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Interferon induces miR-21 through a STAT3-dependent pathway as a suppressive negative feedback on interferon-induced apoptosis

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Abstract

The microRNA miR-21 is overexpressed in many human cancers where accumulating evidence indicate it functions as an oncogene. Here we report that the cytokine; rapidly induces miR-21 expression in human and mouse cells. STAT3 was implicated in this pathway based on the lack of an effect of IFN on miR-21 expression in prostate cancer cells with a deletion in the STAT3 gene. STAT3 ablation abrogated IFN induction of miR-21, confirming the important role of STAT3 in regulating miR-21. Chromatin immunoprecipitation analysis showed that STAT3 directly bound the miR-21 promoter in response to IFN. Experiments in mouse embryo fibroblasts with a genetic deletion of the p65 NF-κB subunit showed that IFN-induced miR-21 expression was also dependent on NF-κB. STAT3 silencing blocked both IFN-induced p65 binding to the miR-21 promoter and p65 nuclear translocation. Thus, IFN-induced miR-21 expression is co-regulated by STAT3 and NF-κB at the level of the miR-21 promoter. Several cell death regulators were identified as downstream targets of miR-21, including PTEN and Akt. Functional experiments in prostate cancer cells demonstrated directly that miR-21 plays a critical role in suppressing IFN-induced apoptosis. Our results identify miR-21 as a novel IFN target gene that functions as a key feedback regulator of IFN-induced apoptosis.

INTRODUCTION

Interferons (IFNs) are antiviral cytokines that have significant effects on cell proliferation, differentiation, apoptosis, and the immune system. Binding of the type I IFNs (such as IFNα, IFNβ and IFNω) to their cognate cell surface receptor leads to the activation of the receptor-associated JAK1 and TYK2 tyrosine kinases. This process results in the recruitment, tyrosine phosphorylation, dimerization, and nuclear translocation of STAT proteins (1,2). In the classical JAK-STAT signaling pathway STAT1 and STAT2, in a complex with IRF9, bind to the conserved IFN-stimulus response element within the promoters of the early response IFN-stimulated genes (ISGs), thereby inducing their expression. However, a number of studies have implicated a role for STAT3 in IFNαβ action (3–7). STAT3, which was originally identified as the transcription factor for acute phase response genes, is

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activated by a wide variety of cytokines suggesting that STAT3 may integrate diverse signals into common transcriptional responses (5,8,9).

Besides inducing ISG expression, IFNs have been found to regulate the expression of miRNAs, which may play a role in IFN-induced antiviral activity (10). miRNAs are an abundant class of small RNAs that control gene expression at the post-transcriptional level through mRNA degradation or repression of mRNA translation. miRNAs are able to regulate the expression of multiple targets by binding to the 3′-untranslated regions of genes. Emerging evidence suggests that miRNAs are involved in many critical biological processes, including development, differentiation, apoptosis, proliferation and the antiviral defense. The ability of IFN to induce apoptosis is somewhat paradoxical, because IFN induces the apoptosis of certain cancer cells but in other cells IFN induces a potent cell survival pathway.

Since STAT3 has also been implicated in IFN action, we examined the promoters of known miRNAs and identified a potential STAT3 binding site in the miR-21 promoter. Expression of miR-21 appears to be upregulated in various human cancers (glioblastoma, breast cancer, prostate cancer), and is upregulated by IL-6 and Toll-receptor signaling (11,12). In this report we examined whether IFN regulates miR-21 expression, and the potential role of STAT3 in its regulation. We found that IFN up-regulated miR-21 expression in a variety of cells. Of particular interest was our finding that IFN induced miR-21 expression in DU145 prostate cancer cells, but not in PC3 prostate cancer cells that have a genetic deletion of the STAT3 gene. STAT3 knockdown of DU145 cells abrogated IFN induction of miR-21 and provided supportive evidence that STAT3 may regulate miR-21. Direct binding of STAT3 to the miR-21 promoter was confirmed by chromatin immunoprecipitation analysis. Moreover, IFN-induced miR-21 expression was dependent on the p65 subunit of NF-κB, which also bound to the miR-21 promoter. A number of downstream targets for miR-21 were also identified, including STAT3 expression itself. Thus, STAT3 regulates IFN-induced miR-21 expression, and in a positive feedback loop miR-21 expression up-regulates STAT3 expression. In addition, we found that miR-21 regulated IFN-induced apoptosis. While IFN-induced apoptosis was inhibited by miR-21 overexpression, miR-21 knockdown enhanced IFN-induced apoptosis. These results identify miR-21 as an IFN target gene and place miR-21 as an important regulator of IFN-induced apoptosis.

MATERIALS AND METHODS

Biological reagents and cell culture

Recombinant human IFNα (IFNα Con1) and murine IFNβ was provided by InterMune (Brisbane, CA) and Biogen-Idec, Inc. (Cambridge, MA), respectively. The biological activity of the IFN preparations was expressed in terms of international reference units/ml using the appropriate NIH reference standard as described previously (3). Human PC3 and DU145 cells (obtained from American Type Culture Collection) were cultured in RPMI 1640 with 10% Bovine Calf Serum (BCS). Cells were maintained in the presence of penicillin (100 IU/ml) and streptomycin (100 mg/ml) at 37°C with 5% CO_2. The cell lines were not authenticated in our laboratory.

Detection of miRNA expression using polyA tailing SYBR green real time PCR

Total RNA (5 μg) was treated with DNase I for 15 min at 22°C (Invitrogen) and then polyA tailed using (polyA) polymerase (NEB; Ipswich, MA) at 37°C for 1 h. The final reaction mixture was extracted with phenol/chloroform, precipitated with isopropanol, and redissolved in 25 μl of diethylpyrocarbonate (DEPC)-treated water. PolyA-tailed RNA (6 μl) was reverse-transcribed into first-strand cDNA using Superscript III transcriptase

(Invitrogen) with the oligo-dT adapter primer 5′GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-3′. For PCR, 30 ng of cDNA was used as a template in each reaction. The reverse primer was from the adapter sequence: 5′GCGAGCACAGAATTAATACGACTCAC3′ and the forward primers were specific to miR-21 mature sequences. U6 small non-coding RNA sequence was amplified as an internal control using the primers 5′-CTCGCTTCGGCAGCACA-3′ (forward) and 5′-AACGCTTCACGAATTTGCGT-3′ (reverse). The SYBR Green-based real-time PCR was performed using MyiQ Real-Time PCR Detection System (Bio-Rad). The relative expression of miRNA was calculated based on the formula: $2^{\Delta tmiRNA\ 21-\Delta tU6}$.

Construction of Lentivirus to overexpress miR-21 or knockdown miR-21

To overexpress the miR-21 gene, we amplified a fragment from mouse genomic DNA using the primers 5′CGCTCTAGATCGTGGTCGTGACATCGCATG (forward) and 5′GCGGATCCGGATGGTCAGATGAAAGATACC(reverse), which contain pre-miR-21 gene with 160bp upstream and 65bp downstream flanking sequence, then inserted it into XbaI and BamHI sites of the pLenti-pgk-puro lentiviral vector, obtained from the viral vector core (VVC) of UTHSC. To knockdown miR-21 expression, oligonucleotides (CGCTCTAGATCAACATCAGTCTGATAAGCTA:forward and GCGGATCCTAGCTTATCAGACTGATCTTGA: reverse) against the mature sequence of miR-21 gene were annealed and cloned into XbaI and BamHI site of lentiviral vector pLenti-U6-pgk-puro (VVC, UTHSC), resulting in the antisense miR-21 gene being driven by polymerase III promoter U6. Lentivirus was produced in 293FT cells by cotransfection of the lentiviral vectors with ViraPower Packaging Mix (Invitrogen). The supernatant was collected 60 h post-transfection and was centrifuged (3,000xg for 15 min at 4°C) to remove cell debris; then the supernatant was passed through a 0.45-μm filter and virus collected by ultracentrifugation (50,000xg for 3 h at 4°C). The pellet was dissolved in phosphate-buffered saline (PBS) and frozen at −80°C. Lentiviral particles were titrated in 293FT cells with a p24 ELISA assay (Perkin Elmer, Boston, MA).

Knockdown of STAT3 using shRNA-miR

To knockdown the human STAT3 gene, we purchased STAT3 shRNA-miR lentiviral vector pGIPZ, which contains a human miR-30 hairpin based shRNA-miR structure against STAT3 (OPEN Biosystems, Huntsville, AL). The target sequence of STAT3 used is AAACGGAAGCTGCAGAAAGATA.

Chromatin Immunoprecipitation—Chromatin Immunoprecipitation (ChIP) was carried outusing the ChIP-IT™ Express Enzymatic kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. In brief, chromatin from cells was cross-linked with 1% formaldehyde (10 min at 22°C), sheared to an average size of 200 bp by enzymatic digestion, and then immunoprecipitated with anti-p65 or STAT3 (Santa Cruz Biotechnology). The ChIP-PCR primers were designed to amplify a proximal promoter region containing putative binding sites in the miR-21 promoter identified by TFSEARCH.

Immunoblot analysis—Total cell lysates (25 μg) 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).

Apoptosis assay—The induction of apoptosis was monitored by DNA fragmentation using the cell death detection $ELISA^{PLUS}$ or by terminal deoxynucleotidyltransferase-mediated UTP end labeling.

Data analysis—The experiments were conducted in at least three independent experiments 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

To examine whether IFN affected miR-21 expression, a variety of normal and cancer cell lines of different origins (prostate cancer, glioma, melanoma and fibroblasts) were treated with IFNα (1000 IU/ml for 6 hr), total RNA was isolated and miR-21 expression was determined by quantitative real time PCR (qPCR). As shown in Figure 1A, although basal miR-21 expression markedly varied among the different cell lines, IFN consistently induced a 3 to 5-fold increase in miR-21 expression. The notable exception was the failure of IFN to upregulate miR-21 in PC3 prostate cancer cells that lack the STAT3 gene (13). We next examined the dose-dependence and time course of IFN-induced miR-21 expression in DU145 cells and found that IFN-induced miR-21 at IFN concentrations greater than 10 IU/ml with near maximal induction observed at 100 IU/ml Fig. 1B). Moreover, IFN induced miR-21 expression within 2 hr after IFN addition (1000 IU/ml), miR-21 levels peaked between 6 and 24 hr after IFN addition and remained elevated at 48 hr after IFN addition. It is of interest that the dose-dependence and time course of IFN induction of miR-21 expression were similar to what is observed for ISG 15 (Fig. 1C). These data indicate that IFNs induce miR-21 expression in a variety of human cell lines, and the kinetics and dose-dependence of induction is similar to that of a "classical" ISG.

The roles of STAT3 and NF-κB in IFN-induced miR-21 expression

Since IFN induced miR-21 in a variety of cell lines but not in STAT3-deficient PC3 cells, we next examined whether STAT3 was involved in IFN-induced miR-21 expression. Activation of STAT3 involves the phosphorylation of tyrosine 705 within its transactivation domain, which is required for STAT dimerization, nuclear translocation, and induction of gene transcription. Expression of a mutant form of STAT3 with the canonical Y705 phosphorylation site mutated to phenylanaine has dominant-negative properties (DN-STAT3) (14). IFN induces STAT3 activation in DU145 cells, while PC3 cells lack STAT3 (Fig. S1). DU145 cells were transfected with DN-STAT3, stable pools were isolated and examined for IFN induction of miR-21 expression. As shown in Fig 2A, IFN induced miR-21 expression in DU145 cells transfected with empty vector (EV) consistent with the results presented in Fig 1. However, IFN did not induce miR-21 expression in DU145 cells transfected with DN-STAT3. Moreover, expression of DN-STAT3 in DU145 cells also reduced the basal expression of miR-21. As a complementary approach, DU145 cells were transduced with a lentivirus-delivered shRNA against STAT3. shRNA knocked down STAT3 protein levels in DU145 cells by ~80% (Fig. S2), and most importantly nearly completely ablated IFN-induced miR-21 expression (Fig. 2B). In contrast, in cells transduced with control shRNA IFN induced miR-21. Taken together these results suggest that STAT3 regulates IFN-induced miR-21 expression.

Since we previously showed that there was crosstalk between STAT3 and NF-κB in the IFN activation pathway (4,6) and LPS-induced miR-21 expression was found to be dependent on the p65 NF-κB protein (15), we next examined whether IFN induced miR-21 was also dependent on p65. IFN induced miR-21 in wild-type mouse embryo fibroblasts (MEFs) but not in MEFs derived from p65-knockout mice (Figure 2C). The induction of miR-21 by LPS was also markedly reduced in p65-knockout MEFs as compared to wild-type MEFs, as reported previously (15). Moreover, we found that two other IFN-induced miRs, miR-296 and miR-351 (10), differed in their p65 dependence (Figure 2D). While IFN induction of

miR-296 was clearly p65-dependent, induction of miR-351 was independent of p65. It is of interest, that we previously showed using p65-knockout MEFs that the NF-κB mediated repression of some ISGs, such as mx1 and nmi, was also p65-dependent (16). These results showed that both the p65 NF-κB protein and STAT3 play important roles in regulating IFN-induced miR21 expression.

Binding of STAT3 and the p65 NF-κB subunit to the miR-21 promoter

Since p65 and STAT3 appear to be required for IFN induction of miR-21 expression, we next examined whether they directly bind to the miR-21 promoter. Examination of putative transcription binding sites by TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) revealed three STAT3 binding sites (hereafter referred to as STAT3-I, -II and -III) and four NF-κB binding sites in the miR-21 promoter (Fig. S3). Specific primers were designed and synthesized for each potential binding site in the miR-21 and the binding of STAT3 and p65 to these putative sites was demonstrated by ChIP analysis. In brief, DU145 cells were treated with IFNα (1000 IU/ml for 1 hr), protein-DNA complexes crosslinked with formaldehyde, chromatin was sheared to average of 500bp and immunoprecipitated with anti-STAT3 or -p65, crosslinking reversed and the resulting DNA sequences detected by PCR and qRT-PCR. As shown in Fig. 3A IFN induced significant binding to the distal STAT3-I binding site (~3.4 kB from the start site of the miR-21 precursor). This STAT3 binding site was previously identified by ChIP analysis in the upregulation of miR-21 by IL-6 (12). In contrast, IFN did not induce significant STAT3 binding to the other putative STAT3 binding sites in the miR-21 promoter. Taken together these results show that STAT3 regulates miR-21 expression in IFN-treated DU145 cells by directly binding to the miR-21 promoter. This led us to examine whether p65 bound to the NF-κB binding site that was nearby the STAT3-I site in the miR-21 promoter. As shown in Fig. 3B, in DU145 cells IFN induced p65 binding to this NF-κB site in the miR-21 promoter. Most interesting, STAT3 knockdown completely ablated NF-κB binding. Moreover, STAT3 knockdown also blocked the IFN-induced translocation of the p65 subunit of NF-κB into the nucleus (Fig. 3C). These results taken together suggest that p65 nuclear translocation and binding to the miR-21 promoter are both STAT3-dependent.

miR-21 expression suppresses IFN-induced apoptosis

Previous studies indicate that miR-21 may regulate apoptosis. Although IFN induces apoptosis in some cancer cell lines, this action appears to be counterbalanced by the induction of a potent cell survival pathway, which is NF-κB dependent. We next examined whether IFN-induced apoptosis in DU145 or PC3 cells. Cells were treated with IFN (1000 IU/ml for 24 hr) and apoptosis measured by a cell-death ELISA. As shown in Figure 4, while IFN induced marked apoptosis in PC3 cells, little apoptosis in DU145 cells was observed upon IFN addition. Since IFN-induced miR-21 expression in DU145 but not in PC3 cells, we hypothesized that miR-21 expression might suppress IFN-induced apoptosis. Therefore, PC3 cells were transduced with miR-21 and stable cell lines were isolated with miR-21 expression levels comparable to DU145 cells (Fig. S4). We then compared PC3 cells overexpressing miR-21 to EV-expressing PC3 cells for IFN-induced apoptosis, and found that miR-21 expression completely blocked IFN-induced apoptosis in PC3 cells as measured by a cell death ELISA assay and by TUNEL staining (Fig. 4). These results suggest that miR-21 expression suppresses IFN-induced apoptosis. We then used a complementary approach to knockdown miR-21 expression in DU145 cells by ~ 85% (Fig. S4) and examined the effect on apoptosis induced by IFN. As shown in Fig 4B, while IFN did not promote significant apoptosis in EV-expressing DU145 cells, IFN induced apoptosis in anti-miR-21 expressing DU145 cells. These results taken together suggest that miR-21 plays an important role in suppressing IFN-induced apoptosis.

We next examined whether miR-21 plays a role in suppressing apoptosis induced by other inducers of apoptosis, camptothecin and staurosporine. As shown in Figure 4B, miR-21 overexpression in PC-3 cells suppresses apoptosis induced not only by camptothecin and staurosporine, but also by IFN. Moreover, miR-21 knockdown in DU145 cells enhances apoptosis induced by camptothecin and staurosporine. These results taken together suggest that induction of miR-21 by IFN is protective against apoptosis induced by a variety of anticancer agents (IFN, camptothecin and staurosporine).

The effect of miR-21 on potential gene targets

Individual miRNA targets genes by regulating their expression at both transcriptional and post-transcription levels. Several studies have identified a number of target genes for miR-21. Since we have shown that miR-21 suppressed IFN-induced apoptosis in PC3 cells, we examined the effect of miR-21 on possible anti-apoptotic effectors in PC3 cells. Activation of the Akt pathway in a variety of cells contributes to cell survival. In addition, we previously showed that Akt activation was critical in the anti-apoptotic pathway induced by IFN (17). As shown in Figure 5A IFN-induced Akt activation in PC3 cells expressing miR-21 but not in control vector-transduced PC3 cells. IFN activated Akt within 30 min of addition, and Akt activity nearly peaked at 2 hr and remained activated 48 hr after IFN addition. In contrast, IFN did not result in Akt activation in empty vector transduced PC3 cells. Moreover, Akt activation was not due to an IFN-induced increase in cellular Akt levels, as the levels remained relatively stable throughout the time-course of IFN addition. The rapid activation of Akt is similar to what we previously reported in Daudi B-lymphoblastoid cells, in which IFN induced a potent cell survival pathway that was dependent on PI3K and Akt (17). Therefore, we next examined whether miR-21 or IFN affected the expression of the p85 subunit of PI3K. As shown in Figure 5B, IFN-induced a time-dependent increase in p85 levels in control vector PC3 cells, but miR-21 expression resulted in a dramatic increase in basal p85 levels, which was further increased by IFN addition. Thus, these results identify the p85 subunit of PI3K, which is an upstream regulator of Akt activation (18), as a miR-21 target.

Previous studies have shown that miR-21 upregulates STAT3 expression (19), and downregulates PTEN expression (20). For these studies we compared expression levels of a number of potential miR-21 targets in DU145 cells and DU145 cells with miR-21 knockdown, since PC3 cells do not express STAT3 and PTEN (Fig. S5). As shown in Figure 5C, STAT3 is downregulated by miR-21 knockdown. Moreover, IFN induced STAT3 activation is similarly downregulated by miR-21 knockdown. In contrast, STAT1 levels are unaffected by miR-21 expression. Moreover, PTEN is upregulated in miR-21 knockdown cells, suggesting that PTEN is a target of miR-21 in DU145 cells. In contrast PDCD4, another known target of miR-21 (15), was unaffected by miR-21 knockdown suggesting that the effects of miR-21 are cell context dependent.

DISCUSSION

By expression profiling of miRNAs that are dysregulated in human cancers, miR-21 was found to be overexpressed in diverse forms of cancer including glioma, breast cancer, colorectal cancer, stomach and gastric cancer, lung cancer, hepatocellular carcinoma and prostate cancer (21). Similar to these findings we found that relative to expression in human fibroblasts, basal expression was higher in various cancer cell lines studied (glioma, prostate, melanoma, etc). Most importantly, we found that irrespective of basal expression IFN induced miR-21 expression. A recent study reported that IFN reduced miR-21 expression in human gliomas (22), which is in conflict with our findings. However, we cannot exclude cell-type specific differences in the effects of IFN on miR-21 expression. Several studies have established that miR-21 is induced by various stimuli. For example,

Toll ligand receptor activation by LPS upregulates miR-21 in many cells types, including macrophages, fibroblasts and PBMCs (15). Moreover, IL-6 promotes the survival of multiple myeloma cells through the induction of miR-21 expression (12). In the present study we show that in common with IFN-stimulated gene induction, miR-21 is induced within several hrs after IFN exposure, and induction is clearly dose dependent. Thus, miR-21 is an IFN target gene in multiple cell types.

The finding that IFN induced miR-21 expression in the DU145 prostate cancer cells but not in PC-3 cells, which have a genetic deletion of the STAT3 locus (13), provided suggestive evidence that STAT3 may regulate miR-21 expression. Expression of a dominant negative STAT3 construct (F705-STAT3) or knockdown of STAT3 expression in DU145 cells ablated IFN-induced miR-21 expression which demonstrated that STAT3 plays an important role in IFN-induced miR-21 expression. ChIP analysis on putative STAT3 binding sites in the miR-21 promoter showed that IFN induced STAT3 binding to the most distal STAT3 binding site, but had no effect on the two other putative STAT3 binding sites. Previous studies found that IL-6 induced STAT3 binding to this same STAT3 site in the miR-21 promoter (12). Furthermore, a reporter construct containing this same STAT3 binding site was shown to be IL-6 responsive. Taken together these results demonstrate that the IFN-induction of miR-21 is STAT3 dependent.

In addition, the miR-21 promoter has four putative NF-κB binding sites. Therefore, we examined the induction of miR-21 by IFN in mouse embryo fibroblasts deficient in the p65 subunit, and found that IFN induction of miR-21 was also dependent on p65. Moreover, we found that IFN induction of a known IFN-induced miR, miR-296, was also p65-dependent. Therefore, we next examined the direct binding of the p65 subunit of NF-κB to the miR-21 promoter by ChIP analysis. IFN induced p65 binding to the upstream region of miR-21 that was nearby the STAT3 site to which IFN induced binding. Most interestingly, STAT3 knockdown ablated IFN-induced p65 binding to this NF-κB binding site, as well as p65 nuclear translocation. These results suggest that IFN induces STAT3 and the p65 subunit of NF-κB subunit to simultaneously bind to mIR-21 promoter, which is of particular interest since we previously reported crosstalk between IFN-induced STAT3 and NF-κB signaling pathways (4,6). Moreover, our recent studies suggest that STAT3 plays a critical role in forming a complex with p65 in the cytoplasm that subsequently translocates into the nucleus to regulate gene expression (manuscript in preparation). Constitutive activation of STAT3 and NF-κB signaling has long been associated with cancer and inflammation. Therefore, it is highly interesting that miR-21 upregulation has also recently been found in various cancers (23) as well as inflammatory conditions, including osteoarthritis (24), psoriasis (25), ulcerative colitis (26), and cardiac hypertrophy (27).

Since miR-21 is involved in the control of tumor cell apoptosis (28,29), we reasoned that miR-21 might contribute to the regulation of IFN-induced apoptosis. We found that while IFN induced significant apoptosis in PC3 cells, little IFN-induced apoptosis was observed in DU145 cells, which was inversely correlated with the IFN-induced expression of miR-21 (i.e. IFN induced miR-21 in DU145 cells but not in PC3 cells). Ectopic overexpression of miR-21 in PC3 cells completely blocked IFN-induced apoptosis, while miR-21 knockdown in DU145 cells sensitized these cells to IFN-induced apoptosis. Moreover, the suppressive effect of miR-21 on apoptosis was also evident for other apoptosis inducers (camptothecin and staurosporine). These results are in agreement with the previous studies that miR-21 knockdown in glioblastoma cells enhances apoptosis (28). One major limitation of IFN as an effective therapy in cancer is its inability to efficiently induce the apoptosis in cancer cells. We propose that the upregulation of miR-21 in various cancers may be a cause of the intrinsic resistance of these cancers to IFN-induced cell death. Thus, manipulation of

miR-21 expression may be a possible means to improve the efficacy of IFN, alone or in combination with other chemotherapeutic agents, as a treatment for prostate cancer.

Since high miR-21 expression was linked to suppression of apoptosis, we examined various proteins that have been previously associated with the induction of an anti-apoptotic pathway by IFN. STAT3 has been shown to regulate apoptosis in various cell lines. Elevated STAT3 activity has been shown to render cells resistant to apoptosis by inducing anti-apoptotic genes such as BCL2, BCL-xL, and Mcl-1 (30). In contrast, inhibition of STAT3 activation by knockdown or expression of dominant negative STAT3 constructs has been shown to sensitize cells to apoptosis (31). Thus, it is of interest that we found that miR-21 expression was found to upregulate STAT3 expression. These results are also consistent with our previous studies that place STAT3 as an important component of a PI3K/Akt-dependent IFN-induced cell survival pathway (3,4). It is of particular interest that miR-21 targeted Akt activation but not Akt protein levels, which is consistent with our previous finding that IFN-induced Akt activation was critical to an NF-κB dependent cell survival pathway (17). Moreover, we found that miR-21 also targets PTEN, which is also dependent on STAT3. Thus, our evidence indicates that miR-21 downregulates PTEN levels through a STAT3-dependent pathway and results in enhanced Akt activity. However, it is important to note that in PC3 cells, which lack PTEN (32), IFN resulted in Akt activation through a miR-21-dependent pathway. Therefore, our results suggest that miR-21 may also activate Akt through a PTEN-independent pathway that involves the upregulation of the p85-subunit of PI3K.

In conclusion, we have found that IFN induced miR-21 expression through a pathway that involves both STAT3 and NF-κB. Most interesting is that miR-21 suppresses IFN-induced apoptosis through upregulating the levels of p85, STAT3 and Akt activity. This is the first report that the oncomir miR-21 can be upregulated by IFN and that manipulation of miR-21 expression by miR-21 knockdown can be employed to enhance IFN's apoptotic action. This finding adds a new dimension to the control of the anticancer action of IFN and provides a new understanding of the means that cancer and other diseases may have evolved means to subvert a normal host defense pathway to suppress IFN action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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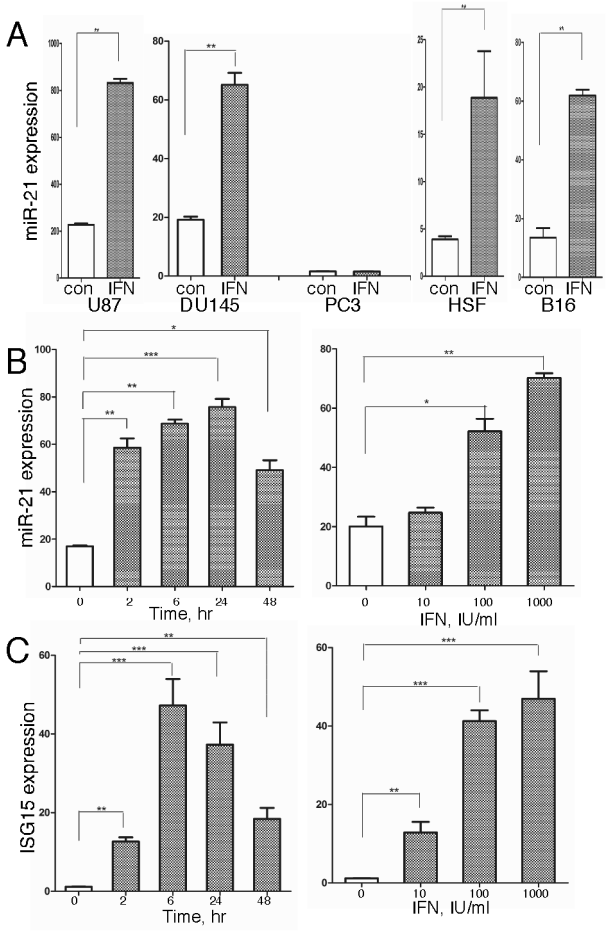

Figure 1. IFN induces miR-21 in a variety of cell types

(A) Human U87 glioma, DU145 and PC3 prostate cancer cells, and skin fibroblasts, and B16 mouse melanoma were treated with IFN (1000 IU/ml, for 6 h) prior to RNA extraction. DU145 cells were treated with IFN (1000 IU/ml) for the indicated times, or with the indicated concentrations of IFN for 6 h prior to RNA extraction and miR-21 (B) or ISG15 (C) expression was determined by qPCR. Expression was normalized to expression of U6 RNA for miR-21 and B-actin for ISG15, respectively. Data represent the mean +/− SD of at least three experiments performed in duplicate.

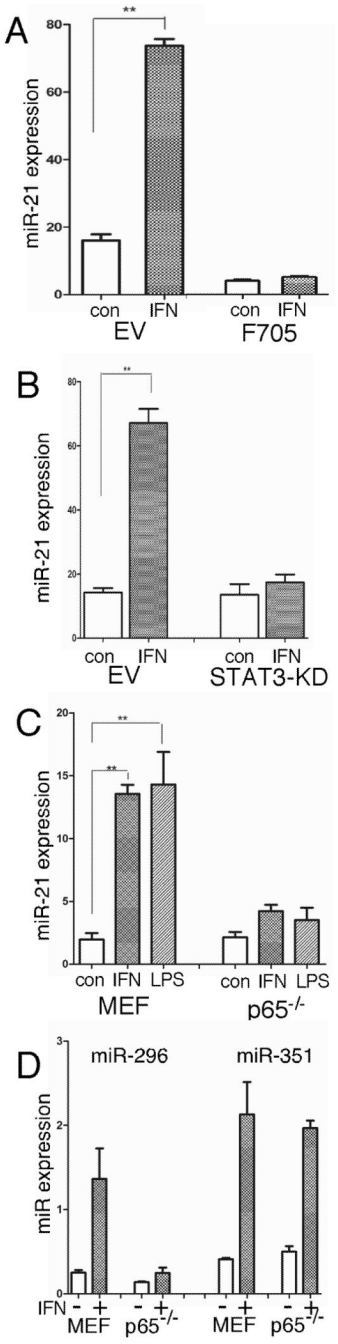

Figure 2. The roles of STAT3 and NF-κB in the induction of miR-21 by IFN

DU145 cells were (A) transfected with DN-F705 STAT3 (F705) or (B) transduced with lentivirus encoding shRNA for STAT3, and treated with human IFNα (1000 IU/ml for 6 h). (C,D) MEFs from wild type mice or mice in which the p65 subunit of NF-κB was knocked out ($p65^{-/-}$) were treated with murine IFN (1000 IU/ml) or LPS (40 ng/ml) as indicated for 6 h. (C) miR-21, or (D) miR-296 or miR-351 expression was determined by qPCR and expressed relative to U6 RNA levels. Data represent the mean +/− SD of at least three experiments performed in duplicate.

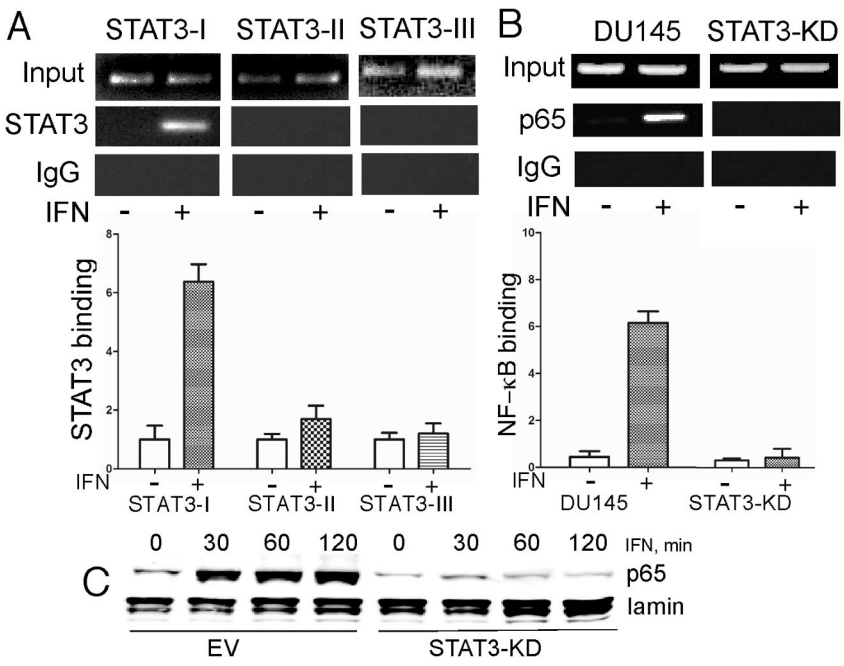

Figure 3. The IFN-induced binding of STAT3 and the p65 subunit of NF-κB to the miR-21 promoter

(A) ChIP analyses of STAT3 binding to the STAT3-I, -II and -III within the miR-21 promoter in DU145 mock treated or treated with IFNα (1000 U/ml) for 1 h. (B) ChIP analysis of p65 binding to κB site nearby the STAT3-I site was performed on DU145 cells and DU145 cells in which STAT3 levels were knocked down by shRNA for STAT3 (STAT3-KD). The ChIP-enriched DNA levels analyzed by Q-PCR were normalized to input DNA, followed by subtraction of non-specific binding determined by control IgG. (C) Immunoblot analysis for p65 in nuclear extracts prepared from control and STAT3-KD DU145 cells treated with IFN (1000 IU/ml) for the indicated times. Lamin serves as a loading control for nuclear extracts.

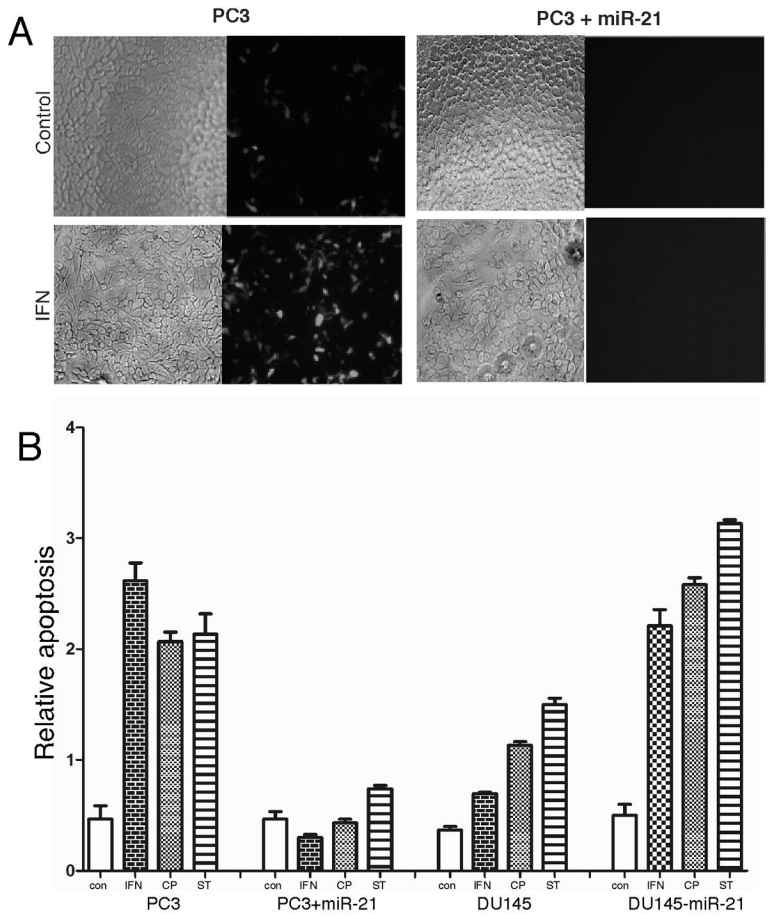

Figure 4. miR-21 suppresses IFN-induced apoptosis

(A) PC3 cells and PC3 cells overexpressing miR-21 (+miR-21) were treated with IFN (1000 IU/ml for 24 h) and analyzed for apoptosis by TUNEL assays. Phase contrast and fluorescent images are on the left and right, respectively. Apoptotic cells are fluorescent and appear green in the photomicrograph. (B) PC3 cells, PC3 cells overexpressing miR-21, DU145 cells, and DU145 cells in which miR-21 was knocked down (−miR-21) were treated for 24 hr with IFN (1000 IU/ml), camptothecin (CP, 1 μM) or staurosporine (ST, 1 μM) and analyzed for apoptosis by cell death detection ELISA assays. Data represent the mean +/− SD of at least three experiments performed in duplicate.

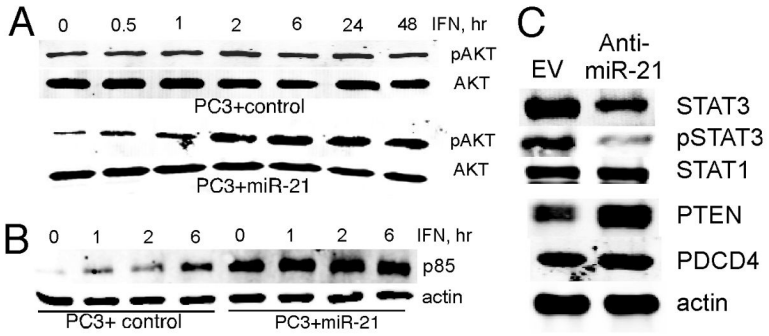

Figure 5. The effect of miR-21 on potential apoptotic effectors

(A and B) PC3 cells transduced with control vector and PC3 cells overexpressing miR-21 (+miR-21) were treated with IFN (1000 IU/ml) for the indicated times, and cell lysates were immunoblotted for total and phosphorylated Akt levels (A) or p85 and actin (B). (C) DU145 cells transduced with anti-miR control (EV) and DU145 cells in which miR-21 was knocked down (Anti-miR-21) were immunoblotted for the indicated proteins. For analysis of STAT3 phosphorylation cells were treated with IFN (1000 IU/ml, 2 hr)