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Regulation of Complement-Dependent Cytotoxicity by MicroRNAs miR-200b, miR-200c, and miR-217

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The impact of microRNAs (miRNAs) known to regulate numerous biologic processes on complement-dependent cytotoxicity (CDC) was investigated in K562 cells. The C5b-9 complex is the executioner of CDC. Cells protect themselves from CDC by C5b-9 elimination, a process involving the mitochondrial chaperone mortalin/GRP75. Potential miR-200 (b and c) and miR-217 regulatory sites were identified in mortalin mRNA. Overexpression of miR-200b/c or miR-217 lowered the expression of mortalin mRNA. miRNA inhibitors for miR-200b, miR-200c, or miR-217 enhanced mortalin mRNA level. Unexpectedly, these miRNA modulators had no significant effect on mortalin protein level. Metabolic labeling analysis demonstrated that, to compensate for reduction in mortalin mRNA level, the cells increased the rate of synthesis of mortalin protein. Cells overexpressing miR-200b/c or miR-217 showed reduced sensitivity to CDC, whereas inhibition of miR-200c and miR-217 enhanced cell death. miR-200b/c overexpression reduced C5b-9 binding and enhanced its release from the cells and promoted mortalin relocation to the plasma membrane. Inhibition of miR-200 (b and c) and miR-217 had no effect on the expression level of the membrane complement-regulatory proteins CD46, CD55, and CD59. However, overexpression of miR-200b/c or miR-217 enhanced expression of CD46 and CD55 (not of CD59). Overall, the data demonstrate miRNA regulation of cell sensitivity to CDC. We identified miR-200b, miR-200c, and miR-217 as regulators of mortalin and, perhaps indirectly, of CD46 and CD55. Cell exposure to a sublytic dose of complement was shown to increase expression of miR-200 (b and c), suggesting that complement C5b-9 exerts a feedback-regulatory effect on these miRNAs. ***The Journal of Immunology*****, 2016, 196: 5156–5165.**

Complement-dependent cytotoxicity (CDC) is an essential immune effector mechanism employed to eliminate from the body pathogens and abnormal cells (1, 2). CDC is activated in cancer patients upon injection of therapeutic anticancer Abs and participates in the overall fight against the cancerous cells (3). However, CDC is also activated accidentally in numerous patients suffering from autoimmune, immune complex, or neurodegenerative diseases and is implicated in pathogenesis. Mastering the factors regulating CDC will permit potentiation of its toxicity toward pathogenic cells and sparing of healthy tissue at risk. The first step in formation of the cytotoxic complement membrane attack complexes (MAC) is a proteolytic cleavage of complement C5 into C5b by the classical or alternative pathway C5 convertase. C5b then binds to C6 and assembles with C7, C8, and C9 to form the C5b-9 membranolytic complex, also known as the MAC (4). The MAC has been shown to induce cell activation, division or proliferation, cell movement, adherence or secretion, and, at higher doses, cell death (5, 6). Short treatment with a sublytic dose of MAC induces in them elevated resistance to lytic doses of MAC (7).

Most cells are protected from the MAC by an array of resistance strategies, headed by the membrane-bound complement-inhibitory proteins CD46, CD55, and CD59 (8). CD46, the membrane cofactor protein, and CD55, the decay-accelerating factor, target C3b and the C3/C5 convertases (9, 10). CD59 prevents MAC formation by binding to C8 and C9 during MAC assembly (11). Overexpression of these membrane regulators has been demonstrated in many primary tumors and tumor cell lines (12). For protection, cells also rapidly eliminate the MAC from their surface by endocytosis, through caveolae, and by ectocytosis (13–15). MAC removal by ectocytosis or exovesiculation has been demonstrated in several cell types. Mortalin, protein kinase C, and the MAPK ERK were implicated in this ectocytic MAC removal (16, 17). Mortalin, also known as GRP75 and mthsp70, is a 75-kDa protein found primarily within mitochondria, but spotted also in other cellular compartments (18, 19). It participates in stress response (20), mitochondrial import (21), intracellular trafficking (22), and cell proliferation (23), and is frequently upregulated in tumors (24, 25). Direct binding of mortalin to two components of the terminal complement pathway, complement C8 and C9, was demonstrated with purified proteins (16).

MicroRNAs (miRNAs) are evolutionarily conserved, small RNA molecules that suppress the expression of protein-coding genes by translational repression, mRNA degradation, or both (26, 27). More than 2000 miRNAs are currently reported in the human genome (28). A single miRNA can regulate the expression of tens or hundreds of mRNAs or proteins within a cell. miRNAs mediate their regulatory action through imperfect binding to the 3′ untranslated region (UTR) of target mRNAs carrying the complementary sites (29). Each mRNA can be targeted by one or multiple miRNAs, and it is predicted that >60% of protein-coding genes undergo direct miRNA regulation (30). Thus, miRNAs play an important role in diverse biologic processes such as development,

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Abbreviations used in this article: CDC, complement-dependent cytotoxicity; Ct, threshold cycle; HIS, heat-inactivated serum; MAC, complement membrane attack complex; miRNA, microRNA; NHS, normal human serum; RQ, relative quantity; UTR, untranslated region.

cell proliferation, differentiation, apoptosis, fat metabolism, and oncogenesis (31, 32). A number of miRNAs, called oncomirs, which appear to function as oncogenes or tumor suppressors, have been identified (33).

In this study, we examined the hypothesis that miRNAs that affect mortalin expression can consequently regulate programmed necrotic cell death activated by the complement MAC. Although predicted binding sites for miR-200 (b and c) and miR-217 were identified in the 3′UTR of mortalin mRNA (TargetScanHuman [www.targetscan.org]; miRanda [http://www.microrna.org/]; DIANA-microT-CDS [http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index]), no targeting has yet been reported. In our study, we show that these three miRNAs regulate mortalin expression and activity and, as a result, regulate the quantity of MACs deposited on target cells during complement activation, and thus contribute to cell resistance to CDC.

Materials and Methods

Cells, sera, and Abs

Cells were cultured in RPMI 1640 (Sigma-Aldrich, Rehovot, Israel) (K562 and Raji) or DMEM (HEK293T) supplemented with 10% (v/v) heat-inactivated FBS (Life Technologies, Grand Island, NY), 1% glutamine, 2% pyruvate, 100 μg/ml penicillin, and 400 μg/ml streptomycin (Bio-Lab, Jerusalem, Israel) at 37˚C and 5% CO_2. Following approval by the Ethics Committee of Tel Aviv University, peripheral blood was drawn from healthy individuals. Normal human serum (NHS) was prepared from the blood and served as the source for the complement proteins. Heat-inactivated serum (HIS) was prepared by heating NHS at 56˚C for 45 min. NHS and HIS were kept frozen at −70˚C in small aliquots. A polyclonal antiserum directed to K562 cells was prepared in rabbits, and anti-human C9 antiserum was prepared in goats. Mouse anti-mortalin Abs were purchased from StressMarq (Victoria, Canada), and mouse anti-actin Abs were purchased from Millipore (Billerica, MA). Peroxidase-conjugated goat anti-mouse IgG, peroxidase-conjugated rabbit anti-goat IgG, and FITC-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Mouse mAb directed to a neoepitope in human C5b-9 (clone aE11) was purchased from Hycult Biotech (Uden, The Netherlands). Mouse anti-human CD46, anti-human CD55, and anti-human CD59 mAbs were purchased from AbD Serotec (Oxford, U.K.).

Analysis of cell death and protein expression by flow cytometry

Cells were treated with diluted anti-K562 Abs for 30 min at 4˚C and then with complement (NHS or HIS, 50%) for 60 min at 37˚C. Percentage of cell death was determined microscopically by trypan blue inclusion or after staining with propidium iodide by flow cytometry (BD Biosciences, Franklin Lakes, NJ).

To measure C5b-9/MAC deposition, cells were incubated with a sublytic dose of anti-K562 Abs (yielding 10–20% dead cells) and then with NHS or HIS for 10 min at 37˚C. The cells were washed with PBS and treated with mouse anti-neo C5b-9 (clone aE11) Abs for 30 min at 4˚C. The cells were washed again, treated with FITC-conjugated secondary Abs, and analyzed by flow cytometry. The level of fluorescence (in 10,000 cells) was analyzed with the Cyflogic 1.2.1 data analysis software, and the mean fluorescence intensity (G-mean) values were determined.

To quantify complement regulator expression, cells were treated with mouse anti-CD46, anti-CD55, or anti-CD59 Abs for 30 min at 4˚C and then with FITC-conjugated secondary Abs for 30 min at 4˚C. The cells were then analyzed by flow cytometry, as described above.

Protein analysis by Western blotting

For collection of proteins secreted from cell undergoing a complement attack, cells were treated with a sublytic dose of Abs and NHS or HIS for 10 min at 37˚C. Then they were washed and suspended in HBSS (Sigma-Aldrich) and incubated at 37˚C. After 20 min, the cells were removed by centrifugation, and cell supernatants were further subjected to centrifugation at 5000 × *g*. The cleared supernatants were collected and kept frozen at −70˚C until tested.

Cell lysates were prepared by incubating 5 min at 95˚C in sample buffer. The lysates and supernatants were subjected to SDS-PAGE under reducing conditions (150 mM DTT) in a 10% acrylamide gel and then transferred onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). The membrane was blocked with 5% skim milk (Tnuva, Rehovot, Israel) in TBS containing 0.05% Tween 20 (TBST) for 1 h at room temperature. The membrane was treated with mouse anti-mortalin Abs or goat anti-C9 Abs and then with peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated rabbit anti-goat IgG, respectively. Bands were developed with an ECL reagent (Pierce, Rockford, IL) and exposed to a SuperRX film (Fuji, Tokyo, Japan). Results were quantified by densitometry scanning in OD units using ImageJ software.

Plasmids, miRNA inhibitors, and transient transfection

Expression plasmids for miR-200c, miR-217, and control plasmid were provided by R. Agami (The Netherlands Cancer Institute). The control plasmid contains 211 nt of noncoding RNA. miR-200c and miR-200b sequences are almost identical and differ in 2 nt outside the seed region. Overexpression of miR-200c is expected to affect both miR-200c and miR-200b targets. Therefore, cells transfected with miR-200c plasmid will be presented as miR-200b,c overexpressors (miR-200-b/c-OE). miRNA inhibitors specific to miR-200b, miR-200c, or miR-217 and a nonspecific oligonucleotide (a negative control) were purchased from Exiqon (Vedbaek, Denmark). The nonspecific oligonucleotide was designed to have no expected microRNA targets in miRBase. Cells were suspended in electroporation buffer (20 mM PIPES, 128 mM glutamate, 10 μM calcium acetate, 2 mM magnesium acetate in RPMI 1640) and mixed with the plasmid or inhibitor, as specified. The cells were subjected to electroporation (250 mV, 1500 μF, 14 ms) in ECM-830 (BTX Harvard Apparatus, Holliston, MA) in 1-mm electroporation cuvette and then suspended in culture medium and incubated at 37˚C. The control plasmid and inhibitor gave a minimal effect (0–10%) on cells relative to sham transfection.

RNA extraction, RT-PCR, and real-time PCR

Total RNA was extracted from cells using TRI reagent (Sigma-Aldrich). The final RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, Wilmington, DE). First-strand cDNA was synthesized from total RNA using MultiScribe reverse-transcriptase reaction with a TaqMan miRNA reverse-transcription kit (Applied Biosystems, Foster City, CA). This reaction contains a specific stem–loop primer for each mature target miRNA. Each stem–loop primer is designed to hybridize to only the fully mature miRNA, and not to precursor forms of its target. PCR amplification was carried out using a Step One Plus Real-Time PCR System (Applied Biosystems) under the following thermal cycler conditions: 2 min at 50˚C, 10 min at 95˚C, and 40 cycles of 15 s at 95˚C and 1 min at 60˚C. Results were analyzed with the SDS software (Applied Biosystems) and the relative quantity (RQ) Manager Software for automated data analysis. miRNA relative levels were calculated based on the comparative threshold cycle (Ct) method. The Ct for each miRNA and endogenous control U6 snRNA in each sample were used to create ΔCt values (CtmiRNA − CtU6 snRNA). Next, ΔΔCt values were calculated by subtracting the ΔCt of the control group from the ΔCt value of the tested group. The RQs were calculated using the following equation: $RQ = 2^{-\Delta\Delta ct}$. For detection of mortalin mRNA expression, HPRT gene was used as endogenous control.

Dual-luciferase reporter assay

A fragment of mortalin 3′UTR that includes the relevant miRNA binding sites was cloned into psiCHECK-2 plasmid (Promega, Madison, WI) downstream to the *Renilla* luciferase reporter gene, into XhoI–NotI restriction sites. Firefly luciferase reporter that is part of psiCHECK-2 plasmid served as an internal control (under a different promoter). The 3′UTR fragment was PCR amplified from human genomic DNA, and XhoI–NotI restriction sites were added. Primers used for this purpose were as follows: forward, 5′-GACGCTGACACCTCGAGGGAGTGAAGAGACTTCCTGAGCAGAA-3′; reverse, 5′-AAGTTTAAGCGGCCGCGCTAGAAAAGTATGCCTTAGGGAAGTTAAAG-3′.

As a control for specific binding of selected miRNA to its binding sites in relevant 3′UTR, mutation for miRNA seed sites was performed. The mutagenesis was carried out by PCR of the plasmid using the enzyme Phusion DNA Polymerase with GC buffer (Thermo Scientific). The primers used for mutagenesis and their reverse complement primers are as follows: 5′-CAGAAATGGGCGAACTTCAGTCTTTTTACTGTGTTTTTGCAACGCTCTATATATAATTTCCTTAATTTGTAAA-3′ and 5′-TTTACAAATTAAGGAAATTATATATAGAGCGTTGCAAAAACACAGTAAAAAGACTGAAGTTCGCCCATTTCTG-3′ for miR-200 (b + c) binding site; and 5′-CAGAAATGGGCGAACTTCAGTCTTTTTACTGTGTTTTTATGATATTCTATATATAATTTCCTTAATTTGTAAATTTAGTGA-3′ and 5′-TCACTAAATTTACAAATTAAGGAAATTATATATAGAATATCATAAAAACACAGTAAAAAGACTGAAGTTCGCCCATTTCTG-3′ for miR-217 binding site. Mutated plasmid was sequenced to verify that the desired mutagenesis was achieved and no additional unexpected mutations were created.

HEK293T cells were seeded in 24-well plates in DMEM. The cells were transfected 24 h after seeding with 485 ng miRVec containing the desired premiRNA and 5 ng psiCHECK-2 containing the desired 3′UTR with or without site-directed mutations. The transfection was done using the Lipofectamine RNAiMAX Transfection Reagent (Invitrogen), according to the manufacturer's protocol. Firefly and *Renilla* luciferase activities were measured 48 h after transfection using the Dual-Luciferase Reporter Assay System kit (Promega) and a Veritas microplate luminometer (Promega), according to Promega's protocol.

Confocal microscopy

K562 cells were transfected with miR-200b/c or a control plasmid. Twenty-four hours posttransfection, cells were incubated with a sublytic dose of Ab for 30 min at 4˚C, followed by NHS or HIS for 10 min at 37˚C. Then the cells were washed and fixed with 2% paraformaldehyde, permeabilized with saponin, and stained with mouse anti-mortalin mAb for 60 min at 4˚C. Next, a secondary goat anti-mouse Ab conjugated to Alexa Fluor 488 was added for 60 min at 4˚C. Cells were imaged under a Leica TCS SP5 II confocal microscope. The largest diameter focal plane of a cell was captured, and all cells were imaged under the same scaling of exposure, applying the same imaging parameters. Quantification of mortalin mean fluorescence intensity at the plasma membrane region and in the whole cell was measured by using the Image J software.

Metabolic labeling of mortalin

K562 cells transfected with a miR-200b/c plasmid or a control plasmid were kept at 37˚C for 2 h in RPMI 1640 lacking methionine and cysteine. Then, ^{35}S-labeled methionine/cysteine (100 μCi) was added to the medium, and the cells were incubated for an additional 1 h at 37˚C. Cells were washed and extracted with lysis buffer (100 mM Tris base, 10 mM EDTA disodium salt, 1% Triton, protease inhibitor mixture). The cells were incubated with mouse anti-mortalin Ab or mouse IgG as control for 4 h at 4˚C. Next, protein A-Sepharose was added into the lysates for 2 h at 4˚C. After washing of the Sepharose beads, they were treated with sample buffer for 5 min at 95˚C and the eluted proteins were analyzed by SDS-PAGE. After electrophoresis, the acrylamine gel was treated with acetic acid and methanol for 15 min at room temperature and dried under vacuum at 80˚C for 30 min. The dried gel was exposed to a phosphor screen (Molecular Dynamic) and analyzed in phosphorplate reader Typhoon 8600 (Amersham Biosciences).

Statistical analysis

Statistical significance was analyzed by using the two-sided unpaired Student *t* test and was assumed when $p < 0.05$. Results are expressed as arithmetic mean ± SD.

Results

Regulation of mortalin mRNA and protein by miR-200b, miR-200c, and miR-217

Using the dual-luciferase reporter assay described above, binding of miR-200b/c and miR-217 to the 3′UTR of mortalin mRNA was confirmed. Expression of the wild-type mortalin mRNA sequence in HEK293 cells, but not that of a mutated mortalin sequence, was largely reduced by transfection of miR-200b/c or miR-217 vectors (Fig. 1A). The role of mortalin in resistance to CDC was characterized with K562 cells (16, 17, 34). Therefore, K562 cells were transfected with miR-200b/c or miR-217 plasmids or a control plasmid, and mortalin mRNA level of expression was quantified. RNA samples extracted from the cells were reverse transcribed into cDNA, and mortalin DNA level was determined by real-time PCR. As shown in Fig. 1B, 3 h after transfection with miR-200b/c or miR-217, mortalin mRNA level was significantly reduced. After 24 h, mortalin mRNA levels returned almost to normal. The effect of miR-200b, miR-200c, or miR-217 inhibitors on mortalin mRNA expression in K562 cells was also examined (Fig. 1C). RNA samples were extracted from the cells 2, 4, and 6 h after transfection and reverse transcribed into cDNA. Two hours after transfection of miR-200b, miR-200c, or miR-217 inhibitors, expression of mortalin mRNA was elevated. After 4 and 6 h, mortalin mRNA level began to decline to normal level and even below normal level.

In contrast to their effect on mortalin mRNA expression, the miRNA plasmids and inhibitors had a minimal effect on total mortalin protein expression in the cells (Supplemental Figs. 1, 2). We, therefore, hypothesized that the cells have means to maintain mortalin protein level through protection of the mortalin translation process. This was verified by measuring the rate of mortalin metabolic labeling as described under *Materials and Methods*. K562 cells transfected with a miR-200b/c plasmid or a control plasmid were labeled with ^{35}S-methionine/cysteine, and mortalin was immunoprecipitated with anti-mortalin Abs and protein A-Sepharose. The quantity of metabolically labeled mortalin in cells is shown in Fig. 1D. The level of mortalin mRNA was quantified in the same cells by real-time PCR (Fig. 1E). As can be seen, synthesis of new mortalin protein was only slightly affected by transfection with miR-200b/c plasmid. In fact, after correction for the reduction in mortalin mRNA level, significantly more mortalin protein was synthesized per mRNA in cells overexpressing miR-200b/c (Fig. 1F).

Cell resistance to complement is regulated by miR-200b/c and miR-217

The effect of miR-200b/c and miR-217 on sensitivity to complement-mediated cell death was examined. K562 cells were transfected with miR-200b/c or miR-217 plasmids or control plasmid. After 3 and 24 h, cells were collected and incubated with Ab and complement, and cell death was determined. As shown, overexpression of miR-200b/c or miR-217 markedly reduced cell sensitivity to complement-mediated cell death both at 3 and 24 h posttransfection (Figs. 2A, 3A).

Next, K562 cells were transfected with miRNA inhibitors specific for miR-200b, miR-200c, and miR-217, or with nonspecific oligonucleotide as negative control. After 24 h, the cells were incubated with Ab and complement, and cell death was measured. As shown in Figs. 2B and 3B, inhibition of miR-200c or miR-217 (but not of miR-200b) enhanced cell sensitivity to complement-dependent cytotoxicity.

Regulation of C5b-9 deposition

The capability of the miRNAs to control cell resistance to complement-dependent cytotoxicity raises the possibility that these miRNAs regulate C3b and C5b-9 deposition on the target cells. K562 cells were transfected with miR-200b/c plasmid (Fig. 2C, 2D) or miR-217 (Fig. 3C, 3D) or control plasmid or with miRNA inhibitors specific for miR-200b, miR-200c (Fig. 2E), and miR-217 (Fig. 3E), or with a nonspecific oligonucleotide as negative control. After 24 h, the cells were incubated with a sublytic dose of Ab and with complement for 10 min at 37˚C (maximal C5b-9 deposition) and labeled with the anti–C5b-9 aE-11 mAb. As shown in Fig. 2, overexpression of miR-200b/c markedly lowered C5b-9 deposition, whereas inhibition of miR-200c enhanced C5b-9 deposition. Inhibition of miR-200b or miR-217 had no effect on level of deposited C5b-9, at 24 h (Figs. 2E, 3E) and 48 h (data not shown) after transfection with a miRNA inhibitor. In addition, miR-200b/c overexpression did not affect significantly C3b deposition (Fig. 2C).

However, miR-217 modulation affected both C3b and C5b-9 deposition (Fig. 3C, 3D). K562 cells were transfected with miR-217 plasmid or control plasmid. After 24 h, the cells were incubated with a sublytic dose of Ab and with complement for 10 min at 37˚C and labeled with goat anti-C3 Ab (Fig. 3C) or with the anti–C5b-9 aE-11 mAb (Fig. 3D). As shown, miR-217 overexpression reduced C3b and C5b-9 deposition on the cells. Inhibition of miR-217 did not have a significant effect on C5b-9 deposition (Fig. 3E).

Expression of the membrane complement-regulatory proteins

Cancer cells evade complement attack by upregulation of their membrane complement-regulatory proteins CD46, CD55, and CD59 (8). To examine the impact of miRNAs on these regulatory proteins, K562 cells were transfected either with miR-200 (b or c)

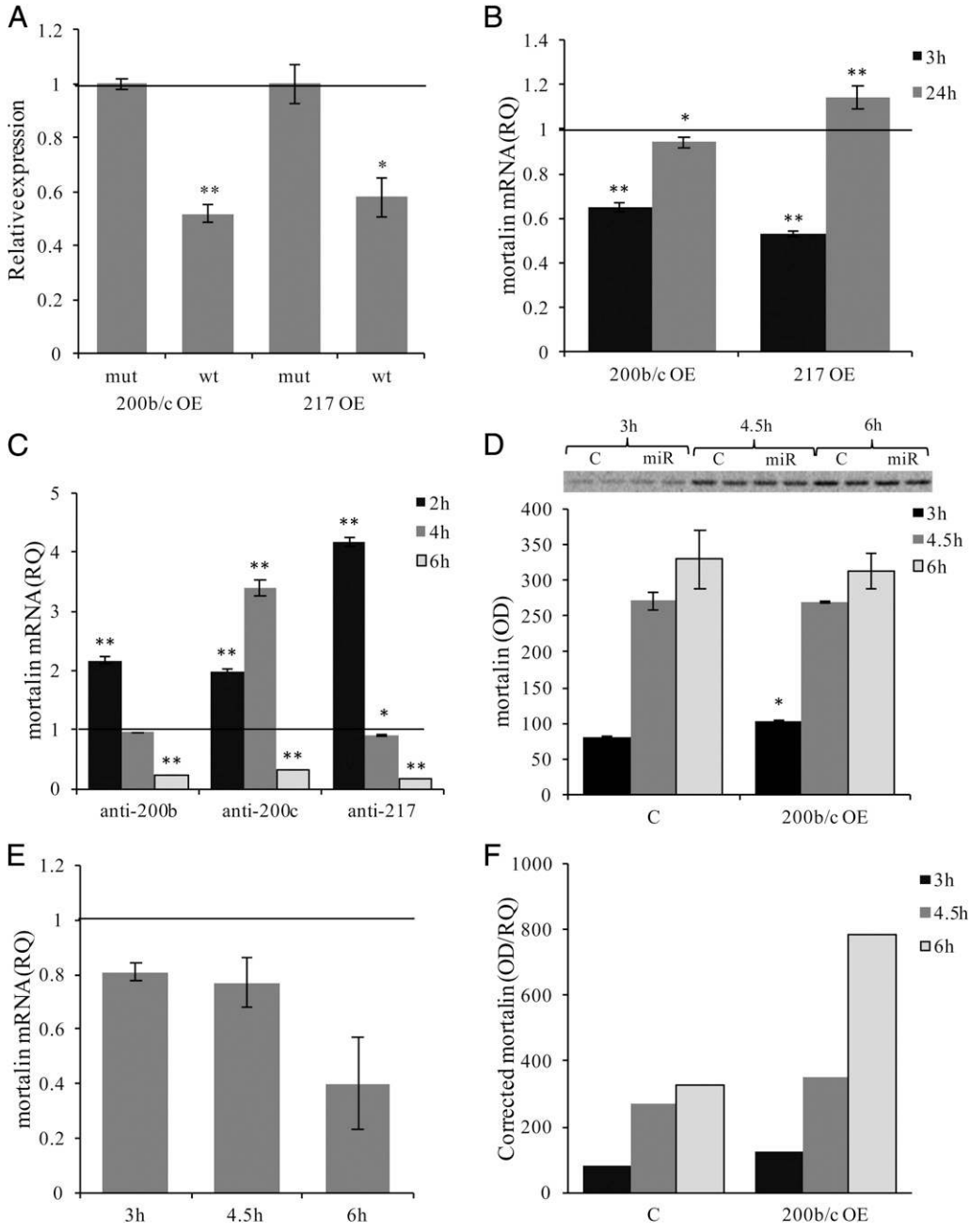

FIGURE 1. miR-200 (b and c) and miR-217 regulate mortalin mRNA expression. (**A**) HEK293T cells were transfected with miRVec containing premiRNA (miR-200b/c or miR-217) and psiCHECK-2 containing the wild-type (wt) or mutated (mut) mortalin 3′UTR. Luciferase luminescence results (arithmetic mean ± SD) are shown in relative luciferase expression units for cells transfected with wild-type UTR and miRVec compared with cells transfected with mutated UTR and miRVec. $*p < 0.05$, $**p < 0.01$ relative to mutated UTR. (**B**) K562 cells were transfected with miR-200b/c, miR-217, or a control plasmid. RNA samples extracted from the cells 3 and 24 h after treatment were reverse transcribed and subjected to quantitative real-time PCR. Expression level of mortalin mRNA is shown in RQ units of cells transfected with miR-200b/c or miR-217, compared with control plasmid (RQ = 1). $*p < 0.05$, $**p < 0.01$ relative to control. (**C**) K562 cells were transfected with miRNA inhibitors specific to miR-200b, miR-200c, or miR-217 or with a nonspecific oligonucleotide as negative control. RNA samples were extracted from the cells 2, 4, and 6 h after treatment, reverse transcribed, and subjected to quantitative RT-PCR. Expression level is shown in RQ units of cells transfected with inhibitors of miR-200b, miR-200c, or miR-217, compared with control (RQ = 1). $*p < 0.05$, $**p < 0.01$ relative to control. (**D**) K562 cells transfected with a miR-200b/c plasmid or a control plasmid were metabolically labeled with ^{35}S-labeled methionine/cysteine, as explained under *Materials and Methods*. Mortalin was immunoprecipitated from cell lysates and analyzed by SDS-PAGE on acrylamine gel. The dried gel was exposed to a phosphor screen, and the OD of mortalin bands was quantified in a phosphorplate reader. A representative gel is shown above the figure presenting the quantified OD (mean ± SD). $*p < 0.05$ relative to control. (**E**) K562 cells were pretreated as in (D), by transfection with miR-200b/c or a control plasmid, and RNA was extracted at 3, 4.5, and 6 h after treatment and reverse transcribed. Quantitative RT-PCR data of mortalin expression level are shown in RQ units of cells transfected with miR-200b/c compared with control plasmid (RQ = 1). (**F**) Mortalin protein synthesis (D) was normalized by mortalin mRNA expression (E) (OD/RQ).

and miR-217 inhibitors or with the miRNA plasmids. Transfection with inhibitors of miR-200b, miR-200c, or miR-217 for 48 h had a minimal effect on CD46, CD55, or CD59 expression (Supplemental Fig. 3). After transfection for 24 h with miR-200b/c (Fig. 2F) or miR-217 (Fig. 3F) plasmids, the levels of expression of CD46, CD55, and CD59 on the cells were measured. As shown, overexpression of miR-200b/c resulted in enhanced expression of CD46 and CD55, whereas overexpression of miR-217 significantly

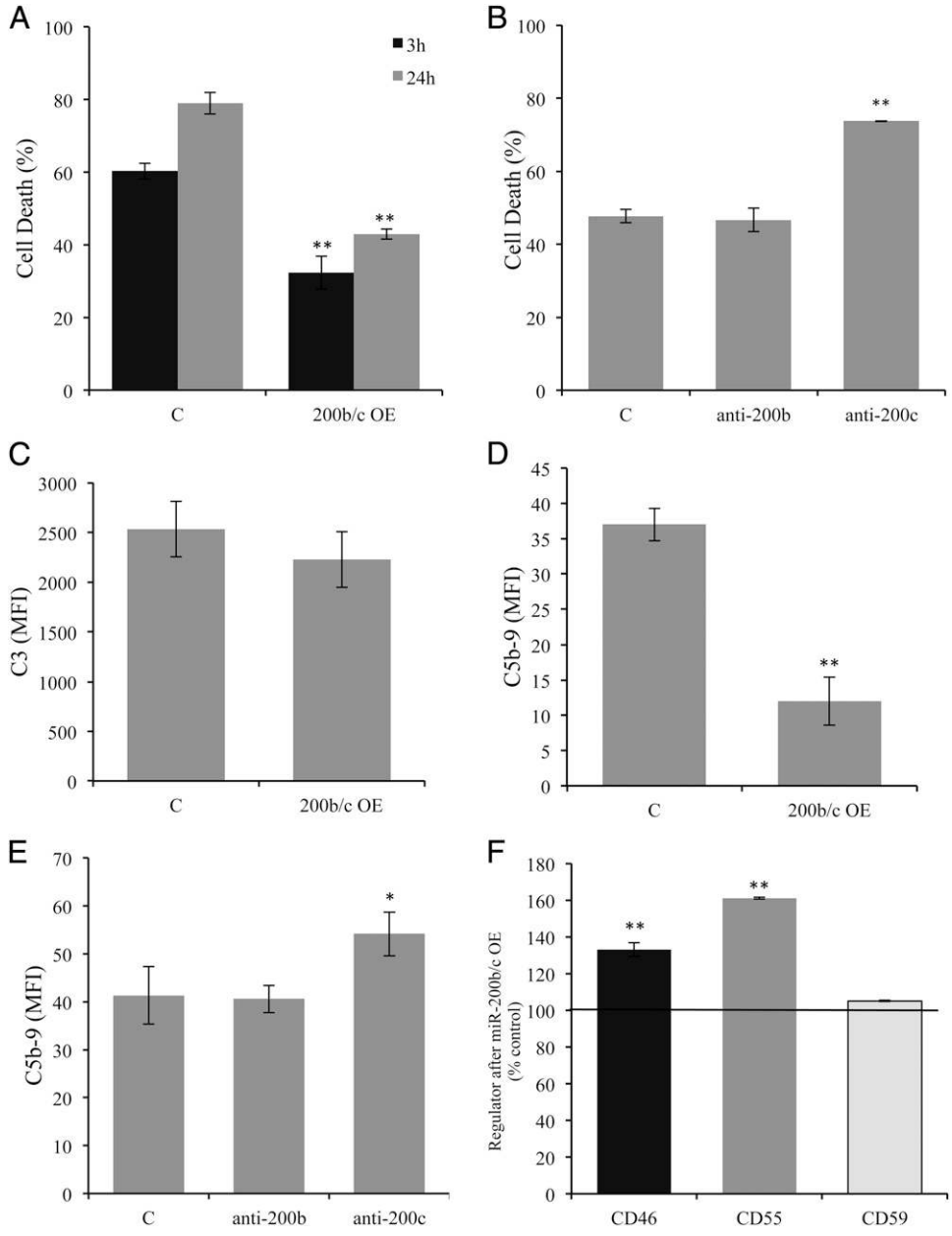

FIGURE 2. miR-200 (b and c) regulates cell sensitivity to complement, C5b-9 deposition, and CD46 and CD55 expression. (**A**) K562 cells were transfected with miR-200b/c or control plasmid. After 3 or 24 h, cells were treated with Ab and NHS. Cell death (%) was measured by trypan blue inclusion. (**B**) K562 cells were transfected with miRNA inhibitors specific for miR-200b and miR-200c, or with nonspecific oligonucleotide as negative control. After 24 h, cells were treated with Ab and complement, as above. Cell death (%) was measured by propidium iodide inclusion. (**C**) K562 cells transfected with miR-200b/c or a control plasmid for 24 h were treated with a sublytic dose of Ab and then with NHS for 10 min at 37°C. Then they were labeled with goat anti-C3 Ab and FITC-conjugated secondary Ab and analyzed by flow cytometry. Mean fluorescence intensity (MFI) values of cell-bound C3b, representative of three independent experiments, are shown. (**D**) K562 cells transfected with miR-200b/c or a control plasmid for 24 h were treated with sublytic complement, as in (C). Then they were labeled with mouse anti–C5b-9 aE-11 Ab and FITC-conjugated secondary Ab and analyzed by flow cytometry. MFI values of cell-bound C5b-9, representative of three independent experiments, are shown. (**E**) K562 cells transfected for 24 h with inhibitors of miR-200b and miR-200c, or with nonspecific oligonucleotide, were incubated with sublytic Ab and NHS for 10 min at 37°C. The cells were labeled with the aE-11 Ab, as above. MFI values of cell-bound C5b-9, representative of three independent experiments, are shown. (**F**) K562 cells were transfected with miR-200b/c or control plasmid for 24 h and were labeled with mouse anti-CD46, anti-CD55, or anti-CD59 Ab and then with FITC-conjugated secondary Ab. Cells were then analyzed by flow cytometry, and MFI values were determined. Results, representative of three independent experiments, present each regulator's level relative to its expression in control cells (set as 100%). $*p < 0.05$, $**p < 0.01$ relative to control.

increased only CD46 expression. However, overexpression of miR-200b/c or miR-217 had no effect on CD59 expression.

miR-200b/c regulates complement-induced mortalin trafficking to the plasma membrane and removal of C9

One of the early events occurring in K562 cells following membrane insertion of C5b-9 complexes is the release of mortalin and C5b-9 out of the cells. This was proposed to be an essential step in the mortalin-dependent cell resistance against CDC. The impact of miR-200b/c on mortalin translocation to the plasma membrane was examined. K562 cells were transfected with miR-200b/c or a control plasmid. After 24 h, the cells were incubated with a sublytic dose of Ab and with NHS or HIS for 10 min at 37°C. Then the cells were fixed, permeabilized, and labeled with mouse anti-mortalin mAb.

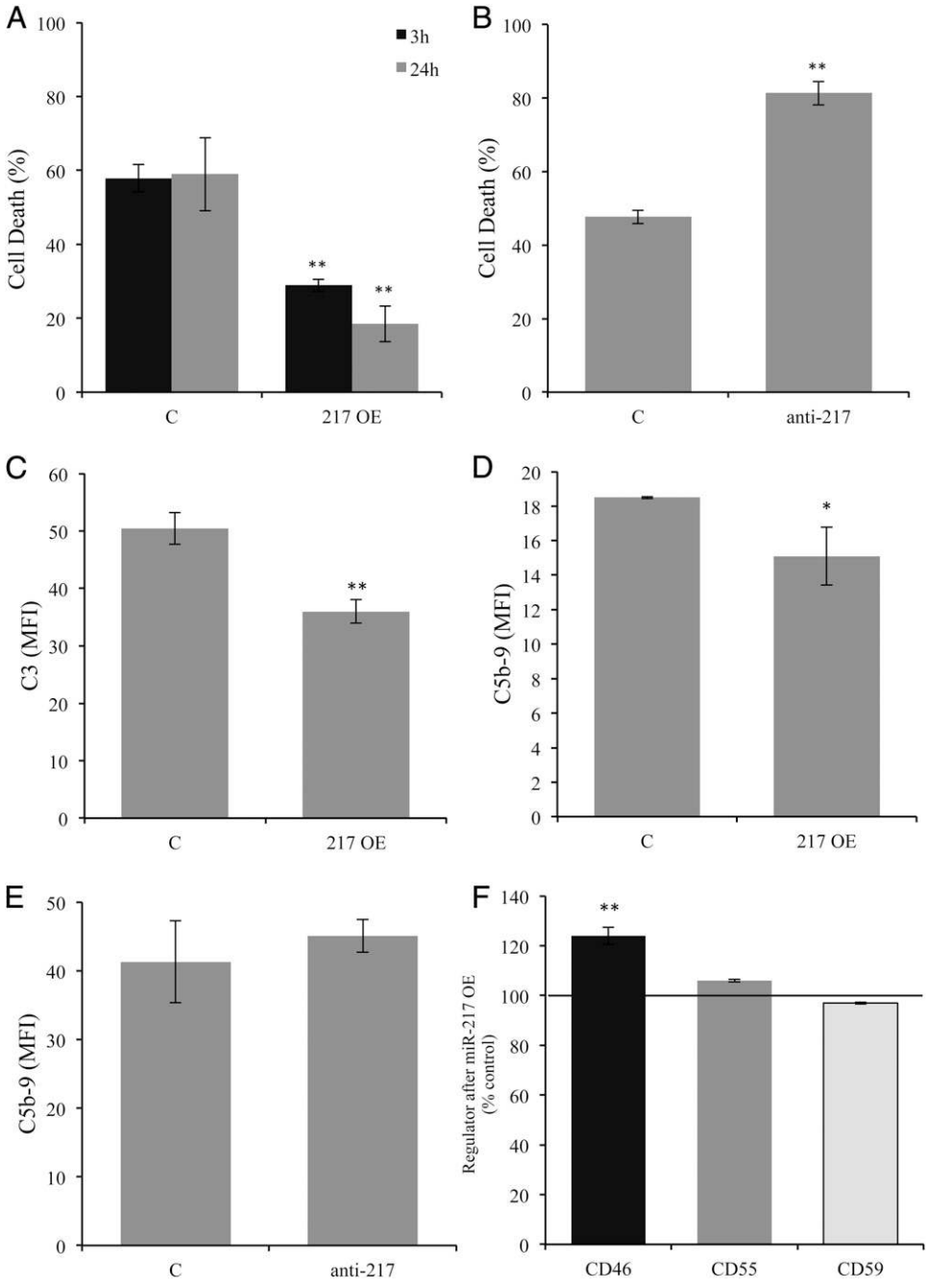

FIGURE 3. miR-217 regulates cell sensitivity to complement, C3 and C5b-9 deposition, and CD46 expression. (**A**) K562 cells were transfected with miR-217 or control plasmid. After 3 or 24 h, cells were treated with Ab and NHS (60 min). Cell death (%) was measured by trypan blue inclusion. (**B**) K562 cells were transfected with miRNA inhibitors specific for miR-217 or with nonspecific oligonucleotide as negative control. After 24 h, cells were treated with Ab and complement, as above. Cell death (%) was measured by propidium iodide inclusion. (**C** and **D**) K562 cells transfected for 24 h with miR-217 or control plasmid were incubated with a sublytic dose of Ab and NHS for 10 min at 37˚C. Cells were labeled with goat anti-C3b Ab and FITC-conjugated secondary Ab (C) or mouse anti–C5b-9 aE-11 Ab and FITC-conjugated secondary Ab (D) and analyzed by flow cytometry. Mean fluorescence intensity (MFI) values of cell-bound C3b or C5b-9, representative of three independent experiments, are shown. (**E**) K562 cells transfected for 24 h with miR-217 inhibitor or with nonspecific oligonucleotide (C) were incubated with a sublytic dose of Ab and NHS for 10 min at 37˚C. The cells were labeled with aE-11 Ab and FITC-conjugated secondary Ab, and MFI values of cell-bound C5b-9 were determined. Results are representative of three independent experiments. (**F**) K562 cells were transfected with miR-217 or control plasmid for 24 h and were then labeled with mouse anti-CD46, anti-CD55, or anti-CD59 Ab and FITC-conjugated secondary Ab. Cells were then analyzed by flow cytometry, and MFI values were determined. Results, representative of three independent experiments, present each regulator's level relative to its expression in control cells (set as 100%). $*p < 0.05$, $**p < 0.01$ relative to control.

As shown in Fig. 4, confocal microscope images (Fig. 4A) and image analysis by Image J (Fig. 4B), overexpression of miR-200b/c enhanced complement-induced translocation of mortalin to the plasma membrane region.

K562 cells can remove the C5b-9 complexes from their surface by exovesiculation (16). The possible regulation of the C5b-9 removal process by miRNAs was next examined. K562 cells were transfected with miR-200b/c or miR-217 plasmids or a control plasmid. After 24 h, cells were incubated with a sublytic dose of Ab and with complement for 10 min at 37˚C. The cells were washed and incubated in HBSS buffer for 20 min at 37˚C and subjected to centrifugation. The supernatants were analyzed by Western

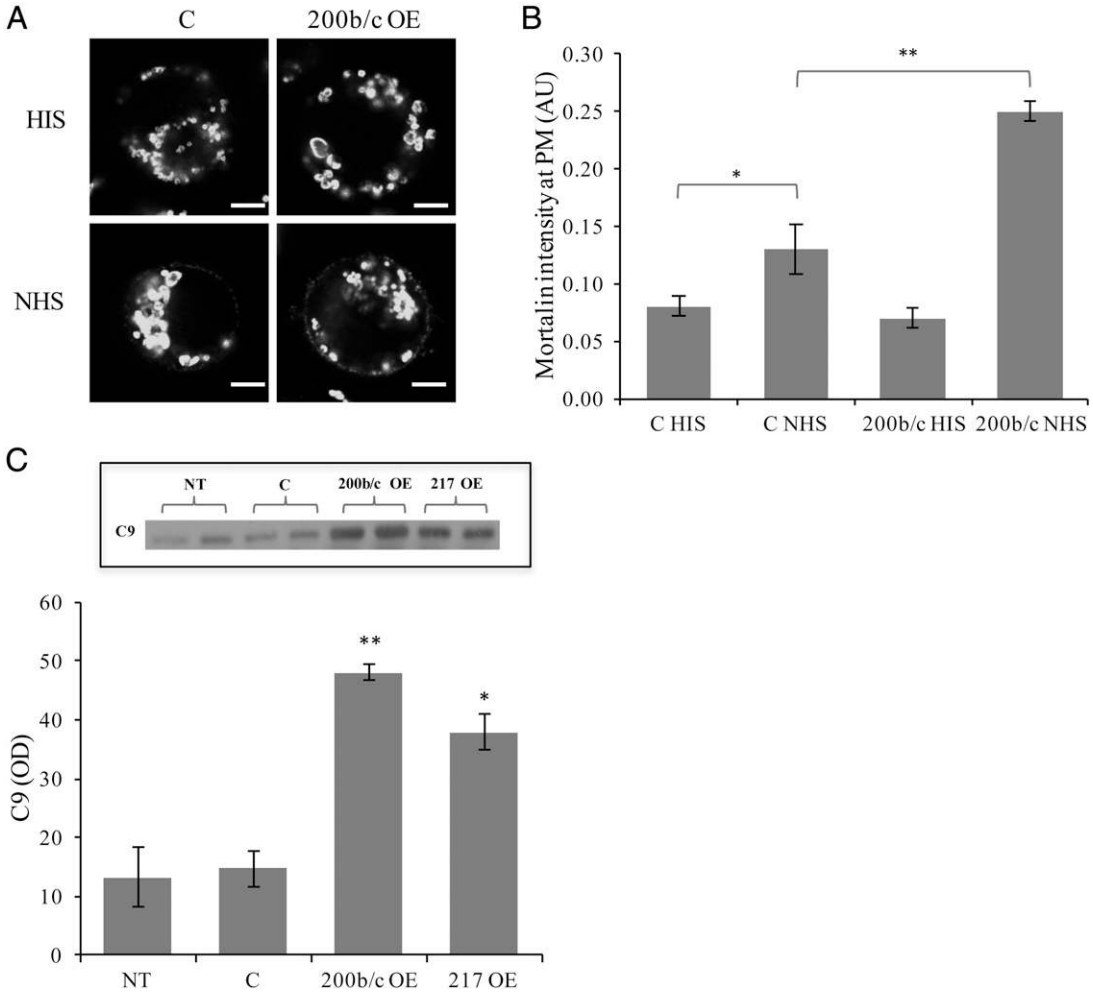

FIGURE 4. miR-200b/c facilitates complement-induced mortalin translocation to the plasma membrane and C9 removal. (**A** and **B**) K562 cells transfected with miR-200b/c or a control plasmid for 24 h were treated with a sublytic dose of Ab and NHS or HIS for 10 min at 37˚C. Then the cells were fixed, permeabilized, and labeled with mouse anti-mortalin mAb and a secondary goat anti-mouse IgG Ab conjugated to Alexa Fluor 488 and analyzed under a confocal microscope. Representative cells are shown in (A). Bar indicates 5 μM. (B) Quantification of mortalin fluorescence signal at the plasma membrane (PM) region (normalized over the whole cell fluorescence) was performed with the Image J software on 40 cells and is presented in arbitrary units (AU) ± SE. Results are representative of three independent experiments. *$p < 0.05$ compared with control HIS, **$p < 0.01$ compared with control NHS. (**C**) K562 cells transfected with miR-200b/c, miR-217, or a control plasmid (C) for 24 h or nontreated cells (NT) were incubated with a sublytic dose of Ab and with NHS for 10 min at 37˚C. The cells were washed and incubated in HBSS for 20 min at 37˚C. Next, the cells were removed by centrifugation, and the supernatants were collected and analyzed by Western blotting with anti-C9 Ab. A representative blot is shown. *Below*, C9 band densities quantified by densitometry scanning and shown in OD units. Results are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$ relative to control.

blotting with anti-C9 Ab. As shown in Fig. 4C, overexpression of miR-200b/c and miR-217 increased release of C9 out of the cells.

Exposure to a sublytic dose of complement enhances miR-200b/c and mortalin expression

To examine the effect of complement activation on miRNA expression, K562 or Raji cells were incubated with a sublytic dose of Ab and complement for 1 h and then kept at 37˚C. RNA was extracted from the cells at different times and reverse transcribed into cDNA. Quantitative real-time PCR was performed on the DNA samples using the ΔΔCT method. Expression level of miR-200b (Fig. 5A) and miR-200c (Fig. 5B) in K562 or Raji cells was quantified. As shown, 1 h after complement activation, expression of both miR-200b and miR-200c was markedly elevated in K562 (1.7- and 6.6-fold enhancement, respectively) and Raji cell (5.6- and 4.8-fold enhancement, respectively) relative to control cells. The levels of miRNAs decreased over time, but miR-200b remained, even after 24 h, higher in complement-treated cells than control K562 and Raji cells. In addition, mortalin protein expression was analyzed after complement activation (Fig. 5C). As shown, mortalin expression was significantly elevated 24 h after exposure to sublytic complement, compared with cells exposed to HIS. Cells treated with HIS had similar miR-200 and mortalin levels as untreated cells.

Discussion

miRNAs are known to regulate numerous, essential cell functions and to be involved in pathogenesis (35, 36). To our knowledge, the data shown in this work demonstrate for the first time that miRNAs regulate cell sensitivity to Ab-based complement-dependent necrotic cell death. Based on their predicted impact on mortalin expression level, we focused on miR-200 (b and c) and miR-217. Overexpression of miR-200b/c or miR-217 confers on K562 cells resistance to complement, whereas inhibition of these miRNAs enhances their sensitivity to CDC. Cell lysis by complement requires the assembly of the C5b-9 (MAC) complex. Indeed, overexpression of miR-200b/c is shown to reduce the amount of

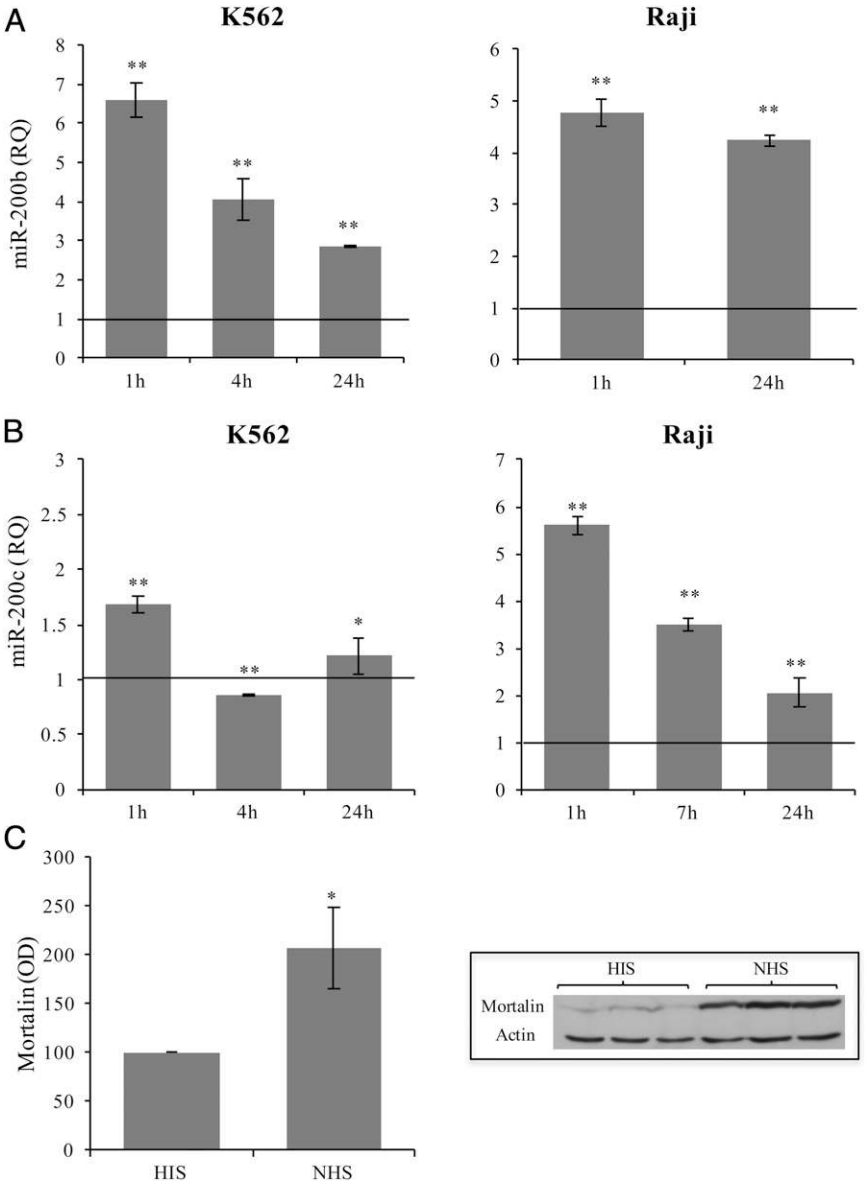

FIGURE 5. Exposure to a sublytic dose of complement enhances expression of miR-200b, miR-200c, and mortalin. K562 or Raji cells were incubated with a sublytic dose of Ab for 30 min at 4°C and then with HIS or NHS for 60 min at 37°C. RNA samples were extracted from the cells, reverse transcribed, and subjected to quantitative RT-PCR. Expression levels of miR-200b (**A**) and miR-200c (**B**) in cells treated with NHS were plotted relative to cells treated with HIS (RQ = 1, line drawn). (**C**) K562 cell lysates were prepared 24 h after treatment with Ab and HIS or NHS and analyzed by Western blotting with anti-mortalin or anti-actin Ab. Mortalin and actin band densities were quantified by densitometry scanning and shown in OD units. Mortalin band density was normalized for actin level. Combined results of three independent experiments are presented. A representative blot is also shown. $*p < 0.05$, $**p < 0.01$ relative to HIS.

C5b-9 deposited on the cells, and its inhibition enhances C5b-9 deposition (Fig. 2D, 2E). Moreover, overexpression of miR-217 is shown to reduce the amount of C3 and C5b-9 deposited on the cells (Fig. 3C, 3D). However, miR-200b/c overexpression had no significant effect on C3 deposition on the cells' membrane (Fig. 2C). For protection, cells can eliminate the MAC from their surface by endocytosis and exovesiculation (6, 13). MAC removal by exovesiculation depends on mortalin (16). Interestingly, release of C5b-9 and mortalin from cells subjected to complement attack is elevated in cells overexpressing miR-200b/c and miR-217 (Fig. 4C). Therefore, apparently, these miRNAs are involved in mortalin-dependent regulation of MAC elimination. The mechanism of action of mortalin in MAC removal is still not understood, yet within minutes after MAC formation on K562 cells, mortalin translocation from the mitochondria to the plasma membrane is evident. This process is amplified in cells overexpressing miR-200b/c (Fig. 4).

miR-200b, miR-200c, and miR-217 were studied because of their predicted targeting of mortalin. This was confirmed by running a luciferase assay (Fig. 1A). Overexpression of miR-200b/c or miR-217 in K562 cells is shown, as expected, to transiently reduce mortalin mRNA level, whereas inhibition of miR-200b, miR-200c, or miR-217 transiently enhances mortalin mRNA levels (Fig. 1). The foreseen subsequent effect would have been a correlation between mRNA level and protein level in the cells, that is, reduced mortalin protein level in cells having reduced mortalin mRNA. To our surprise, despite their impact on mortalin mRNA, these miRNAs did not significantly affect the overall mortalin protein level in the cells (Supplemental Figs. 1, 2). This unexpected finding led us to investigate the effect of miRNA overexpression on the rate of mortalin translation regulation. Metabolic labeling analysis demonstrated that mortalin translation is significantly elevated in K562 cells overexpressing miR-200b/c (Fig. 1D–F). Thus, despite the loss of mortalin mRNA and with fewer mortalin mRNA copies, these cells succeed to maintain a normal mortalin protein level and mortalin-dependent protection from CDC. This suggests that a drop in mortalin mRNA level constitutes a stress response leading to positive translational regulation of mortalin synthesis. Several supportive publications are indicated below.

As exception to the rule, recently accumulated evidence has shown that miRNAs may also upregulate expression of their specific targets. Some miRNAs and their associated RNA-induced silencing complexes can posttranscriptionally stimulate gene expression by direct and indirect mechanisms (37). Vasudevan et al.

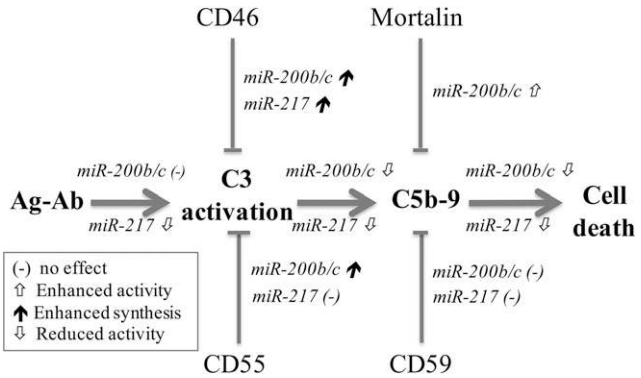

FIGURE 6. Schematic presentation of the proposed regulation of CDC by miR-200-b/c and miR-217. Three key steps in complement activation by Abs bound to cellular Ags (Ag-Ab) are shown, as follows: C3 activation and binding, C5b-9 membrane insertion, and cell death. Effects of the miRNAs on each of these activation steps and on the level of expression of mortalin and the complement membrane regulators are indicated.

(38) showed that when cells are serum starved, binding of miR-369-3 to a reporter mRNA (containing the TNF-α 3′UTR) stimulates translation. In pancreatitis, increased expression of miR-21 was found to be associated with connective tissue growth factor (CCN2) upregulation (39). Overexpression of miR-542-3p in U2OS cells enhanced p53 expression and stimulated the expression of p53 targets (40). It was also found that miR-145 mediates myocardin gene upregulation during muscle differentiation (41). Cell type and growth conditions may dictate gene expression upregulation or downregulation (42). Upregulation of mRNA transcription or translation may involve miRNA binding to 5′UTR of the mRNA (43–45). miR-122 binding was shown to increase ribosome loading of the mRNA and thus to enhance protein synthesis (45). Through which mechanism miR-200 (b and c) and miR-217 promote mortalin translation remains to be investigated.

miRNAs can potentially regulate CDC through the membrane complement-regulatory proteins CD46, CD55, and CD59. These proteins block the complement cascade at distinct stages and restrict MAC formation. Expression of CD46, CD55, and CD59 is elevated in many cancer types (12). Involvement of miRNAs in basal and abnormal membrane regulator expression awaits clarification. In K562 cells, miR-200b, miR-200c, and miR-217 inhibitors showed no effect on these regulators' expression level (Supplemental Fig. 3), indicating that the death-promoting effect of these inhibitors on CDC (Figs. 2B, 3B) cannot be explained by abnormal expression of the complement regulators. In contrast, overexpression of miR-200b/c leads to a small elevation in expression of CD46 and CD55, and overexpression of miR-217 enhances CD46 expression (Figs. 2F, 3F), which could account at least partially for the marked reduction in CDC observed after overexpression of these miRNAs in K562 cells (Figs. 2A, 3A). Apparently, CD59 expression is not regulated in K562 cells, directly or indirectly, by miR-200 (b and c) or miR-217 (Figs. 2F, 3F). A role for miR-224 in regulation of CD59 expression was recently reported in large B cell lymphoma cells (46). In breast cancer cells, miR-520 (b and e) is reportedly regulating expression of CD46, and overexpression of these miRNAs lowers CD46 expression (47).

miR-200b and miR-200c levels increase in K562 and Raji cells subjected to treatment with a sublytic dose of Ab and complement (Fig. 5). Elevated miRNA expression of 1.7- to 6.5-fold is observed within 1 h of treatment and remains above basal level even after 24 h. In addition, mortalin protein expression was elevated 24 h after treatment with sublytic complement (Fig. 5C). Overexpression of miR-200b/c enhances cell resistance to CDC (Fig. 2A). Considering the induction of resistance to CDC by pretreatment with a sublytic dose of complement (7), it is conceivable that complement-induced protection is mediated in part by the newly synthesized miR-200b/c that enhances mortalin, CD46, and CD55 activities, thus jointly promoting resistance to CDC. At the same time, activation of protein kinase C and ERK by sublytic MAC doses (48) also sets into motion mortalin-dependent release of MAC from cells attacked by complement (16).

miR-200 family members are major regulators of epithelial–mesenchymal transition (49) and are believed to be generally involved in cancer progression, metastasis, and chemoresistance (50). Downregulation of miR-200 family members was proposed to be essential for progression and invasiveness of breast (51, 52) and ovarian cancer, and its high expression is associated with improved survival of ovarian (53) and pancreatic cancer patients (54). In female reproductive cancers, *miR-200c* was reported to be a marker of aggressiveness and chemoresistance (55). Decreased miR-200b expression in lung adenocarcinoma appears to lead to resistance to docetaxel therapy and to poor prognosis (56). miR-217 was also shown to act as a tumor suppressor in pancreatic ductal adenocarcinoma (57), lung cancer (58), osteosarcoma (59), and esophageal squamous cell carcinoma (60), and accordingly its expression level is reduced in these tumors. In contrast, miR-217 was found to be overexpressed in breast cancer and to enhance tumor proliferation via promoting cell cycle progression (61). Based on the findings reported in this work, it is reasonable to assume that the aberrant expression of miR-200 family members and miR-217 in cancer is also affecting sensitivity of those cancer cells to CDC, but this remains to be determined.

In conclusion, this study demonstrates miRNA involvement in CDC regulation. We identified miR-200b, miR-200c, and miR-217 as potential regulators of mortalin, CD46, and CD55 expression in leukemia/lymphoma, and thus as regulators of cell resistance to complement (Fig. 6). Therefore, inhibition of these miRNAs, much like blocking mortalin and complement membrane regulators, may be advised as a mean to increase cancer cell sensitivity to CDC. Taking into account also the involvement of miR-200b in cancer metastasis and chemoresistance, the role of miR-200c in apoptosis regulation, and the role of miR-217 in cell senescence, it is conceivable to assume that targeting these miRNAs during cancer therapy will sensitize the cancer cells to therapy in a number of ways.

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Disclosures

The authors have no financial conflicts of interest.

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