Introduction

MicroRNAs (miRNA) are single-stranded 17- to 27-nucleotide RNA molecules that regulate gene expression by posttranscriptional silencing of target mRNAs. Here, we transformed rat 9L gliosarcoma cells to express cel-mir-67, a miRNA that lacks homology in rat. Coculture of these cells with cells that expressed a luciferase reporter that contained a complementary sequence to cel-miR-67 resulted in significant suppression of luciferase expression. This effect was also observed in the U87-MG human glioma cell line. Moreover, luciferase suppression was inhibited by the addition of carbonic anhydrase to cocultures, suggesting that gap junction communication regulates intercellular transfer of miRNA. Finally, in situ hybridization revealed the presence of cel-miR-67 in cel-miR-67–null 9L cells after coculture with cel-miR-67–expressing cells. Our data show that miRNA transcribed in glioma cells can be transferred to adjacent cells and induces targeted inhibition of protein expression in the acceptor cells. These findings reveal a novel mechanism of targeted intercellular protein regulation between brain tumor cells. Cancer Res; 70(21); 8259–63. ©2010 AACR.

Materials and Methods

Cell culture

The 9L rat gliosarcoma, U87-MG human glioblastoma, and U251 human glioblastoma cell lines were obtained from the American Type Culture Collection (ATCC) in 2003, 2007, and 2000, respectively. Cells were resuscitated and cultured in accordance with ATCC guidelines for less than 4 months before use in these experiments. ATCC performs authentication of all cell lines through short tandem repeat profiling (human cells), karyotyping, and cytochrome c oxidase I testing; we did not reauthenticate these lines in our laboratory. Cells were maintained at 37°C in DMEM containing 10% fetal bovine serum as previously described (6).

Cell cocultures

Cells were cultured for 24 hours in 96-well plates. 9L cells were added at 2:1 ratio of C. elegans miRNA-expressing cells (1.4 × 10⁴) to luciferase-expressing cells (7 × 10⁴). For in situ hybridization experiments, 4 × 10⁴ C. elegans miRNA-expressing cells were cocultured for 24 hours with 2 × 10⁴ enhanced green fluorescent protein (eGFP)–expressing cells in eight-well glass chamber slides.

Cell transfection

Cel-mir-67 pMIR-report luciferase and control plasmids (Signosis) and cel-miR-67 and cel-miR-239 expression plasmids (GenScript) were used. Transfection was carried out for 12 hours using Lipofectamine 2000 (Invitrogen) with 4 μg of DNA per transfection. Twenty-four hours after transfection, cells were washed three times in PBS, collected by trypsinization, and resuspended in culture medium before use in experiments. Stably transfected cells were isolated under puromycin selection, tested for miRNA expression, and subsequently used in experiments. We verified that only 9L-mir67 cells expressed cel-miR-67 using a cel-miR-67 TaqMan miRNA assay (CT value = 28.7; Applied Biosystems).

Luciferase assays

Luciferase activity was determined using a Luciferase Assay System kit (Promega). Luminescence was determined
on a Fusion plate reader (Perkin-Elmer). For all wells, each experimental group (e.g., 9L-miR-67) was the result of one transfection per experiment. Each luciferase experiment was performed four times, with six or more replicates per group.

Inhibition of gap junctions
To inhibit the function of gap junctions in 9L cells, we incubated the cells with 150 μmol/L carbenoxolone for 7 hours. Carbenoxolone is a broad-spectrum gap junction antagonist, and incubation for 7 hours in 150 μmol/L has been shown to disrupt the function of gap junctions (7).

In situ hybridization and immunostaining
Probes against rno-miR-21 and cel-miR-67 (Exiqon) were hybridized to miRNAs according to a published protocol that was modified for adherent cells (8). Cells were counterstained with a monoclonal antibody against eGFP (Aves Labs). The probe for rno-miR-21 was used as a positive hybridization control because miR-21 is highly expressed in gliomas (9).

Statistical analysis
Data are shown as mean ± SD. P values were calculated using one-way ANOVA or Student’s t test.

Results
Cel-miR-67 is not expressed in rat, mouse, or human and, for this reason, is often used as an inert miRNA negative control in experiments using mammalian cells (10). Using real-time reverse transcription-PCR with a cel-miR-67 specific primer, we confirmed that 9L rat gliosarcoma cells did not express cel-miR-67 miRNA.

To test whether functional miRNA can be transferred from one tumor cell to another, we cocultured 9L cells transfected with an expression vector for cel-miR-67 (9L-mir67) or cel-miR-239 (9L-mir239) or an empty expression vector (9L-mirNeg), with 9L cells transfected with a luciferase reporter encoding for mRNA with a 3′ untranslated region containing a complementary sequence to cel-miR-67 (9L-Luc67). When 9L-mir67 cells were cocultured for 24 hours with 9L-Luc67 cells, we observed a 25% signal attenuation compared with the 9L-mirNeg control group. However, when 9L-mir239 cells were cocultured with 9L-Luc67 cells, luminescence was not significantly altered compared with 9L-mirNeg/9L-Luc67 control. The luciferase activity detected in the 9L-mir67/9L-Luc67 coculture cells was also significantly less than that detected in the 9L-mir239/9L-Luc67 group (Fig. 1A). These data show that the downregulation of luciferase in the 9L-Luc67 cells was due to the presence of cel-miR-67, produced by the 9L-mir67 cells with which they were cocultured.

We next posited that intercellular regulation of luciferase might be dependent on the expression of cel-miR-67 DNA that had not incorporated into the genome. To test this hypothesis, we established, through puromycin selection, 9L cell lines that stably expressed either cel-miR-67 (9L-mir67s) or cel-miR-239 (9L-mir239s), with 9L cells transfected with a luciferase reporter encoding for mRNA with a 3′ untranslated region containing a complementary sequence to cel-miR-67 (9L-Luc67). When 9L-mir67s cells were cocultured for 24 hours with 9L-Luc67 cells, we observed a 25% signal attenuation compared with the 9L-mirNeg control group. However, when 9L-mir239s cells were cocultured with 9L-Luc67 cells, luminescence was not significantly altered compared with 9L-mirNeg/9L-Luc67 control. The luciferase activity detected in the 9L-mir67/9L-Luc67 coculture cells was also significantly less than that detected in the 9L-mir239/9L-Luc67 group (Fig. 1A). These data show that the downregulation of luciferase in the 9L-Luc67 cells was due to the presence of cel-miR-67, produced by the 9L-mir67 cells with which they were cocultured.
As mentioned above, there is evidence that transfer of RNA between cardiac myocytes occurs and that it is mediated by gap junctions (5). To determine if transfer of functional miRNA between brain tumor cells is regulated by gap junctions, we inhibited them by incubating the cells with carbenoxolone, a broad-spectrum connexin channel antagonist. The addition of carbenoxolone to 9L cocultures significantly blocked the effect of cel-miR-67–expressing cells on luciferase expression in Luc67-expressing cells (Fig. 1C). These findings indicate that gap junction intercellular communication significantly mediates the transfer of functional miRNA between 9L cells.

To confirm that cel-miR-67 was present in cocultured 9L acceptor cells that did not express cel-mir67, we cocultured 9L-mir67s cells with 9L gliosarcoma cells that express eGFP (9L-eGFP). We used in situ hybridization to visualize cel-miR-67. We observed colocalized green fluorescent protein (GFP) and cel-miR-67 signals in 9L-mir67/9L-eGFP coculture.
∼75% of GFP-reactive cells were positive for cel-miR-67), but no cel-miR-67 in 9L-mir239/9L-eGFP controls (Fig. 2A–D). These results confirmed the presence of cel-miR-67 in 9L-eGFP acceptor cells. Thus, 9L gliosarcoma cells can transfer functional miRNA between cells, and this miRNA can regulate protein expression in the acceptor cells.

Finally, we tested whether miRNA could be transferred between other glioma cells. To this end, we transfected the human glioma cell lines U87-MG and U251 with the cel-mir67, cel-mir239, or Luc67 plasmid and performed the same coculture experiment. Here, we found that luciferase expression in the U87-MG cells was significantly reduced by coculture with cel-miR-67 expressing U87-MG cells (U87-mir67); however, no effect was observed in the U251 cocultures (Fig. 3A and B). These results indicate that miRNA transfer may occur in human as well as rat glioma cells; however, a lack of luciferase suppression in the U251 cocultures suggests that miRNA transfer may not occur or may be limited in some glioma cell types.

Discussion

Alterations in the expression of miRNA contribute to the pathogenesis of human cancers (11). Dysregulation of miRNAs promotes malignancy of glioblastoma and contributes to cell proliferation, invasion, and angiogenesis and glioma stem cell multipotency and survival (12, 13). Intercellular transfer of RNA was hypothesized as early as 1971 (14). Recent evidence indicates that gliomas can shed microvesicles that contain functional miRNAs, mRNAs, and receptors (3, 15). These microvesicles have been detected in biological fluids including blood, urine, and cerebral spinal fluid (3, 16). Proteins, RNAs, and miRNAs transported by microvesicles are now believed to play a critical role in tumor invasion and metastases (17). Previous studies showed that cells exposed to tumor-shed microvesicles take up and incorporate proteins and nucleic acids contained within (3, 4, 15). These prior experiments provided compelling evidence that intercellular protein regulation through transferred miRNA is possible, if not likely. However, using a vector encoding for alien miRNA, we provide direct evidence that gliosarcomas exchange functional miRNA, and importantly, that this transferred miRNA leads to significant alterations in protein expression in the acceptor cells.

Previously, Valiunas and colleagues showed that oligonucleotides the size of siRNA are permeable to gap junctions (18). More recently, it was shown that cardiac myocytes exchange small RNAs through a gap junction–dependent mechanism (5). These studies are important because they suggest a route of intercellular RNA transfer independent of microvesicles. Our findings indicate that gap junctions mediate the transfer of miRNA between 9L cells. However, it remains to be determined whether miRNAs are transported through these intercellular channels directly or if gap junctions influence processes such as microvesicle release. It is also possible that carbenoxolone affects mechanisms other than gap junctions, and future experiments targeting specific connexins with siRNA could test for this.

An obvious next step will be to determine if tumors can manipulate protein expression in neighboring nontumor cells, which, if true, may contribute to recruitment or transformation of nontumor cells. Interestingly, miRNA transfer either does not occur in U251 cells or elicits an effect below the sensitivity of our assay. This finding raises the possibility that intercellular miRNA transfer varies between glioma cell types. Of note, it has been reported that connexin 43 is highly expressed in cultured U87-MG cells, but not in U251 cells (19). As gap junctions contribute to miRNA transfer, it would be interesting to test if the difference we observed was due to
the differential connexin 43 expression between the two cell lines. Furthermore, we have not established whether cell-to-cell contact is necessary for miRNA transfer or effective suppression of protein expression in the acceptor cells. Nevertheless, this study shows direct and targeted regulation of protein expression between brain tumor cells. These findings have wide-ranging implications for our understanding of tumorigenesis and progression, as well as for the development of miRNA-based antitumor therapies.

Acknowledgments

We thank Zheng Gang Zhang and Feng Jiang for comments and reagents, and Ann Hozeska-Solgot for image analysis.

Grant Support

NIH grant ROI CA129446 and a Henry Ford Hospital Research Proposal Development Program Grant (M. Katakowski).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Functional MicroRNA Is Transferred between Glioma Cells

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Cancer Res 2010;70:8259-8263. Published OnlineFirst September 14, 2010.

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