The Modulation of MicroRNAs by Type I IFN through the Activation of Signal Transducers and Activators of Transcription 3 in Human Glioma

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Abstract
Type I IFNs are involved in double-stranded RNA responses. Here, we investigated the possibility that IFN-β may induce or downregulate cellular microRNAs (miRNA) in human neoplasms and thereby use the RNA interference system to show antitumor effects. Because of its known connection to glioma biology, we focused on miR-21 among seven miRNAs influenced by IFN-β. We analyzed the effect of IFN-β treatment on miR-21 expression in glioma cells and intracranial glioma xenografts. IFN-β treatment reduced miR-21 expression in glioma cells markedly, and IFN-β administration suppressed the growth of glioma-initiating cell–derived intracranial tumors. The levels of primary miR-21 gene transcripts, precursor miR-21, and mature miR-21 decreased 6 hours after the addition of IFN-β, indicating that the reduction in miR-21 levels was due to transcriptional suppression. We did reporter assays to elucidate the IFN-β-mediated suppression of miR-21; the addition of signal transducers and activators of transcription 3 (STAT3)–expressing vectors induced the IFN-β–mediated suppression of miR-21, whereas STAT3–inhibiting agents inhibited the miR-21 suppression. Thus, the results of our study show that the downregulation of miR-21 contributes to the antitumor effects of IFN-β and that miR-21 expression is negatively regulated by STAT3 activation. These results highlight the importance of understanding the transcriptional regulation of the miRNAs involved in oncogenesis. (Mol Cancer Res 2009;7(12):2022–2030)

Introduction
MicroRNAs (miRNA) are small noncoding RNAs consisting of 20 to 22 nucleotides that participate in the posttranslational regulation of gene expression by means of RNA interference (RNAi). The miRNA genes are transcribed by RNA polymerase II in the nucleus to form large pri-miRNA transcripts. These pri-miRNA transcripts are processed by Drosha to release the pre-miRNA precursor product, which is less than 70 nucleotides in length. After the pre-miRNA is transported into the cytoplasm, Dicer processes the intermediate to generate a mature 22-nucleotide miRNA. This mature miRNA is integrated into the RNA-induced silencing complex (1) and forms double-stranded RNA with complementary miRNAs (mRNA). Depending on the degree of homology between the miRNA and the mRNA, the RNA-induced silencing complex could inhibit mRNA function by either promoting its cleavage or by inhibiting its translation (2, 3). Emerging evidence suggests that miRNAs are involved in crucial biological processes, including the development, differentiation, apoptosis, and proliferation of mammalian cells (4). In humans, miRNAs have been proposed to contribute to oncogenesis because they possess multifaceted functions either as tumor suppressors or as oncogenes (5).

RNA interference induces a multitude of responses in addition to the knockdown of a gene. This is best understood in the context of an antiviral immune response. In particular, double-stranded RNA, a nucleic acid associated with viral replication, is involved in numerous interactions contributing to the induction, activation, and regulation of antiviral mechanisms. It is especially responsible for stimulating important protective responses such as the activation of dicer-related antiviral pathways, induction of type I IFN (IFN-α/β), and stimulation of double-stranded RNA–activated protein kinase and oligoadenylate synthase (6). IFN-α/β regulates the levels of crucial mediators of the antiviral response, such as protein kinase R, the 2′-5′ oligoadenylate synthase/RNase L system, the adenosine deaminase ADR1, or the Mx GTPase (7, 8). Thus, RNA interference might be involved in the IFN-mediated antiviral response. It was recently reported that the levels of liver-specific miRNA, i.e., miR-122, and several other miRNAs are regulated by IFN-β in human hepatoma cells, and that IFN-β rapidly modulates the expression of miRNAs, which target the hepatitis C virus genomic RNA, and thus, inhibits viral replication (9, 10).

In addition to its ability to interfere with viral replication, IFN-β is also known for its antiproliferative effects in a variety of neoplasms such as hepatocellular carcinoma, chronic myeloblastic leukemia, melanoma, renal cancer, and glioma (11, 12). However, the possibility that IFN-β might induce or downregulate cellular miRNAs in human neoplasms and thereby use the RNA interference system in its action against tumor progression has been left unexplored.
Malignant glioma represents ~20% of all intracranial tumors. Despite the advances in radiation therapy and chemotherapy administered after the surgical resection of the tumor, the prognosis of malignant glioma remains poor, with a median survival time of <10 months (13). In the treatment of malignant gliomas, IFN-β exhibits pleiotropic biological effects such as antiproliferation, immunomodulation, induction of differentiation from glioma-initiating cells (GIC), and drug sensitization by classically activating Janus kinase/signal transducers and activators of transcription (STAT) pathways (14, 15). However, there is no report on IFN-mediated modulation of cellular miRNAs as an antitumor mechanism.

In the present study, we test whether IFN-β can alter the expression of cellular miRNAs in human glioma cells by using the data obtained from genomewide microarray technology. On the basis of the initial screening efforts identifying several increased or attenuated miRNAs, we show that in cultured glioma cells and orthotopic glioma xenograft, IFN-β treatment leads to STAT3-mediated reduction in the expression of miR-21, an antiapoptotic miRNA that has been shown to be overexpressed in gliomas (16).

Results

Differential miRNA Expression in Human Glioma Cells Treated with IFN-β

To investigate which miRNAs are induced or downregulated by IFN-β, we used a microarray containing 662 mammalian miRNAs. We identified a total of two overexpressing and five underexpressing human miRNAs in the IFN-β-treated glioma cells. The expression of miRNAs, including miR-187 and miR-194, was increased >2-fold, whereas that of miR-100, let-7a, let-7b, let-7c, and miR-21 was decreased <0.5-fold in the T98 cell line treated with IFN-β as compared with the expression levels in cells without any treatment (Fig. 1A; Supplementary Fig. S1 and Table S1). To confirm the accuracy of microarray data, we examined the changes in the expression of these miRNAs following IFN-β treatment by performing quantitative reverse transcription-PCR (qRT-PCR). The findings were similar to the pattern of expression observed in the miRNA microarray analysis (Table 1). Among these miRNAs influenced by IFN-β treatment, miR-21 was pursued because of its known connection to glioma biology. Indeed, IFN-β treatment of T98 glioma cells recovered the expression of programmed cell death 4 (PDCD4), a well-known target of miR-21 (refs. 17-19; Fig. 1B).

miR-21 Overexpression in Glioma Cells, Particularly in the GICs

Previously, miR-21 was suggested to be aberrantly expressed and to be one of the major antiapoptotic factors in malignant gliomas (16, 18, 20-22). To our knowledge, we have shown, for the first time, the overexpression of miR-21 in a surgical specimen of glioblastoma by performing in situ hybridization (Fig. 2A-F). The in situ hybridization was optimized to distinguish between the areas of high (blue) and low expression of miR-21. The locked nucleic acid–enhanced miR-21–specific probe clearly stained the glioblastoma tissue but did not stain the normal cortex tissue. Tumor cells expressed significant amounts of miR-21, as seen at high magnification, whereas nontumor tissue showed no expression of miR-21 (Fig. 2C and E). In contrast, neither tumor nor nontumoral tissues in the section adjacent to that hybridized with the miR-21 probe showed positive staining with the scramble probe (Fig. 2D and F). Next, we compared the miR-21 expression levels in glioma cell lines, GICs, and the normal brain tissue. The miR-21 was overexpressed in glioma cells compared with the normal brain. Notably, the amount of miR-21 was greater in GICs than in the glioma cell lines (Fig. 2G). This finding may indicate that miR-21 plays a crucial role in the initiation and progression of glioma.

IFN-β Downregulates miR-21 Transcription

Previous evidence reported by our group and others has shown that IFN-β induced growth inhibition or apoptosis in a variety of neoplasms (12, 23-25). Recently, we reported that IFN-β induced the differentiation of GICs to cells with an oligodendrocyte-like phenotype (26). In this study, we investigated the possibility that IFN-β might downregulate oncomir miR-21 in human gliomas. We compared the expression of miR-21 in IFN-β-treated and untreated glioma cells by using qRT-PCR. IFN-β treatment reduced the expression of miR-21 by ~40% to 60% in all the glioma cells tested, including the SKMG1, T98, U87, U251 glioma lines, and 0222-GIC (Fig. 3A). On the other hand, miR-21 was not affected by temozolomide, an alkylating agent commonly used in the treatment of glioma (Supplementary Fig. S2). At 4 weeks after the intracerebral inoculation of GICs, the mice received either PBS or IFN-β i.p. We previously showed that IFN-β suppressed the growth of GIC-derived intracranial tumor (26). In this study, the systemic delivery of IFN-β reduced the level of miR-21 in the tumor (Fig. 3B). The regulation of miRNA expression has been documented at the transcriptional level and RNA-mediated posttranscriptional processing (19). Therefore, we next analyzed the time course of reduction of primary miR-21 gene transcripts (pri-miR-21), precursor miR-21 (pre-miR-21), and mature miR-21 in response to IFN-β treatment (Fig. 3C). The pri-miR-21 transcript levels decreased 6 hours after the addition of IFN-β, and began to recover at ~48 hours. Similar to these findings, the reduction of pre-miR-21 and mature miR-21 occurred as early as 6 hours. However, the recovery of premiR-21 began later than that of pri-miR-21. These results indicate that the decrease in the levels of miR-21 is the result of transcriptional suppression.

STAT3 Negatively Regulates miR-21 Transcription

To examine the molecular mechanisms involved in miR-21 expression, we analyzed the structure of the pri-miR-21 gene by studying its promoter and primary transcripts. As previously reported (27), several conserved enhancer elements were found in the consensus sequence upstream of the transcription start site of the pri-miR-21 on the basis of TRANSFAC matrices, including Ets/PU.1, activator protein-1, serum response factor, CAAT/enhancer-binding protein-α, p53, and STAT3 (4). This suggests that highly conserved transcriptional regulatory mechanisms may operate on the pri-miR-21 promoter (Fig. 4A). Of all these transcription factors, we focused on STAT3 in this study because IFN-β phosphorylates the tyrosine and, in part,
the serine of STAT3 in both T98 and SKMG1 glioma cells (Fig. 4B). Furthermore, a STAT3-specific inhibitory peptide increased the level of miR-21 expression and inhibited IFN-β-mediated suppression of miR-21 (Fig. 4C).

We constructed a reporter plasmid in which the full-length pri-miR-21 promoter was fused to the 5′-end of the luciferase gene. The plasmid was transfected into T98 and SKMG1 cells. The reporter gene system displayed high basal activity in untreated cells and clearly reduced activity in response to IFN-β treatment. In contrast, the addition of the STAT3 inhibitor prior to IFN-β treatment returned the activity of the promoter to the basal level (Fig. 5A). To further determine if STAT3 is responsible for the reduction of the promoter activity, the reporter construct harboring the pri-miR-21 promoter was cotransfected into SKMG1 cells with a STAT3-expressing plasmid, which was found to increase the total amount as well as the phosphorylation of STAT3 (Fig. 5B). As shown in Fig. 5C, the STAT3-expressing vector significantly reduced the promoter activity, and the addition of IFN-β further suppressed it.

**Discussion**

In this study, we hypothesized that type I IFN might regulate the expression of specific miRNAs in gliomas and that these
modulations lead to antiproliferative effects. By performing initial screening by the microarray method, we observed an increase in the miR-187 and miR-194 levels and a decrease in the levels of miR-100, miR-21, and let-7 family miRNAs in response to IFN-β treatment. The biological functions and putative targets of each miRNA, except the let-7 family miRNAs and miR-21, in cancer remain unclear (Table 1). miR-187 was reported to be overexpressed in thyroid tumors (28). The level of miR-194 was decreased in colon cancer and oral cancer, but increased in prostate cancer. Therefore, it is unclear whether miR-194 acts as an oncomir or a tumor suppressor. Similarly, miR-100 was reported to be highly expressed in ovarian carcinoma and hepatocellular carcinoma, but its expression was lower in oral cancer. Let-7 is one of the first identified miRNAs. The biological functions of let-7 in animals include the regulation of stem cell differentiation, organ development, and cell proliferation and differentiation. Moreover, many let-7 family members function as tumor suppressors in a variety of cancers (29). However, there is no report suggesting that let-7 functions as a tumor suppressor in gliomas. Of the miRNAs regulated by IFN-β, we focused on miR-21 because it is one of the most well known miRNAs associated with tumorigenesis and progression in gliomas. miR-21 also modulates tumorigenesis through the regulation of genes, such as bcl-2, PTEN, tropomyosin-1, and PDCD4 (17, 30-32). Indeed, IFN-β treatment of T98 glioma cells recovered the expression of PDCD4 (Fig. 1B). These results suggest that miR-21 is one of the major antiapoptotic factors. Our results showed miR-21 overexpression in a glioblastoma surgical specimen by performing in situ hybridization with the miR-21–specific probe, and in glioma cell lines and GICs by performing qRT-PCR. To our knowledge, this is the first report to show the expression of miR-21 in situ in a human glioma surgical specimen.

We found that IFN-β downregulated miR-21 in cultured glioma cells and intracranial glioma xenograft in mice. In the time course experiments, miR-21 expression was reduced as early as 6 hours after IFN-β treatment. The IFN-β treatment showed a relatively fast response in reducing miR-21 levels, suggesting that the negative regulation of miR-21 might be mediated directly by IFN-β, for example, through phosphorylation of Janus kinase/STAT. Our finding that IFN-β also suppressed the expression of pri-miR-21 and pre-miR-21 suggests that it regulates miR-21 transcription. The putative regulatory region of the miR-21 gene is located within an intron of the overlapping transmembrane protein 49 (TMEM49) gene, and contains two consensus STAT3-binding sites at −800 bp upstream from the transcription start site (33). The results of a recent study (26), similar to our findings, showed that IFN-β induces the phosphorylation of STAT3 in glioma cells and thereby activates STAT3-mediated miR-21 transcription in a luciferase reporter gene system. Our findings support the hypothesis that STAT3 activation exerts a cytoprotective or antiproliferative effect in some types of cells (34-37); however, the role of STAT3 activation is debatable because its overactivation has been reported to be oncogenic in some cell lines (38, 39). Loffler et al. showed that IL-6–dependent STAT3 activates the transcription of miR-21 in multiple myeloma cells. Whereas IL-6 induces proliferation of myeloma cells, IFN-β reduces the growth of glioma cells or induces apoptosis in these cells (33). The possible explanation of this seemingly paradoxical role of STAT3 activation is that the STAT pathway is context-dependent and that various intracellular and/or environmental cues play a pivotal role in determining the outcome of pathway activation. This discrepancy may arise from the difference in cytokine stimulus and cell type (33).

An unresolved question that needs to be addressed is why the recovery of mature miR-21 occurred earlier than that of pri-miR-21 and pre-miR-21, as shown in Fig. 3C. miR-21 may form a family and possess isoforms similar to those of let-7 family miRNAs, in which the mature let-7a sequence is produced by three separate precursors (pre-let-7a-1, pre-let-7a-2, and pre-let-7a-3). Similarly, mature miR-21 could be produced by precursor(s) as well as the known pre-miR-21. One other possibility is that the maturation of miR-21 may be involved in the recovery of mature miR-21 after IFN-β treatment.

In conclusion, the downregulation of miR-21 in response to IFN-β treatment contributes to the antitumor effects of this cytokine. This is the first report demonstrating that an oncomir miR-21 is downregulated in cancer by endogenous stimulation

### Table 1. Differentially Expressed miRNAs in T98 Glioma Cells Treated with IFN-β

<table>
<thead>
<tr>
<th>Probe no.</th>
<th>MiRNA Fold change (microarray)</th>
<th>Mean fold change (q-PCR*)</th>
<th>Chromosome location</th>
<th>No. of putative targets†</th>
<th>Connection to cancer in previous reports</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2269</td>
<td>hsa_miR_187</td>
<td>3.05</td>
<td>1.37 ± 0.13</td>
<td>18q12.2</td>
<td>0</td>
</tr>
<tr>
<td>2395</td>
<td>mmu_miR_187</td>
<td>3.00</td>
<td>NE</td>
<td>18A2</td>
<td>NA</td>
</tr>
<tr>
<td>2218</td>
<td>hsa_miR_194</td>
<td>2.68</td>
<td>1.74 ± 0.34</td>
<td>1q41, 11q13.1</td>
<td>35</td>
</tr>
<tr>
<td>2603</td>
<td>hsa_miR_100</td>
<td>0.16</td>
<td>0.7 ± 0.15</td>
<td>11q24.1</td>
<td>6</td>
</tr>
<tr>
<td>2285</td>
<td>hsa_let_7b</td>
<td>0.17</td>
<td>0.57 ± 0.08</td>
<td>22q13.31</td>
<td>12</td>
</tr>
<tr>
<td>2431</td>
<td>hsa_let_7c</td>
<td>0.20</td>
<td>0.6 ± 0.02</td>
<td>21q21.1</td>
<td>6</td>
</tr>
<tr>
<td>2556</td>
<td>hsa_let_7a</td>
<td>0.32</td>
<td>0.66 ± 0.07</td>
<td>9q22.32, 22q13.31</td>
<td>164</td>
</tr>
<tr>
<td>2220</td>
<td>mmu_let_7a</td>
<td>0.32</td>
<td>NE</td>
<td>13A5, 9A5.1</td>
<td>NA</td>
</tr>
<tr>
<td>2083</td>
<td>hsa_miR_21</td>
<td>0.33</td>
<td>0.44 ± 0.14</td>
<td>17q23.1</td>
<td>46</td>
</tr>
</tbody>
</table>

*The expression changes in mature miR-21, miR-187, miR-194, miR-100, and let-7a,b,c were validated in triplicate by using the LightCycler TaqMan Master and TaqMan MicroRNA assays. Values expressed as mean ± SD.
†Putative target genes were investigated by the prediction software programs, Targetscan (http://genes.mit.edu/tscan/targetscan2003.html; ref. 41), Miranda (http://www.microrna.org/microrna/getMirnaForm.do; ref. 42) and PicTar (http://www.pictar.org/) software (43). The name of common genes is listed in Supplementary Table S1.
with a cytokine or a growth factor. This finding adds a new dimension to the anticancer mechanism of IFNs. In addition, although there is little evidence supporting a direct or immediate transcriptional regulation of miRNAs by IFNs, this study shows for the first time that miR-21 expression is negatively regulated by STAT3 activation. Our results highlight the importance of understanding the transcriptional regulation of miRNAs, which would be involved in oncogenesis.

Materials and Methods
Glioma Cell Lines and Primary Tumor Sphere Cultures
We used human glioma cell lines (T98, SKMG1, U251MG, and U87MG) in this study. The source of the cell lines and the culture conditions have been reported previously (23). Glioma tissue samples were obtained from patients (nos. 0222, 0316, and 0125) undergoing surgical treatment at the Nagoya University Hospital, Nagoya, Japan, after they provided written informed consent. The procedures for the derivation of GICs were described in our recent article (26). Briefly, dissociated tumor cells were cultured in NBE media comprising neurobasal medium, with N2 and B27 supplements (Invitrogen), and human recombinant basic fibroblast growth factor and epidermal growth factor (20 ng/mL each; R&D Systems). We have previously shown that as few as $10^5$ GICs could form a tumor in the brain of nonobese diabetic/severe combined immunodeficient mice (26).

Treatment
Human IFN-β (kindly provided by Toray, Kamakura, Japan) was added to the culture medium at 12 h after $2 \times 10^5$ cells were placed in a 25 cm$^2$ cell culture flask (BD Falcon). To examine the effect of STAT3 inhibition on miR-21 expression, we treated the cells with 50 μmol/L of the STAT3 inhibitor (PpYLKTK-mts; Calbiochem) 30 min before the addition of IFN-β. We confirmed that the STAT3 inhibitor specifically inhibited the IFN-β–mediated phosphorylation at tyrosine-705 of STAT3 (data not shown).

miRNA Microarray Analysis
We used mirVana miRNA Bioarray V2 (Ambion) containing 662 miRNAs in four copies. We treated T98 cells with IFN-β at a concentration of 100 IU/mL or with PBS for 12 h. To isolate miRNA fractions from total RNA, we fractionated and cleaned up 30 μg of total RNA by using the flashPAGE fractionator system and reagents (Ambion). Chemically synthesized oligoribonucleotides (Ambion) or purified miRNAs were labeled by using the mirVana miRNA Labeling Kit (Ambion) and amine-reactive dyes as recommended by the manufacturer. Poly(A) polymerase and a mixture of unmodified and amine-modified nucleotides were first used to append a poly-nucleotide tail. The amine-modified miRNAs were then cleaned up and coupled to N-hydroxysuccinimide ester–modified Cy3 or Cy5 dye (GE Healthcare). We removed the unincorporated dyes by using a second glass fiber filter–based cleaning procedure. A 3× miRNA hybridization buffer (Ambion) was added to the fluorescently labeled miRNAs, and the solution was heated at 95°C for 3 min. Slides were hybridized from 12 to 16 h at 42°C in sealed cassettes by using a water bath. Following hybridization, the slides were washed and dried before performing a high-resolution scan on a GenePix 4000B (Axon Instruments). Raw data were normalized and analyzed using Array-Pro Analyzer Version 4.5 (Media Cybernetics) to determine the signal intensity of each spot and its local background on the microarrays. We calculated the net intensity by subtracting the mean intensity of all pixels within the local background area from the mean intensity of all pixels within the spot areas. We normalized differences in net intensity between the two fluorescent dye channels in a microarray by using the local regression (loess) normalization. We selected...
the analyzed data by using the MicroArray Data Analysis Tool (Filgen).

qRT-PCR

Total RNA, including miRNA, was isolated by using the mirVana RNA Isolation Kit (Ambion). The analyses of mature miR-21, miR-187, miR-194, miR-100, and let-7a, let-7b, and let-7c were carried out in triplicate by using the LightCycler TaqMan Master (Roche) and TaqMan MicroRNA assays (Applied Biosystems) on a LightCycler ST300 (Roche). PCR conditions were as follows: 95°C for 10 min and 45 cycles of 95°C for 10 s, 60°C for 30 s, and 40°C for 30 s. The relative level of miRNA expression was calculated by the 2-ΔΔCt method. The data were normalized to the expression of U6B small nuclear RNA. In some assays, samples from the normal brain tissue were used as a calibrator, whereas in others, untreated samples were used.

To quantify the pri-miR-21 and pre-miR-21 expression, we treated the isolated RNA with DNase and reverse-transcribed it using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The LightCycler-FastStart DNA Master SYBR Green I kit (Roche) was used for real-time PCR applications. The primer sets for pri-miR-21 and pre-miR-21 qRT-PCR were 5′-TTTTGGTTTTGGTGGAGGA-3′ and 5′-AGCAGACAGTCAGAGGAT-3′, and 5′-TGTCAGACAGCCCAATCGACT-3′, respectively. The PCR conditions were as follows: 95°C for 15 min, and 40 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s.

Western Blotting

Cell lysis and immunoblotting were done as described previously (23). Antibodies against the following proteins were used: PDCD4 (ab51495; Abcam), phosphorylated STAT3 (p-Ser727

FIGURE 3. IFN-β downregulates miR-21 transcription. A. The expression of miR-21 in IFN-β–treated and untreated glioma cells was compared by using qRT-PCR. In all the glioma cells analyzed, IFN-β treatment reduced the expression of miR-21 by ~40% to 60%, including the cells of the SKMG1, T98, U87, and U251 glioma lines, and 0222-GIC cells. B. At 4 wk after intracerebral inoculation of GICs, the mice received i.p. injection of either PBS or IFN-β. IFN-β suppressed the growth of the GIC-derived intracranial tumor. In particular, the systemic delivery of IFN-β reduced the level of miR-21 in the tumor. C. We analyzed the time course of reduction of the levels of pri-miR-21 gene, pre-miR-21, and mature miR-21 transcripts in response to IFN-β treatment. The levels of pri-miR-21 transcript were 0.46 ± 0.02, 0.44 ± 0.05, 0.74 ± 0.33, 0.55 ± 0.17, and 1.37 ± 0.82; pre-miR-21 levels were 0.23 ± 0.03, 0.17 ± 0.01, 0.26 ± 0.04, 0.19 ± 0.02, and 0.89 ± 0.32; and miR-21 levels were 0.53 ± 0.04, 0.69 ± 0.03, 0.78 ± 0.05, 1.11 ± 0.09, and 1.26 ± 0.16 at 6, 12, 24, 48, and 72 h, respectively. Columns, mean; bars, SD (*, P < 0.05).
and p-Tyr705), STAT3 (Cell Signaling), and β-actin (AC-15; Sigma-Aldrich).

Intracranial Glioma Xenograft and IFN-β Treatment

Animal experiments were done according to the principles described in the Guide for the Care and Use of Laboratory Animals prepared by the Office of the Prime Minister of Japan. We stereotactically injected 0316-GICs (1 × 10⁵ cells) resuspended in 5 μL of PBS into 5- to 6-wk-old nonobese diabetic/severe combined immunodeficient female mice (SLC, Shizuoka, Japan) as described previously (40). IFN-β (2 × 10⁵ IU/animal) was given i.p. at 4 wk after tumor inoculation. The control mice received PBS. Treatments were repeated at 24-h intervals for a total of five doses.

In situ Hybridization

Tissues were fixed with Tissue Fixative (Genostaff), embedded in paraffin, and sectioned at 6 μm. For performing in situ hybridization, the tissue sections were deparaffinized with xylene, and rehydrated using a series of ethanol washes and PBS. The sections were fixed with 4% paraformaldehyde in PBS for 15 min and then washed with PBS. They were then treated with 10 μg/mL of Proteinase K in PBS for 30 min at 37°C, washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 mol/L of HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1 mol/L of triethanolamine-HCl (pH 8.0) and 0.25% acetic anhydride for 10 min. After washing with PBS, the sections were...
dehydrated using a series of ethanol washes. Hybridization was done with either the locked nucleic acid–enhanced miR-21 probe (5′-TCAACATCAGTCTGAATAAGCTA-3′; Exiqon) or a scramble probe (5′-GTGTAACACGTCTATACGCCCA-3′) at concentrations of 18 nmol/L in the Probe Diluent (Genostaff) at 50°C for 16 h. After hybridization, the sections were washed in 5× HybriWash (Genostaff; equivalent to 5× SSC) at 50°C for 20 min and then in 50% formamide, 2× HybriWash at 50°C for 20 min. Subsequently, the sections were washed thrice with 2× HybriWash at 50°C for 20 min, and once with TBS Tween 20 (TBST; 0.1% Tween 20 in TBS). After treatment with 0.5% blocking reagent (Roche) in TBST for 30 min, the sections were incubated with anti–DIG-AP conjugate (Roche) diluted 1:1,000 with TBST for 2 h. The sections were washed twice with TBST and then incubated in 100 mmol/L of NaCl, 50 mmol/L of MgCl₂, 0.1% Tween 20, and 100 mmol/L of Tris-Cl (pH 9.5). Coloring reactions were done using nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate, an alkaline phosphatase color substrate. The sections were counterstained with Kernechtrot stain solution (Muto, Tokyo, Japan), dehydrated, and then mounted with Malinol (Muto).

Luciferase Reporter Assay

The miR-21 promoter/enhancer region was amplified by PCR from human genomic DNA using the primers 5′-TTTGGTACCTTGCTAATGCATTCT-3′ and 5′-TTTAGATCTAGTTCA GCTATGGTAAGAGC-3′ and inserted into the KpnI and BglII sites of pGL3-Enhancer vector (Promega) immediately downstream of the luciferase gene to form a pGL-miR-21 promoter/enhancer construct. The overexpression of STAT3 in the cells was achieved by transfection with pcDNA3.1-STAT3 that was provided by Dr. Takeshi Senga (Department of Tumor Biology, Nagoya University School of Medicine, Nagoya, Japan). The SKMG1 and T98 cell lines were seeded in 24-well plates for 24 h and then transfected with 1 μg of pGL-miR-21 promoter/enhancer construct with or without pcDNA 3.1-STAT3 for 48 h.
Statistical Analysis

The statistical significance of the differences observed was determined by ANOVA (StatView; SAS Institute), and Bonferroni’s correction was applied for multiple comparisons.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest are disclosed.

References

Molecular Cancer Research

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