Marek's Disease Virus Type 1 MicroRNA miR-M3 Suppresses Cisplatin-Induced Apoptosis by Targeting SMAD2 of the Transforming Growth Factor Beta Signal Pathway[▽]

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Viruses cause about 15% of the cancers that are still the leading causes of human mortality. The discovery of viral oncogenes has enhanced our understanding of viral oncogenesis. However, the underlying molecular mechanisms of virus-induced cancers are complex and require further investigation. The present study has attempted to investigate the effects of the microRNAs (miRNAs) encoded by Marek's disease virus 1 (MDV1), a chicken herpesvirus causing acute T-cell lymphomas and solid visceral tumors in chickens, on anti-cancer drug-induced apoptosis and identify the targets of the miRNAs. The results showed that of the total 14 miRNAs encoded by MDV1, MDV1-miR-M3 significantly promoted cell survival under treatment with cisplatin, a widely used chemotherapy drug. MDV1-miR-M3 suppressed cisplatin-induced apoptosis by directly downregulating expression at the protein but not the mRNA level of Smad2, a critical component in the transforming growth factor β signal pathway. Our data suggest that latent/oncogenic viruses may encode miRNAs to directly target cellular factors involved in antiviral processes including apoptosis, thus proactively creating a cellular environment beneficial to viral latency and oncogenesis. Furthermore, the knowledge of the apoptosis resistance conferred by viral miRNAs has great practical implications for improving the efficacy of chemotherapies for treating cancers, especially those induced by oncogenic viruses.

The relevance of viral infection to tumor development has aroused widespread attention since the identification of the first tumor-causing virus, Rous sarcoma virus (RSV), in 1911 (32). Viruses are now accepted as an important etiologic factor of human tumors (3, 21), including Epstein-Barr virus (EBV) (39), hepatitis B virus (HBV) (15), hepatitis C virus (HCV) (40), human papilloma virus (HPV) (19), human T-cell lymphotropic virus (HTLV-1) (30), and Kaposi's sarcoma-associated herpesvirus (KSHV) (42). It is estimated that about 15% of human cancers worldwide are caused by these viruses (20). Marek's disease virus 1 (MDV1) is a chicken-oncogenic virus and belongs to the herpesvirus family including EBV and KSHV. MDV1 causes acute T-cell lymphomas and solid visceral tumors in chickens within 4 to 8 weeks of infection; the reliable kinetics of disease induction and progression makes MDV1 unique as a natural virus-host model system for studying herpesvirus diseases and oncogenesis (26).

MicroRNAs (miRNAs) are a large class of short noncoding RNAs generated from hairpin-shaped transcripts through the Drosha/Dicer process, which negatively regulates gene expression posttranscriptionally by imperfect base pairing with target mRNA 3' untranslated regions (UTRs), leading to mRNA cleavage or translational repression (1, 12). miRNAs have been found in all multicellular organisms and some viruses and are involved in diverse biological processes including proliferation, apoptosis, development, immunity, and oncogenesis (1,

5, 8, 9, 12, 29, 38). Most viral miRNAs identified belong to the herpesviruses, including human alphaherpesviruses herpes simplex virus 1 (HSV-1) and HSV-2, avian alphaherpesviruses MDV1 and MDV2, betaherpesvirus human cytomegalovirus (HCMV), and gammaherpesviruses EBV and KSHV (7, 9, 24, 41). While viral miRNAs supposedly have diverse functions, the identification and validation of their functions and targets have had extremely limited success (7).

Fourteen MDV1-encoded miRNAs (MDV1-miR-M1 to -miR-M13 and MDV1-miR-M31) have been identified so far (miRBase [http://microrna.sanger.ac.uk/]), and six of them (MDV1-miR-M4, -miR-M2, -miR-M3, -miR-M12, -miR-M5, and -miR-M9) were sequentially adjacent to the upstream sequence of *meq* (2). They were expressed in MDV1-infected chicken embryo fibroblasts (CEF), an MDV1-transformed lymphoblastoid cell line (MSB-1), and MDV1-induced tumors (2, 16, 23, 44), implying their roles in latent infection and oncogenesis. miR-M3, miR-M4, and miR-M5 were abundantly expressed in MSB-1 cells while miR-M2, miR-M12, and miR-M9 were scarcely expressed. In addition, miR-M4 was shown to be an orthologue of cellular miR-155, which regulates an array of cellular mRNAs but is not implicated in apoptosis (46).

Rapid growth of a tumor causes an insufficient blood supply, which renders cancer cells consistently under some survival pressure as nutritional starvation and hypoxia can evoke apoptosis. Apoptosis serves as a barrier to cancer formation; thus, cancer cells should acquire the capability to confer resistance toward apoptosis induced in such situations (10). In this study, we sought to shed light on the roles of miRNAs encoded by MDV1 in modulating apoptosis, elucidating the molecular mechanisms in virus-induced oncogenesis. We present data

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TABLE 1. Sequences of RNA and DNA oligonucleotides

Group or function and name	Unidirectional or sense strand/primer/probe sequence (5'–3')	Antisense strand/primer sequence (5'–3')
miRNA and siRNA duplexes		
miR-M1	UGCUGUUCACUGUGCGGCA	CCGCACAGUGAACAAAGCCUU
miR-M2	GUUGUAUUCUGCCCGUAGUCCG	GACUACCGGGCAGAAUACAAGUU
miR-M3	AUGAAAUGUGAAACCUCUCCCG	GGGAGAGGUUUCACAUUUUACGUU
miR-M4	UUAAGUCUGUACGGAAACCCUUC	AGGGUUCGGAUACAGCAUUACUU
miR-M5	UGUGUACGUGGUCGUCUACUGU	AGUAGACGACCACGAUACACCUU
miR-M6	UCUGUUGUUCGGUAGUUCUC	GAACACUACGGAACAACAGCUU
miR-M7	UCGAGAUUCUCACGAGAUUACAG	GUAAUCUCGAGAGAUACGCUU
miR-M8	UAUUGUUCUGUGGUUGGUUUCG	AAACCAACCACAGAACAACUUCU
miR-M9	UUUUCUCCUUCUCCCGGAGUU	CUCCGGGGGGAAGGAGAAACUU
miR-M10	GCGUUGUCUCGUAAGGUCCAG	GGACCUCUACGAGACAACGAUU
miR-M11	UUUCCUUAACCGUGUAGCUAGA	UAAGCUACACGGUAAAGGAAACUU
miR-M12	UGCAUAAUACGGAGGGUUCU	AACCCUCCGUAAUUGCCUU
miR-M13	GCAUGGAAACGUCCUGGGAAA	UCCCAGGACGUUCCAUGAUU
miR-M31	UGCACAGUCGUGAGCAGAUCAA	GAUCUGCUCACGACUGUAGCCUU
si-Smad2	GAGGAGAAGUGGUGAAAUU	UUUCACACCAUUCUCCUUCU
miR-C	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
Primers for gene or 3' UTR cloning		
Smad2	AGTGAATTCGAGAGGAGAGGTTGGTGTGC	AGTTCTAGAAAGTCTGTTGATGGGCCTTG
Smad2 3' UTR	AGTGAATTCGCACCTTGTGGATTCTGTTTC	AGTTCTAGAGATTTCATTTAGGCCCAT
Primers for RT-PCR		
Smad2	AACGAGTGACCAGCAGTTGA	CCACAGTCAGGGAAGGTTGT
GAPDH	GCACGCCATCACTATCTTCC	CATCCACCGTCTTCTGTGTG
Stem-loop RT	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGACGCGGGA	
miR-M3	GTGCGGATGAAAATGTGAAACCTC	GTGCAGGGTCCGAGGT
Probes and primers for qRT-PCR		
miR-M3 LNA TaqMan probe	5'-(FAM)-AT+T C+GC A+CT G+GA+TA+C G+AC-(Eclipse)-3' ^a	
miR-M3 primer	GTGCGGATGAAAATGTGAAACCTC	GTGCAGGGTCCGAGGT
GAPDH TaqMan probe	5'-(FAM)-CACGCCATCACTATCTTCCAGGA GC-(Eclipse)-3'	
GAPDH primer	AGGCTGAGAACGGGAAACTTG	CACCTGCATCTGCCCATTG

^a In the sequence of the miR-M3 LNA probe, the plus sign indicates the position of the locked nucleic acid (LNA) insert.

herein that MDV1-miR-M3 substantially abrogated apoptosis triggered by proapoptotic stimuli and that miR-M3 directly targeted Smad2, a critical component in the transforming growth factor β (TGF- β) signal pathway. Our findings highlight the important role of viral miRNAs in virus-induced oncogenesis.

MATERIALS AND METHODS

Virus and cell lines. The China standard J1 strain of MDV1 was obtained from Yu Zhou (Beijing Institute of Animal Husbandry and Veterinary Science). DF-1 is an immortalized chicken embryo fibroblast cell line, and 293T is a simian virus 40 (SV40)-transformed embryonic kidney cell line. Both cell types were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco). Chicken peripheral blood mononuclear cells (PBMCs) were prepared from specific-pathogen-free (SPF) chicken blood using OptiPrep (Axis-Shield PoC, Norway) density gradient centrifugation and cultured in RPMI 1640 medium supplemented with 10% FBS (Gibco).

RNA oligoribonucleotides and cell transfections. The RNA duplexes mimicking 14 MDV1-encoded miRNAs (sequences are listed in Table 1) were designed as described previously (18). The small interfering RNA (siRNA) targeting chicken Smad2 mRNA (GenBank accession number NM_204561.1) was designated si-Smad2 (sense strand, GAGGAGAAGUGGUGUGAAAUU). The control RNA duplex (named miR-C) (sense strand, UUCUCCGAACGUGUCACGUTT) was nonhomologous to any chicken genome sequences and used for both miR-M3 mimics and si-Smad2. All RNA oligoribonucleotides were purchased from Genescript (China).

Transfection of RNA oligoribonucleotide(s) was done using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. A 50 nM concentration of RNA duplex was used for each transfection, unless otherwise indicated. The transfection efficiency, examined by a 6-carboxyfluorescein (FAM)-conjugated siRNA and flow cytometry analysis, was about 80% in DF-1 cells (data not shown). In the experiment expressing exogenous SMAD2 protein, 24 h after RNA transfection cells were transfected with 400 ng of plasmids in a 24-well plate, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Cell viability assays. Cell viability was determined by an Alamar blue assay (AbD Serotec, Oxford, United Kingdom). As an initial test, cells were treated with cisplatin at different concentrations, and 5 μ M cisplatin was selected for its ability to induce significant but not severe cell death. Briefly, cells were seeded in a 96-well plate at 50% confluence; after 24 h in culture, cells were transfected with miR-C or miR-M3 RNA duplex, and 24 h later cells were treated with cisplatin (5 μ M) followed by the Alamar blue assay at 0 h, 24 h, and 48 h after cisplatin treatment. The transfection efficiency was confirmed by stem-loop quantitative reverse transcription-PCR (qRT-PCR) (data not shown). Fluorescence of the reduced Alamar blue dye was measured using an M200 microplate fluorescence reader (Tecan) at an excitation wavelength of 540 nm and an emission wavelength of 590 nm.

Colony formation assay. Twenty-four hours after transfection, 1,000 transfected cells were placed in a fresh six-well plate and maintained in DMEM containing 10% FBS for 2 weeks. Colonies were fixed with methanol and stained with 0.1% crystal violet in 20% methanol for 15 min.

Apoptosis assays. Apoptosis was evaluated by apoptotic morphology, the activity of caspase-3/caspase-7 (caspase-3/7), and/or annexin V-fluorescein isothiocyanate/propidium iodide (FITC/PI) assay; cells were treated as for the cell

viability assay. For morphological examination, 48 h after cisplatin treatment, cells were stained with 4',6'-diamidino-2-phenylindole (DAPI; Sigma-Aldrich), and those with fragmented or condensed nuclei in deep staining were counted as apoptotic cells. At least 500 cells were counted for each sample. The activity of caspase-3/7 was detected in a 96-well format using a Caspase-Glo3/7 Assay (Promega). One hundred microliters of the Caspase-Glo3/7 reagent was added to each well and then incubated at room temperature for 1 h. Luminescence was detected using a Synergy 2 Multi-Mode Microplate reader (BioTek). The background luminescence associated with cell culture and assay reagent (blank reaction) was subtracted from the experimental value. For the treated cells, 48 h after cisplatin treatment, an annexin V-FITC/PI assay (BD Biosciences Pharmingen) was performed according to the manufacturer's protocol; after staining, cells were analyzed by a FACSCalibur instrument (Becton Dickinson, San Jose, CA).

RNA isolation, semiquantitative RT-PCR, and stem-loop qRT-PCR. To obtain cDNA, 2 µg of total RNA was reverse transcribed using a reverse transcription kit (Takara) according to the manufacturer's protocol. Transcripts of *Smad2* and the internal control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were specifically amplified. The primers used for *Smad2* and *GAPDH* are listed in Table 1. PCR products were then separated on 1.5% agarose gels containing SYBR green and visualized under UV transillumination. The intensity of each product was quantified using GeneTools software (version 3.07; SynGene). The intensity ratios between *Smad2* and *GAPDH* (*S/G*) were calculated as an indication of mRNA expression changes.

For stem-loop qRT-PCR of miR-M3, 2 µg of total RNA was reverse transcribed using a reverse transcription kit (Takara) according to the manufacturer's protocol with the modification that the random primer was replaced by stem-loop RT primer which was designed specifically for miR-M3 (50 nM), and the reaction mixture was incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C and then held at 4°C. For the internal control *GAPDH*, the total RNA was reverse transcribed according to the manufacturer's protocol (Takara). miR-M3 and *GAPDH* were then specifically amplified. The primers used for miR-M3, including the stem-loop RT primer, are listed in Table 1. Real-time PCR was performed using a standard TaqMan PCR kit protocol (Takara) on a Light-Cycler 480 (Roche). The sequences of probes and primers used are listed in Table 1. The reactions were performed in a 384-well plate at 95°C for 5 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. All reactions were run in triplicate.

Western blotting. Cell protein lysates were separated on 10% SDS polyacrylamide gels, electrophoretically transferred to polyvinylidene difluoride membranes (45-µm pore size; Roche), and then detected with rabbit polyclonal antibody specific for SMAD2 (ab47083; Abcam) and a commercial enhanced chemiluminescence (ECL) kit (Pierce). Protein loading was estimated using mouse anti-β-actin monoclonal antibody (sc-47778; Santa Cruz Biotechnology). The intensity of each band was quantified using GeneTools software (version 3.07; SynGene). The intensity ratios between SMAD2 and β-actin (*S/A*) were calculated as an indication of endogenous SMAD2 protein expression changes.

Vector construction. To construct a luciferase reporter vector (pGL3cm-Smad2-3'UTR-wt), a wild-type (wt) 3' UTR fragment of *Smad2* containing the putative binding site for miR-M3 was amplified and then inserted downstream of the stop codon of firefly luciferase in pGL3cm as described previously (37) (designated *Smad2-3'UTR-wt*). pGL3cm-Smad2-3'UTR-mut, which carried a mutated sequence in the complementary site for the seed region of miR-M3, was generated using the fusion PCR method (designated *Smad2-3'UTR-mut*).

To construct the *Gallus gallus* *Smad2* expression vector (pcDNA3.0-Smad2Δ3'UTR), the full-length coding sequence of *Smad2* (GenBank accession number NM_204561.1) without the 3' UTR region was amplified and then cloned into the *EcoRI* and *XbaI* sites in pcDNA3.0 (Invitrogen).

To construct *Smad2* expression vectors with 3' UTR sequences, a DNA fragment containing the complete coding sequence and a 504-nucleotide (nt) wild-type 3' UTR with the putative binding site for miR-M3 was amplified and then cloned into the *BamHI/EcoRI* sites and *EcoRI/XbaI* sites, respectively, in pcDNA3.0 (designated *Smad2 wt*). When the seed region of the putative binding site for miR-M3 was mutated (see Fig. 3A), the expression vector was designated *Smad2 mut*.

Dual luciferase reporter assay. The dual luciferase reporter assay was comprised of two reporters: a *Renilla* luciferase expression construct, pRL-TK, and a firefly luciferase expression construct in pGL3cm containing the assayed 3' UTR sequences. For the luciferase reporter assay, HK293T cells (4×10^4) were plated in a 48-well plate and then cotransfected with 10 nanomoles per liter of either miR-M3 or miR-C, 20 ng of either *Smad2-3'UTR-wt* or *Smad2-3'UTR-mut*, and 4 ng of pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were collected 48 h after transfection and analyzed using a Dual Luciferase Reporter Assay System (Promega).

Luciferase activity was detected by a Lumat LB 9507 Ultra Sensitive Tube Luminometer (Berthold). The pRL-TK vector that provided the constitutive expression of *Renilla* luciferase was cotransfected as an internal control to correct the differences in both transfection and harvest efficiencies. Firefly luciferase activity of each sample was normalized to *Renilla* luciferase activity. Transfections were done in duplicates and repeated at least three times in independent experiments.

Chicken splenic tissues. One-day-old specific-pathogen-free chickens were infected with the China standard J1 strain of MDV1. Forty-eight days after infection, splenic tissues containing tumors were obtained. The ethical guidelines for animal protection rights in China were observed.

Immunohistochemistry (IHC). The splenic tissues containing tumors obtained from J1-infected SPF chickens were formalin fixed and paraffin embedded for immunostaining of SMAD2 protein. Briefly, tissues were sectioned (5 µm), deparaffinized in xylene, rehydrated through graded ethanol, quenched for the endogenous peroxidase activity, and permeabilized with 1% Triton X-100. Sections were incubated at 4°C overnight with SMAD2-specific rabbit antibody (ab47083; Abcam) or β-actin-specific mouse antibody (sc-47778; Santa Cruz Biotechnology). Immunostaining for SMAD2 or β-actin was performed using a ChemMate Dako EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (code K 5007; DakoCytomation, Glostrup, Denmark), which resulted in a brown precipitate at the antigen site. Subsequently, sections were counterstained with hematoxylin (Zymed Laboratories, South San Francisco, CA) and mounted in nonaqueous mounting medium for photographs.

Statistical analysis. Data are presented as means ± standard errors of the means (SEM) from at least three separate experiments. Unless otherwise noted, the differences between groups were analyzed by using a Student's two-tailed *t* test when only two groups were compared or by one-way analysis of variance (ANOVA) when more than two groups were compared. Differences were considered statistically significant at a *P* value of <0.05.

Nucleotide sequence accession numbers. GenBank accession numbers for *Smad2* mRNA sequence (NM_204561.1) and the MDV genome sequence (AF243438.1) are found at the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>). The sequences of 14 mature MDV1-encoded miRNAs described in this paper were deposited in miRBase (<http://www.mirbase.org/>) under accession numbers MI0005093 to MI0005100, MI0006987 to MI0006991, and MI0008337.

RESULTS

MDV1-miR-M3 promoted cell survival under cisplatin treatment. To unveil the probable functions of MDV1-encoded miRNAs, we first performed a functional screening assay to identify the miRNAs that might promote cell survival under adverse treatment. Initially, DF-1 cells (an immortalized chicken embryo fibroblast cell line) were found to lose their viabilities and manifest evident apoptosis under treatment with cisplatin, an apoptosis inducer and widely used chemotherapy drug for cancer treatment (36). Then, we transfected DF-1 cells with each the 14 MDV1 miRNAs or miR-C oligonucleotides; miR-C was nonhomologous to chicken genome sequences (Table 1). Twenty-four hours after transfection, cells were treated with cisplatin and evaluated for their viabilities using an Alamar blue assay. In the absence of cisplatin treatment, the RNA mimics did not have evident effects on cell viability (Fig. 1A and B). Under treatment cisplatin, of the 14 MDV1-encoded miRNAs, miR-M3 gave significantly (*P* < 0.05) higher viability than miR-C or nontransfected (NT) groups at 48 h in the presence of cisplatin (Fig. 1A and C). These results demonstrated that miR-M3 promoted cell survival under cisplatin treatment. Therefore, miR-M3 was selected for further investigation.

MDV1-miR-M3 suppressed cisplatin-induced apoptosis. The increased cell viability with miR-M3 under cisplatin treatment might be the result of reduced apoptosis and/or enhanced cell proliferation. The possibility that miR-M3 might

FIG. 1. MDV1-miR-M3 promoted cell survival under cisplatin treatment. (A) The relative effects of all MDV1-encoded miRNAs on DF-1 cell viabilities. Influence of each miRNA on DF-1 cell viability with or without cisplatin treatment is shown as a continuum of blue (inhibition) to yellow (promotion). The color is determined by the ratio between the viability values of miRNA- and miR-C-transfected cells. (B) Effect of miR-M3 on DF-1 cell viability in the absence of cisplatin. (C) Effect of miR-M3 on DF-1 cell viability under cisplatin treatment. NT, nontransfected.

enhance cell proliferation was excluded by a colony formation assay; all three groups (NT, miR-C, and miR-M3) displayed similar sizes and numbers of colonies (Fig. 2A and B). We then investigated the apoptosis-blocking capability of miR-M3 by apoptotic morphology examination and annexin V-FITC/PI staining. As revealed by DAPI staining, miR-M3 remarkably reduced cisplatin-induced apoptosis in DF-1 cells ($P < 0.001$) (Fig. 2C) and showed a dose-dependent effect (data not shown) though no significant difference was observed without cisplatin treatment (Fig. 2C). The effects of miR-M3 on cisplatin-induced apoptosis were visually apparent (Fig. 2D). Analyses with annexin V-FITC/PI staining confirmed the miR-M3-induced diminishment of the cisplatin-induced apoptosis from 60% to 21% (Fig. 2E). These results collectively demonstrated that miR-M3 promoted cell survival by suppression of apoptosis under cisplatin treatment.

Apoptosis rates were decreased in freshly isolated chicken PBMCs infected with MDV1. To further confirm the biological significance of the antiapoptotic function of miR-M3, freshly isolated chicken peripheral blood mononuclear cells (PBMCs) were infected with the J1 strain of MDV1 and analyzed for miR-M3 expression and resistance to cisplatin-induced apoptosis. Chicken PBMCs were prepared from uninfected SPF chicken blood using OptiPrep density gradient centrifugation and then infected with the J1 strain of MDV1. Stem-loop qRT-PCR was performed 48 h after MDV1 infection. For the apoptosis assay, 24 h after MDV1 infection, cells were treated

with cisplatin. The caspase-3/7 assay and annexin V-FITC/PI staining were performed after 48 h of cisplatin treatment. Chicken PBMCs infected with MDV1 manifested expression of miR-M3 (Fig. 3A) and a decrease in caspase-3/7 activity and cisplatin-induced apoptosis (Fig. 3B and C). However, MDV1 infection showed a smaller antiapoptosis effect in PBMCs (27% to 20%) (Fig. 3C) than miR-M3 transfection in DF-1 cells (60% to 21%) (Fig. 2E). One possible explanation is the lower expression of miR-M3 in MDV1-infected PBMCs as the stem-loop qRT-PCR revealed that the expression of miR-M3 in MDV1-infected PBMCs was about 64 times lower than that in miR-M3-transfected DF-1 cells (Fig. 3A). In addition, chicken PBMCs appeared more resistant to cisplatin treatment than DF-1 cells (27% versus 60%). While various reasons can be speculated for these differences between chicken PBMCs and DF-1 cells, these data demonstrated that MDV1 infection did show protection against cisplatin-induced apoptosis.

Smad2 is a direct target of MDV1-miR-M3. We then attempted to identify the cellular targets of miR-M3 in order to unravel the molecular mechanisms by which miR-M3 exerts its antiapoptotic effect. Using a target prediction program (TargetScan, version 4.2 [http://www.targetscan.org/vert_42/seedmatch.html]), putative cellular targets showed complementarity with the "seed region" of miR-M3. A total of 224 candidate genes were initially identified, and some of them have been reported to be associated with tumor development or to be involved in related signal transduction

FIG. 2. miR-M3 blocked cisplatin-induced apoptosis in DF-1 cells. (A and B) Effects of miR-M3 on colony formation of DF-1 cells. Representative photographs of colony formations are shown (A). Cells were either transfected with miR-C or miR-M3 or not transfected (NT). The experiments were repeated at least three times. The average colony counts from three experiments are shown in panel B. Apoptotic rates of DF-1 cells were evaluated by apoptotic morphology examination (C) and annexin V-FITC/PI staining (E) 48 h after cisplatin treatment. Data are the means of at least three independent experiments; error bars indicated SEM. **, $P < 0.01$, compared with miR-C-transfected and NT cells as indicated. (D) Exemplary photographs corresponding to panel C.

pathways, such as ZEB2, FOXD1, CDK2AP1, SMAD2, TOB1, TOB2, and ACVR1. Among the predicted targets, Smad2 had a perfect match with the seed region of miR-M3 (Fig. 4A) and stood out as an attractive candidate for being a transcription factor belonging to the Smad family involved in the TGF- β signal pathway (22), essential for early embryonic development (11), and involved in *in vitro* cell transformation and TGF- β -induced apoptosis (43). The 3' UTR of Smad2 was verified to be 1,862 bp by 3' full rapid amplification of cDNA ends (RACE), perfectly matching with the genomic sequence and containing the binding site of miR-M3 (data not shown). Using a dual luciferase reporter system for the primary screen, the inhibition of SMAD2 was most significant among the tested candidate genes. Therefore, Smad2 was selected for further studies.

Smad2 as a direct target of miR-M3 was explored in three

ways. First, DF-1 cells were transfected with miR-M3 or miR-C, and Smad2 mRNA and protein expression was assayed 48 h after transfection. miR-M3 but not miR-C significantly suppressed the expression of endogenous Smad2 at the protein but not mRNA level (Fig. 4B). Second, a dual luciferase reporter system was prepared by cloning a chicken Smad2-3'UTR-wt or Smad2-3'UTR-mut (no complementarity with miR-M3) (Fig. 4A) fragment (~500 bp with a predicted target site of miR-M3) downstream of the firefly luciferase reporter, and DF-1 cells were then cotransfected with the dual reporters and miR-M3 or miR-C. miR-M3 significantly diminished the relative luciferase activity of the firefly luciferase reporter containing Smad2-3'UTR-wt ($P < 0.001$) but not Smad2-3'UTR-mut while miR-C had no effect on either reporter (Fig. 4C). Furthermore, two additional Smad2 expression vectors were constructed containing the 3' UTR sequences used in the lu-

FIG. 3. Apoptotic rates in chicken PBMCs infected with MDV1. (A) The expression level of miR-M3 in MDV1-infected cells and miR-M3-transfected cells. Stem-loop qRT-PCR was performed to monitor the expression level of miR-M3 48 h after MDV1 infection or 24 h after transfection with miR-M3 or miR-C. (B) Chicken PBMCs were infected with the J1 strain of MDV1 and 24 h later treated with cisplatin. Caspase-3/7 activity was measured using a caspase-Glo3/7 assay 48 h after cisplatin treatment. Data are the means of at least three independent experiments; error bars indicate SEM. **, $P < 0.01$ (comparison between two groups as indicated). (C) Annexin V-FITC/PI staining and fluorescence-activated cell sorter analysis of cells treated as in panel B.

ciferase assays (Fig. 4A), one with the 3' UTR sequence containing the miR-M3 binding site (designated Smad2 wt) and the other with the same 3' UTR sequence of Smad 2 wt except that the miR-M3 binding site was mutated (designated Smad2 mut). Then, Smad2 wt or Smad2 mut was cotransfected, respectively, with miR-M3 or miR-C into DF-1 cells. Western blotting data showed that M3 significantly inhibited SMAD2 expression mediated by the Smad2 wt vector but not the Smad2 mut vector (Fig. 4D). Taken together, these results indicate that Smad2 is a direct and authentic target of miR-M3.

Smad2 was significantly underexpressed in MDV1-induced tumor tissues. The previous studies have established the expression of miR-M3 in MDV1-infected cell lines and tumor tissues (2, 16, 23, 44, 46); thus the relevance of the identification of Smad2 as a direct target of miR-M3 from our *in vitro* experiments to MDV1-induced latent infection and tumorigenesis would pass the first but critical test only if Smad2 protein expression in MDV1-induced tumor tissues were indeed reduced. We infected SPF chickens with the J1 strain of MDV1, and the MDV1-induced splenic tumors were taken 48 days after infection. The expression of miR-M3 in MDV1-induced tumors was confirmed by stem-loop qRT-PCR (data not shown). We then detected the SMAD2 expression level in four splenic tumor tissues by utilizing Western blotting;

the results showed that Smad2 was greatly underexpressed in splenic tumor tissues compared with the nontumor splenic tissues (Fig. 5A, T1 to T4 versus N1 to N4). We further validated the reduction of SMAD2 in MDV1-induced tumors by immunohistochemistry (IHC) (Fig. 5B). These data strongly suggested that Smad2 is a direct and authentic target of miR-M3.

MDV1-miR-M3 blocked cisplatin-induced apoptosis via targeting of Smad2. Smad2 was shown to be an authentic target of miR-M3, but further investigation was required to determine whether miR-M3 blocked cisplatin-induced apoptosis through direct downregulation SMAD2. We first investigated whether reduction of SMAD2 expression might mimic the antiapoptotic effect of miR-M3 overexpression. siRNA is another class of small, noncoding RNAs that specifically degrade their target mRNAs (13). Thus, we specifically knocked down Smad2 expression using the designed siRNA si-Smad2. DF-1 cells were transfected with si-Smad2 or miR-C and 48 h later analyzed for endogenous Smad2 mRNA and protein expression. si-Smad2 almost completely abrogated Smad2 mRNA and protein expression in DF-1 cells (Fig. 6A). The knockdown of Smad2 significantly blocked cisplatin-induced apoptosis, similar to the result with miR-M3 ($P < 0.01$) (Fig. 6B), confirming that Smad2 is integrally involved in cisplatin-induced apoptosis.

FIG. 4. Smad2 was a direct target of MDV1-miR-M3. (A) Alignments of Smad2-3'UTR, miR-M3, and Smad2-3'UTR-mut, where the complementary site for the seed region of miR-M3 is indicated. (B) Suppression of endogenous SMAD2 protein (bottom) but not mRNA (top) expression by miR-M3. *S/G* denotes the intensity ratio between Smad2 and GAPDH, and *S/A* is the intensity ratio between SMAD2 and β -Actin. *S/A*, the average value of *S/A*; *n*, the number of times the experiment was repeated. (C) Analysis of luciferase activity. HK293T cells were cotransfected with Smad2-3'UTR-wt with either miR-M3 or miR-C (left), and Smad2-3'UTR-mut with either miR-M3 or miR-C (right). Data are means of at least three independent experiments done in duplicate; error bars indicated the SEM. ***, $P < 0.001$, compared with miR-C-transfected cells. (D) The ectopic expression of exogenous Smad2 by the Smad2 expression vector with mutated 3' UTR. The intensity of each band was densitometrically quantified. *S/A* denotes the intensity ratio between SMAD2 and β -actin.

Notably, in comparison with miR-M3, si-Smad2 was less potent in blocking cisplatin-induced apoptosis (33% versus 18%) (Fig. 6C).

Furthermore, we examined whether constitutive expression of Smad2 could counteract the antiapoptotic function of miR-M3. The Smad2 expression vector pcDNA3.0-SMAD2 Δ 3'UTR was constructed with complete deletion of

the 3' UTR, and its overexpression in DF-1 cells was confirmed (Fig. 7A). DF-1 cells were first transfected with miR-M3 or miR-C; 24 h later they were transfected with pcDNA3.0 or pcDNA3.0-SMAD2 Δ 3'UTR and after another 24 h treated by cisplatin for 48 h before the apoptosis assay. Ectopic overexpression of exogenous Smad2 by pcDNA3.0-SMAD2 Δ 3'UTR substantially reversed the anti-

FIG. 5. SMAD2 was significantly underexpressed in MDV1-induced tumor tissues. (A) Western blotting was used to monitor the expression levels of SMAD2 in MDV1-induced splenic tumor tissues (lanes T1 to T4) compared with normal ones (lanes N1 to N4). *S/A* denotes the intensity ratio between SMAD2 and β -actin. Data are means of at least three independent experiments; error bars indicate SEM. **, $P < 0.01$ for the comparison between two groups as indicated. (B) Immunohistochemistry staining of SMAD2 in paired MDV1-induced splenic tumor tissues (Tumor) and nontumor splenic tissues (Normal). The staining of β -actin in both tumor and normal tissues was used as an internal control. Images were captured at a magnification of $\times 400$.

FIG. 6. Smad2 siRNA (si-Smad2) blocked cisplatin-induced apoptosis. (A) si-Smad2 efficiently suppressed Smad2 mRNA (top) and protein (bottom) expression in DF-1 cells. *S/G* denotes the intensity ratio between Smad2 and GAPDH, and *S/A* is the intensity ratio between SMAD2 and β -actin. (B) si-Smad2 blocked cisplatin-induced apoptosis. DF-1 cells were transfected with miR-C, miR-M3, or si-Smad2 and 24 h later treated with cisplatin. After a 48-h cisplatin treatment, cells were stained by DAPI, and positive cells were counted. Data are means of at least three independent experiments; error bars indicate SEM. *, $P < 0.05$ (miR-M3 compared with si-Smad2); **, $P < 0.01$ (si-Smad2 compared with miR-C). (C) Cells were treated as described for panel B. Annexin V-FITC/PI staining was performed 48 h after cisplatin treatment, and then stained cells were analyzed by FACSCalibur.

apoptotic function of miR-M3 ($P < 0.01$) (Fig. 7B and C). In line with the results of Smad2 knockdown (Fig. 6B and C), overexpression of exogenous Smad2 could only partially reverse the effect of miR-M3, which suggests that miR-M3 targets more cellular proapoptotic genes than Smad2 for its full apoptosis-blocking potency even though Smad2 is a direct and authentic target of miR-M3.

DISCUSSION

Viral miRNAs have recently become an emerging hot spot in the study of host-virus interaction (8, 24, 31, 33, 34). Although an increasing number of viral miRNAs have been identified, the molecular mechanisms by which these miRNAs modulate the process of viral pathogenesis and the behavior of host cells are largely unknown. Our data demonstrated that miR-M3 promoted cell viability under cisplatin treatment and that miR-M3 had an antiapoptotic function. Our results also identified and validated that Smad2 was a direct target of miR-M3.

Apoptosis is readily assayable and a major barrier for latent infection and tumorigenesis (25). For tumor-inducing viruses, apoptosis is a major barrier not only for virus survival but also for the malignant transformation of host cells. Thus, viruses must evolve to contribute host cells to evade apoptosis so that they can escape from being eliminated by the surveillance

system and can survive in the rigorous tumor microenvironment. The MDV1-encoded oncoprotein MEQ, which could transform DF-1 cells, has been reported to protect DF-1 cells from apoptosis (17). In this study, we showed that an MDV1-encoded miRNA, miR-M3, which is located just upstream of Meq and is highly expressed in MSB-1 cells (2, 44), might contribute to MDV1 latent infection and lymphomagenesis by directly suppressing apoptosis.

The demonstration that miR-M3 blocked intrinsic apoptotic stimulus-induced apoptosis by directly downregulating Smad2 protein expression is in line with the literature about the roles of viral miRNA in manipulating apoptosis. For example, one group reported that EBV miR-BART5 was involved in blocking etoposide-induced apoptosis by targeting PUMA (6). Another group showed that KSHV miR-K5 was able to block etoposide-induced apoptosis in human umbilical vein endothelial cells (HUVEC) cell (47). Moreover, the newly identified miRNA encoded by HIV-1 TAR RNA (27, 45) was reported to protect against apoptosis by downregulating expression of cellular genes involved in apoptosis such as ERCC1 and IER3 (14).

Methodologically, we have employed a function-first approach that could narrow the scope of potential targets, mitigating the challenge of identifying authentic cellular targets for viral miRNAs. Two other current approaches for identifying miRNA targets are being widely used without consideration of

FIG. 7. Ectopic expression of exogenous Smad2 substantially reversed the antiapoptotic effect of miR-M3. (A) Overexpression of Smad2 in DF-1 cells. Reverse transcription-PCR and Western blotting were used to monitor the expression level of Smad2 in DF-1 cells 48 h after transfection with pcDNA3.0-Smad2Δ3'UTR or empty pcDNA3.0 vector, and GAPDH or β-actin was used as an internal control. The intensity for each band was densitometrically quantified. S/G denotes the intensity ratio between Smad2 and GAPDH. S/A denotes the intensity ratio between SMAD2 and β-actin. (B) Expression of exogenous SMAD2 proteins significantly abrogated miR-M3-conferred apoptosis resistance. Data are means of at least three independent experiments; error bars indicate SEM. **, $P < 0.01$ for comparison between two groups as indicated. (C) Annexin V-FITC/PI staining and fluorescence-activated cell sorter analysis of cells treated as described for panel B.

miRNA functions; one uses algorithms for target prediction, mainly relying on seed region matches between miRNAs and targets (6); another is microarray analysis of cellular gene expression with/without miRNA treatments (47). It is apparent that the function-first approach is a useful tool to take on the challenge of identifying the viral miRNA functions and targets.

The conservation of viral miRNAs and their targets is intriguing since EBV and rhesus lymphocryptovirus are the only ones retaining similar miRNAs (35). miR-M3 shared no sequence homology with any known viral miRNAs. Nonetheless, both MDV1 and HSV-1 encoded their miRNAs in their homologous genomic regions, as is the case for rhesus rhadinovirus (RRV) and KSHV (4). Interestingly, predicted cellular targets for HSV-1 miR-H2-3p included multiple apoptosis-related genes such as BCL2, PTEN, SMAD4, and SMAD3. It is plausible that miRNA-mediated repression of cellular genes required for different steps in a single pathway might have similar biological consequences; consequently, viral miRNAs may have very similar functions yet show no sequence identity (9).

Viruses cause about 15% of all human cancers, having evolved multiple strategies to prevent infected cells from be-

coming apoptotic and to evade the innate and adaptive immune responses of their hosts (28). Latent infections are presumably requisites for cancer development. Our data that miR-M3 directly targeted Smad2 and endowed host cells with apoptosis resistance suggest that viruses might be proactive in manipulating their host cells' cellular environment so as to control their own fate and further promote tumorigenesis. The identification of cellular genes that are involved in affecting the apoptosis sensitivity of cancer cells has great practical implications, providing new candidates for improving chemotherapy efficacy.

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