

# MicroRNA-1/206 Targets c-Met and Inhibits Rhabdomyosarcoma Development\*

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MicroRNAs (miRNAs) are endogenous short (~22) nucleotide RNAs that regulate gene function by modification of target mRNAs. *miRNA-1* (*miR-1*) and *miRNA-206* (*miR-206*) are highly expressed in skeletal muscle. Due to the tissue-specific nature of *miR-1/206* for skeletal muscles, we investigated the role of *miR-1/206* in the development of rhabdomyosarcoma. Initially, we demonstrated that *miR-1/206* expression was suppressed in rhabdomyosarcomas and found at very low levels in a rhabdomyosarcoma RD cell line. Transient transfection of *miR-1/206* into cultured RD cells led to a significant decrease in cell growth and migration. Using bioinformatics, we identified two putative *miR-1/206* binding sites within the 3'-untranslated region of the human *c-Met* mRNA. *miR-1/206* was then shown to have activity on mRNA expression by targeting the *c-Met* 3'-untranslated region. The expression of *c-Met* protein was shown to be down-regulated by subsequent Western blot analysis. Conversely, up-regulation of *c-Met* was confirmed in tissue samples of human rhabdomyosarcoma, with its level inversely correlated with *miR-1/206* expression. *In vivo*, *miR-1/206*-expressing tumor cells showed growth delay in comparison with negative control. Our results demonstrated that *miR-1/206* suppressed *c-Met* expression in rhabdomyosarcoma and could function as a potent tumor suppressor in *c-Met*-overexpressing tumors. Inhibition of *miR-1/206* function could contribute to aberrant cell proliferation and migration, leading to rhabdomyosarcoma development.

Soft tissue sarcomas are a heterogeneous group of mesenchymal tumors that carries a guarded prognosis due to the aggressive local invasion and metastatic potential of these tumors. Progress in the search for etiology and treatment of soft tissue sarcomas is hampered by the fact that they represent a small proportion of all malignancies. Rhabdomyosarcoma (RMS)<sup>3</sup> is

a distinct soft tissue sarcoma that likely originates from cells of the myogenic lineage (1). On the basis of histology, three main subgroups are often described: alveolar, embryonal, and pleomorphic RMS (2). Alveolar RMS consists of small, round, and densely packed cells and occurs mainly in the trunk and extremities and carries with it a more unfavorable prognosis (1, 3). Embryonal RMS typically consists of spindle-shaped cells and occurs mainly in the head and neck region (1, 3). Pleomorphic RMS is uncommon and usually occurs in the adult population (2). Until now, treatment of RMS has largely been based on local regional control and toxic systemic chemotherapy regimens without directed cellular therapy (2, 3). Recent investigations, however, are improving our understanding of its tumor biology and are helping to identify novel prognostic factors and targets for clinical therapy (1).

MicroRNAs (miRNAs) are endogenously expressed, non-protein-coding RNAs that can influence a wide variety of biological processes including development, metabolism, proliferation, differentiation, and oncogenesis (4). Aberrant post-transcriptional regulation of mRNAs by miRNAs can lead to oncogenesis with increased cell proliferation, decreased apoptosis, and enhanced metastatic potential of affected cells (5). Following the discovery of miRNA *let-7*, multiple miRNAs were linked to oncogenes and tumor suppressor genes including the *Ras* proto-oncogene, the antiapoptotic gene *BCL2*, the potent *p53* tumor suppressor gene, and the *MET* oncogene (5, 6). The *MET* oncogene encodes a cell surface receptor tyrosine kinase, *c-Met*, that is up-regulated in a variety of tumors including rhabdomyosarcoma (7, 8). *c-Met* is a disulfide-linked heterodimer consisting of an extracellular  $\alpha$ -subunit and a  $\beta$ -subunit that spans the plasma membrane and contains the catalytic region with tyrosine kinase activity (7). Binding of hepatocyte growth factor (HGF)/scatter factor induces *c-Met* dimerization and autophosphorylation, which leads to cellular activation (7). *c-Met* activation, through aberrant HGF stimulation, can contribute to tumor growth, invasiveness, and metastasis (9). *c-Met* has been predicted and shown to be the target gene of multiple miRNAs including *miRNA-206* (*miR-206*) (10).

co's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; ERK1/2, extracellular signal-regulated kinases 1 and 2; FAK, focal adhesion kinase; NC, negative control.

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<sup>3</sup> The abbreviations used are: RMS, rhabdomyosarcoma; miRNA, microRNA; HGF, hepatocyte growth factor; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; UTR, untranslated region; DMEM, Dulbeco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; ERK1/2, extracellular signal-regulated kinases 1 and 2; FAK, focal adhesion kinase; NC, negative control.

*miR-206* and *miRNA-1* (*miR-1*) are members of the muscle-specific *miR-1* family of so-called myomiRs that currently consists of six members (10). Based on sequence conservation of the seed region, the *miR-1* family can be divided into *miR-1/206* or *miR-133a/b* subgroups (11, 12). Although *miR-1* is highly enriched in both cardiac and skeletal muscle, *miR-206* is exclusively expressed in skeletal muscle (11, 12). Therefore, it can be deduced that these miRNAs play an important role in the myogenesis and development of cardiac and skeletal muscles. *miR-1* knock-out mice confirmed that this miRNA is necessary for cardiac development and physiology (13). *miR-206*, which is specifically expressed in skeletal muscle and rarely detected in the heart, plays an important role in skeletal muscle development (12, 14). However, development and progression of skeletal muscle tumors such as rhabdomyosarcoma based on the presence or absence of *miR-1* and *miR-206* remain largely unknown.

In this study, we attempted to decipher the biological function of *miR-1/206* in human rhabdomyosarcoma specimens and rhabdomyosarcoma RD cells. By causal association following the identification of cell specificity for *miR-1/206*, we surmised that *miR-1/206* acted as a suppressor of RD cell proliferation and migration. Furthermore, we set out to elucidate the cellular mechanisms responsible for its activity and to identify its target, *c-Met*, so that it may one day serve as a potential target in the treatment of rhabdomyosarcoma.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Tumor Specimens**—The human rhabdomyosarcoma cell line, RD, purchased from ATCC (Manassas, VA), was grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and incubated at 37 °C in a humidified incubator containing 5% CO<sub>2</sub>. HEK-293 cells were grown under the same conditions. Eight rhabdomyosarcoma specimens and normal donor skeletal muscle tissues were obtained from the Eye Hospital and the First Affiliated Hospital of Wenzhou Medical College (Wenzhou, China). All studies and procedures involving human tissue samples were approved by the Wenzhou Medical College Institutional Review Board.

**Northern Blot Analysis**—Total RNA was extracted from cell lines or tissue samples with TRIzol reagent (Invitrogen), and the integrity was confirmed using spectrophotometry and formaldehyde/agarose gel electrophoresis. 10 µg of total RNA were dissolved in gel loading buffer II (Ambion, Austin, TX), heated at 95 °C for 3 min, loaded onto denaturing 15% Tris borate-EDTA-urea gels, and separated on a 15% denaturing urea-PAGE gel for 1 h and then transferred onto positively charged nylon membranes (GE Healthcare) followed by a cross-linking with UV irradiation. The RNA blots were prehybridized at 68 °C for 1 h using ULTRAhyb ultrasensitive hybridization buffer (Ambion) and subjected to hybridization with 3'-digoxigenin (DIG)-labeled locked nucleic acid probe for *miR-1* or *miR-206* (100 ng/ml) overnight at 42 °C. The locked nucleic acid-modified oligonucleotide probe was obtained from Exiqon (Vedbaek, Denmark). 100 pmol of the probe were DIG-labeled

using DIG oligonucleotide 3'-end labeling kit (Roche Applied Science, Mannheim, Germany). Following hybridization, membranes were rinsed and then washed three times using a low stringency buffer (2× SSC and 0.1% SDS). Detection was performed using the DIG luminescent detection kit (Roche Applied Science) according to manufacturer's instructions. In brief, membranes were blocked in blocking buffer for 30 min and then incubated with alkaline phosphatase-conjugated anti-DIG antibody for 60 min followed by washing three times in washing buffer. After equilibration in detection buffer, blots were incubated with chemiluminescent substrate CDP-Star and exposed to a Kodak Biomax MR film (Eastman Kodak Co.). DIG-labeled U6 small nuclear RNA probe was used as an internal control.

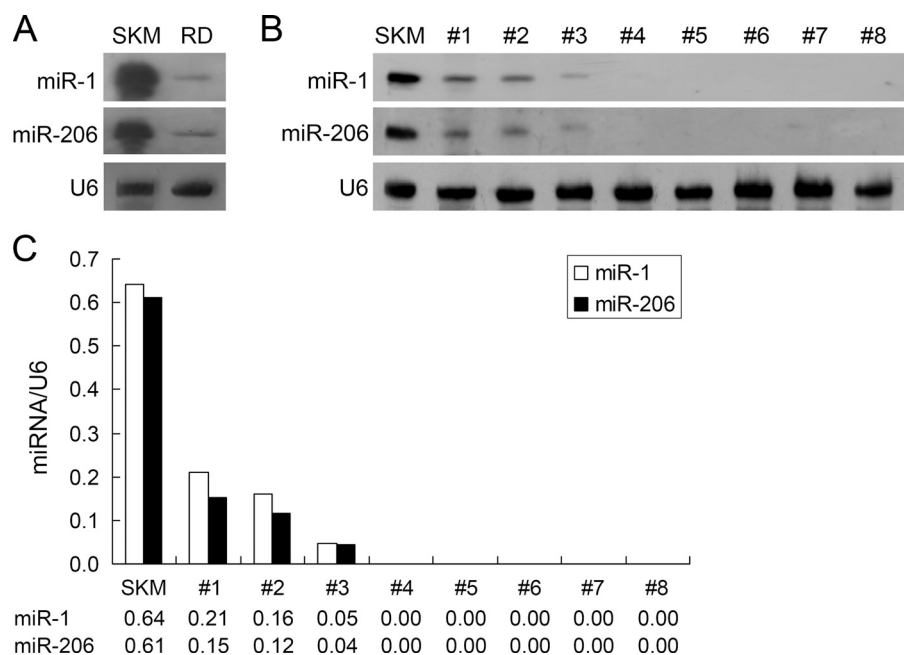
**Cell Proliferation Assay**—RD cells were plated at  $3 \times 10^3$  cells/well in 96-well plates for each transfection. Transfections were performed using Lipofectamine 2000 (Invitrogen). For each well, 50 nM *miR-1* or *miR-206* precursor molecule or a negative control precursor miRNA was employed. For convenience, the *miR-1* precursor or the *miR-206* precursor is termed *miR-1* or *miR-206*, respectively, following transfection throughout this report. Pre-miR<sup>TM</sup> miRNA precursor molecules (Ambion) are small, chemically modified double-stranded RNA molecules designed to mimic endogenous mature miRNAs once properly transfected and expressed by recipient cells. The negative control is a scrambled oligonucleotide that has been validated to not produce identifiable effects on known miRNA function (Ambion). After a 24-h culture, cell proliferation was assessed using the CellTiter 96 AQueous MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay (Promega, Madison, WI) according to the manufacturer's instructions. Briefly, the CellTiter 96 AQueous one solution reagent was added to each well and incubated at 37 °C for 3 h. Cell proliferation was assessed by measuring the absorbance at 490 nm using a microtiter plate reader (Molecular Devices, Sunnyvale, CA).

**Hoechst Staining**—RD cells were plated in 24-well plates and transfected with different miRNAs as described above. 24 h later, doxorubicin (0.1 µg/ml; Sigma) was added to each well prior to staining with Hoechst 33342 (5 µg/ml; Sigma) to visualize nuclear condensation and DNA fragmentation. After 20 min of staining at 25 °C, the cells were examined under fluorescence microscopy (Zeiss, Oberkochen, Germany).

**Caspase Activity Assay**—Apoptosis in RD cells was determined using the Caspase-Glo 3/7 assay kit according to the manufacturer's instructions (Promega). RD cells were first plated in triplicates in 96-well plates and transfected with different miRNAs as described above. Samples were then incubated with the caspase substrate for 2 h followed with measurements by a microtiter plate reader (Molecular Devices).

**Fluorescence-activated Cell Sorting of Cell Cycling**—RD cells were transfected with 50 nM *miR-1* or *miR-206* precursor molecule or a negative control. After 48 h, the cells were collected, washed with phosphate-buffered saline, and stained with propidium iodide using the BD Cycletest Plus DNA reagent kit (BD Biosciences). The stained cells ( $1 \times 10^5$ ) were then analyzed for DNA content with a flow cytometer (FACScaliber, BD Biosciences).

## MicroRNA-1/206 Down-regulates *c-Met* in Rhabdomyosarcoma



**FIGURE 1. *miR-1/206* expression is down-regulated in human RD rhabdomyosarcoma cell line and human rhabdomyosarcoma specimens.** *A*, Northern blot analysis was performed to detect the expression of *miR-1* and *miR-206* in the RD cell line (RD), in comparison with human skeletal muscle (SKM). *miR-1* and *miR-206* were highly expressed in human skeletal muscle tissues but were down-regulated in RD cells. U6 small nuclear RNA (U6) was used as an internal control. *B*, *miR-1* and *miR-206* were expressed at low levels in eight human rhabdomyosarcoma specimens. *C*, quantitative representation of the data from panel *B* by measuring the intensities of each band and then normalizing to the intensity of the internal control U6 small nuclear RNA.

**Luciferase Reporter Assays**—The 3′-untranslated region (UTR) of human *c-Met* was amplified from human genomic DNA and individually cloned into the pMIR-REPORT vector (Ambion) by directional cloning. Seed regions were mutated to remove all complementarity to nucleotides 1–7 of *miR-1/206* by using the QuikChange XL mutagenesis kit (Stratagene, La Jolla, CA). HEK-293 cells were co-transfected with 0.4 μg of firefly luciferase reporter vector and 0.02 μg of the control vector containing *Renilla* luciferase, pRL-SV40 (Promega), using Lipofectamine 2000 (Invitrogen) in 24-well plates. Each transfection was carried out in four wells. For each well, 50 nM *miR-1* or *miR-206* precursor molecule (Ambion) or a negative control precursor miRNA (Ambion) was co-transfected with the reporter constructs. Luciferase assays were performed 24 h after transfection using the Dual-Luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

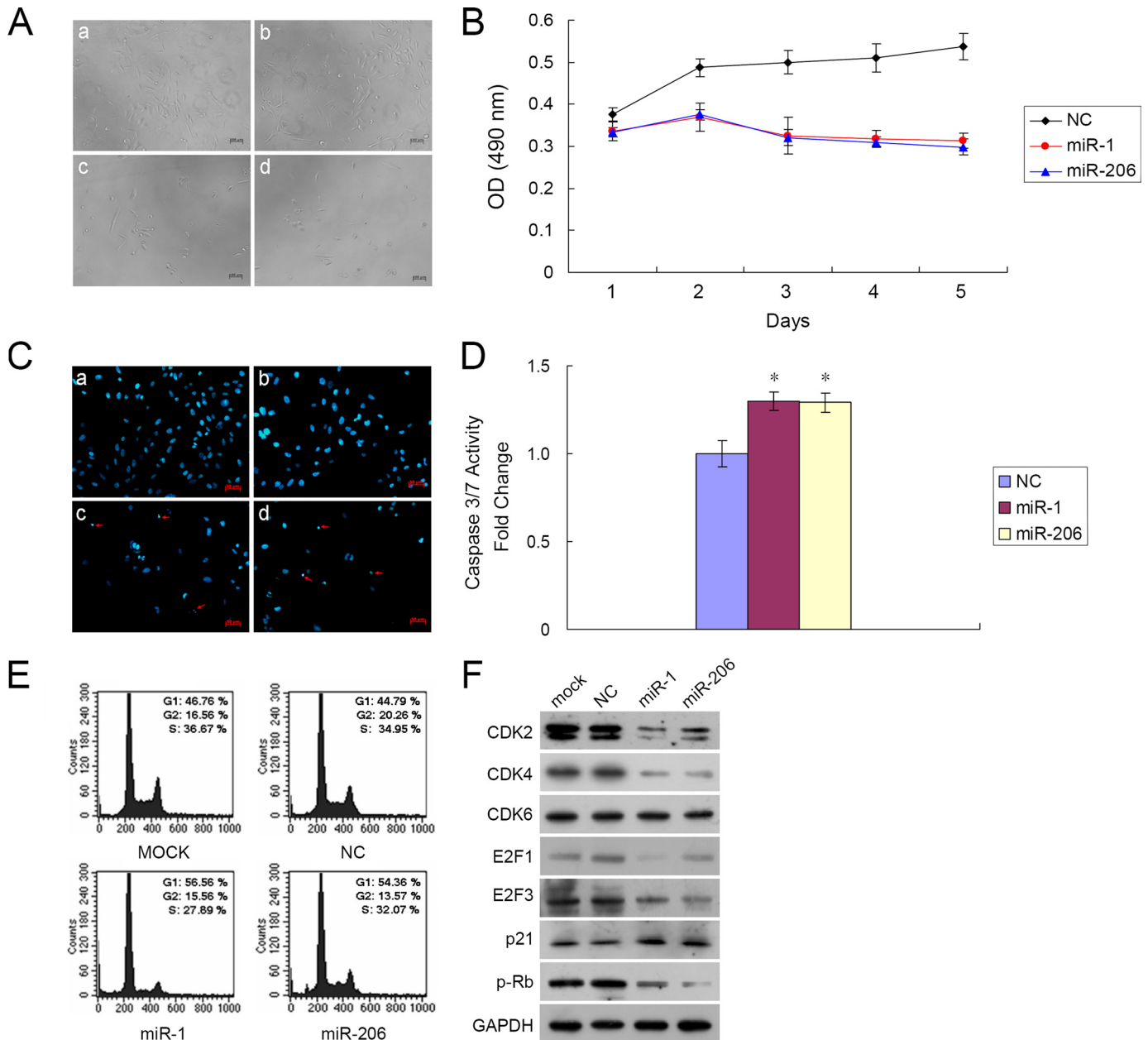
**Transwell Migration Assay**—RD cells were grown in DMEM containing 10% fetal bovine serum to ~60% confluence and transfected with 50 nM *miR-1* or *miR-206* precursor molecule or a negative control. After 24 h, the cells were harvested by trypsinization and washed once with Hanks' balanced salt solution (Invitrogen). To measure cell migration, 8-mm pore size culture inserts (Transwell; Costar, High Wycombe, UK) were placed into the wells of 24-well culture plates, separating the upper and the lower chambers. In the lower chamber, 400 μl of DMEM containing recombinant human hepatocyte growth factor (HGF, 20 ng/ml) were added. HGF was purchased from R&D Systems (Minneapolis, MN). Then, 1 × 10<sup>5</sup> cells were added to the upper chamber. After 24 h of

incubation at 37 °C with 5% CO<sub>2</sub>, the number of cells that had migrated through the pores was quantified by counting 10 independent visual fields under the microscope (Zeiss) using a ×20 objective, and cell morphology was observed by staining with hematoxylin and eosin.

**Western Blot Analysis**—RD cells (1 × 10<sup>5</sup>) were seeded and grown in DMEM with 10% fetal bovine serum in 6-well plates for 24 h. After transfection, the cells were washed with cold phosphate-buffered saline and subjected to lysis in a lysis buffer (50 mM Tris-Cl, 1 mM EDTA, 20 g/liter SDS, 5 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride). Equal amounts of protein lysates (50 μg each) and rainbow molecular weight markers (GE Healthcare) were separated by 10% SDS-PAGE and then electrotransferred to nitrocellulose membranes. The membranes were blocked with a buffer containing 5% nonfat milk in phosphate-buffered

saline with 0.05% Tween 20 for 2 h and incubated overnight with antibody at 4 °C. After a second wash with phosphate-buffered saline containing 0.05% Tween 20, the membranes were incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and developed with an enhanced chemiluminescence (ECL) detection kit (Pierce). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Antibodies for total extracellular signal-regulated kinases 1 and 2 (ERK1/2), phosphorylated ERK1/2, total Akt, phosphorylated Akt, total focal adhesion kinase (FAK), phosphorylated FAK, CDK2, CDK4, CDK6, phosphorylated Rb, and p21 were from Cell Signaling Technology (Beverly, MA), and those for *c-Met*, E2F1, and E2F3 were from Santa Cruz Biotechnology.

**In Vivo Tumor Growth Assay**—The pre-microRNA expression constructs lenti-*miR-1*, lenti-*miR-206*, and pCDH-CMV-MCS-EF1-copGFP control vector were purchased from System Biosciences (Mountain View, CA). The lentivirus was produced according to the manufacturer's instructions. RD cells were infected with lentivirus expressing *miR-1*, *miR-206*, or negative control, respectively. Female nude mice 6 weeks of age were used for xenograft studies. RD cells (8 × 10<sup>6</sup>) expressing *miR-1/206* or negative control were inoculated subcutaneously into the flanks of nude mice. All mice were sacrificed 8 weeks later after injection of tumor cells. Tumor size was measured with a caliper, and volume was calculated using the following formula:  $(L \times W^2) \times 0.5$  (where *L* = length and *W* = width), according to the method previously reported (15). All studies and procedures were approved by the Wenzhou Medical College Animal Care and Use Committee.



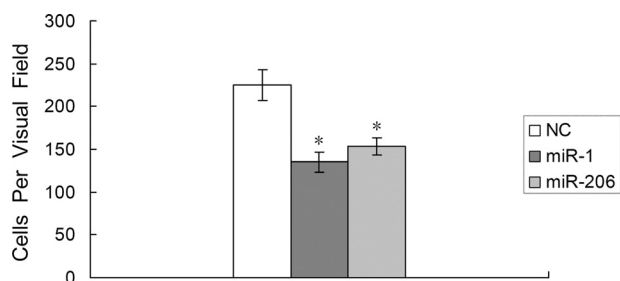
**FIGURE 2. Exogenous miR-1/206 inhibits RD cell proliferation through induction of apoptosis and G<sub>1</sub> cell cycle arrest.** *A*, RD cell growth was assessed after transfection with *miR-1* or *miR-206*. The negative control (NC) scrambled oligonucleotide does not encode for any known miRNA. The number of cells was dramatically reduced after transfection with *miR-1* (panel *c*) or *miR-206* (panel *d*) as compared with mock (panel *a*) and NC (panel *b*) transfection. Photos were taken 72 h after transfection. *B*, the MTS cell proliferation assay was carried out and plotted from days 1 to 5 as indicated after Lipofectamine transfection of RD cells with *miR-1* (50 nM), *miR-206* (50 nM), or NC. Cells transfected with *miR-1/206* were less metabolically active than cells transfected with NC. The data are expressed as the mean value  $\pm$  S.E. (error bars) of the results obtained from triplicates in one experiment. Results are representative of three separate experiments. *C*, transfection with *miR-1/206* induced DNA fragmentation in RD cells as demonstrated by Hoechst staining. Photographs of cells following transfection are shown as indicated: panel *a*, mock; panel *b*, negative control; panel *c*, *miR-1*; and panel *d*, *miR-206*. Red arrows denote cells with fragmented nuclei. *D*, caspase 3/7 activity assay was performed on RD cells transfected with either NC or *miR-1/206*. Relative caspase 3/7 activity is indicated in comparison with negative control. Results are expressed as the mean value  $\pm$  S.E. (error bars) of the results obtained from triplicates in one experiment. Results are representative of three separate experiments. \*, differences between *miR-1/206* and negative control transfected cells were significant,  $p < 0.05$ . *E*, cell cycle arrest was evaluated using flow cytometry. RD cells, collected 48 h after transfection, were stained with propidium iodide and analyzed by flow cytometry. Cells transfected with *miR-1* and *miR-206* had a higher proportion of cell cycle arrest at the G<sub>1</sub> phase in comparison with mock or NC. 10,000 cells were evaluated in each sample. Results depicted are representative of three independent experiments. *F*, *miR-1/206* regulated cell cycle-related proteins that are important for cell cycle G<sub>1</sub> phase progression and G<sub>1</sub>/S transition. RD cells were transfected with *miR-1/206*, mock, or a negative control. Cell lysates were prepared and used for Western blot analysis with CDK2, CDK4, CDK6, E2F1, E2F3, p21, and phosphorylated Rb (p-Rb) antibodies. GAPDH was used as a loading control.

**Statistical Analysis**—All data were shown as the mean  $\pm$  S.E. Differences between samples were analyzed using the Student's *t* test. Statistical significance was accepted at  $p < 0.05$ .

**RESULTS**

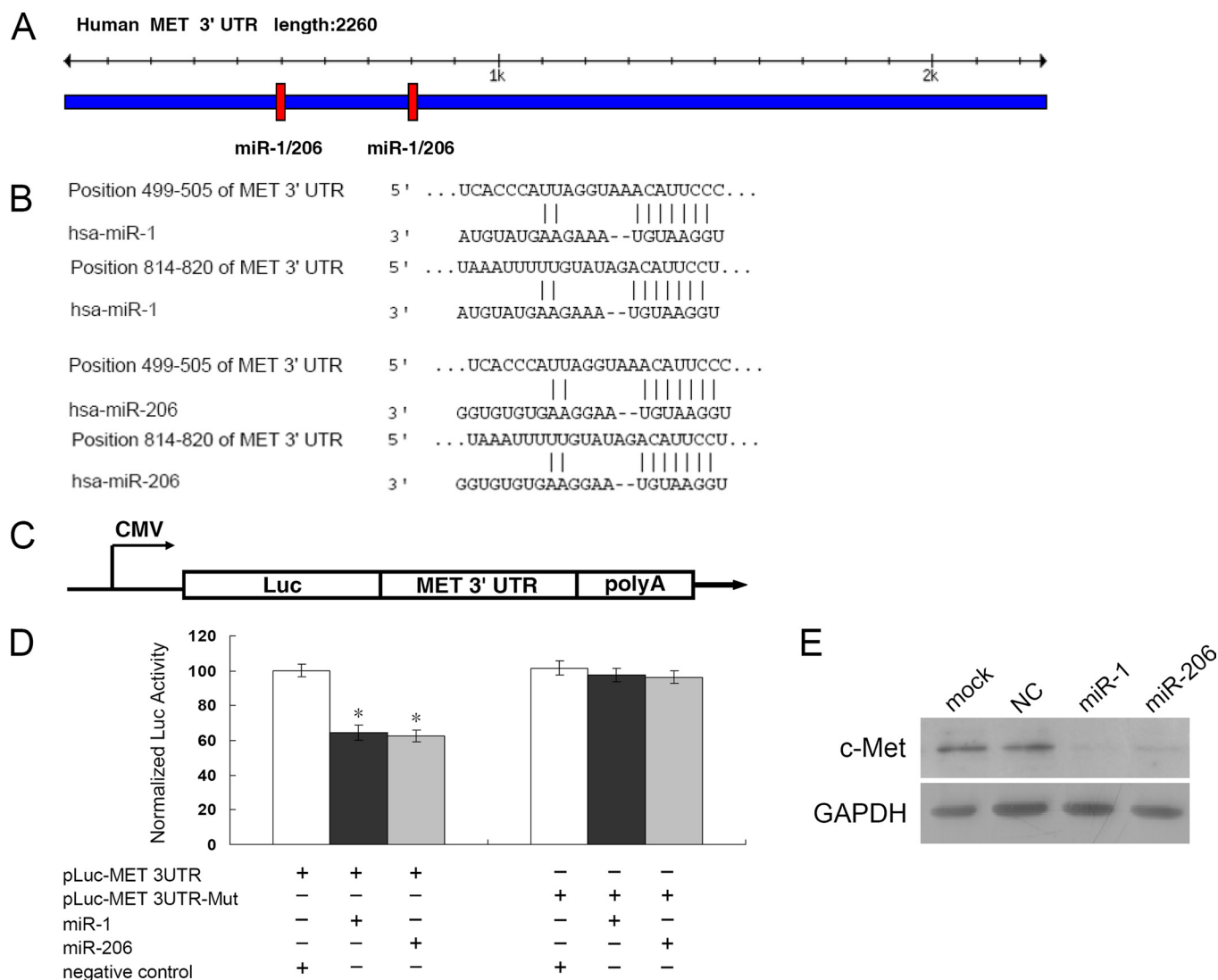
*miR-1/206 Expression Is Down-regulated in Rhabdomyosarcoma Specimens and RD Tumor Cells*—To determine whether miRNA was involved in the tumorigenesis of rhabdomyosar-

## MicroRNA-1/206 Down-regulates c-Met in Rhabdomyosarcoma



**FIGURE 3. Transfection of *miR-1/206* reduces the migration of RD cells.** RD cells were transfected with *miR-1/206* or NC for 24 h and plated on culture inserts in DMEM containing 20 ng/ml HGF to assess the number of migrating cells. The number of cells that had migrated through the pores was quantified by counting 10 independent visual fields using a  $\times 20$  microscope objective. Results are expressed as the mean  $\pm$  S.E. (error bars) for the data obtained from three independent assays. \*, differences in cell migration between *miR-1/206* and negative control transfected cells were significant,  $p < 0.01$ .

coma cells, we first compared *miR-1/206* expression in normal skeletal muscle and rhabdomyosarcoma. Northern blot analysis was performed using DIG-labeled locked nucleic acid probe for *miR-1* or *miR-206* to detect its expression in tissue specimens and the immortalized rhabdomyosarcoma RD cell line. Both *miR-1* and *miR-206* were highly expressed in normal skeletal muscle (Fig. 1A). In contrast, expression of *miR-1/206* was decreased significantly in RD cells (Fig. 1A). To determine the expression pattern of *miR-1/206* in primary human rhabdomyosarcoma, total RNA from eight human rhabdomyosarcoma specimens was analyzed by Northern blotting. Consistent with results from the RD cells, *miR-1/206* expression was suppressed or not detected in all eight samples (Fig. 1, B and C). These results indicate that *miR-1/206* expression is down-regulated in human rhab-



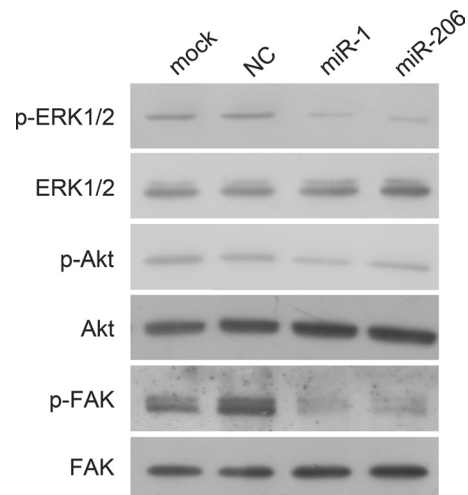
**FIGURE 4. c-Met is a direct target of *miR-1/206*.** A, predicted *miR-1/206* binding sites in *c-Met* 3'-UTR. Specific locations of the binding sites were marked with red color, and *c-Met* 3'-UTR was marked with blue color. B, alignment between the predicted *miR-1/206* target sites and *miR-1/206* is shown. The conserved, 7-bp seed sequence for *miR-1/206*:mRNA pairing is also indicated. C, diagram depicting the pMIR luciferase reporter constructs, containing a cytomegalovirus (CMV) promoter, which was utilized to verify the putative *miR-1/206* binding sites (see "Experimental Procedures"). *Luc*, luciferase; *poly A*, poly(A) tail. D, HEK293 cells were co-transfected with *miR-1* or *miR-206*, pLuc-MET 3'-UTR, along with a pRL-SV40 reporter plasmid. After 24 h, the luciferase activity was measured. Values are presented as relative luciferase activity after normalization to *Renilla* luciferase activity. The data are expressed as the mean value  $\pm$  S.E. (error bars) of the results obtained from three independent experiments. \*, differences in luciferase activity between *miR-1/206* and negative control transfected cells were significant,  $p < 0.01$ . E, *c-Met* expression levels in RD cells after transfection with *miR-1/206* were determined by Western blot analysis. As compared with the NC miRNA, *miR-1/206* expression dramatically reduced the levels of *c-Met* in RD cells. GAPDH was used as an internal control.

domyosarcoma and imply a possible role of *miR-1/206* in rhabdomyosarcoma development.

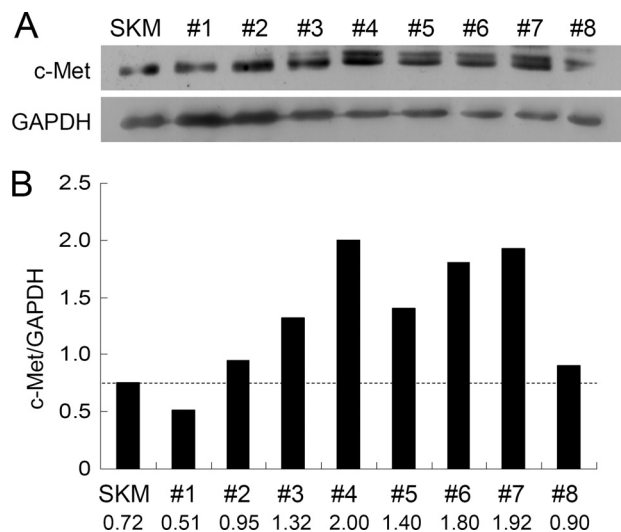
***miR-1/206* Inhibits RD Cell Proliferation through Apoptosis and Cell Cycle Arrest**—Because *miR-1/206* expression was down-regulated in rhabdomyosarcoma, we sought to establish its biological activity on cell growth. RD cells were first transfected with the *miR-1*, the *miR-206* precursor molecule, or a negative control. Although transfection with the negative control did not affect RD cell viability, both *miR-1* and *miR-206* caused a significant inhibition of RD cell growth as compared with that of control (Fig. 2A). Subsequently, the MTS assay was carried out to assess growth inhibition at days 1–5 after transfection. *miR-1* or *miR-206* transfected cells caused a dramatic inhibition of RD cell proliferation as compared with that of control over a 5-day interval (Fig. 2B). The decrease in cell number was statistically significant between cells transfected with *miR-1/206* and cells transfected with a negative control at day 5 (*miR-1* led to  $41.5 \pm 3.2\%$ , whereas *miR-206* led to  $44.4 \pm 3.6\%$  decrease,  $p < 0.01$ ). To further characterize *miR-1/206*-mediated inhibition of cell proliferation, we measured the amount of cell death using Hoechst staining. *miR-1/206*-transfected RD cells showed diminished viability and higher incidence of DNA fragmentation following Hoechst staining (Fig. 2C). RD cells transfected with *miR-1* or *miR-206* had a  $12 \pm 1.5\%$  ( $n = 3$ ,  $p < 0.01$ ) and  $11 \pm 2\%$  ( $n = 3$ ,  $p < 0.01$ ) increase in cell death as compared with control. We then investigated apoptosis as a mode of cell death by examining caspase activity. Caspase 3/7 activity was significantly increased in *miR-1/206* transfected cells in comparison with negative control after 48 h (Fig. 2D). Complementary to the finding that *miR-1/206* inhibited cell proliferation, these cells were found to have  $G_1$  cell cycle arrest. 48 h after transfection, cells were stained with propidium iodide and analyzed by flow cytometry. Cells transfected with *miR-1* or *miR-206* showed 56.56 and 54.36%  $G_1$  arrest in comparison with 46.76% with mock transfection and 44.79% with negative control (Fig. 2E). Concurrently, introduction of *miR-1/206* dramatically down-regulated cell cycle-related proteins, including CDK2, CDK4, E2F1, E2F3 and phosphorylated Rb, which are important for cell cycle  $G_1$  phase progression and  $G_1/S$  transition (Fig. 2F). In contrast, p21, which mediates the p53-dependent cell cycle  $G_1$  arrest, was up-regulated (Fig. 2F). Overall, these results suggest that *miR-1/206* expression suppressed RD cell growth by increasing apoptosis and by cell cycle  $G_1$  arrest.

***miR-1/206* Inhibits Migration of RD Cells**—Cell migration, a prerequisite for malignant transformation and metastasis, was assessed using a Transwell migration assay. RD cells were first transfected with either the *miR-1/206* precursor or a control precursor. Cells were seeded on culture inserts, and the ability of cells to migrate to the underside of the inserts was determined in the presence of HGF. As shown in Fig. 3, the HGF-induced migration was significantly decreased when comparing *miR-1/206*-transfected cells with negative control ( $135 \pm 12$  versus  $225 \pm 18$  for *miR-1*,  $153 \pm 10$  versus  $225 \pm 18$  for *miR-206*,  $n = 3$  each,  $p < 0.01$ ). Therefore, introduction of *miR-1/206* resulted in reduced cell motility in response to HGF.

***c-Met* Is a Target of *miR-1/206***—Having demonstrated a functional role for *miR-1/206* in RD cells, we attempted to



**FIGURE 5. Introduction of *miR-1/206* down-regulates targets of c-Met.** RD cells were transfected with *miR-1*, *miR-206*, or NC. Cell lysates were prepared and used for Western blot analysis with antibodies specific for phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, phosphorylated Akt (p-Akt), total Akt, phosphorylated FAK (p-FAK), and total FAK. *miR-1/206* down-regulated expression levels of p-ERK1/2, p-Akt, and p-FAK, but not total ERK1/2, Akt, or FAK.



**FIGURE 6. The expression levels of c-Met in human rhabdomyosarcoma specimens are up-regulated.** A, tissue samples were prepared and used for Western blot analysis. SKM, skeletal muscle. B, the majority of samples examined (samples 2–8) showed an up-regulation of c-Met. Expression of c-Met was shown after normalization to GAPDH.

explore the cellular mechanisms underlying *miR-1/206*-mediated cell proliferation and migration. TargetScan was conducted for *miR-1/206* target prediction. Two potential binding sites of *miR-1/206* were predicted in the *c-Met* 3'-UTR (Fig. 4A). Alignment between the predicted *miR-1/206* target sites and *miR-1/206*, the conserved 7-bp seed sequence for *miR-1/206*:mRNA pairing, is shown (Fig. 4B). To test the specific regulation of *c-Met* through the two predicted binding sites, we amplified the *c-Met* 3'-UTR sequence and inserted it downstream of the firefly luciferase coding region of a pMIR-Luc vector (Fig. 4C). Mutants with the putative binding sites were prepared as described (see "Experimental Procedures"). As indicated, introduction of *miR-1* or *miR-206* in HEK293 cells with the wild-type 3'-UTR (pLuc-MET 3UTR) construct

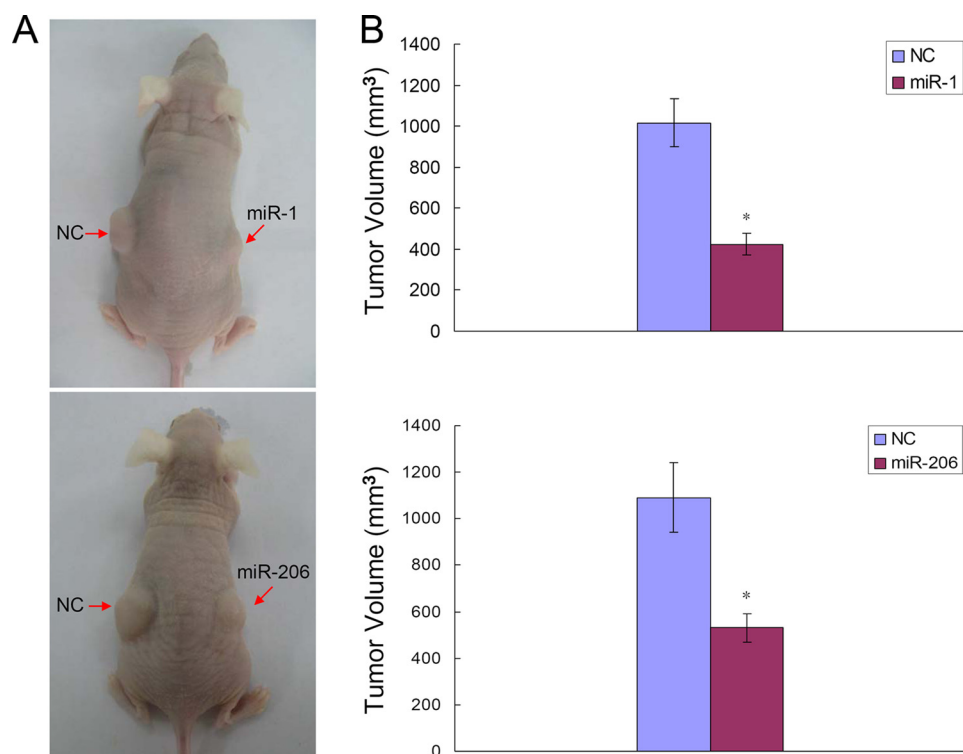


FIGURE 7. Introduction of *miR-1/206* in RD cells suppresses tumor growth in nude mice. *A*, mice were inoculated with lentivirus-infected RD cells expressing *miR-1*, *miR-206*, or NC. Representative photographs of nude mice 8 weeks after inoculation are shown. *B*, average volume of tumors derived from RD cells infected with NC or *miR-1/206* in nude mice. \*, differences in tumor volume between *miR-1/206* and NC infected cells were significant,  $n = 6$  each,  $p < 0.01$ . Error bars indicate S.E.

had significant inhibition of luciferase activity as opposed to negative control (Fig. 4D). Mutations of the two binding sites, using a mutant vector (pLuc-MET 3UTR-Mut), completely abolished the ability of *miR-1* or *miR-206* to regulate luciferase expression (Fig. 4D). These results demonstrated that *c-Met* was a potential target of *miR-1/206*. To further confirm that *miR-1/206* was indeed responsible for the down-regulation of *c-Met*, RD cells were transfected with the *miR-1/206* molecule or a negative control. Western blot analysis showed that although *c-Met* expression was not affected by the transfection of a negative control, *c-Met* expression was dramatically reduced when transfected with either *miR-1* or *miR-206* (Fig. 4E).

**Introduction of *miR-1/206* Down-regulates Activation of Intracellular Targets ERK1/2, Akt, and FAK**—*c-Met* has been shown to activate diverse intracellular signaling pathways (7, 8). To examine intracellular expression patterns affected by *c-Met* in RD cells, we next determined the activation status of ERK1/2, Akt, and FAK after down-regulation of *c-Met* by *miR-1/206*. RD cells were transfected with *miR-1*, *miR-206*, or a negative control. Cells were solubilized and subjected to Western blot analysis with phosphorylation-specific antibodies to ERK1/2, Akt, and FAK, molecules involved in the three major signaling pathways previously shown to be stimulated by HGF (7, 8). As shown in Fig. 5, down-regulation of *c-Met* by *miR-1* or *miR-206* led to significant reduction of phosphorylated ERK1/2 and FAK in RD cells but had less obvious effect on phosphorylated Akt. Total ERK1/2, Akt, and FAK were not affected when comparing *miR-1/206* transfection with negative control transfection (Fig.

5). Taken together, these results demonstrated that introduction of *miR-1/206* in RD tumor cells suppressed *c-Met* and in turn targets of *c-Met*, leading to inhibition of cell proliferation and migration.

***miR-1/206* Suppresses Rhabdomyosarcoma Development through *c-Met* Expression**—Having demonstrated that *c-Met* is a potential target of *miR-1/206* in RD cells (Fig. 4), we next evaluated the interaction of *miR-1/206* with *c-Met* on tissue specimens. As shown in Fig. 1, *B* and *C*, both *miR-1* and *miR-206* were highly expressed in normal skeletal muscle but not in rhabdomyosarcoma. Moreover, down-regulation of *miR-1/206* in human rhabdomyosarcoma was associated with an elevated *c-Met* level in most of the tissue samples analyzed. Expression of *c-Met* was up-regulated in all the samples except one (Fig. 6, *A* and *B*).

**Expression of *miR-1/206* Suppresses Tumor Growth in Vivo**—Because *miR-1/206* expression inhibited the proliferation and migration of RD cells *in vitro*, it was

reasonable to speculate that ectopic *miR-1/206* could also repress tumor growth *in vivo*. To confirm this, RD cells infected with lentivirus expressing *miR-1*, *miR-206*, or negative control were injected subcutaneously into the flanks of nude mice. After 8 weeks, all mice were sacrificed, and the tumor volumes were measured (Fig. 7A). The average tumor volumes were significantly decreased in cells infected with *miR-1* or *miR-206*, as compared with negative control (Fig. 7B). The results indicated that introduction of *miR-1* or *miR-206* suppressed the growth of RD rhabdomyosarcoma cells *in vivo*.

## DISCUSSION

Emerging evidence suggests that in addition to protein-encoding genes, miRNAs have more than a cursory role in the pathogenesis of human cancer development by acting as agents of the RNA interference pathway (16). Following transcription and post-transcriptional modification by the ribonuclease Dicer, miRNA becomes associated with the RNA-induced silencing complex to repress expression (16). Preceded by the insight that global suppression of miRNA leads to increased tumorigenesis, studies to identify cancer-related miRNAs have unveiled a plethora of potential candidates important for human tumors (5, 17). *miR-1/206*, which plays a key role in heart and muscle development, was evaluated for its role in the pathogenesis of rhabdomyosarcoma within the context of this study.

In this study, we were able to illustrate for the first time that *miR-1/206* can regulate cell proliferation and migration

through its target gene *c-Met* in rhabdomyosarcoma. *miR-1/206*, which is highly expressed in human skeletal muscle, is down-regulated in rhabdomyosarcoma tissue specimens and the RD cell line (Fig. 1). We computationally identified the *c-Met* mRNA as a potential target of *miR-1/206* and demonstrated that *miR-1/206* decreased endogenous *c-Met* protein levels in RD cells by Western blotting. We also provided evidence that *miR-1/206* can directly regulate mRNA expression by targeting the *c-Met* 3'-UTR. The inverse relationship between *miR-1/206* expression and *c-Met* production is illustrated by Western blot analysis of the original human rhabdomyosarcoma tissue specimens (Fig. 6). Functionally, *miR-1/206* may have a role in the treatment of rhabdomyosarcoma. Tumor cell invasiveness, a cornerstone capability necessary for tumor metastasis, has been shown to be affected by the presence of miRNAs (18). A series of Transwell experiments outlined under "Results" illustrated that RD cell migration can be inhibited with the restoration of *miR-1/206* activity (Fig. 3). *miR-1/206* inhibited RD cell migration by targeting *c-Met* in an HGF-dependent fashion. Finally, introduction of *miR-1/206* through transfection into RD rhabdomyosarcoma cells led to a significant decrease in cell growth both *in vitro* and *in vivo* (Figs. 2 and 7).

*c-Met* is a particularly interesting target of investigation due to its involvement in many types of cancer (7). *c-Met* expression has previously been confirmed to be up-regulated in the two most common forms of rhabdomyosarcoma: alveolar and embryonal types (19–22). Other investigations into *c-Met* revealed that it is an essential mediator of the oncogenic properties of *PAX3/7-FKHR* gene translocation, thereby resulting in a number of different tumors including rhabdomyosarcoma (3). *c-Met* was also demonstrated to be the common denominator for aberrant cell growth using a fusion protein *PAX3-FKHR* (3). Despite histological differences between several types of RMS, studies using an inducible lentivirus expressing anti-*c-Met* short hairpin RNA validated dependence of RMS cell proliferation, survival, and invasiveness on the presence of *c-Met* (3). Concordant to all these findings, RMS samples evaluated in this study showed an up-regulation of *c-Met* (Fig. 6). In addition, we provided evidence that this up-regulation is inversely proportional to *miR-1/206* through direct inhibition of *c-Met* 3'-UTR by *miR-1/206*.

Recent studies have revealed that both *miR-1* and *miR-206* were down-regulated in primary human cancers (23, 24), including sarcomas (25). Antitumor effects of *miR-1* were seen in lung cancer cells with its targets linked to MET and Pim-1, a serine/threonine kinase commonly seen in lung cancer (23). Likewise, *miR-206* was found to be inversely correlated with levels of estrogen receptor  $\alpha$  mRNA in estrogen receptor  $\alpha$  positive human breast cancer and could also act as a therapeutic agent in a dose-dependent fashion (24). We demonstrated that *c-Met* levels were directly down-regulated by introduction of *miR-1/206* in rhabdomyosarcoma cells (Fig. 4E). Concomitantly, the downstream targets of *c-Met* including phosphorylated ERK1/2, Akt, and FAK were reduced (Fig. 5). Previous studies have demonstrated that inactivation of *INK4a/ARF*, along with aberrant *c-Met* signaling, led to rhabdomyosarcomagenesis in mice (26). The *INK4a/ARF* locus contains tumor

suppressor genes encoding p16<sup>INK4a</sup> and p14<sup>ARF</sup> that affect downstream target proteins Rb and p53, respectively. Therefore, it would be very interesting to examine whether Rb and p53 are affected in human rhabdomyosarcoma tissue specimens as well.

In the present study, we identified a mechanism for regulation of *c-Met* gene expression through the miRNAs *miR-1* and *miR-206* in rhabdomyosarcoma. *c-Met* overexpression following *miR-1/206* down-regulation seems to be the common etiology for the pathogenesis of RMS in the majority of samples examined in this study. Although other mechanisms of tumorigenesis surely exist, overexpression of *c-Met* can be an etiology for RMS development with *c-Met* being reported as the target gene of *miR-1/206*. In summary, we demonstrated that *miR-1/206* negatively modulates the *c-Met* signaling pathway involved in cell proliferation and migration. These findings suggest that *miR-1/206* plays an important role in regulating the development of rhabdomyosarcoma. Our studies will hopefully have important clinical consequences in the treatment of rhabdomyosarcoma.

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