory pathways, namely the Notch pathway and the JAK-dependent JAK1/STAT1/STAT3 pathway, respectively (Fig. 7). The Notch pathway is indispensable for the maintenance and proliferation of muscle satellite cells (i.e. muscle stem cells) during normal muscle development and muscle regeneration (74–82). It also inhibits myogenic differentiation by repressing MEF2 and MyoD (55, 57, 58). As to LIFR, our previous work showed that both LIF and oncostatin M potently inhibit myogenic differentiation by activating the LIFR-dependent JAK1/STAT1/STAT3 pathway (65, 66). Consistently, we find that specific down-regulation of either pathway in RMS cells could promote myogenic differentiation (Figs. 4 and 5), which contributes to the tumor-suppressive effect of miR-203.

Cooperation of miR-203 with Other Tumor-suppressive miRNAs in Promoting Myogenic Differentiation in RMS Cells—For known tumor-suppressive miRNAs identified in RMS, they have different effects on myogenic differentiation when re-expressed in RMS cells. Many of them (e.g. miR-1, miR-29, and miR-206) enhance myogenic differentiation (27, 30), although miR-133a does not (26). miR-203 clearly belongs to the former group. However, unlike miR-1 that effectively induced the expression of both myogenin (an early differentiation marker) and especially myosin heavy chain (a late differentiation marker), miR-203 mainly induced the expression of myogenin. Its induction of MHC was not as effective as miR-1 as judged by Western blot analysis (Fig. 2F). Because different miRNAs act on distinct subsets of targets, it is possible that some of these tumor-suppressive miRNAs may cooperate with each other to induce better differentiation in RMS cells. Indeed, we found that co-expression of miR-1 with either miR-206 or especially miR-203 had an obvious synergistic effect on MHC induction in RMS cells (Fig. 2, F and G). In contrast, miR-203 and miR-206 had less cooperative effect, suggesting that such a cooperative effect on MHC induction can only be achieved by specific sets of miRNAs.

Potential Application of miRNAs and 5-Aza-deoxycytidine in RMS Therapies—Because of the prominent roles of miRNAs in tumor development, a number of miRNA-based therapies have been tested in animal models and proved to be effective (23, 24, 83). Although technical obstacles (e.g. unintended off-target effects, in vivo delivery methods, etc.) still exist that hinder a quick and widespread application of miRNA-based therapies in human patients, the encouraging results of the first miRNA-based clinical trial indicate that it is a feasible and effective approach (84). Thus, miR-203 along with other tumor-suppressive miRNAs identified in RMS has the potential to be used in miRNA-based therapeutics for treatment of RMS in the future. As many genes, including both protein-coding and miRNA-coding genes, are known to be down-regulated in various tumors by epigenetic mechanisms, including promoter hypermethylation and histone hypoacetylation, drugs that are capable of altering the epigenetic state of cancer cells are expected to be effective by re-activating multiple tumor-suppressor genes (85, 86). Indeed, several such drugs (e.g. azacytidine and sub-
eralylidine hydroxamic acid) are now in clinical use to treat cancer patients (86). Consistently, in a mouse model of RMS, a combined treatment of 5-aza-dC and valproic acid was shown to be effective in preventing tumor formation (87). In support of this approach, we showed here that 5-aza-dC greatly reduced the growth of RMS in both cell cultures as well as in the nude mouse-based animal model (Figs. 1B and 6D). Therefore, application of such drugs together with conventional therapy represents a promising direction in RMS treatment.

In summary, our work here shows that miR-203 functions as a tumor suppressor in the development of RMS. It is frequently down-regulated by promoter hypermethylation and can be reactivated by 5-aza-dC treatment. miR-203 negatively regulates the Notch and the JAK1/STAT1 pathways by directly targeting p63 and LIFR. As both pathways potently inhibit myogenic differentiation, re-expression of miR-203 in RMS cells promotes myogenic differentiation, which results in decreased proliferation and migration of RMS cells. Thus, miR-203 represents a promising target in therapeutic treatment of RMS in the future.

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