Abstract

Aberrant microRNA (miRNA) expression has been identified in various human solid cancers. However, whether the levels of miRNA expression in tumor cells have any effect on tumor progression has not been determined. In this proof-of-concept study, the restoration of high-level expression of the miR17–92 cluster of miRNAs reveals its function as a tumor suppressor in murine solid cancer cells. Specifically, genetically engineered expression of higher levels of miR17/20a in the miR17–92 cluster in both murine breast cancer and colon cancer cells triggered natural killer (NK)–cell recognition by inhibiting the expression of MHC class I (H-2D) through the Mekk2–Mek5–Erk5 pathway. Results from the mouse tumor studies were recapitulated using samples of human solid tumors. Together, these data indicate that miR17/20a miRNAs function as tumor suppressors by reprogramming tumor cells for NK cell-mediated cytotoxicity. Cancer Immunol Res; 2(8); 789–99. ©2014 AACR.

Introduction

microRNAs (miRNA) are known to play key regulatory roles in physiologic processes, including cell differentiation, apoptosis, proliferation, metabolism, organ development, and tumor progression (1–4). Aberrant expression of miRNAs is a common feature in hematologic malignancies (5, 6) and solid cancers (7, 8), suggesting a potential role for miRNAs in cancer. It has been proposed that miRNAs function as integral parts of the molecular architecture of oncogene and tumor-suppressor networks (9). Although a small, specific subset of oncogenic miRNAs is upregulated in cancer, the expression and processing of most of these regulators are downregulated during tumorigenesis (10–12).

The miR17–92 cluster produces a single polycistronic primary transcript that is processed to yield six individual mature miRNAs including miR17, miR18a, miR19a, miR20a, miR19b, and miR92a (13). There is evidence indicating that the miR17–92 cluster inhibits tumor-cell proliferation and invasion in human solid cancers, including breast cancer (14), hepatomas (15), and gastrointestinal stromal tumors (16). However, other studies support a role for miR19a,b in promoting tumorigenesis in hematologic malignancies, including B-cell lymphomas (5, 17). The molecular mechanisms underlying the miR17–92 cluster-mediated protumorigenic or antitumorigenic effects are not fully understood.

We hypothesize that miRNAs play dual regulatory roles in cancer progression. Depending on their expression levels in tumor cells, miRNAs could be either protumorigenic or antitumorigenic. To test this hypothesis, we used a novel approach, restoring the expression levels of miRNAs in tumor cells to the same levels as those in the corresponding adjacent nontumor tissue. The outcome of this approach could provide a rationale to investigate the therapeutic potential of using miRNAs to treat human cancers.

In this study, we demonstrate that as the expression level of the miR17–92 cluster reached the level found in adjacent nontumor cells, the miR17–92 cluster serves as a tumor suppressor in the tumor cells. Specifically, we have delineated a potential mechanism by which miR17/20a functions as a tumor suppressor. It mediates the inhibition of the Mekk2-regulated pathways leading to the downregulation of MHC class I (H-2D) molecules expressed on tumor cells, and enhances the antitumor activity of natural killer (NK) cells.

Materials and Methods

Clinical specimens

Human breast and colorectal specimens (primary lesion and adjacent normal) were collected at the time of surgery. All samples were collected with written informed consent from the patients, and the experiments were performed with the approval from the Institute Research Ethics Committee of Nanjing Medical University (Nanjing, Jiangsu, China).

Reagents, cell lines, and plasmids

All cell lines were obtained from the American Type Culture Collection (ATCC). Mouse CT26 and CMT-93 colon cancer cell...
lines were maintained in RPMI-1640 (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen). Mouse 4T1 and 4T07 breast cancer cell lines were maintained in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen). All cell lines were tested regularly for potential Mycoplasma contamination using a PCR-based assay and validated to be Mycoplasma free. No other authentication assays were performed.

To generate cell lines stably expressing the miR17–92 cluster miRNAs, mouse cancer cells were seeded into 6-well plates, cultured overnight, and then transfected with the following expression vectors: MSCV-Pig-miR-Ctrl, MSCV-Pig-miR17–92 WT, MSCV-ΔPig-miR17/20a, MSCV-ΔPig-miR18a, MSCV-ΔPig-miR19a,b, and MSCV-ΔPig-miR92a. (MSCV-Pig is MSCV-Puro-IRES-GFP; kindly provided by Dr. Andrea Ventura, Memorial Sloan-Kettering Cancer Center, New York, NY; ref. 5). Six hours after transfection, cells were returned to regular culture media containing 7.5 μg/mL puromycin (CT26) or 5 μg/mL puromycin (4T1; Sigma). Two weeks after drug selection, GFP+ cells were sorted using a FACSaria II Flow Cytometer (BD Biosciences) and then seeded into 96-well plates for monoclonal selection by serial dilution. pEh-Mekk2-YFP was kindly provided by Dr. Brian C. Schaefer (Uniformed Services University of the Health Sciences, Bethesda, MD; ref. 18). pGL3-B250 and pGL3-pEGFP-C1 (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) were injected s.c. per mouse. Tumors were measured with a caliper, and tumor volumes were calculated using the formula length × width^2 and presented as the mean ± SD. To generate a mouse tumor metastasis model, 1 × 10^6 tumor cells per mouse were injected i.v. via the tail vein. To generate a mouse model of breast cancer, 5 × 10^4 4T1 tumor cells per mouse were orthotopically injected into the mammary fat pads. Female BALB/c mice, C.B-17/SCID (CB17-Prkdc^scid/J) mice, and NSG (NOD-scid IL2Rγ^−/−) mice were purchased from The Jackson Laboratory. All animal studies were performed in accordance with protocols approved by the University of Louisville Institutional Animal Care and Use Committee (Louisville, KY).

Immune-cell depletion

Groups of BALB/c mice were depleted of specific immune-cell populations. Briefly, for NK-cell depletion, BALB/c mice were injected i.p. with 50 μg of anti-asialo-GM1 Ab (eBioscience) for 3 consecutive days beginning 5 days before implantation of tumor cells and continued every 3 days thereafter for the duration of the experiment. NK-cell depletion was confirmed by FACS analysis.

In vivo imaging of tumor metastasis

To monitor tumor-cell metastasis in vivo, tumor cell lines were first labeled with a near-infrared lipophilic carboxylic dye-dioctadecyl-tetramethylindocarbocyanine iodide (DiR; Invitrogen). Cells were incubated with the DiR dye [1 × 10^5 cells in 10-μL 1:1 phosphate-buffered saline (PBS) containing 3.5 μg/mL dye and 0.5% ethanol] for 30 minutes at 37°C (20). Thereafter, cells were washed twice with PBS and the viability of labeled cells was verified by Trypan blue staining. Each mouse received 1 × 10^6 DiR-labeled tumor cells administered i.v. via the tail-vein. Mice were then imaged at scheduled time points using a Carestream Molecular Imaging system (Carestream Health, Woodbridge, CT).

Real-time PCR

Total RNA was isolated from tumor tissues or cells using RNeasy Mini Kit (Qiagen). Quantitative real-time PCR (qRT-PCR) analysis of the mature miR17–92 cluster in tumor tissues was performed using a Bulge-Loop miRNA qPCR system (Guangzhou RiboBio Co., Ltd.). Relative quantification of selected miRNA and pre-miR17–92 was performed using a CFX96 Real-time System (Bio-Rad Laboratories) and SsoFast EvaGreen Supermix (Bio-Rad Laboratories), according to the manufacturer’s instructions. All primers were obtained from Eurofins MWG Operon. Expression values were normalized to cell number. PCRs for murine Gadh were performed as positive controls. Sequences of the primers are listed as follows: pre-mumu-miR17 forward: 5′-CGCGTCAAGAATACGTGT-3′; pre-mumu-miR17 reverse: 5′-CCGCACATATGGCACAAA-3′; pre-mumu-miR18a forward: 5′-CCGACATGCTGCTTTTCTTACT-3′; pre-mumu-miR18a reverse: 5′-CGCACCCCTTCTTTCAGG-3′; pre-mumu-miR19a forward: 5′-CGCGTCCTGCCACTACCA-3′; pre-mumu-miR19a reverse: 5′-CGGACCTTCCATGGTTAT-3′; pre-mumu-miR20a forward: 5′-CTCGCCCTCTAGCCACACAC-3′; pre-mumu-miR20a reverse: 5′-ACCGAGCCATACCCAGT-3′; pre-mumu-miR20b forward: 5′-CGGTGATGTGACGCAG-3′; pre-mumu-miR20b reverse:

Mouse models

To generate mouse tumor models, 1 × 10^6 colon tumor cells were injected s.c. per mouse. Tumors were measured with a
Flow cytometry

Cell lines were digested and centrifuged at 800 × g and cell pellets were resuspended in FACS buffer (PBS, 1% BSA, and 0.1% EDTA). Cells were pretreated with the FcγR-blocking mAb (eBioscience) on ice for 10 minutes, followed by incubation on ice for 30 minutes with anti-mouse MHC class I (H-2D/H-2K; eBioscience) or H-2D mAb (BioLegend) on ice for 30 minutes. Whole tumor, lung, or liver from each mouse was minced and digested for 30 minutes at 37°C in the presence of 30 μg/mL collagenase mix (Blenzyme) in Hank’s Balanced Salt Solution. Completely digested tissues were centrifuged at 800 × g and pellets were resuspended in FACS buffer (PBS, 1% BSA, and 0.1% EDTA). Cells were pretreated with the FcγR-blocking mAb, followed by incubation on ice for 30 minutes with Abs against DX5, CD3, CD4, CD8, F4/80, CD11c, CD11b, Gr-1, Perforin, Granzyme B, or IFNγ (eBioscience). All samples were analyzed with the Accuri C6 Flow Cytometer System (Accuri Cytometers). After an initial gating on forward-versus-side scatter plots, cell populations were gated on all viable leukocytes. Subsets of populations were defined using Abs against DX5, CD3, CD4, CD8, F4/80, CD11c, CD11b, or Gr-1. Intracellular-labeled cells were pretreated for intracellular staining (eBioscience). Data were analyzed using FlowJo FACS software (TreeStar, Inc.).

Luciferase assays

CT26 cells (5 × 10⁴) were seeded into individual wells of a 24-well plate, cultured overnight, and then transfected with Renilla luciferase constructs together with different doses of 20 μmol/L. miRNA mimics (miScript MiRNA Mimic; Qiagen) for mmu-miR17 and/or mmu-miR20a mimics using Lipofectamine 2000 (Invitrogen). After 24 hours of incubation, Renilla luciferase activities were evaluated using the Dual-Luciferase Reporter Assay system (Cat#1910; Promega). For MHC 1 promoter reporter assay, 5 × 10⁴ CT26, miR-Ctrl, or miR17–92 cells were seeded into individual wells of a 24-well plate, cultured overnight, and then transfected with MHC 1 promoter reporters, pGL3-B250 or pGL3-ß2m, or together with plasmids encoding pre-miR17/20a or and Mekk2, or together with plasmids encoding shMekk2 or and Mekk2 using Lipofectamine 2000 (Invitrogen). After 24 hours of incubation, luciferase activities were evaluated using the Luciferase Reporter Assay system (Cat#E1500; Promega).

Cytotoxicity assay

DX5⁺ or DX5⁻ effector cells were purified from mouse spleens using MACS sorting as described previously (22). Splenic leukocytes were stained with biotinylated anti-DX5 (eBioscience) followed by incubation with anti-PE streptavidin MACS beads. Thereafter, DX5⁺ or DX5⁻ cells were isolated by magnetic cell sorting using the MACS system (Miltenyi Biotec). To determine NK-cell cytotoxicity in vitro, target cells were digested and washed three times, and adjusted to 1 × 10⁶ cells/mL. Serially diluted splenic leukocytes or purified DX5⁺-positive/negative cells were mixed with target cells at various effectortarget (E:T) ratios and incubated for 3 hours at 37°C. Cells were harvested and stained with anti-PI Ab (eBioscience). Thereafter, GFP⁺ PI⁻ subsets were analyzed with the Accuri C6 Flow Cytometer System (Accuri Cytometers). Data were analyzed using FlowJo FACS software (TreeStar, Inc.).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections. Serial 4-μm sections were stained with hematoxylin and eosin (H&E) for morphologic analysis. For tissue immunofluorescence staining, slides were washed three times (5 minutes each) with PBST (PBS, 0.1% Tween 20). Tissues were permeabilized by incubating the slides in 1% Triton X-100 in PBS at 25°C for 15 minutes and then washed three times in PBST. After blocking for 1 hour at 25°C in blocking buffer (PBS containing 10% BSA), slides were incubated overnight in a humidity chamber with anti-mouse/ human Abs. Abs were diluted 1:50 in blocking buffer. The following Abs were used: anti-DX5-PE, anti-CD4-PE, anti-CD8-PE, anti-F4/80-PE, anti-Gr-1-PE, anti-CD11c-PE, anti-MHC class I-PE (BD Biosciences), anti-Mekk2, and anti-p-Erk5 Abs (Santa Cruz Biotechnology). Following another three PBST washes, slides were incubated with Alexa Fluor 594- or Alexa Fluor 647–conjugated goat anti-mouse/rabbit secondary Ab at a 1:500 dilution (Molecular Probes). Slides were then washed and nuclei were counterstained with DAPI. For cell immunofluorescence staining, cell lines were seeded into a chamber slide (Lab-Tek II Chamber Slide System; Thermo Fisher Scientific) and cultured overnight. Slides were then processed as described previously for tissue immunofluorescence staining.

Results

Restoration of the expression levels of the miR17–92 cluster inhibits the growth of solid tumor in immunocompetent but not in immunodeficient mice

To address the potential role of the miR17–92 cluster in regulating tumor progression, we analyzed the expression pattern of this cluster in human colon tumor tissues. Expression levels of the miR17–92 cluster in human adjacent non-tumor colon tissues were almost 50-fold higher than those in the highly metastastic tumor tissues (Supplementary Fig. S1A). To determine whether the expression levels of miR17–92 correlate with tumor progression, we analyzed the potential correlation between the expression levels of the miR17–92 cluster and the metastatic capacity in mouse colon and breast cancers. We found that the expression levels of the miR17–92 cluster were significantly lower in CT26 and 4T1 cancer cells
with higher metastatic capacity (Supplementary Fig. S1B) than in the nonmetastatic CMT-93 colon cancer and 4T07 breast cancer cells.

To determine whether restoring the expression levels of the miR17–92 cluster has an impact on tumor growth, we stably transfected CT26 cells with PIG-miR17–92, an expression vector encoding the miR17–92 cluster miRNAs (Supplementary Fig. S1C). We generated subclones of transfected CT26 cells expressing high levels of the miR17–92 cluster (CT26/miR–92) that are close to the levels expressed in the adjacent nontumor tissue. After implantation into BALB/c mice, the CT26/miR17–92 tumor cells grew much more slowly than the CT26/miR-Ctrl cells (Fig. 1A, top). In contrast, when the transfected tumor cells were s.c. injected into NSG mice (NOD-scid IL2Rg−/−), NK, and T cell-null immunodeficient mice, no significant difference in the growth of the tumor cells was observed (Fig. 1A, bottom). This result was replicated using the 4T1 mouse breast cancer cells (Fig. 1B). In summary, when compared with that of the miR-Ctrl, expression of the miR17–92 cluster significantly inhibited the growth of tumor cells in immunocompetent mice but not in immunodeficient mice lacking NK and T cells, indicating that higher levels of the miR17–92 cluster increase the sensitivity of tumor cells to attack by the host’s immune cells.

Recruitment and activation of NK cells through downregulation of MHC class I molecules expressed on tumor cells by miR17–92

To determine which subset of the host’s immune cells plays crucial roles in the prevention/elimination of tumor growth, we analyzed tumor-infiltrating immune cells in the 4T1 breast and CT26 colon tumors. There was a significant increase in the number of DX5+ CD3− IFNγ+ NK cells infiltrating the 4T1/miR17–92 tumors and CT26/miR17–92 tumors when compared with miR-Ctrl tumors (Fig. 2A and B). Furthermore, when compared with that of the miR-Ctrl, the expression of the miR17–92 cluster (CT26/miR17–92) resulted in significant inhibition of tumor growth in T cell–deficient but not in NK cell–sufficient C.B-17/SCID mice (data not shown). We used anti-asialo-GM1 Abs to deplete NK cells in BALB/c mice to assess the role of NK cells on tumor growth in vivo. Anti-asialo-GM1 Ab-mediated depletion of NK cells in CT26/miR17–92 tumor-bearing mice led to a significant increase in tumor growth in comparison with CT26/miR17–92 tumor-bearing mice injected with a control isotype IgG Ab (Fig. 2C), suggesting that NK cells play critical roles in the miR17–92–mediated suppression of tumor growth in vivo. These results also suggest that high levels of miR17–92 are required for NK cell–mediated inhibition of tumor growth as there was no difference in tumor growth in CT26/miR-Ctrl tumor-bearing mice treated with NK cell–depletion Ab or a control isotype IgG Ab (Fig. 2C).

NK cells are innate immune cells with effector functions via the recognition of MHC class I molecules. Therefore, we tested whether miR17–92 cells had impaired expression of MHC class I molecules, which subsequently triggered the activation of NK cells. To determine the role of miR17–92 in the regulation of MHC class I expression, cell lines with varying levels of miR17–92 were analyzed for their expression of MHC class I surface molecules. As expected, there was a significant decrease in the expression of MHC class I (H-2D) in CT26/miR17–92 cells

Figure 1. miR17/20a inhibits tumor growth in vivo. A, growth curves of control (miR-Ctrl) and miR17–92 cluster (miR17–92) tumors by s.c. injection of CT26/miR-Ctrl and CT26/miR17–92 monoclonal cells into BALB/c mice (top) or NOD-scid IL2Rg−/− mice (bottom, 5 mice/group). Error bars represent mean ± SD (two-way ANOVA; **, P < 0.01). B, growth curves of control (miR-Ctrl) and miR17–92 cluster (miR17–92) tumors by orthotopic injection of 4T1/miR-Ctrl and 4T1/miR17–92 monoclonal cells into the mammary fat pads in BALB/c mice (top) or NOD-scid IL2Rg−/− mice (bottom, 5 mice/group). Error bars represent mean ± SD (two-way ANOVA; **, P < 0.01).
compared with that in CT26/miR-Ctrl cells (Supplementary Fig. S2A).

To determine which miRNAs in the miR17–92 cluster contributed to the suppression of MHC class I expressed on tumor cells, we analyzed the expression levels of MHC class I (H-2D) in CT26 cells expressing various deleted forms of miR17–92 (DmiR17/20a, DmiR18a, DmiR19a,b, and DmiR92a). We found that the deletion of miR17/20a, but not that of other members of the miR17–92 cluster, increased the expression level of MHC class I (H-2D) (Fig. 3A), indicating that miR17/20a is responsible for the miR17–92–mediated suppression of MHC class I (H-2D) expression. CT26 colon cancer cells expressing a deleted form of miR17/20a (DmiR17/20a) did not exhibit suppressed tumor growth, further supporting the role of miR17/20a in the inhibition of tumor growth (Fig. 3B).

To determine whether MHC class I is critical for the escape of tumor cells from NK-cell recognition and killing in vivo, we analyzed the expression levels of MHC class I in tumor cells from immunocompetent mice. There was a dramatic increase in the expression levels of MHC class I (H-2D) in the surviving miR17–92–expressing tumor cells recovered from the tumors (Fig. 3C). Furthermore, the surviving miR17–92–expressing cells recovered from tumors implanted in BALB/c mice showed significant increases in MHC class I promoter activity compared with that of nonimplanted parental miR17–92–expressing tumor cells (Supplementary Fig. S2B). The expression levels of miR17/20a were reduced significantly in surviving tumor cells isolated from miR17–92 tumors (Fig. 3D). These data suggest that higher levels of miR17/20a in tumor cells result in the downregulation of MHC class I and the subsequent activation of NK cells that kill tumor cells.
miR17/20a suppresses MHC class I via the Erk5 signaling pathway by targeting Mekk2

To define the molecular mechanism underlying the down-regulation of MHC class I expression by miR17/20a, we analyzed miR17/20a targets predicted by public algorithms (TargetScan and RNAbridi). We did not find any genes predicted to be associated with the regulation of MHC class I. However, the sequence of Map3k2 encoding Mekk2, an upstream regulator of the Erk5 pathway, was predicted to have four potential conserved miR17/20a binding sites within its 3'-UTR with lower minimum free energy (Fig. 4A and Supplementary Fig. S3A). As shown in previous findings, the Erk5 pathway transcriptionally regulates the MHC class I promoter (23, 24); therefore, we hypothesized that miR17/20a suppresses Erk5 signaling by directly binding to sites within the 3'-UTR of Map3k2, thereby suppressing MHC class I (H-2D) expression.

To test this hypothesis, we constructed luciferase reporter vectors encoding the top two predicted wild-type miR17/20a binding sites (Fig. 4A), and assessed their activity in CT26 cells. The wild-type miR17/20a binding site reporters showed reduced luciferase expression in cells expressing miR17/20a but not in cells expressing miR-Ctrl. This was further confirmed by transfection of mutant miR17 or mutant-miR20a in which the Map3k2-3'-UTR binding sites were mutated (Fig. 4B). We then examined whether forced expression of miR17-92 decreased the levels of Mekk2, phosphorylated-Mek5 (p-Mek5), and phosphorylated...
Erk5 (p-Erk5; Fig. 4D), but it had no effect on the Mek4–Mek7–Jnk pathways (data not shown). We performed luciferase-reporter assays to determine whether the activation of the Mekk2–Erk5 pathway directly affects the promoters of the MHC class I genes. Transient expression of miR17/20a in CT26 cells was sufficient to reduce luciferase expression from the promoters of HLA-B and β2M, whereas restoration of Mekk2 expression reversed the suppressive effect of miR17/20a (Supplementary Fig. S3B). Together, our data suggest that tumor cells escape NK-cell recognition by upregulating MHC class I expression via the Mekk2–Erk5 pathway.

Mekk2–Erk5 pathway is targeted by miR17/20a in NK cell–mediated immunosurveillance in vivo

Restoration of Mekk2 expression reversed miR17/20a-mediated inhibition of MHC class I expression, followed by decreased tumor-cell killing by purified DX5+ splenic leukocytes in vitro (Fig. 5A). Furthermore, forced expression of Mekk2 in miR17–92 cells promoted tumor growth and metastasis in vivo (Fig. 5B and C). Taken together, these observations indicate that miR17/20a suppresses MHC class I via the Erk5 signaling pathway by targeting Mekk2.

We next determined whether the expression of Mekk2 has a role in the downregulation of MHC class I in tumor cells. We evaluated the surface expression of MHC class I (H-2D) on cells transfected with shRNA targeting Mekk2 (shMekk2) or a control scrambled sequence (shNC). As expected, there was a significant reduction of MHC class I on shMekk2 cells compared with that on control shNC cells (Supplementary Fig. S3B). Together, our data suggest that tumor cells escape NK-cell recognition by upregulating MHC class I expression via the Mekk2–Erk5 pathway.

Figure 4. miR17/20a suppresses the expression of MHC class I via the Erk5 signal pathway by targeting Mekk2. A, schematic representation of the mouse Map3k2 3′-UTR. miR17/20a complementary sites are indicated (vertical red lines). B, schematic representation of mutant sites of miR17 and miR20a (top). HEK293T cells were cotransfected with wild-type Map3k2 3′-UTR luciferase reporter plasmids, together with miR17 mimic, miR20a mimic or mutant miR17, mutant miR20a (Thermo scientific) as indicated. Renilla luciferase activity was measured 24 hours after transfection. Error bars represent mean ± SD (one-way ANOVA; **, *P < 0.01). C, Western blot analyses showing expression of Mekk2 in CT26 cells after transient transfection with miR-Ctrl, miR17, miR20a, or miR17/20a for 24, 48, or 72 hours. β-Actin was used as a loading control. D, Western blot analyses showing expression of Mekk2, p-Mek5, Mek5, p-Erk5, and Erk5 in CT26/miR-Ctrl and CT26/miR17–92 cell lines (left) or 4T1/miR-Ctrl and 4T1/miR17–92 cell lines (right). β-Actin or Gapdh was used as a loading control.
leads to the activation of NK cells in vivo, we analyzed the profiles of cytotoxic markers released from NK cells of mice injected with CT26 cells transfected with either shMekk2 or shNC. Knockdown of Mekk2 in tumor cells decreased tumor size and caused a higher number of Granzyme B$^+$Perforin$^+$IFN$\gamma$$^-$ activated NK cells in the tumor (Fig. 5D and E), in agreement with the in vivo results shown in Fig. 5B and C. When tumor cells with an inactivated Erk5 pathway were injected into immunocompetent mice, the tumor cells were mostly eliminated in comparison with tumor cells transfected with the control vector (Fig. 5F). Taken together, these data demonstrate that inhibiting Erk5 activation via a knockdown of Mekk2 leads to prevention of tumor growth and metastasis through the activation of NK cells in vivo.

**miR17/20a is significantly downregulated in human solid cancers**

To determine the potential role of the miR17–92 cluster in regulating human tumor progression, we assessed the
expression levels of miR17/20a in 14 tumor samples of human colorectal cancer and 12 corresponding adjacent nontumor colon tissues by qRT-PCR. Results from analysis of human samples recapitulated our mouse tumor data; we showed a significant reduction of the levels of both miR17 and miR20a in human colorectal cancer tumor samples compared with those in the corresponding adjacent normal colon tissues (Fig. 6A). Finally, we evaluated whether the Mekk2/Erk5 activity was associated with the expression levels of the miR17/20a in human colon tissues. We found that the Mekk2–Erk5 pathway was highly activated in tumors with low expression levels of miR17/20a; in contrast, the Mekk2/Erk5 activity was suppressed in the corresponding adjacent normal colon tissues, which expressed higher levels of miR17/20a (Fig. 6B). Results from the human studies are in agreement with the data generated from mouse cancer models, strongly supporting that miR17/20a is a negative regulator of the Mekk2–Erk5 pathway.

Discussion

In this study, we demonstrated that the expression of higher levels of miR17/20a in the miR17–92 cluster inhibits tumor growth in vivo. We also showed that miR17/20a suppresses tumor growth by enhancing NK-cell recognition via targeting the Mekk2–Mek5–Erk5 pathway. Our findings provide a rationale for developing a therapeutic strategy to treat certain cancers by targeted-delivery of miR17/20a.

The antitumor effect of NK cells has been shown in clinical studies. Results from an epidemiologic study have also suggested that low peripheral blood NK-cell activity is correlated with increased cancer risk (26). NK-cell infiltration into colorectal tumor tissue is correlated with a better disease prognosis (27). NK-cell activation is controlled by a balance of stimulatory and inhibitory signaling incurred when target-cell engagement occurs.

The relationship between immunosuppression and imbalanced miRNA expression has been observed in various human cancer cell lines and tumor samples (28, 29). However, the potential role of restoring miRNA expression in tumor cells on the modulation of NK-cell tumor cytotoxicity has rarely been reported (30, 31). Our data demonstrate that higher levels of miR17/20a suppress the expression of MHC class I on tumor cells by targeting the Mekk2–Mek5–Erk5 pathway, which in turn, enhances NK-cell recognition. This finding is in agreement with results reported by Charni and colleagues showing that knockdown of Erk5, which is downstream of the Mekk2-mediated pathway in leukemia cells, effectively attenuates their tumor activity by downregulating MHC class I expression, leading to activation of NK cells (23). Our observations delineate the mechanism of suppression and reveal that the

Figure 6. Clinical association between the expression levels of miR17–92 and the activity of the MEKK2–ERK5 pathway in human colon tumor samples. A, the expression levels of the hsa-miR17 and hsa-miR20a in 14 human colorectal tumor tissues and 12 adjacent nontumor colon tissues using the Student t test. B, immunofluorescent staining showing the expression of MEKK2 and p-ERK5 in paired human colon cancer and adjacent nontumor tissues. Original magnification, ×10.
Mekk2–Mek5–Erk5 pathway is the major pathway that contributes to the suppression of MHC class I expression in miR17–92-expressing cells. Moreover, we found that higher levels of miR17/20a mediated the targeting of MHC class I more efficiently and rapidly for inducing NK cell–mediated tumor cytotoxicity compared with shRNA knockdown of Erk5 reported by others (23). This suggests that Mekk2 may regulate more molecules/pathways that play a role in modulating the expression of MHC class I. Our finding that miR17/20a targets MHC class I provides a rationale to develop targeted delivery of miR17/20a into tumor tissue for cancer therapy as well as other diseases associated with dysregulated MHC class I expression.

Aberrant miRNA expression has been identified in various human solid cancers (10). Whether restoration of the miRNA levels expressed in tumor cells to the levels expressed in the adjacent nontumor tissue has any effect on tumor progression has not been studied. In this report, we describe for the first time that the restoration of the expression levels of the miR17–92 cluster miRNAs in both murine breast cancer and colon cancer cells also restores its function as a tumor suppressor. Specifically, the restoration of miR17/20a within the miR17–92 cluster plays a dominant role in the inhibition of tumor growth by enhancing NK-cell recognition via directly targeting the Mekk2–Mek5–Erk5 pathway. From a therapeutic standpoint, this finding is significant. An appealing property of miRNAs as therapeutic agents is their capacity to target multiple genes, making them extremely efficient in regulating distinct biologic processes in the context of a network. Therefore, developing therapeutic strategies to restore homeostasis by modifying the levels of miRNA expression would be more efficient than targeting individual genes or proteins.

Results from this study raised several new challenging questions for future studies. We found that surviving miR17–92–expressing tumor cells recovered from the tumors still expressed miR17–92-GFP (data not shown), indicating that the transgene was not lost. However, the machinery in the tumor microenvironment that prevents miR17–92-mediated downregulation of MHC class I expression on tumor cell is not yet known. We have demonstrated that miR17/20a suppressed Map3k2 expression by directly binding to the Map3k2 3′-UTR. Although there are four potential miR17/20a binding sites in the Map3k2 3′-UTR, we were only able to amplify via PCR the genomic DNA fragment covering the first two sites, which have a lower energy requirement for binding. Because we failed to apply PCR to the fragment covering all four computationally predicted binding sites for the Map3k2 3′-UTR (more than 10 kb in full), we cannot exclude the regulatory effect of additional binding sites on the expression of the Map3k2 protein.

Also, there is some discrepancy between the results we present in this article and data published by others in terms of miR17–92–mediated effects on either protumor or antitumor progression. Previous observations support the dominant role of miR19a and miR19b in promoting tumorigenesis and in tumor angiogenesis (1, 11, 13, 17). Recently, miR20a has been reported to promote tumor metastasis by downregulating Fas expression in osteosarcoma tumor cell lines (32). However, there is also increasing evidence to support the function of the miR17–92 cluster in tumor suppression in breast cancer (14), hepatomas (15), and human gastrointestinal stromal tumors (16). Loss of heterozygosity at 13q12-q13 (the locus of the miR17–92 cluster) is associated with tumor progression and poor prognosis in breast cancer, squamous cell carcinoma of the larynx, retinoblastoma, and hepatocellular carcinoma (33, 34). Moreover, using high-resolution array-based comparative genomic hybridization in human tumor specimens, the miR17–92 cluster was deleted in 16.5% of ovarian cancers, 21.9% of breast cancers, and 20.0% of melanoma (35, 36). A number of possibilities could explain the discrepancies of these studies. Unlike siRNAs, one miRNA could regulate a number of genes, and the potential effects on targeted genes could be dependent on the levels of that miRNA. In addition, the accessibility and availability of the miRNA-targeted genes that reside in the same compartment of the cells is dependent on many factors. It is conceivable that different tumor microenvironments, such as those in osteosarcomas and lymphomas tested in other studies, could be different from the colon cancer and breast cancer microenvironment we tested in our study. Therefore, the results generated from these studies support the need to further identify factors that regulate miR17 or miR20a as a protumorigenic or antitumorigenic miRNA.

In summary, restoration of the expression level of miR17/20a enhances NK-cell recognition by targeting the Mekk2–Mek5–Erk5 pathway. This finding not only uncovers the molecular mechanism underlying the miR17/20a enhancement of immune responses against tumor growth, but it also opens up a new approach for developing a therapeutic strategy to treat certain types of cancer by targeted delivery of miR17/20a.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: H. Jiang, D. Miller, H.-G. Zhang


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