MicroRNA miR-21 Regulates the Metastatic Behavior of B16 Melanoma Cells

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Background: miRNAs are involved in many critical biological processes.

Results: miR-21 induction is STAT3-dependent, and miR-21 knockdown inhibited melanoma cell proliferation and migration and enhanced apoptosis. Although B16 cells produced large lung metastases in mice, miR-21 knockdown cells only formed small lung lesions.

Conclusion: miR-21 regulates the metastatic behavior of melanoma.

Significance: miR-21 is identified as a potential drug target in melanoma.

MicroRNA-21 (miR-21) is overexpressed in many human tumors and has been linked to various cellular processes altered in cancer. miR-21 is also up-regulated by a number of inflammatory agents, including IFN, which is of particular interest considering the close relationship between inflammation and cancer. Because miR-21 appears to be overexpressed in human melanoma, we examined the role of miR-21 in cancer development and metastasis in B16 mouse melanoma cells. We found that miR-21 is a member of an IFN-induced miRNA subset that requires STAT3 activation. To characterize the role of miR-21 in melanoma behavior, we transduced B16 cells with lentivirus encoding a miR-21 antagonir and isolated miR-21 knockdown B16 cells. miR-21 knockdown or IFN treatment alone inhibited B16 cell proliferation and migration in vitro, and in combination they had an enhanced effect. Moreover, miR-21 knockdown sensitized B16 cells to IFN-induced apoptosis. In B16 cells miR-21 targeted tumor suppressor (PTEN and PDCD4) and antiproliferative (BTG2) proteins. To characterize the role of miR-21 in vivo, empty vector- and antagonir-21-transduced B16 melanoma cells were injected via tail vein into syngeneic C57BL/6 mice. Although empty vector-transduced B16 cells produced large lung metastases, miR-21 knockdown cells only formed small lung lesions. Importantly, miR-21 knockdown tumor-bearing mice exhibited prolonged survival compared with empty vector tumor-bearing mice. Thus, miR-21 regulates the metastatic behavior of B16 melanoma cells by promoting cell proliferation, survival, and migration/invasion as well as by suppressing IFN action, providing important new insights into the role of miR-21 in melanoma.

MicroRNAs (miRNAs) are an abundant family of small RNAs (~22 nucleotides) that act as crucial post-transcriptional regulators of genes involved in fundamental biological processes, including cell differentiation, proliferation, and apoptosis (1, 2). miRNAs are able to regulate the expression of multiple targets by binding to the 3’-untranslated regions of genes. miR-21 is frequently up-regulated in various human tumors and appears to play an important role in the oncogenic process as indicated by its association with high cell proliferation, low apoptosis, high invasion, and metastatic potential (3–9). We recently found that the cytokine IFN enhanced miR-21 expression in human prostate cancer cells, which diminished the sensitivity of cancer cells to IFN-induced apoptosis (10). In contrast, knocking down miR-21 expression enhanced sensitivity to apoptosis.

Although originally discovered as a potent inhibitor of virus replication, IFN also regulates cell proliferation, differentiation, survival, angiogenesis, and the immune system (11). IFN is the most commonly used cytokine for the treatment of patients with cancer, including hairy cell leukemia, melanoma, chronic myeloid leukemia, Kaposi sarcoma, renal carcinoma, and non-Hodgkin lymphoma (12). The JAK-STAT signaling pathway leading to the expression of IFN-stimulated protein-encoding genes has been extensively studied (13). In contrast, whereas several IFN-induced noncoding miRNAs that play roles in the antiviral action of IFN have been described (14), the mechanisms leading to IFN-induced miRNA expression and the roles of these miRNAs in cancer models are relatively uncharacterized. Consequently, it is essential to elucidate the signaling pathways regulating the expression of IFN-induced miRNAs as well as the cellular pathways that these miRNAs regulate to determine their role in cancer. One possibility is that, whereas some IFN-induced pathways may inhibit tumorigenesis, other pathways are protumorigenic. For example, the apoptotic action of IFN is balanced by the induction of a potent IFN-induced cell survival pathway (15–17).
In this study we examined the induction of miR-21 expression by IFN and the role of miR-21 in tumorigenesis by employing the well characterized B16 murine melanoma model, which \textit{in vivo} has the inherent propensity to metastasize to the lung (18–20). We report that IFN-induced miR-21 expression in B16 cells requires STAT3 activation and that miR-21 regulates cell proliferation, apoptosis, and migration \textit{in vitro}. We also show that miR-21 regulates the metastatic behavior of B16 melanoma cells \textit{in vivo}.

**EXPERIMENTAL PROCEDURES**

**Biological Reagents and Cell Culture**—The biological activity of recombinant murine IFNβ provided by Biogen-Idec, Inc. (Cambridge, MA) was expressed in terms of international reference units/ml using the mouse National Institutes of Health reference standard (21). B16 melanoma cells were cultured as monolayers at 37 °C, 5% CO2, in RPMI 1640 medium with 10% FCS (Hyclone) supplemented with glutamine, penicillin, and streptomycin and subcultured every 3 days at 10–30% confluence.

**Construction of Lentivirus to Express F705-STAT3 or AntagomiR-21**—The cDNA for the F705-STAT3 with a substitution of phenylalanine for the tyrosine at amino acid 705 was digested with SpeI and BamH1 and then inserted into Xbal and BamH1 sites of pcFUW-IREs-puro lentiviral vector to construct the lentiviral vector pcFUW-F705-STAT3 (22). To knock down miR-21 expression, oligonucleotides GCCTCTA-GATCAACATCAGTCTGATAAGCTA (forward) and GCGGATCTCTACGTACACTGCTTGCA (reverse) (reverse) against the mature sequence of miR-21 gene were annealed and cloned into Xbal and BamH1 site of lentiviral vector pLenti-U6-pkg-puro, resulting in antagomiR-21 being driven by polymerase III promoter U6 (10). Lentivirus was produced in 293FT cells by transfection and was centrifuged (3,000 \( g \)) to remove cell debris; the supernatant was then passed through a 0.45-\( \mu \)m filter and virus collected by ultracentrifugation (50,000 \( \times \) \( g \) for 3 h at 4 °C). The pellet was dissolved in PBS and frozen at −80 °C. Lentiviral particles were titrated in 293FT cells with a p24 ELISA (PerkinElmer Life Sciences).

**Detection of miRNA Expression**—Poly(A)-tailed total RNA (5 \( \mu g \)) was prepared as described previously (10) and reverse-transcribed into first-strand cDNA using Superscript III transcriptase (Invitrogen) with the oligo(dT) adapter primer 5′-GCGGCACAGAATTAATACGCTACATATAAGTTTGGTTTTGN-3′. For PCR, 40 ng of cDNA was used as a template in each reaction. The reverse primer was from the adapter sequence 5′-GCGGAGCACAGAATTAATACGCTACATAC-3′, and the forward primer was specific to the mature miRNA sequence (supplemental Table S1). U6 small noncoding RNA sequence was amplified as an internal control using the primers 5′-CTGGCTTCCGGCAGCACA-3′ (forward) and 5′-AACGCTTACGAATTGCGCT-3′ (reverse). The SYBR Green-based real-time PCR was performed using the MyiQ Real-Time PCR Detection System (Bio-Rad) and analyzed as described previously (10).

**Transwell Migration Assay**—B16 melanoma cells were transduced with lentivirus pcFUW and pcFUW-F705-STAT3 (F705) and subjected to transwell migration assays as described previously (22).

**Apoptosis Assay**—The induction of apoptosis was monitored by DNA fragmentation using the cell death detection ELISAPLUS or by flow cytometry (Accuri Model 6C) with the Annexin V-FITC apoptosis detection kit (BD Pharmingen), according to the manufacturer’s instructions.

**Immunoblot Analysis**—Proteins from total cell lysates (25 \( \mu g \)) were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes (Millipore), and immunoblotted with the indicated antibodies, followed by IRDye800CW goat anti-mouse IgG or IRDye680 goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE). Blots were visualized on an Odyssey Infrared Imaging System (LI-COR Biosciences).

**Quantitative Real-time PCR**—Total RNA was isolated using RNeasy Mini kit (Qiagen). Quantitative real-time PCR (qPCR) was performed using gene-specific primers (sequences in supplemental Table S2) on the iCyclerIQ detection system (Bio-Rad) using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad). Reaction parameters were as follows: cDNA synthesis at 50 °C for 10 min, iScript reverse transcriptase inactivation at 95 °C for 5 min, PCR cycling at 95 °C for 10 s and 60 °C for 30 s for 40 cycles. Gene expression data were normalized to the expression of the β-actin housekeeping gene. The relative units were calculated from a standard curve, plotting three different concentrations against the PCR cycle number at the cycle threshold (with a 10-fold increment equivalent to 3.1 cycles).

**Mice**—C57BL/6 mice were obtained from the Jackson Laboratory, Bar Harbor, ME, and housed in a pathogen-free animal facility. All experimental procedures involving animals in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Tennessee Health Science Center. Mice were injected via tail vein with a single cell suspension of B16 melanoma cells (1 \( \times \) 106) in 0.2 ml of culture medium. For survival analysis mice were observed daily after injection and were sacrificed at the first sign of shortness of breath, decreased locomotion, or reduced body weight (>20% of total body weight). For bioluminescence imaging, mice were injected intraperitoneally with \( \beta \)-luciferin, anesthetized in an O2-rich induction chamber with 2% isoflurane, and imaged using the Xenogen \textit{in vivo} imaging system (IVIS; Xenogen, Alameda, CA). Photonic emissions were assessed using Living image® 3.2 software, and the data are presented as pseudocolor representations of light intensity.

**Data Analysis**—A minimum of three independent experiments was performed at least in duplicate. Statistical analysis was performed by Student’s \( t \) test. \( p \) values of <0.05 (*), <0.01 (**), and <0.001 (***)) were considered significant.

**RESULTS**

**IFN Induces miR-21 Expression via a STAT3-dependent Pathway**—We previously showed that IFN induced miR-21 expression in various mammalian cells including mouse B16 cells (10). To characterize IFN induction further, RNA was prepared from B16 cells treated with murine IFN (1,000 units/ml)
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for varying times, and miR-21 expression was determined by qPCR. IFN rapidly induced miR-21 expression in B16 melanoma cells with a time course of induction similar to that of the IFN-induced gene ISG15 (Fig. 1A). IFN induced miR-21 and ISG15 expression within 2 h after IFN addition, with maximal levels attained by 6 h, and expression remained high for up to 48 h. To characterize the dose dependence of IFN induction of miR-21, RNA was prepared from B16 cells treated with varying IFN concentrations for 6 h, and gene expression was determined by qPCR. As shown in Fig. 1B, both miR-21 and ISG15 levels were increased by IFN at concentrations between 10 and 1,000 units/ml.

Because STAT3 appears to play a role in regulating miR-21 expression (10), we next examined the role of STAT3 in IFN-induced miRNA expression by expressing a construct where the canonical tyrosine 705 phosphorylation site was mutated to phenylalanine (F705-STAT3). F705-STAT3 expression in B16 melanoma cells blocked not only IFN-induced tyrosine phosphorylation of STAT3 (Fig. 2A), but also IFN-induced miR-21 expression (Fig. 2B). miR-196a, miR-296, and miR-351 were previously found to be induced by IFN in Huh7 human hepatoma cells (14), but the role of STAT3 in their IFN-induced expression had not been explored. We found that although expression of F705-STAT3 also blocked the induction of miR-351 and miR-296 in B16 melanoma cells (Fig. 2B), it had no effect on IFN-induced miR-196a expression. Therefore, these results indicate that the expression of a subset of IFN-inducible miRNAs (miR-21, miR-351, and miR-296) is highly dependent on STAT3 activation.

miR-21 Regulates Melanoma Cell Proliferation, Apoptosis, and Migration—To characterize the biological role of miR-21, B16 cells were transduced with an antagoniR-21 lentivirus (10) or a lentivirus containing empty vector (EV), and stable pools of cells were isolated. AntagoniR-21 transduced cells (miR-21KD) showed an ~75% knockdown in miR-21 expression relative to EV-transduced cells (Fig. 3A). In contrast, knockdown of miR21 had little effect on the basal and IFN-induced expression of other miRNAs (miR-196a, miR-296, and miR-351), as well as on the IFN-induced expression of MyD88, Ifi203, Cxcl11, and Il6 (Fig. 3B). These results demonstrate that antagoniR-21 was highly specific, i.e. selectively knocked down miR-21 expression, but had little (or no) effect on the expression of other miRNAs or IFN-induced genes.

We then studied the effects of miR-21 knockdown on B16 cell proliferation, apoptosis, and migration. As shown in Fig. 4A, knockdown of miR-21 in B16 cells reduced cell proliferation, indicating that miR-21 plays an important role in melanoma cell proliferation. Because IFN has known potent antiproliferative activity (11), it was not surprising that IFN treatment inhibited B16 cell proliferation. However, the combination of miR-21 knockdown and IFN treatment markedly reduced the proliferation of B16 cells. Because we previously found that miR-21KD sensitized prostate cancer cells to IFN-induced apoptosis (10), we next examined the effect of miR-21 expression on B16 cell apoptosis as determined by ELISA and Annexin V staining. Although IFN treatment or miR-21KD alone had only negligible effects on the level of apoptosis in B16 cells, IFN treatment of miR-21KD B16 cells induced significant apoptosis (Fig. 4B). We next determined the roles of miR-21 expression and IFN treatment on cell migration by transwell assays and found that both miR-21KD and IFN treatment significantly inhibited B16 cell migration (Fig. 4C). Moreover, the combination of miR-21 knockdown and IFN treatment further reduced B16 cell migration. Taken together, our results indicate that miR-21 knockdown enhances the sensitivity of B16 cells to IFN-induced apoptosis, as well as basal and IFN-induced inhibition of cell proliferation and migration.

miR-21 Targets Tumor Suppressors and Antiproliferative Proteins—miRNAs negatively regulate the expression of multiple genes post-transcriptionally by binding to the 3'-untranslated region of target miRNAs, thereby decreasing the expression of selective target proteins. A number of potential miR-21 targets have been described in the literature, including the tumor suppressors PTEN and PDCD4, and the antiproliferative proteins BTG2 and SPRY2 (23–26). To identify miR-21 targets in vitro, cell lysates were prepared from EV and miR-21KD B16 cells and subjected to immunoblotting. As shown in Fig. 4D,
miR-21 knockdown in B16 cells resulted in increased levels of PTEN, PDCD4, and BTG2 but had no effect on SPRY2 levels, indicating that PTEN, PDCD4, and BTG2 are miR-21 targets in B16 cells, whereas SPRY2 is not. It is important to note that, whereas miR-21 knockdown did not affect PDCD4 levels in DU145 cells (10), miR-21 knockdown resulted in enhanced PDCD4 levels in B16 melanoma cells, demonstrating that miR-21 targets are cell type-dependent.

miR-21 Regulates the Metastatic Behavior of B16 Melanoma Cells in Vivo—B16 melanoma cells have the propensity to form lung metastases when injected in mice and thus are a well studied model for melanoma metastasis (18–20). Therefore, we sought to characterize the role of miR-21 further by examining the metastatic properties of B16 cells in vivo. In brief, B16 cells (EV and miR-21KD) were transduced with Luc-2 for bioluminescent imaging and introduced directly into the bloodstream of syngeneic C57BL/6 mice by tail vein injection. Bioluminescent imaging at 1 day after injection showed that equivalent numbers of B16 melanoma cells could be detected in the lungs of mice injected with EV or miR-21KD cells (1.55 ± 0.52 versus 1.14 ± 0.15 × 10⁴ photons/s per cm², respectively). At 16 days after injection a strong luciferase signal was detected in the lungs of 8 of 8 mice injected with EV-transduced B16 cells, representing a 2,360-fold increase in bioluminescent signal intensity relative to that of the day 1 signal (Fig. 5A). In contrast, at 16 days after injection a relatively weak signal was detected in the lungs of mice injected with miR-21KD B16 cells, which represented only a 4-fold increase in signal intensity relative to the day 1 values. As further evidence of the altered metastatic potential of miR-21KD cells, knockdown of miR-21 markedly

**FIGURE 2.** Role of STAT3 in IFN-induced miRNA expression in B16 melanoma cells in vitro. B16 cells transduced with EV or F705-STAT3 were treated with IFN (1,000 units/ml) and cell lysates were prepared and immunoblotted as indicated (A), or after 6 h of IFN treatment the indicated miRNAs were quantified by qPCR (n = 3) (B). Error bars, S.D., **p < 0.01; NS, not significant.

**FIGURE 3.** Effects of miR-21KD on miRNA expression and IFN-induced gene expression in B16 cells in vitro. B16 cells transduced with anti-miR-21 lentivirus (miR-21KD) or with empty vector (EV) were treated with IFN (1,000 units/ml), and total RNA assayed for miRNA expression (A) or IFN-induced gene expression by qPCR (n = 3) (B). Error bars, S.D., **, p < 0.01; ***, p < 0.001.

**FIGURE 4.** Effects of miR-21KD on B16 melanoma cell proliferation, apoptosis, migration, and protein expression in vitro. A–C, EV and miR-21KD B16 cells were treated with IFN (1,000 units/ml), and at daily intervals cell numbers were determined in a Coulter Counter (n = 3) (A), or after 48 h apoptosis was determined by cell death ELISA or by Annexin V-staining (n = 3) (B), or after 24 h cell migration was determined by transwell migration assays (n = 3) (C). D, EV and miR-21KD B16 cells were lysed, and the expression of key target proteins was determined by immunoblotting with anti-PTEN, PDCD4, BTG2, and SPRY2. Error bars, S.D., *, p < 0.05; **, p < 0.01; ***, p < 0.001.
prolonged the survival of mice injected with B16 cells (Fig. 5B). Mice injected with EV-transduced cells had a mean survival of 18 days, whereas mice injected with miR-21KD B16 cells had a mean survival of 29.5 days. As additional evidence of tumor burden, the mean body weight of mice injected with EV-transduced cells was reduced by ~20% at 18 days after injection, whereas mice injected with miR-21KD cells exhibited a 25% increase in body weight (Fig. 5C).

Upon necropsy, mice injected with EV-transduced B16 cells exhibited large melanocytic tumors in their lungs, whereas mice injected with miR-21KD cells exhibited only small melanocytic nodules (supplemental Fig. S1A). Histological staining of lung tissue with H&E showed that EV-tumor bearing mice had widespread replacement of the pulmonary parenchyma with the metastatic neoplasm (supplemental Fig. S1B). In contrast, lung tissue of mice injected with miR-21KD cells exhibited relatively normal bronchial and vascular structures with minor metastatic deposits that measured 1–2 mm in diameter.

**DISCUSSION**

In the present study, we identified a new link between miR-21 and the metastatic behavior of melanoma cells. We show that upon tail vein injection, vector control B16 melanoma cells selectively home to the lung, forming rather large tumors, which is entirely consistent with previous studies (18–20). In contrast, B16 cells with miR-21 knocked down also home to the lung but only form micrometastases. Moreover, there was a significant increase in the mean survival of the mice injected with miR-21KD B16 cells. Taken together, these data demonstrate that miR-21 knockdown results in a diminished metastatic potential of B16 cells. Metastasis is a major cause of cancer-related death, especially in melanoma. Thus, our finding that miR-21 promotes the metastatic properties of melanoma has important clinical implications. Previous studies have indicated that miR-21 plays an important role in tumor metastasis. For example, tail vein injection of miR-21KD MDA-MB-231 breast cancer cells showed a significant reduction in the number of lung metastases, but the survival of the injected mice was not examined (8).

Relatively high miR-21 levels have been found in a variety of solid cancers (lung, prostate, breast, colon, stomach, liver) and hematological malignancies (1–3). The role of STAT3 in the expression of the oncomir miR-21 as well as in other STAT3-dependent miRNAs such as miR-296 and miR-351 is particularly important because constitutive activation of STAT3 has...
been demonstrated in a large number of diverse human tumors, and considerable evidence suggests that constitutive STAT3 activation actively participates in tumor formation and progression (27, 28).

The role of miR-21 in skin cancer is relatively uncharacterized. The oncogenic properties of miR-21 have been extensively studied in vitro in various cell lines, by strategies involving transfection and selection of stable miR-21-overexpressing or -underexpressing cells, or by short term knockdown of miR-21 expression by antisense oligonucleotides. To overcome the inherent problems related to either of these approaches we resorted to lentiviral transduction of cells with antagomiR-21 and have shown that the IFN-induced miRNA, miR-21, has potent effects on the behavior of melanoma cells in vitro and in vivo.

In vitro miR-21 promotes the proliferation, survival, and migration of tumor cells. The oncogenic effects of miR-21 on B16 melanoma cells in vitro may be mediated by down-regulation of the tumor suppressors PTEN and PDCD4 as well as the antiproliferative protein BTG2. Moreover, the protein targets of miR-21 appear to be highly cell type-dependent. Although SPRY2 is a reported target of miR-21 in colon cancer cells (29), we did not observe an effect of miR-21 on SPRY2 levels in B16 cells. In addition, we found that PDCD4 was a target of miR-21 in B16 cells, but PDCD4 was unaffected by miR-21 knockdown in DU145 prostate cancer cells (10). Most interestingly, when B16 melanoma cells were injected into the bloodstream of syngeneic C57BL/6 mice, miR-21 knockdown markedly enhanced animal survival as well as inhibited lung metastases. Taken together, our results indicate that miR-21 programs the metastatic behavior of cells, rendering miR-21 knockdown cells largely unable to colonize efficiently in the lung, and thus results in the formation of only micrometastases. Our findings that miR-21KD inhibited lung metastasis are in agreement with the recent studies showing that miR-21 plays a critical role in pre-B cell lymphoma and K-ras-dependent lung tumorigenesis (30, 31). These findings indicate that miR-21 is an important therapeutic target in cancer and suggest that selective inhibition of miR-21 can reprogram the proliferative, migratory, invasive, and metastatic characteristics of melanoma and potentially other cancers. Consistent with the critical role that we report herein that miR-21 plays in melanoma, a recent report showed that the loss of miR-21 reduced tumorigenesis in a mouse model of skin carcinogenesis (32). Overexpression of miR-21 in vivo leads to pre-B cell lymphoma and enhances K-ras-mediated lung tumorigenesis, whereas genetic ablation of miR-21 partially protects against tumorigenesis (30, 31).

Finally, our results also suggest that although some IFN-induced pathways may be antitumorigenic, the IFN-induced miR-21 pathway is protumorigenic. For example, although IFN has been shown to inhibit the proliferation, survival, and migration of cancer cells (11), we demonstrate that miR-21 promotes the proliferation, survival, and invasiveness of tumor cells. Thus, the anticancer action of IFN apparently is opposed by the IFN induction of the oncomir miR-21. Better characterization of these IFN-induced pathways could lead to novel strategies to improve the efficacy of IFN as an anticancer agent.

**Acknowledgments—**We thank Dr. Darren Baker (Biogen-Idec) for recombinant murine IFN, Dr. Levy Kopelovich (NCI, National Institutes of Health) for critical reading of the manuscript, and Zixuan Chen for help with Xenogen imaging.

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