Targeting the miR-221–222/PUMA/BAK/BAX Pathway Abrogates Dexamethasone Resistance in Multiple Myeloma

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Abstract

Despite recent therapeutic advances that have doubled the median survival time of patients with multiple myeloma, intratumor genetic heterogeneity contributes to disease progression and emergence of drug resistance. miRNAs are noncoding small RNAs that play important roles in the regulation of gene expression and have been implicated in cancer progression and drug resistance. We investigated the role of the miR-221–222 family in dexamethasone-induced drug resistance in multiple myeloma using the isogenic cell lines MM1R and MM1S, which represent models of resistance and sensitivity, respectively. Analysis of array comparative genome hybridization data revealed gain of chromosome X regions at band p11.3, wherein the miR-221 of array comparative genome hybridization data revealed gain of RNAs that play important roles in the regulation of gene expression and have been implicated in cancer progression and drug resistance. We investigated the role of the miR-221–222 family in dexamethasone-induced drug resistance in multiple myeloma using the isogenic cell lines MM1R and MM1S, which represent models of resistance and sensitivity, respectively. Analysis of array comparative genome hybridization data revealed gain of chromosome X regions at band p11.3, wherein the miR-221–222 resides, in resistant MM1R cells but not in sensitive MM1S cells. DNA copy number gains in MM1R cells were associated with increased miR-221–222 expression and downregulation of p53-upregulated modulator of apoptosis (PUMA) as a likely proapoptotic target. We confirmed PUMA mRNA as a direct target of miR-221–222 in MM1S and MM1R cells by both gain-of-function and loss-of-function studies. In addition, miR-221–222 treatment rendered MM1S cells resistant to dexamethasone, whereas anti-miR-221–222 partially restored the dexamethasone sensitivity of MM1R cells. These studies have uncovered a role for miR-221–222 in multiple myeloma drug resistance and suggest a potential therapeutic role for inhibitors of miR-221–222 binding to PUMA mRNA as a means of overcoming dexamethasone resistance in patients. The clinical utility of this approach is predicated on the ability of antisense miR-221–222 to increase survival while reducing tumor burden and is strongly supported by the metastatic propensity of MM1R cells in preclinical mouse xenograft models of multiple myeloma. Moreover, our observation of increased levels of miR-221–222 with decreased PUMA expression in multiple myeloma cells from patients at relapse versus untreated controls suggests an even broader role for miR-221–222 in drug resistance and provides a rationale for the targeting of miR-221–222 as a means of improving patient outcomes. Cancer Res; 75(20): 1–14. ©2015 AACR.

Introduction

Despite recent advances in treatment, multiple myeloma remains incurable due to tumor progression and the emergence of resistance (1). Therefore, to develop more effective treatments and improve patient outcome, it is imperative to better understand the cellular and molecular mechanisms mediating drug resistance in multiple myeloma. Many treatment regimens include novel agents in combination with dexamethasone; unfortunately, however, multiple myeloma cells often become dexamethasone resistant (1).

The exact basis for the beneficial mechanism of action of glucocorticoids in cancer treatment has not been fully and definitively elucidated, although the apoptotic pathway is considered to be the main target. It is thought that glucocorticoid-induced apoptosis is initiated via activation of transcription of death-specific genes, and inhibition of the apoptotic cascade is believed to occur via negative modulation of proinflammatory cytokines that block transcription of death-specific genes (2). During prolonged exposure to dexamethasone, it is believed that resistance apparently stems from downregulation of glucocorticoid receptor (GR) gene expression (3). Although only one GR gene has been identified, several GR proteins (e.g., GRα and GRβ) can be generated by alternative splicing of the mRNA. GRα is expressed at relatively higher levels than GRβ in most tissues and plays a major role in dexamethasone-induced apoptosis. However, the mechanism of GRα downregulation in dexamethasone-resistant...
patients remains somewhat ambiguous (3). Other mechanisms of induced dexamethasone resistance have been proposed and include the following: (i) overexpression of the ABC transporter that will decrease intracellular dexamethasone levels, leading to resistance (4); (ii) blocking of the proapoptotic effect of dexamethasone by cytokines secreted by the bone marrow microenvironment, or via binding of multiple myeloma cells to bone marrow stroma, in either case inducing cell adhesion–mediated resistance (5).

miRNAs are small (~22nt) noncoding RNAs that negatively regulate protein-coding gene expression by enhancing degradation or inhibiting translation of mRNAs (6, 7). Dysregulation of miRNA expression is frequently detected in multiple myeloma and has been associated with increased metastatic potential and poor clinical outcome, suggesting an important role for miRNAs in multiple myeloma disease progression (8). miR-221 and miR-222 are highly homologous miRNAs encoded on the X-chromosome (9) and designated as the miR-221–222 cluster. This cluster has been found to be overexpressed in a large variety of human cancers, including hematologic malignancies such as multiple myeloma (10). It has been shown that miR-221–222 promotes oncogenesis by downregulating the expression of tumor suppressors such as the proapoptotic protein p53–upregulated modulator of apoptosis (PUMA) and MM1S, which represent resistance and sensitivity, respectively, to dexamethasone, to delineate a pathogenetic role of the miR-221–222 cluster in promoting dexamethasone resistance in multiple myeloma via downregulation of PUMA, and inhibition of apoptosis.

Materials and Methods
Cell lines and human tumor tissues and RNA isolation.
MM1S and MM1R cell lines were obtained from the ATCC.

miRNA profiling in MM1S and MM1R cells
Total RNA was isolated from MM1R and MM1S cells grown in tissue culture or isolated from xenografts using TRizol reagents (Invitrogen), and subjected to quantitative RT-PCR (qRT-PCR) analysis or whole-genomic miRNA profiling using TagMan Low Density microRNA Array (AM1792, Invitrogen). Expression of miRNAs was determined using the X-chromosome (9) and designated as the miR-221–222 cluster. This cluster has been found to be overexpressed in a large variety of human cancers, including hematologic malignancies such as multiple myeloma (10). It has been shown that miR-221–222 promotes oncogenesis by downregulating the expression of tumor suppressors such as the proapoptotic protein p53–upregulated modulator of apoptosis (PUMA) and MM1S, which represent resistance and sensitivity, respectively, to dexamethasone, to delineate a pathogenetic role of the miR-221–222 cluster in promoting dexamethasone resistance in multiple myeloma via downregulation of PUMA, and inhibition of apoptosis.

Argonaute 2 binding RNA immunoprecipitation quantitative RT-PCR
Argonaute 2 (AGO2) binding RNA immunoprecipitation analysis were performed as described (14). MM1S V- GFP and MM1S V-miR-222–222–GFP stable transduced cells (3 × 10^7 per RNA immunoprecipitation experiment) were washed with ice-cold PBS and dispersed into 1 mL of cell lysis buffer (50 mmol/L Tris–HCl Ph 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1% NP 40) containing Protease inhibitor (#5871S, Cell Signaling Technology) and RNase inhibitor (#N808-0119, Invitrogen) for 20 minutes on ice. Lysates were microcentrifuged at maximum speed for 20 minutes at 4°C, and supernatants were collected and pre-clearced with 20 μL of Protein A and G Dynabeads coupled with 5 μg of normal Rabbit IgG (#SC-3888, Santa Cruz Biotechnology). The beads were removed by centrifugation and the supernatants incubated with Dynabeads A and G coupled with 5 μg of anti-AGO2–specific antibody (#2897S; Cell Signaling Technology) or 5 μg IgG isotype control antibody (#SC-3888; Santa Cruz Biotechnology) overnight at 4°C, followed by three time washes with washing buffer (50 mmol/L Tris–HCl Ph 7.5, 300 mmol/L NaCl, 1 mmol/L EDTA, 1% NP 40, Protease inhibitor). After pulldown by centrifugation, RNA was extracted using 1000 μL TRizol reagent (Invitrogen). qRT-PCR detection of pulled-down PUMA

Plasmids and 2′-OMe-modified anti-miR-221/222
Viral expression vectors of miR-221–222 (V-miR-221–222) were created by PCR utilizing normal human DNA and the following pair of primers: V-miR-221–222 (F) (EcoRI) TACCAGAACATCCGTCCTCCAGACAGGAAAGAT, and V-miR-221–222 (R) (NotI) CTTGCGGCGCGTGTTGAAGACCAATGGTAGC, then cloned into the EcoRI and NotI restriction sites of pCDH-CMV-EF1-GFP lentiviral vector (#CD511B-1; System Biosciences). The packaging system was used according to the manufacturer’s protocol. The sequences of as-miR-221 and as-miR-222 were 5′-ACCACGAGGAGCAAGUGCCUGGUU-3’, and 5′-GAACCAGACAGGAGCAUGCUUGUGA-3’, respectively. Scrambled 2′-OMe-modified RNA (5′-AAGCGACGCCGACGCGCGCU-3’) was used as a negative control.

As-miR-221–222 sponge plasmid
The following oligos were obtained from Integrated DNA Technologies: as-221–222–EN(F) 5′-AATTCGAAACCCAGCAGAATCCGATCAAGCTCCAGAGGAAAGC-GACAGTGAAGAGCTGGGGT-3′, and as-221–222–EN(R) 5′-TGTCCGAGACCCAGAGGCAGCAGGCGAAGATCCCG-3′. The oligos were dispensed into annealing buffer and inserted into the EcoRI site of pCDH-CMV-EF1-GFP (#CD511B-1, System Biosciences). The construct is pCDH-CMV-as-miR-221–222–EF1-GFP with 3 repeat of antisense of miR-221 and miR-222 sequences (5′-GAAAACGACAGCAGCAAGUGCCUGG-3′), and is referred to as V-as-miR-221–222–GFP.

qRT-PCR for evaluating miR-221, miR-222, and PUMA mRNA levels was performed as previously described (12). The primers for miR-221(#000524), miR-222(#000525), U44(#001094), PUMA (#Hs00248075_m1), and GAPDH (#Hs99999905_m1) were purchased from Applied Biosystems. ImmunobLOTS were obtained as previously described (13). Primary antibodies included: anti-PUMA (#4976, Cell Signaling Technology), anti-BAX (#2772; Cell Signaling Technology), anti-cleaved caspase-3 (#9661; Cell Signaling Technology), anti-PARP (#2897; Cell Signaling Technology), anti-BID (#sc-11423; Santa Cruz Biotechnology), anti-BAK (#06-536; Millipore), anti-P53 (#SC-126; Santa Cruz Biotechnology), and anti-BIM (#202000; Calbiochem). Horseradish peroxidase (HRP)-conjugated secondary antibodies included: anti-rabbit IgG (#W0411; Promega), anti-mouse IgG (#W04021; Promega), and anti-actin (#sc-1615; Santa Cruz Biotechnology).
mRNA and miR-221/222 was performed as described in the section of qRT-PCR.

PUMA open reading frame overexpression virus

PUMA cDNA lacking a 3′UTR was amplified from pHAPUMA plasmid (Plasmid #16588, Addgene; ref. 15) using the following pair of primers: PUMA-V-dsRed-F-EcorI, 5′-GCTAGCGGATTCGCCGCCACCATGGCCCGCAGCGCAA-3′ and PUMA-V-dsRed-R-BamH1, 5′-CCGGGATCCATTGCGGCTCATTCCATC-3′. The PCR products were purified and cloned into the EcoR1 and BamH1 restriction sites of the pCDH-CMV-EF1a-dsRed viral vector (herein referred as V-PUMA-dsRed), and to allow sorting by flow cytometry using the red fluorescent protein dsRed as a marker.

BH3 domain profiling in multiple myeloma cells

MM1R V-dsRed and MM1R V-PUMA-dsRed stable transduced cells were subjected to BH3 profiling as previously described (16). Briefly, cells were permeabilized with digitonin and exposed to BH3 peptides on a 384-well plate. Loss of mitochondrial transmembrane potential loss induced by the peptides was measured over a period of 3 hours with the help of the radiometric dye JC-1. The mitochondrial membrane depolarization, expressed as a percentage of control values, was calculated and a Tecan plate reader. Mitochondrial membrane potential loss induced by the peptides was measured over a period of 3 hours with the help of the radiometric dye JC-1.

Locked nucleic acid miRNA in situ hybridization and immunostaining

Locked nucleic acid miRNA in situ hybridization (LNA-ISH) was performed and analyzed as previously described (12) following the instruction of the DIG Nucleic Acid Detection Kit (#11175041910; Roche). The sequences of digoxigenin (DIG)-labeled oligos were LNA-miR-222 (5′-DIG-accCaGacAgaTgg-3′), and LNA-miR-221 (5′-DIG-gaaCaGacGacAaTGt-3′). Capital letters indicate LNA modification.

Immunostains were performed according to our routine procedures (12). Briefly, formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Sections (4 μm) of formalin-fixed tissue were used for immunohistochemical analysis after baking at 60°C for 1 hour, deparaffinization, and rehydration. The sections were then blocked for peroxidase activity with 3% hydrogen peroxide in methanol for 10 minutes, washed under running water for 5 minutes, and finally pressure-cooked at 123°C in citrate buffer (DAKO Target Retrieval Solution, S1699) for antigen retrieval. The slides were cooled for 15 minutes, transferred to Tris-saline (TBS), and incubated with primary antibodies (5 μg/ml) or the corresponding IgG fraction of preimmune serum overnight at 4°C in blocking solution consisting of containing 3% BSA in PBS. Anti-human primary specific antibodies included PUMA (#AB9643, AbCam), BAX (#5023P; Cell Signaling Technology), and BAK (#6947S; Cell Signaling Technology). Cleaved caspase-3 (#9664; Cell Signaling Technology), and were visualized with the aid of the corresponding biotinylated antibody coupled to streptavidin-peroxidase complex (Vector Labs). Optimal antibody concentrations were used according to recommendations of the manufacturer. Incubations were carried out under a CO2 humidified atmosphere at room temperature, and slides were incubated with VECTASTAIN Universal ABC Kit (Vector) for 30 minutes and rinsed with PBS between each incubation. The sections were developed using 3,3-diaminobenzidine (DAB) (Sigma-Aldrich) as the substrate, and were counterstained with Mayer hematoxylin. Frozen and formalin-fixed paraffin-embedded human primary multiple myeloma cells were obtained from the Tissue Procurement Facility at Dana-Farber Cancer Institute (DFCI, Boston, MA) in accord with Institutional Review Board protocols. For cleaved caspase-3 immunostains, cells were first spun down onto slides using a cytocentrifuge (Shandon), then fixed in methanol-acetone for 2 minutes, and washed in PBS prior to immunostaining.

Cell viability assay

Cell viability was assessed with MITT, as previously described (17). A total of 1 × 10⁴ MM1S V-GFP and MM1S V-miR-221–222-GFP stable transduced cells were seeded onto a 96-well plate, and after 24 hours of incubation were treated with dexamethasone (20 μg/ml, Sigma-Aldrich), bortezomib (PS-341; Velcade; 10 nmol/l LC Laboratories), lenalidomide (1 μmol/l, AVA Chem), melphanal (1 μmol/l, Sigma-Aldrich), doxorubicin (25 ng/ml, Sigma-Aldrich), or DMSO/Ethanol alone as control for 48 hours and then subjected to the MITT assay.

In vitro luciferase reporter assay

Two reporter plasmids that could recognize the miR-221–222 target PUMA 3′UTR were constructed into pmIR-REPORT plasmid (#AM5795, Life Technologies), pmIR-PUMA-wt and pmIR-PUMA-mut, that served as conserved and nonconserved target sites of PUMA 3′-UTR, respectively. The sequences were as follows: pmIR-PUMA-wt(F), CCGGTACCTCTCTGACACCATGAGCAGATCTGAGTACGCAACATGTCGCAGATCCA; pmIR-PUMA-wt(R), AGCTTGGATCCACTGTCGTCAACAGCAGGCAGATCCA; pmIR-PUMA-Mut(F), CCGGTGACCTCTCTGACCACTATGTCGTCAACAGCAGGCAGATCCA; pmIR-PUMA-Mut(R), AGCTTGGATCCACTGTCGTCAACAGCAGGCAGATCCA; pmIR-PUMA-Mut. The primers were annealed and inserted into the pmIR-Reporter construct (Ambion). Empty pmIR plasmid (pmIR-0) served as a negative control. Triplicate samples of 1 × 10⁴ MM1S and MM1R cells in 24-well plates were transfected using Lipofectamine 2000 (Invitrogen) with 0.1 μg of the reporter plasmids and 0.05 μg of Renilla control plasmid (Promega). Six hours after transfection, the cells were fed with fresh DMEM with 10% FBS and incubated overnight. Cell extracts were then prepared, and luciferase assays were performed using the Dual Luciferase Reporter Assay System (Promega). Luciferase activities were normalized with respect to parallel Renilla activities.

Mouse xenograft models of tumor burden and metastasis

A total of 5 × 10⁴ MM1S cells stably transduced with V-miR-221–222-GFP or V-GFP and MM1R cells, stably transduced with V-as-miR-221–222-GFP or V-GFP, were injected via the tail vein into CB17.Cg-PrkdcscidLystbg-J/Crl mice (Code 250, Charles River) to establish a disseminated human multiple myeloma xenograft model as previously described (12). Survival was evaluated from the first day of tumor injection until death. All mice were intraperitoneally injected with 9 mg/kg
dexamethasone-21-phosphate disodium salt (Sigma-Aldrich) on days 1–4, 9–12, and 17–20. Mice were observed daily and sacrificed when hind limb paralysis was detected. Hind limb paralysis and tumor burden were used as an endpoint. To assess in vivo cell proliferation, apoptosis, and expression of miR-221–222 target genes, GFP-positive tumor samples were excised for immunohistochemical analysis, as in previous studies (12). All experiments involving animals were preapproved by the DFCI Institutional Animal Care and Use Committee.

The MM1R-Luc-GFP tumor dissemination mouse model

Totally 15 NOD/SCID mice were injected via the tail vein with 5 × 10⁶ MM1R-Luc-GFP cells (Gift from Dr. Constantine S. Mitsiades, at DFCI), and one week after injection, the mice were randomized to separate to control and treated groups and treated by intraperitoneal injection once a week with RNA-LANCErII (BioScience) to control mice, or 1:1 as-miR-221 and as-miR-222 (as-miR-221–222 mix; 100 pmol total, pre-mixed in RNA-LANCErII) to treatment group mice. All mice were intraperitoneally injected with 9 mg/kg dexamethasone-21-phosphate disodium salt (Sigma-Aldrich) on days 2–5, 9–12, 17–20, and 25–28. Tumor development was monitored by whole-body imaging using a Xenogen system. Mice were evaluated every week after initiation of treatment, and survival was evaluated from the day of tumor injection until death.

Statistical analysis

Differences between groups were analyzed by the unpaired Student t test (with the exception of survival curves). Kaplan–Meier survival curves were generated using Prism software and compared using a log-rank test. In all statistical analyses, P ≤ 0.05 considered statistically significant.

Results

Differential expression of miR-221–222 in MM1S and MM1R myeloma cell lines

To study the mechanism of dexamethasone resistance in multiple myeloma, two isogenic cell lines were previously generated (18): the parental cell line MM1S and the resistant MM1R subline. To investigate a possible role of miRNAs in promoting dexamethasone resistance in MM1R cells, we first performed genome-wide miRNA expression analysis in MM1R and MM1S cells using miRNA arrays. We found that 10 miRNAs were upregulated (Fig. 1A), whereas 12 others were downregulated in MM1R cells (Fig. 1B) versus MM1S cells. These results prompted us to examine CGH array data from the Broad Institute’s Multiple Myeloma Genomics Portal (http://www.broadinstitute.org/mmgp/home; ref. 19). Copy numbers were log-transformed prior to plotting using the Broad’s Integrative Genome Browser. Interestingly, analysis of the data revealed copy number gains in the X-chromosome of MM1R as compared with MM1S cells, without significant copy number differences in other chromosomal regions (Fig. 1C, top). Interestingly, miR-222 was among the upregulated miRNAs in MM1R cells. Increased levels of miR-221 and miR-222 expression in MM1R cells were confirmed using qRT-PCR (Fig. 2F, see below). More detailed examination of the X-chromosome revealed that band p11.3, in which the miR-221–222 cluster resides, has a normal gene copy number in MM1S cells but is tetraploid in MM1S cells (Fig. 1C, bottom). Taken together, these findings indicate that gene copy numbers contribute to higher expression of the miR-221–222 in MM1R cells than in MM1S cells, suggesting a possible pathogenetic role for this cluster in dexamethasone resistance.

PUMA plays a key role in the dexamethasone resistance in MM1R cells

As dexamethasone promotes multiple myeloma cell death through induction of apoptosis (20); we next examined expression levels of proapoptotic factors in MM1S and MM1R cells using immunoblot analysis. As shown in Fig. 2A and Supplementary Fig. S1, PUMA expression was significantly decreased in MM1R cells as compared with MM1S cells. In agreement with the wild-type status of p53 in these two cell lines (21), equal levels of p53 protein were detected by immunoblots. To further verify that PUMA plays a major role in promoting dexamethasone resistance in MM1R cells, we lentivirally transduced MM1R cells with vectors expressing dsRed protein alone as control (V-dsRed) or dsRed in combination with PUMA (V-PUMA-dsRed). To exclude possible endogenous miRNA regulation of transduced PUMA, the mRNA encoding PUMA lacked 3’UTR sequences. After flow cytometric sorting of dsRed-positive cells, stably V-PUMA-dsRed- and V-dsRed–transduced cells were first examined by Western blot analysis (Fig. 2B) and immunohistochemical (Fig. 2C, top) analysis to confirm increased PUMA expression in V-PUMA-dsRed cells, and then evaluated them for apoptosis in the absence or presence of dexamethasone. As shown in Fig. 2C, apoptosis observed by cleaved caspase-3 staining was greater in MM1R V-PUMA-dsRed cells than in V-dsRed cells, especially after treatment with dexamethasone. In addition, BH3 profiling revealed that MM1R V-PUMA-dsRed cells were more primed to undergo apoptosis than V-dsRed cells (Fig. 2D), further highlighting the role of PUMA as a mediator of dexamethasone resistance in MM1R cells.

The 3’UTR of PUMA mRNAs contains two binding sites for miR-221–222

We next investigated whether PUMA mRNA expression is regulated by miRNAs. We first used miRNA target prediction databases (TargetScan) to identify possible miRNAs targeting PUMA among the upregulated miRNAs in our MM1S and MM1R miRNA profiling data (Fig. 1A). Interestingly, miR-221 and miR-222 were the top candidates that could target PUMA. Bioinformatics analysis indicated that miR-221 and miR-222 share a single binding site on the 3’UTR of PUMA mRNA, which turns out to be conserved across different species (Fig. 2E), suggesting a possible role for these miRNAs in regulating PUMA expression. The inverse correlation of miR-221–222 and PUMA mRNA expression in MM1S and MM1R cells, as evaluated by qRT-PCR, further support this possibility (Fig. 2F).

To directly demonstrate a physical and functional interaction between miR-221–222 and the 3’UTR of PUMA, we made reporter constructs containing empty (pmir-0), wild-type (pmir-PUMA-wt), and mutant (pmir-PUMA-mut) sequences of the 3’UTR of PUMA mRNA (Fig. 2E). The constructs were transfected into MM1S cells, which were subsequently mock-treated with cells or treated miR-221–222 (Fig. 3A). After documenting enforced expression of both miR-221 (Fig. 3A, top) and miR-222 (Fig. 3A, middle) by qRT-PCR, we observed that wild-type, but not mutant, PUMA reporter activity was inhibited by miR-221 (Fig. 3A, bottom). To further define the role of targeting miR-221–222 as a way to abrogate dexamethasone resistance, MM1R cells transduced with pmir-0, pmir-PUMA-wt, or pmir-PUMA-mut reporter vectors were treated with control (scrambled miR) or
anti-sense (as) miR-221–222 (2’-OMe as-miR-221–222) oligos (Fig. 3B). After confirming by qRT-PCR that expression of both miR-221 (Fig. 3B, top) and miR-222 (Fig. 3B, middle) was efficiently knocked down, we observed that wild-type, but not mutant, PUMA reporter activity was restored (Fig. 3B, bottom).

As the AGO2/Dicer complex is known to recruit functional miRNAs (22), we performed the AGO2 pull-down RNA qRT-PCR assay to further evaluate whether PUMA mRNA is the target of miR-221–222 in multiple myeloma cells. As expected from our previous studies (Fig. 4), after ectopic upregulation of miR-221 and miR-222 expression in MM1S cells and transduction with V-miR-221–222-GFP, there was significant downregulation of PUMA mRNA in comparison with MM1S cells transduced with control V-GFP (Supplementary Fig. S2A). In addition, we observed that miR-221–222 and PUMA mRNA levels increased in pulled-down AGO2 complexes from MM1S cells transduced with V-miR-221–222-GFP (Supplementary Fig. S2B), indicating that upregulation of miR-221–222 recruits PUMA mRNA to the AGO2/Dicer complex.

miR-221–222 downregulates both PUMA mRNA and protein expression in MM1S cells in vitro

To examine whether miR-221 and miR-222 could induce dexamethasone resistance in multiple myeloma cells, MM1S cells
were lentivirally transduced with vectors expressing GFP alone (V-GFP) or GFP and miR-221–222 (V-miR-221–222-GFP). After flow cytometric sorting of GFP-positive cells, stably transduced MM1S V-miR-221–222-GFP and MM1S V-GFP cell lines were expanded, and expression of both miR-221 and miR-222 was verified by qRT-PCR (Fig. 4A and B). PUMA mRNA expression and
protein levels were also evaluated by qRT-PCR (Fig. 4C) and immunoblot analysis (Fig. 4D), respectively. Most importantly, ectopic expression of miR-221–222 can render MM1S cells resistant to dexamethasone as documented by PARP and caspase-3 activation detected by Western blot analysis (Fig. 4E), as well as by viability assays (Fig. 4F). Stably transduced cells were also examined by immunohistochemical analysis to assess the extent of apoptosis in V-miR-221–222-GFP cells in the absence or presence of dexamethasone. As shown in Fig. 4G, apoptosis as evaluated by cleaved caspase-3 expression, decreased in MM1S cells lentivirally
transduced with V-miR-221–222-GFP versus V-GFP, especially after treatment with dexamethasone.

Knockdown of miR-221 and miR-222 in MM1R cells partially restores PUMA expression and dexamethasone sensitivity in vitro

To evaluate the therapeutic role of miR-221–222 in vitro, we next generated sponge lentivirus to obtain long-lasting knockdown of mature miR-221 and miR-222 expression in vitro. We transduced MM1R cells with either V-GFP as a control or antisense miR-221–222 (V-as-miR-221–222-GFP) and assessed knockdown efficiency by qRT-PCR and Western blot analysis. As shown in Fig. 5A and B, expression of both mature miR-221 and mature miR-222 was efficiently knocked down. PUMA expression was found to be restored in V-as-miR-221–222-GFP cells as compared with stably V-GFP-transduced MM1R cells checked by qRT-PCR (Fig. 5C) as well as immunoblot (Fig. 5D) analysis. Most importantly, knockdown of miR-221–222 was able to resensitize MM1R cells to dexamethasone as assessed by apoptosis (Fig. 5E and G) and viability (Fig. 5F) assays. Stably transduced cells were also examined by immunohistochemical analysis to confirm restoration of PUMA expression in V-as-miR-221–222-GFP MM1R cells, and to document apoptosis in the absence or presence of dexamethasone. As shown in Fig. 5G, cleaved caspase-3 immunostaining for...
apoptosis was increased in MM1R cells lentivirally transduced with V-as-miR-221–222-GFP versus V-GFP–transduced controls, especially after treatment with dexamethasone.

**miR-221–222 induces dexamethasone resistance in vivo**

To further confirm the role of miR-221–222 in promoting dexamethasone resistance in vivo, MM1S cells stably transduced with V-GFP or V-miR-221–222-GFP, as well as MM1R cells stably transduced with V-GFP or V-as-miR-221–222-GFP, were injected via the tail vein in our mouse model of multiple myeloma dissemination (12). As in our in vitro studies showing that MM1S V-miR-221–222-GFP cells became dexamethasone-resistant (Fig. 4), mice injected with MM1S V-miR-221–222-GFP cells and treated with dexamethasone were observed to have shorter survival times than control mice injected with MM1S V-GFP cells (Fig. 6A). The levels of miR-221 (Supplementary Fig. S3A), miR-222 (Supplementary Fig. S3B), and PUMA (Supplementary Fig. S3C) in excised GFP-positive tumor xenografts were analyzed by qRT-PCR. As PUMA is a proapoptotic factor that may be involved in other drug responses, we evaluated whether miR-221–222 could enhance resistance to other drugs used in multiple myeloma. Interestingly, we found that overexpression of miR-221–222...
increased MM1S cell survival after treatment with valcade, lenalidomide, melphalan, and doxorubicin (Supplementary Fig. S4).

As-miR-221–222 abrogate dexamethasone resistance of MM1R cells in vivo

In contrast to preceding experiment, mice transplanted with MM1R V-as-miR-221–222-GFP cells and treated with dexamethasone showed increased survival when compared with control mice transplanted with MM1R V-GFP cells (Fig. 6A). These changes in survival were associated with a lower tumor burden in mice transplanted with V-as-miR-221–222-GFP cells than in mice transplanted with V-GFP cells as evaluated by whole-body imaging (Fig. 6B, top) as well as histologic examination (Fig. 6B, middle). We also observed that downstream target genes of miR-221–222, including PUMA, BAX, and BAK, were downregulated in V-miR-221–222-GFP MM1S tumors and upregulated in V-as-miR-221–222-GFP MM1R tumors in vivo (Fig. 6B, bottom). These results demonstrated that low miR-221–222 expression increased MM1S cell survival after treatment with valcade, lenalidomide, melphalan, and doxorubicin (Supplementary Fig. S4).

As-miR-221–222 decreases survival of MM1S cells whereas as-miR-221–222 increases survival of MM1R cells in vivo

Figure 6.

miR-221–222 decreases survival of MM1S cells whereas as-miR-221–222 increases survival of MM1R cells in vivo. A, Kaplan–Meier survival plots of mice injected via the tail vein with lentivirally transduced MM1S V-GFP, MM1S V-miR-221–222-GFP, MM1R V-GFP, or MM1R V-as-miR-221–222-GFP cells and treated with dexamethasone (red arrows). *, \( P < 0.05 \). B, tumor burden at day 30 after tumor injection of mice, as evaluated by whole-body fluorescence imaging (top); standard histology (middle); and immunohistochemical (bottom) analysis of PUMA, BAX, and BAK expression in GFP-positive tumors. As-miR-221–222 treatment increases survival and decreases tumor burden in MM1R-Luc-GFP bearing mice. C, Kaplan–Meier survival plots of mice treated with vehicle or with 2′-OMe-modified as-miR-221–222 plus dexamethasone (red arrows) tail vein injection of MM1R-Luc-GFP cells. *, \( P < 0.05 \). D, Xenogen images of vehicle and as-miR-221–222-treated mice at different time points (7, 21, and 35 days) after vein tail injection of cells.
sensitized the MM1R cells to dexamethasone-induced growth arrest and apoptosis, thereby prolonging survival in tumor-bearing mice.

As-miR-221–222 treatment increases survival of MM1R cells in vivo

We next performed a mouse xenograft experiment to evaluate the therapeutic effect of in vivo delivery of 2′-OMe–modified antisense-miR-221–222 (as-miR-221–222). We evaluated tumor growth in mice transplanted with MM1R-luc-GFP cells after intraperitoneal delivery of as-miR-221–222 using lipid nanoparticles in combination with dexamethasone. As shown in Fig. 6C, survival increased in mice treated with as-miR-221–222 compared with mice treated with vehicle alone (32.4 ± 10.2 days vs. 36.1 ± 4.1 days, \( p < 0.05 \)), and was associated with decreased tumor burden as evaluated by Xenogen imaging (Fig. 6D).

High levels of miR-221–222 expression correlates with low levels of PUMA expression in multiple myeloma patient samples

The above results prompted us to investigate the relationship between miR-221–222 and PUMA mRNA levels in a large set of multiple myeloma cells from patients to assess the role of these molecules in drug resistance more broadly. We used published datasets GSE16558 (23) for which both miRNA and mRNA gene expression profiling were available. Interestingly, this analysis showed a significant \( (P = 0.001) \) and inverse relationship between miR-221 and PUMA mRNA expression (Fig. 7A). We further investigated this relationship using qRT-PCR in eighteen multiple myeloma patients for whom clinical information, mRNA samples, and bone marrow biopsies were available (Supplementary Table S1). Of these, 8 were from untreated, newly diagnosed patients, and 10 were from patients with relapsed refractory multiple myeloma. As shown in Fig. 7B, higher levels of miR-221 with corresponding lower levels of PUMA mRNA were observed in multiple myeloma cells from relapsed refractory versus newly diagnosed patient. In the case of miR-222 and PUMA mRNA levels, this inverse correlation was less evident; however, samples from two refractory patients had relatively high levels of miR-222 expression with lower levels of PUMA mRNA expression. One sample (MM2) had high levels of both miR-221 and miR-222. To further investigate the relationship between miR-221–222 and the downstream targets PUMA, Bak and Bax, we performed LNA-ISH and immunohistochemical stains, respectively, on bone marrow biopsies. Interestingly, an inverse correlation was observed between miR-221–222 expression and PUMA, BAK, and BAX expression. Two representative cases, a newly diagnosed multiple myeloma (MM2) and a refractory multiple myeloma (MM10), are show in Fig. 7C. Taken together, the results revealed higher levels of miR-221 and/or miR-222 expression with corresponding lower levels of PUMA expression in a subset of samples from refractory multiple myeloma patients as compared with those from untreated, newly diagnosed multiple myeloma. Therefore, the miR-221–222 cluster, by regulating expression of the proapoptotic regulator PUMA, may more broadly mediate drug resistance leading to disease progression in multiple myeloma.

Discussion

Disease progression in multiple myeloma is due partly to development of drug resistance. Dexamethasone is an effective therapeutic agent against multiple myeloma; however, resistance eventually develops, signaling the arrival of unwelcome relapse. Previous studies have shown that tumor cells from steroid-resistant multiple myeloma patients have lower expression of the GR than those from steroid-sensitive patients, but the molecular mechanisms for clinical dexamethasone resistance remain poorly understood. In this study, we offer the first evidence for a role of the miR-221–222 cluster in mediating dexamethasone resistance and disease progression in multiple myeloma. Importantly, we provide compelling preclinical proof-of-concept experiments setting the stage for a novel antisense pharmacologic strategy to abrogate dexamethasone resistance specifically linked to enhanced expression of the miR-221–222 cluster that downregulates PUMA expression. The latter is a Bcl-2 homology 3 (BH3)-only Bcl-2 family member, and a critical mediator of p53-dependent and -independent (FOXO3a and p73) apoptosis induced by a wide variety of stimuli, such as: genotoxic stress, deregulated oncogene expression, toxins, altered redox status, growth factor/cytokine withdrawal, and infection while at the same time triggering drug resistance (24). Our experiments show that direct targeting of the miR-221–222 cluster can abrogate this general mechanism of drug resistance, and because its target PUMA is a downstream regulator of apoptosis, it is likely that as-miR-221–222 therapy should not only overcome dexamethasone resistance but also resistance to other drugs, including novel agents such as Lenolinamide, as well as p53-dependent and -independent mechanisms of drug resistance (Fig. 7D).

miR-221 and miR-222 are highly homologous miRNAs encoded as a cluster from a genomic region on the X-chromosome (9). They are widely overexpressed and involved in the pathogenesis of many human cancers including thyroid papillary carcinoma (25), glioblastoma (26), colorectal (27), lung (28), pancreas (29), ovarian (30), breast (17), gastric (31), liver (32), and chronic lymphocytic leukemia (33) and lymphoma (34). Accumulating in vitro evidence implicates the miR-221–222 cluster as an oncogene that bypasses cell quiescence and increases the survival (35), proliferation (36), and metastatic potential of cancer cells (37). This cluster has also been shown to promote oncogenesis via downregulation of several tumor suppressor proteins including p27 (38, 39), p57 (35), PTEN (31), PUMA (40), BIM (11), TIMP3 (41), and many others (http://www.ncbi.nlm.nih.gov/pubmed/21743492; refs. 26, 42). We and others have also found that miR-221–222 is highly expressed in multiple myeloma (10, 43), and we first time found that miR-221–222 targets PUMA in multiple myeloma cells. However, in the referred study (44), it was found that PTEN, BIM, p27, and p57 were also targets of miR-221–222 in other multiple myeloma cell lines, indicating that the target of these miRNAs are cell type–specific and/or dependent on the physiologic or pathologic state of the cells.

Our studies further indicate that inhibition of miR-221–222 via antisense therapy offers an enticing approach by which to overcome drug resistance more broadly. In dexamethasone resistance as it operates in the MM1S/MM1R cellular model, the target PUMA is downstream of the GR. Therefore, downregulated expression of PUMA mRNA via as-miR-221–222 therapy could trigger apoptosis independently of the status of the GR receptor. In addition, p53 mutations, which are highly recurrent in multiple myeloma, and are associated with primary drug resistance (45), as-miR-221–222 treatment may abrogate drug resistance and promote apoptosis even in the presence of p53 mutation by
restore the expression of PUMA mRNA. Furthermore, our studies documenting higher levels of miR-221–222 in refractory than in newly diagnosed multiple myeloma patients suggest that as-miR-221–222 therapy may prevent, or at least delay, dexamethasone resistance in particular and perhaps resistance more broadly in multiple drugs such as valcade, lenalidomide, melphalan, and doxorubicin. With regard to the possibility of overcoming multiple myeloma resistance to Lenolinamide (46) using as-miR-221–222 therapy, it is worth mentioning that this effect is also expected to take place by regulating PUMA levels and apoptosis rather than regulating the levels of Cerebron (CRBN) and the binding proteins (IKZF-1 and IKZF-3) as no consensus bindings...
sites for miR-221–222 are present in the 3′ UTR of CRBN mRNA (data not shown). Although our studies indicate that as-miR-221–222 could be used as a therapy to overcome drug resistance in multiple myeloma patients, additional preclinical studies will be needed to further validate this hypothesis. In addition, these studies should include the development of better delivery systems that could provide tumor specific uptake of miRNAs and avoid off-target effects.

Finally, miR-221–222 expression may be a useful prognostic marker in diffuse large B-cell lymphoma (47), papillary thyroid carcinoma (48), and lung cancer (49). In light of our findings, use of these miRNAs to predict prognosis and clinical response to dexamethasone and/or other drugs warrants similar evaluation in patients with multiple myeloma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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