miR-137 and miR-197 Induce Apoptosis and Suppress Tumorigenicity by Targeting MCL-1 in Multiple Myeloma

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Abstract

Purpose: Deregulation of miRNA has been implicated in the pathogenesis of multiple myeloma. We identified miR-137 and miR-197, mapped to the chromosome 1p (12–21) deletion region, and examined their antimiema activity as tumor suppressors.

Experimental Design: The expression of miR-137/197 was examined in multiple myeloma and normal plasma cells by qRT-PCR. Functional effect of miR-137/197 was analyzed by cell viability, apoptosis, clonogenic, and migration assays. Antimyeloma activity of miR-137/197 was further evaluated in vivo by lentiviral-based or lipid-based delivery in a mouse xenograft model of multiple myeloma.

Results: miR-137/197 expression was significantly lower in multiple myeloma cell lines and multiple myeloma patient samples compared with normal plasma cells. Transfection of miR-137/197 resulted in reduction of MCL-1 protein expression, as well as alteration of apoptosis-related genes, and induction of apoptosis, inhibition of viability, colony formation, and migration in multiple myeloma cells. MCL-1 was further validated as a direct target of miR-137/197. Conversely, overexpression of MCL-1 partially reverted the effect of miR-137/197. Importantly, in vivo lentiviral-mediated or intratumor delivery of miR-137/197 induced regression of tumors in murine xenograft models of multiple myeloma.

Conclusions: Our study reveals a novel role of miR-137/197 as tumor suppressors in mediating apoptosis in multiple myeloma cells by targeting MCL-1. Our findings provide a proof-of-principle that lentivirus-based or formulated synthetic miR-137/197 exerts therapeutic activity in preclinical models, and support a framework for development of mirR-137/197-based treatment strategies in patients with multiple myeloma. Clin Cancer Res; 21(10); 2399–411. ©2015 AACR.

Introduction

Multiple myeloma is a plasma cell malignancy characterized by the aberrant expansion of plasma cells within the bone marrow (1). Even though much progress in the treatment of this disease has been made in the past decade, myeloma remains largely incurable by current therapeutic strategies (2, 3). Therefore, the development of novel treatment options is urgently required for the treatment of patients with multiple myeloma. Because multiple myeloma is characterized by very complex cytogenetic aberrations that affect prognosis (4), it is likely that these aberrations also affect the expression of miRNAs, a class of regulatory noncoding RNAs. miRNAs have become essential regulators of cellular functions, such as proliferation, differentiation, and apoptosis (5). Deregulated expression of miRNAs in hematologic malignancies, including multiple myeloma, has been widely demonstrated, thus eliciting interest for these molecules as antitumor therapeutic agents (6–8). Impaired processing of miRNAs has also been reported to be associated with high-risk multiple myeloma (9).

Emerging evidence suggests that a great majority of miRNAs are mapped to chromosomal instability regions (10). We previously showed that chromosome 1 abnormalities, including 1p deletions and 1q amplifications, are frequent events and associated with poor prognosis in multiple myeloma (11–13). For 1p deletions, one of the minimal common deletion regions has been defined on 1p12–1p21 locus based on SNAP arrays and Karyotype analysis (14, 15). We demonstrated that 1p21 deletion was an independent risk factor for progression-free survival and overall survival in multiple myeloma (13). However, the underlying mechanisms remain to be elucidated. In an aim to identify potential tumor suppressor genes in the 1p deletion region, we searched for all miRNA genes that are mapped to the minimal deletion region 1p12–1p21. We detected miR-137 and miR-197 in this region and evaluated their functional role as tumor suppressors and identified MCL-1 as their direct target in multiple

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Translational Relevance

Despite the introduction of new treatment regimens, the development of effective strategies to inhibit multiple myeloma cell growth and proliferation without unwanted side effects has proven to be a challenge. miRNAs represent an emerging class of biomolecules considered as an alternative therapeutic approach in different types of cancers. In this study, we identified two miRNAs, miR-137 and miR-197, with low abundance in multiple myeloma cell lines and patient samples relative to normal controls that have not been previously described in multiple myeloma. Importantly, both miRNAs with tumor suppressor function are located in the 1p21 deletion region associated with poor prognosis. Our in vitro and in vivo studies revealed potential antitumor activities of miR-137/197 by targeting of MCL-1. Thus, our data have relevance for the future therapeutic use of miR-137/197 in the treatment of patients with multiple myeloma, including the high-risk group with 1p21 deletion.

Materials and Methods

Cell lines and primary cultures

The multiple myeloma cell lines, MM1.S, MM1.R, and OCI-My5 (harboring 1p deletions), NCI-H929 and U266 (without 1p deletions), were cultured in complete RPMI-1640 medium (harboring 1p deletions), NCI-H929 and U266 (without 1p deletions), MM.1S cells transduced with miRNAs or empty vector was analyzed by RT2 Profiler PCR Array Data Analysis v3.5, and fold changes in miRNA treatments relative to scrambled treatments were calculated by using the 2^(-ΔΔCT) algorithm.

Overexpression of miR-137/197 in multiple myeloma cells

MM1.S and My5 cells were transiently transfected with either pre-miR-137/197 or scrambled miRNA (Gene Copeia) using the HiPerFect transfection kit (Qiagen). The transfected cells were examined for cell viability using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Biobasic) assay after transfection treatment for 72 hours.

Cell viability, apoptosis, colony formation, and migration assays

Cell viability in MM.1S and My5 cell lines transfected with miR-137/197 or scrambled control was assessed using MTT assay as described previously (16–18). An annexin V–FITC/propidium iodide apoptosis detection kit (BD Biosciences) was used to quantify apoptosis and data were analyzed with a FACS Calibur flow cytometer (BD Biosciences; refs. 16, 17). Colony formation was measured by methyl cellulose method as described previously (16, 17). Migration was performed using 24-well Transwell plate (Millipore) in the presence of 10% FBS and the migration of cells was quantified as described previously (16, 17).

Protein extraction and Western blotting analysis

Cells were lysed in cold cell lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with protease inhibitor cocktail tablets (Roche). Equal amounts of proteins were resolved by 12% SDS-PAGE and transferred onto PVDF membranes. Membranes were blocked by incubation in 5% nonfat dry milk in PBS (0.05% Tween-20 in PBS) and probed with anti-MCL-1, anti-Bad, anti-Bad (phospho), anti-Bcl-2, anti-Bcl-2, anti-Bcl-2, anti-caspase-9, anti-caspase3, anti-PARP, anti-tubulin, and anti-GAPDH (signalway Antibody). Blots were then developed by enhanced chemiluminescence kit (Millipore).

Reporter assay

pEZX-Reporter plasmids for the miR-137/197 putative target MCL-1 were constructed. 293T, MM.1S, and My5 cell lines were transiently cotransfected with reporter plasmids containing Renilla luciferase (pEZX-MT-Control, pEZX-3'-UTR/MCL-1-WT), or pEZX-3'-UTR/MCL-1-MT), miRNAs (miR-137, miR-197, or control miRNA), with Qiagen HiPerFect kit. Cells were harvested 48 hours after transfection and lysed, then firefly and Renilla relative activities were measured using the Dual-Luciferase Reporter Assay System following the manufacturer’s instruction. Luciferase activities were analyzed as the activity of firefly relative to Renilla.

Stable cell line generation

To achieve stable expression of miR137, two rounds of transduction of MM.1S cells in the presence of 8 μg/mL of polybrene (Sigma) were performed with lentiviral vector pEZX-MR03 miRNA constructs or with an empty vector (pEZX). Three days after transduction, transduced cells were selected with 2.5 μg/mL puromycin (Sigma). Transduction efficiency (>95%) was evaluated by flow-cytometric GFP expression. Expression of miR137 in MM.1S cells transduced with miR197 or empty vector was analyzed by qRT-PCR.

Animals and in vivo models of human multiple myeloma

SCID mice (6–8 weeks old; OGI) were housed and monitored in our Animal Research Facility. All experimental procedures and protocols had been approved by the Institutional Ethical Committee (University Health Network, Toronto, Ontario, Canada). To establish subcutaneous human multiple myeloma xenograft models, 5 × 10⁶/100 μL of pre-miR-137–transduced MM.1S cells or pre-miR-scramble–transduced MM.1S cells were injected subcutaneously into SCID mice (n = 5, 5 mice/group). The mice started to develop subcutaneous tumors approximately 25 days after injection. Tumor size was monitored and measured every 3 days in two dimensions using calipers, and tumor volume was...
Lipid-based delivery of synthetic miRNA

Mice were randomized in two groups and treated with synthetic miR-137 or miR-197 mimics or miR-scramble (negative control). Each dose contained 20 μg synthetic oligo, which equals 1 mg/kg per mouse with an average weight of 20 g. Administration of miRNA mimics was performed using the novel formulation of neutral lipid emulsion (NLE; MaxSuppressor miRNA mimics) according to the manufacturer’s instructions. Treatments were performed intratumorally by using the formulation and dosage as described above. Tumors were then collected and placed in 10% formalin for histologic assessments.

Statistical analysis

Each experiment was performed at least three times and all values are reported as mean ± SD. Comparisons between groups were made with the Student t test, and statistical significance of differences among multiple groups was determined by GraphPad Prism software. P value of less than 0.05 was considered as statistically significant.

Results

Expression of miR-137/197 in multiple myeloma primary samples and cell lines

In an aim to identify potential tumor suppressor genes in the 1p21-deletion region, we searched for all miRNA genes that are mapped to the minimal deletion region 1p12–1p21. According to NCBI MapViewer, minimal deletion region is located between nucleotides 94,500,000 and 124,300,000. Defining these nucleotides as start and end locations in miRBase search lists (19) identifies five miRNAs, including miR-137/197, located in this deletion region (Supplementary Fig. S1).

We first evaluated the miR-137/197 expression in a series of five multiple myeloma cell lines (H929, U266, MM.1R, MM.1S, and My5), six multiple myeloma patient bone marrow samples, and 4 normal donor plasma cell samples (PC). By qRT-PCR analysis, we found that the basal expressions of miR-137 and miR-197 were significantly higher in normal PCs samples than those derived from myeloma patients or multiple myeloma cell lines (Fig. 1A and B). Moreover, miR-137/197 expression was reduced in My5, MM.1S, and MM.1R cell lines harboring del (1p12–1p21) compared with H929 and U266 cells without such deletion (Fig. 1C–E). In addition, miR-553 expression in above cell lines followed the same pattern as for miR-137 and miR-197, however, we could not observe a significant effect of miR-553 overexpression on viability and apoptosis in multiple myeloma cell lines (Supplementary Fig. S2).

miR-137/197 regulates cell viability, colony formation, and migration of multiple myeloma cells

To determine the functional role of miR137/197 in multiple myeloma cells, pre-miR137/197 or control probes were transiently transfected into MM.1S and My5 cells and cell viability and apoptosis were measured. Overexpression of miR-137/197 triggered significant inhibition of cell viability in both dose and time-dependent manner in MM.1S (Fig. 2A and B) and My5 (Fig. 2C and D). Moreover, overexpression of miR-137/197 in MM.1S and My5 cells significantly decreased the number of colonies and the number of migrating cells induced by serum (Fig. 2E–I). The inhibition of viability, colony formation, and migration was accompanied by induction of apoptosis (Fig. 2J and K). Furthermore, transfection of oligonucleotide inhibitors against miR-137/197 in H929 cells with normal level of both miRNAs increased cell viability, and appeared to induce resistance to the cytotoxic effect of velcade (Supplementary Fig. S3). On the other hand, overexpression of these two miRNAs in U266 or H929 cells resulted in a decrease in cell viability (Supplementary Fig. S4). These data further confirm the role of miR-137/197 as negative regulators of cell growth in multiple myeloma cells, and strengthen the rationale for our experimental strategy of enforced expression in these cells.

Expression of apoptosis-related genes by qPCR and Western blot analysis

In an attempt to identify the differentially expressed apoptosis genes between miRNAs-treated and untreated multiple myeloma cells, we conducted a customized human apoptosis RT² Profiler PCR Array assay to monitor the expression of 84 key genes, which were further validated by qRT-PCR analysis. We observed upregulation of proapoptotic genes, indicating that these two miRNAs could sensitize cells for apoptotic events (Fig. 3A–C). In cells treated with miR-137 or miR-197, the expression of the antiapoptotic gene MCL1 showed no noticeable changes, whereas the other genes coding for proapoptosis-related products such as BAD, BAX, BID, and BIM were markedly increased (Fig. 3B and C). In addition, transfection with miR-137 or miR-197 in MM.1S cells resulted in an increase of the cleavages of caspase-9, caspase-3, and PARP, but not caspase-8, at 48 hours posttransfection (Fig. 3D). Importantly, MCL-1 protein in MM.1S and My5 cells at 72 hours of treatment with miR-137 or miR-197 was significantly decreased compared with scrambled treatment (Fig. 3D). The results suggest that apoptosis induced by these two miRNAs in multiple myeloma cells may be mediated through an intrinsic apoptotic pathway by targeting MCL-1.

miR-137 and miR-197 directly target MCL-1

To further identify miR-137 and miR-197 targets, we first used bioinformatics analysis. Comparing the results obtained from the different searches, we found that the MCL-1 protein was predicted as a target of miR-137 and miR-197 by the miRanda algorithm. RNAhybrid also predicted a possible binding region of miR-137 and miR-197 in the 3’ untranslated region (UTR) of MCL-1 (Fig. 4A). To validate MCL-1 as a target of miR-137 and miR-197, we cloned the 3’UTR sequence of human MCL-1 into the luciferase-expressing vector pEZK-MF01 to the downstream of the luciferase stop codon. 293T, MM.1S, and My5 cells were transiently transfected with this construct in the presence of pre-miR-137 or pre-miR-197, or a scrambled oligonucleotide acting as a negative control. As shown in Fig. 4, miR-137 or miR-197 significantly reduced luciferase activity compared with the scrambled control miRNA (Fig. 4B–D). This indicates that miR-137 and miR-197 bind to the 3’UTR of MCL-1 and impair its mRNA translation. To confirm that the region was specific for binding with miR-137
and miR-197, we generated the deletion mutants of 3'UTR of MCL-1 lacking the binding site for miR-137 and miR-197, respectively. The mutants were subsequently cloned into the luciferase gene following Renilla gene, and then cotransfected with pre-miR-137 or miR-197 into 293T, MM.1S, and My5 cells. miR-137 or miR-197 did not significantly reduce luciferase activity in the presence of the 3'UTR of MCL-1–mutated sequence (Fig. 4E–J). Thus, our data strongly suggest that miR-137 and miR-197 induce apoptosis in myeloma cells at least in part through controlling the expression of MCL-1 protein.

Overexpression of MCL-1 partially reverts apoptosis and cell viability induced by miR-137/miR-197 in multiple myeloma cells

To establish a more definitive functional link between MCL-1 and miR-137/miR-197, MCL-1 was overexpressed in multiple myeloma cell lines (MM.1S) using transient transfection. MM.1S cells were transiently cotransfected with expression plasmid of MCL-1 with 3'UTR and miRNAs (miR-137, miR-197, or scrambled miRNA) using Qiagen HiPerFect transfection reagent, and cells were analyzed for apoptosis, cell viability, and expression of apoptotic genes at the indicated time points. Results show that overexpression of miR-137/197 induced about 70% cell death in MM.1S cells. However, apoptosis induction in these cells was significantly inhibited when MCL-1 was overexpressed (Fig. 5A and B). MCL-1 overexpression by Western blot analysis was shown (Fig. 5C). In parallel, inhibition of viability induced by overexpression of miR-137 or miR-197 was also prevented by MCL-1 overexpression in both MM.1S (Fig. 5D) and My5 (Fig. 5E) cells. These results indicate that overexpression of MCL-1 reverts the effect of miR-137/197 in multiple myeloma cells and miR-137/197-mediated cell death in multiple myeloma cells is regulated by MCL-1.

miR-137 lentiviral transduction inhibits multiple myeloma xenograft formation in SCID mice

For in vivo studies, we examined the effect of transduced miR-137 on the tumorigenic potential of multiple myeloma cells engrafted in SCID mice. As shown in Fig. 6A, enhanced expression of miR-137 caused a significant inhibition (P < 0.05) of tumor formation.
without showing any untoward toxicity as indicated by the body weight (Fig. 6B). Importantly, mice receiving miR-137–overexpressing MM.1S cells achieved significantly longer life span than mice receiving empty vector-MM.1S cells (Fig. 6C, $P < 0.011$). Quantitative analysis of the retrieved tumors showed a significant decrease in MCL-1 and increase in BAD and PARP in miR-137–transduced tumors (Fig. 6D). IHC analysis of excised tumors showed lower expression of Ki67 and higher expression of Tunel in tumors harvested from miR-137–transduced mice compared with that of empty vector-transduced mice (Fig. 6E). Taken together, these results indicate that miR-137 overexpression inhibits proliferation and stimulates the apoptotic cascade in MM.1S xenografts.

Figure 2.
Role of miR-137 and miR-197 as tumor suppressors in multiple myeloma cells. MM.1S and My5 cells were transiently transfected with either pre-miR-137/197 or scrambled miRNA using the HiPerFect transfection kit (Qiagen). The transfected cells were examined for cell proliferation (A and C) and viability (B and D) using the MTT assay after transfection treatment for 72 hours. E and G, miRNA–overexpressing MM.1S and My5 cells transfected with miR-137, miR-197, and scrambled miRNA were used in forming colonies in microcellulose layer containing complete RPMI-1640 medium. The colony number was counted 4 weeks later under microscope, and the morphology image of MM.1S cell colony was obtained at $4 \times 10$ magnifications (E). F and H, MM.1S and My5 cells transiently expressing either scrambled or miR-137 and miR-197 were analyzed for the effects of these miRs on serum-induced migration ability. The migration assay was performed using a 24-well Transwell plate. The migration index was calculated by counting the cells in the lower chamber. (Continued on the following page.)

Synthetic miR-137/197 mimics exert anti–multiple myeloma activity in vivo

To further support the therapeutic potential of miR-137 or miR197 in vivo, we evaluated the growth of multiple myeloma xenografts in SCID mice by intratumor delivery of synthetic miR-137 or miR-197 mimics. MM.1S cells were injected into a cohort of 10 mice and when tumors became palpable, mice were randomized into two groups and treated intratumorally with synthetic miR-137 or miR-NC. To achieve an efficient delivery of miR-137 or miR-NC, we formulated synthetic miR-137 or miR-197 mimics with NLE particles (20–22), a novel in vivo delivery system for oligonucleotides.

A significant inhibition of tumor growth was detected following five injections (3 days intervals) of miR-137 formulated in NLE particles in MM.1S xenografts (Fig. 6F). Importantly, after 39 days, we observed a significant tumor regression in mice treated with formulated miR-137 mimics (Fig. 6F). Notably, miRNAs administration did not produce any significant weight loss in treated animals (Fig. 6G). We observed a prolongation of survival ($P < 0.0127$) of mice treated with miR-137 mimics compared with control groups when our observation ended at 50 days (Fig. 6H). Moreover, protein expression in
Excised tumors exhibited inhibition of expression of MCL-1 and enhanced expression of BAD and PARP in tumors excised from mice treated with miR-137 (Fig. 6I). Moreover, increased expression of Tunel and lower expression of ki67 were also observed in tumor samples of mice treated with miR-137 compared with samples collected from mice treated with miR-NC (Fig. 6J), indicating that miR-137 treatment induced inhibition of proliferation and triggered apoptosis in multiple myeloma xenografts. In addition, treatment with synthetic miRNA-197 mimics achieved similar results in the xenograft model (Supplementary Fig. S5A–S5E).

Discussion

Multiple myeloma is characterized by various cytogenetic abnormalities, including 1p21 deletion, which has prognostic significance (11–13). Previous studies identified and validated a few other genomic regions of loss in chromosome 1 such as 1p12, 1p36-23, and 1p31-32 associated with poor survival in multiple myeloma (23, 24). Boyd and colleagues demonstrated that loss of FAM46C gene at 1p12 and CDKN2C gene at 1p32.3 was associated with poor prognosis in multiple myeloma (24). However, critical genes involved in the pathogenesis and prognoses of
multiple myeloma in 1p12–21 deletion regions have not been identified. Because miRNAs play an important role in multiple myeloma pathogenesis (25), in this study, we explored the role of miRNA and their regulation in relation to 1p12–1p21 locus deletion region in multiple myeloma. In our initial screening, we identified five miRNAs, including miR-137 and miR-197, in this region. Taking into consideration the significance of these two miRNAs in some solid tumor types (26–28), and their location within or closer to 1p21 deletion region that has shown to be associated with poor prognosis in multiple myeloma (13), we then focused our study on miR-137/197.

Our studies revealed significant lower expression of miR-137/197 in multiple myeloma cell lines and patient samples compared with normal donor plasma cells. Analysis of a Gene Expression Omnibus (GEO) database (GSE16558) from Gutiérrez and colleagues (29) indicated that miR-137/197 expression was significantly lower in a cohort of 60 multiple myeloma patients compared with normal donor plasma cells \( (n = 6; \text{miR-137 } P = 0.0447, \text{miR-197 } P = 0.0027) \), which independently confirmed our findings. In addition, further analysis of the GEO database revealed that miR-137/197 low expression was significantly correlated with 13q/Rb deletions \( (P = 0.031) \), suggesting...
**Figure 4.**
miR-137 and miR-197 directly target MCL-1. A, sequence alignment of the miR-137 and miR-197 seed sequence with MCL-1 3′-UTR. Matched nucleic acid–base pairs were linked as “-”. B–D, 293T, MM.1S, and My5 cells were transiently cotransfected with reporter plasmids (pEZX-MT-Control, pEZX-3′-UTR/MCL-1-WT), miRNAs (miR-137, miR-197, or control miRNA) using the HiPerFect kit. Cells were harvested and lysed to measure the fluorescence of firefly and Renilla luciferin according to manufacturer’s instruction. Luciferase activities were analyzed as the relative activity of firefly to Renilla. Readings from the empty plasmid (pEZX-MT-control) were used for normalization. E–J, 293T, MM.1S, and My5 cells were transiently cotransfected with control and mutated reporter plasmids (pEZX-MT-Control and pEZX-3′-UTR/MCL-1-MT), miRNAs (miR-137, miR-197, or scrambled miRNA) using the HiPerFect kit. Cells were harvested and lysed 48 hours after transfection to measure the relative fluorescence intensity of firefly and Renilla luciferin following manufacturer’s instruction. Luciferase activities were analyzed as the relative activity of firefly to Renilla. Readings from the empty plasmid (pEZX-MT-control) were used for normalization. Results shown are mean±SD (n = 3). *, P < 0.05; **, P < 0.01; and ***, P < 0.001.
that low expression of these miRNAs in multiple myeloma might be associated with a poor survival. The lower expression of miR-137 and miR-197 as tumor suppressors in multiple myeloma patients (Fig. 1), which was further verified in 23 patient samples with or without 1p21 deletion (data not shown). As expected, samples with 1p21 deletion (n = 6) showed significantly lower expression of miR-137 compared with the samples without 1p21 deletion (P = 0.0329). Similar to our studies, several groups have reported the antiproliferative role of other miRNAs, including miR-19a/b, miR-21, miR-29b, miR-34a, in multiple myeloma (21, 22, 30). However, these studies lack information about miRNAs located in deletion or amplification regions. To our knowledge, this is the first report to demonstrate two different miRNAs in the del(1p12–21) region with potential antimyeloma activities.

To investigate the potential role of miR-137/197 as antimyeloma agents, we selected multiple myeloma cell lines with lower levels of miR-137/197 as compared with normal donor plasma cells. We found a reduction of miR-137/197 expression in cell lines with 1p12–21 deletion. Our studies demonstrated that the enforced expression of miR-137/197 triggered antiproliferative and proapoptotic effects in multiple myeloma cells. In addition, suppression of miR137/197 using synthetic miRNA inhibitors in multiple myeloma cells (H929) with relatively higher levels of these two miRNAs led to a higher than baseline cell proliferation and some level of drug resistance. Furthermore, enforced expression of miR-137/197 resulted in significant downregulation of MCL-1, which was validated as a direct target of miR-137/197 in multiple myeloma cells. Repression of MCL-1 protein upon enforced expression of miR-137/197 occurred together with a reduced colony formation and cellular migration. Although MCL-1 protein was downregulated in cell lines and retrieved tumors, overexpression of miR-137/197 was unable to produce the same effect at its mRNA level (Fig. 3). This is likely because miR-137/197 may prevent the translation of MCL-1 protein by binding with the complementary sequences in 3’UTR of MCL-1 rather than promoting its mRNA degradation. MCL-1 is dysregulated in multiple myeloma cells and
overexpression of MCL-1 is associated with relapse and poor survival (31). MCL-1 plays essential roles for the survival of multiple myeloma cells by its ability to oppose a wide variety of proapoptotic stimuli. Downregulation or reduction of MCL-1 has been proposed to play important roles in response to drug-induced apoptotic stimuli (32). Similar to the changes of MCL-1 antiapoptotic protein, we also observed that overexpression of miR-137/197 could result in upregulation of several BH3-only proapoptotic proteins such as BAD, BAX, and BID and downregulation of other antiapoptotic proteins, BCL-2, BCL-XL, and survivin. Because a single miRNA can target more than one gene at the same time, we cannot exclude the possibilities for the association of other antiapoptotic proteins targeted by these miRNAs. Further investigations will be required to decipher the contribution of these miRNAs and/or any cooperation with other miRNAs in the regulation of other potential targets.

Moreover, miRNA genes functioning as tumor suppressors can be downregulated because of deletions, epigenetic silencing, or loss of the expression of one or more transcription factors as previously shown in mantle cell lymphoma, prostate cancer, and multiple myeloma (33, 34). Interestingly, low expression of miR-34/b/c in 75% of multiple myeloma cell lines and 52% of multiple myeloma relapsed primary samples were due to promoter methylation of these miRNAs genes (34). Furthermore, a different study showed that the promoters of the miR-34a gene subject to inactivation by CpG methylation may induce the loss of the expression of miR-34a (35). However, in preliminary studies using gene expression analysis after decitabine treatment of eight
multiple myeloma cell lines (5 without 1p deletion, 3 with 1p deletions), we could not find any change in miR-137/197 gene expression (data not shown). This suggests that it is unlikely that hypermethylation plays a major role in dysregulation of miR-137/197 in multiple myeloma, although besides gene deletions, more comprehensive study in a larger multiple myeloma cohort is required to further clarify the role of epigenetic silencing in dysregulation of miR-137/197 in multiple myeloma.

The results of the present study demonstrate that overexpression of miR-137/197 inhibits tumor growth and prolongs survival in subcutaneous mouse xenograft model indicating that findings of our in vitro study can be successfully translated in vivo. Importantly, formulated miR-137/197 was safely administered to mice bearing multiple myeloma tumors by intratumor injection, suggesting a favorable therapeutic index. Our data are in agreement with reports by Trang and colleagues (20), and Di Martino and colleagues (22, 30) on the safe use of formulated NLE-miR-137/197 in experimental animals, and strongly support clinical development of miR-137-based strategies in patients with multiple myeloma. Notably, formulated miRNA mimics are distinct from molecularly targeting drugs whose antitumor activity relies on the modulation of a wide range of genes rather than inhibition of individual gene products. In particular, miRNA-based therapeutics can be relevant both for safety issues and to abrogate late onset of resistance, because of the complexity of miRNA-targeted pathways and the

Figure 6.
(Continued.) F–J, the effect of intratumor injection of synthetic miRNAs on tumor growth. F, miR-137 inhibited tumor growth in vivo. G, body weight was measured every 3 days until day 39, and data are presented as mean±SD. H, survival was evaluated using Kaplan-Meier curves and log-rank analysis from the first day of tumor cell injection until death or occurrence of an event. I, samples retrieved from tumors were analyzed by immunoblotting for MCL-1, BAD, and PARP protein expression. J, IHC analysis of tumor sections showed the same patterns as explained in E.
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consequent low chance of developing individual "escape" mutations in the treated cells.

**Conclusion**

Our investigation provides evidence on miR-137/197 involvement in inhibition of cell proliferation, colony formation, migration, and induction of apoptosis and suggests a critical role for miR-137/197 in the pathogenesis of multiple myeloma. Our data support the development of miR-137/197 mimics as potential pharmacologic intervention strategy in multiple myeloma. The important interaction with the MCL-1 pathway opens new opportunities for combinatory therapeutic approaches that may result in a selective and highly efficient targeting of pathways crucially involved in the control of multiple myeloma cell growth and survival.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors' Contributions**

Conception and design: Y. Yang, L. Qiu, H. Chang

Development of methodology: Y. Yang, F. Li

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Yang, F. Li, L. Qiu, H. Chang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Yang, F. Li, L. Qiu, H. Chang

Writing, review, and/or revision of the manuscript: Y. Yang, M.N. Saha, J. Abdi, H. Chang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Yang, F. Li, L. Qiu, H. Chang

Study supervision: L. Qiu, H. Chang

*Other (performed parts of the experiments):* J. Abdi

**References**


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