Higher miRNA Tolerance in Immortal Li-Fraumeni Fibroblasts with Abrogated Interferon Signaling Pathway

Qunfang Li1 and Michael A. Tainsky1,2

Abstract

The IFN pathway is abrogated in fibroblasts from Li-Fraumeni syndrome (LFS) patients during spontaneous cellular immortalization, a necessary step in carcinogenesis. Microarray profiling of differentially expressed microRNAs (miRNA) revealed that most miRNAs were upregulated in IFN pathway–defective MDAH087-10 fibroblasts compared with MDAH087-N cells with relatively normal IFN signaling. Overexpression of Dicer, a critical enzyme in miRNA biogenesis, promoted cell growth and colony formation in MDAH087-10 cells. However, double-stranded miRNA produced by Dicer enhanced the expression of IFN-stimulated genes in MDAH087-N cells resulting in significant cell death and reduced cell growth. Furthermore, manipulation of the IFN pathway in immortal LFS fibroblasts through transcription factor IRF7 reversed their response to Dicer overexpression due to changed IFN pathway activity. Dicer overexpressing MDAH087-N cells contained lower levels of miRNA than vector control, and conversely much higher miRNA expression was detected in Dicer-transfected MDAH087-10 cells. Therefore, cells with a defective IFN pathway have a higher miRNA tolerance than cells with normal IFN pathway. This work indicates for the first time that the IFN pathway as mediated through the transcription factor IRF7 must be disrupted to permit miRNA upregulation to occur in early carcinogenesis. The IFN pathway appears to provide a checkpoint for miRNA level tolerance and its abrogation leads to cellular immortalization. Cancer Res; 71(1); 255–65. ©2011 AACR.

Introduction

MicroRNAs (miRNA) are small, [19–25 nucleotides (nt)] noncoding single-strand RNAs, which function as negative regulators of gene expression at the posttranscriptional level by miRNA degradation or translational repression depending on the degree of complementarity between the miRNA and its target (1, 2). MiRNAs are initially transcribed by RNA polymerase II into primary miRNAs (3) and then are processed by RNase III endonuclease Drosha to generate approximately 70-nt precursor miRNAs (pre-miRNA). Pre-miRNAs are transported to the cytoplasm by Exportin-5 and further cleaved by another RNase III endonuclease, Dicer, releasing mature double-strand miRNAs (4, 5). One strand of this duplex is subsequently selected and incorporated into the effector complex RISC that mediates target gene silencing (6).

MiRNA expression is regulated developmentally and in a tissue-specific manner; therefore, miRNAs are implicated in a variety of important biological processes such as development, proliferation, cellular differentiation, maintenance of stem cell character, apoptosis, and stress responses (7). Multiple reports have indicated a close relationship between deregulation of miRNA gene expression and carcinogenesis (8–10). MiRNA genes are frequently located at fragile sites or genomic regions involved in cancer that exhibit a high frequency of genomic alterations (11) and can thus serve as either tumor suppressors or oncogenes (12, 13). Apart from the individual roles played by specific miRNAs in tumorigenesis, signatures of global miRNA expression have been shown to distinguish cancer from normal cells in a tissue-specific manner. Alterations in miRNA expression profiles are important for carcinogenesis and may be used to identify key tumorigenic pathways or clinical outcome.

Normal mammalian somatic cells in vitro reach cellular senescence at the "Hayflick limit" (14) and abrogating replicative senescence is a necessary early step in cancer formation. Fibroblasts from patients with Li-Fraumeni syndrome (LFS), who develop a wide variety of early onset tumors, carry a germline mutation in 1 allele of the tumor suppressor gene p53 (15) and spontaneously immortalize in culture losing the remaining wild-type p53 (16). Previously, we have shown that the IFN signaling pathway was disrupted in spontaneously immortalized LFS cell lines by epigenetic silencing (17, 18) and demethylation treatment restored IFN-stimulated genes (ISG) in the IFN pathway inducing a senescence-like state (19).

As part of the innate immunity process, type I IFNs play critical roles in host defense against viral infections...
and immune surveillance against cancer, as well as in cell-cycle control, induction of apoptosis, and differentiation (20, 21). Activation of Toll-like receptor 3 (TLR3) by viral double-stranded RNA induces the nuclear translocation of IRF3 and NF-κB, which leads to the upregulation of primary early genes including IFNβ. IFNβ binds to its receptors in an autocrine manner to activate STAT1 and induces expression of a set of secondary antiviral response genes, including IRF7, thus activating the expression of another wave of ISGs. Finally, the tertiary transcriptional wave takes place involving almost all IFNα genes (22). The IFN-inducible transcription factors, IRFs, are crucial for the induction of other ISGs by IFNs. We previously reported that overexpression of IRF5 and/or IRF7 could reactivate ISGs, thereby suppressing cell proliferation and inducing senescence (18). Silencing of this growth-suppressive IFN pathway may be a necessary early event in the development of cancer, particularly associated with immortalization.

In this study, we hypothesized that abrogation of the IFN signaling pathway is required for an upregulation of small, noncoding RNAs that are necessary for the establishment of immortal cells. We found that the majority of miRNAs differentially expressed between precrisis and immortal LFS fibroblasts were upregulated in IFN pathway–defective MDAH087-10 fibroblasts compared with MDAH087-N cells with normal IFN signaling. Depending on the status of their IFN pathway, opposite effects were observed when we overexpressed Dicer to increase total miRNA production in 2 immortal LFS fibroblast cell lines derived from the same early passage cell strain. In MDAH087-10 cells with impaired IFN signaling, Dicer upregulation promoted colony formation and induced cell growth. Conversely, double-stranded miRNAs produced by Dicer overexpression could mimic viral infection to enhance the expression of ISGs in MDAH087-N fibroblasts that led to cell death and growth inhibition. Furthermore, manipulation of the IFN pathway in those 2 immortal LFS fibroblasts through transcription factor IRF7 reversed their response to Dicer overexpression. Disruption of IFN pathway signaling by IRF7 siRNA rescued MDAH087-N fibroblasts from cell death caused by Dicer transfection, whereas cell viability was considerably decreased upon Dicer overexpression in MDAH087-10 cells once ISGs were reactivated by IRF7 upregulation. This observation was consistent with the lower level of miRNA contents in MDAH087-N cells even after Dicer overexpression and substantially higher miRNA expression in Dicer-transfected MDAH087-10 cells. Therefore, cells with a defective IFN pathway have a higher miRNA tolerance than cells with normal IFN pathway. This work indicates that the growth-suppressive IFN pathway is a checkpoint that must be abrogated for cancer cells to be able to upregulate miRNAs during early tumorigenesis.

Materials and Methods

Cell culture

The immortal MDAH087 cell lines used in this study and their culture conditions were described previously (18). The MDAH087 fibroblast cells were derived from a male LFS patient and in their early lifespan are referred to as precrisis cells (MDAH087PC) in contrast to the 2 spontaneously immortalized cell lines (MDAH087-10 and MDAH087-N).

Polynosinic:polycytidylic acid treatment

Polynosinic-polycytidylic acid (PolyI:C; Amersham Biosciences Corp.) was diluted according to manufacturer’s instructions. Fresh media as control or 100 μg/mL polyI:C was applied on the LFS fibroblast cells for 24 hours before total RNA and protein were harvested.

MiRNA expression profiling by microarray

MiRNAs were isolated using the mirVana miRNA Isolation kit (Ambion Inc.) according to manufacturer’s instructions. Micro RNA microarrays were printed using mirVana miRNA probe set (Ambion Inc.) on glass sides. Micro RNAs were labeled and hybridized to microarrays according to manufacturer’s instructions. Data analysis was done by ANOVA method with P value < 0.05.

Quantitative reverse transcription PCR

Two micrograms of total RNA was reverse transcribed into cDNA using Superscript II (Invitrogen). Quantitative reverse transcription PCR (Q-RT-PCR) was performed using SYBR Green PCR Detection kit (PE Biosystems) as described previously (18). The primer sets used are listed in Supplementary Table 1.

Western blots

Western blots were performed as described (18) using 50 μg of LFS cell extracts. The primary antibodies used in our study were anti-Dicer, anti-V5, and anti-OAS1 (Abcam Inc.); anti-STAT1, anti-IRF7, and anti-α-tubulin (Santa Cruz Biotechnology, Inc.); antiphospho-STAT1 (S727; Cell Signaling Technology, Inc.).

Transfection of Dicer in precrisis and immortal MDAH087-10 and MDAH087-N cells

pCK-Dicer-V5 vector was a kind gift from Dr. V. Narry Kim. pCK-Dicer-V5 and control vector pcDNA3.1 were transfected into MDAH087PC, immortal MDAH087-10, and MDAH087-N cells using Lipofectamine in combination with Plus Reagent (Invitrogen Life Technologies). For stable transfections, the cells were selected in growth medium with 200 μg/mL of G418 (Sigma-Aldrich) after 72 hours of transfection. Transient transfections were also harvested 6, 24, or 48 hours after post-transfection.

Manipulation of IFN pathway by siRNA disruption or IRF7 overexpression

IRF7 siRNA and control siRNA were transfected into MDAH087-N cells using siRNA transfection reagent (all from Santa Cruz Biotechnology, Inc.). pCMV-IRF7 and control vector pCMV were stably transfected into MDAH087-10 cells by 200 μg/mL of G418 selection as described previously (18). Transient transfections of pCK-Dicer-V5 and control vector pcDNA3.1 were then performed on those transfected MDAH087-10 and MDAH087-N cells.
Cell viability, proliferation, lifespan, and colony formation assay

Precrisis and immortal MDAH087 LFS cells were seeded at 2 × 10^5 cells per 100-mm plates for Dicer/pcDNA3.1 transfection. Cell numbers were then counted by a hemocytometer after 24 and 48 hours of transfection to determine cell viability of those cells. Dicer- and vector control–transfected MDAH087-10 and MDAH087-N cells were seeded at 1 × 10^5 and 2 × 10^5 cells per well in 24-well plates right after G418 selection. A modified version of the MTT assay (23) was used to quantify cell growth every 3 days for the next 18 days. The results were shown as absorbance directly proportion to the number of cells. Lifespan assay was also performed by continuously culturing those transfected cells over PD 35 as described earlier (18). To study colony formation, stably transfected cells were seeded at a density of 1,000 cells per 100-mm plate. Twenty days later colonies were fixed in MeOH and stained with Giemsa (Sigma-Aldrich).

MiRNA quantitation by Small RNA assay

MiRNA contents were quantified by Agilent Small RNA Assay according to manufacturer’s instructions. One hundred nanograms of total RNA from Dicer and pcDNA3.1 stably transfected MDAH087-10 and MDAH087-N cells was loaded in small RNA chips and quantified using Agilent 2100 Bioanalyzer (Agilent Technology).

Results

Differentially regulated IFN signaling pathway in immortal LFS fibroblasts

We previously demonstrated that the IFN signaling pathway has been epigenetically silenced in several spontaneously immortalized LFS fibroblast cell lines (17, 18). Two immortal cell lines were used for this study, which were independently derived from the same patient’s early passage cells. MDAH087-N is unique among immortal LFS fibroblasts in that these cells display normal levels of IFNα and IFNβ mRNA expression (18). To further investigate whether the IFN pathway is functionally intact in precrisis and immortal MDAH087 cell lines, we applied 100 μg/mL of polyIC to the LFS cells for 24 hours, which mimics the effect of double-strand viral RNA to activate the IFN pathway. We then examined the expression of a representative set of ISGs using Q-RT-PCR. All of the genes tested in precrisis MDAH087PC70 cells were fully activated after 24-hour polyIC treatment. Although to a lesser extent, most of the ISGs except IRF5 were also induced by polyIC treatment in immortal MDAH087-N cells (Supplementary Table 2) that was similar to precrisis cells. However, polyIC failed to activate any of these ISGs in MDAH087-10 cells.

Consistent with the mRNA levels, polyIC treatment upregulated IRF7 protein levels in both MDAH087PC and immortal MDAH087-N cells but not in immortal MDAH087-10 cells (Fig. 1). STAT1 protein levels were also increased with the most significant enhancement in STAT1 phosphorylation (Ser727) after polyIC treatment of MDAH087PC and MDAH087-N cells. In contrast, neither phosphorylated-STAT1 nor STAT1 protein levels changed appreciably after polyIC treatment of in MDAH087-10 cells (Fig. 1). Furthermore, polyIC treatment induced more IRF5 protein expression only in MDAH087PC70 cells; whereas, we did not detect any IRF5 protein in either untreated or treated immortal MDAH087 cells that was consistent with Q-RT-PCR (Fig. 1). OAS1 expression pattern was similar to STAT1 with a small upregulation in both MDAH087PC and MDAH087-N cells but not in MDAH087-10 cells. Hence, the MDAH087-N cells have relatively normal IFN response comparing with the MDAH087-10 cells that have lost their IFN response pathway.

MiRNA deregulation in immortal LFS fibroblasts

Alteration of miRNA expression has been connected with cancer development and maintenance; we suspected that miRNAs have also been deregulated in LFS fibroblasts after spontaneous immortalization. Using miRNA microarray analysis, we examined miRNA expression profiles in both precrisis and immortal MDAH087 cells. Comparisons between MDAH087PC versus MDAH087-10 or MDAH087-N were performed using 3 independent preparations of total RNA from these cells. The comparison revealed several differentially expressed miRNAs in the immortal cell lines (Supplementary Table 2B and C). Many differentially regulated miRNAs were indicated in carcinogenesis and their expression levels were confirmed using Q-RT-PCR (data not shown). Interestingly, among those deregulated miRNAs identified in IFN pathway deficient MDAH087-10 cells, 15 of 16 showed increased levels of MDAH087-10 cells.
of expression compared with MDAH087PC cells, whereas opposite changes (2 upregulated and 8 downregulated) were found in MDAH087- N cells that have a functional IFN pathway (Supplementary Table 3A). Similar to MDAH087-10 fibroblasts, all but 1 of the differentially regulated miRNAs is upregulated in another IFN signaling–defective immortal cell line, MDAH087-1 cells compared with precancerous cells (data not shown). Because alterations in miRNA levels are often caused by deregulation of the biogenesis machinery components, we tested whether the level of Dicer, the enzyme critical for miRNA maturation varied among those cells. However, no significant change at Dicer mRNA or protein level was revealed in immortal LFS fibroblasts. Similarly, results were found in Drosha and Exportin-5 mRNA levels (data not shown), indicating that other mechanisms must contribute to miRNA deregulation in immortal LFS cells.

**Overexpression of Dicer has opposing growth effects on MDAH087-10 (defective IFN pathway) and MDAH087-N (relatively normal IFN pathway) cell lines**

Although no change in Dicer expression was observed in immortal LFS fibroblasts, several reports revealed that widespread miRNA overexpression resulted from an increase in the essential components of the miRNA machinery, including Dicer, Drosha, and Exportin-5, in premalignant lesions or cancers at an early stage (24, 25). Moreover, opposite miRNA expression patterns in our immortal LFS cells indicated that the IFN/innate immunity pathway might determine the tolerance to miRNA expression level in immortalization.

To further address the relationship between deregulation of miRNAs and IFN signaling pathway in cellular immortality, we studied the effects of Dicer upregulation that directly increases the amount of double-strand miRNA content, on immortal MDAH087-10 and MDAH087-N fibroblasts with different IFN pathway activity. pCK-Dicer-V5 was transfected into the 2 immortal MDAH087 cells as well as their parent MDAH087PC cells. Western blots using antibodies specifically to V5 and Dicer showed that Dicer protein levels started to go up at 24 hours after transfection and continued to be expressed at 48 hours (Fig. 2A). To our surprise, transient transfection of Dicer triggered significant cell death (~40%) in both MDAH087PC and MDAH087-N cells by 48 hours after transfection. In contrast, no loss in cell numbers was observed in MDAH087-10 cells during the same time period when compared with vector control (Fig. 2C).

MDAH087PC failed to generate stably transfected cells because cells were either killed or went into senescence. However, stable cell populations expressing Dicer were achieved after 14 days of G418 selection of the 2 immortal LFS cells. Those stable transfections were harvested to measure the RNA and protein expression levels of Dicer. As expected, Dicer mRNA and protein expression were upregulated compared with the vector control (Table 1 and Fig. 2B). Although increased in expression, Dicer protein was always expressed at a lower level in stably transfected MDAH087-N cells as compared with Dicer-transfected MDAH087-10 cells. More interestingly, continuous Dicer overexpression was observed for over 30 passages after the establishment of stably transfected MDAH087-10 cells, whereas the increase in Dicer expression diminished to a level similar to the vector control–transfected MDAH087-N cells by 20 passages (data not shown), which indicated the selection against Dicer expression.

To investigate whether Dicer elicited variable growth responses in the 2 immortal LFS cells with differing IFN signaling pathway, cell proliferation assays were carried out on both transfected cells immediately following drug selection. The overexpression of Dicer in MDAH087-10 cells resulted in enhanced cell proliferation compared with vector control cells at both low and high plating conditions (Fig. 3A). Conversely, elevated Dicer reduced cell growth rate to approximately 50% to 60% of that of vector control for MDAH087-N cells (Fig. 3B). The 2 transfected immortal cells were cultured for additional 35 population doublings (PD; PD 0 started at day 14 after transfection) to examine the effect of Dicer on cellular lifespan. Consistent with cell proliferation, the Dicer overexpressing MDAH087-10 cells maintained a slightly increased population-doubling rate (Fig. 3C), whereas decreased lifespan was evident beyond PD 15 in Dicer-transfected MDAH087-N cells (Fig. 3D). In addition, elevated Dicer expression increased colony formation by more than 2-fold and to a larger size in MDAH087-10 cells, whereas Dicer transfection of MDAH087-N cells reduced colony size and number to approximately 65% of that in vector control–transfected MDAH087-N cells (Fig. 3E and F). The same effects on cell viability and colony formation after Dicer overexpression were observed in a parallel transfection study of MDAH087-1 cells (data not shown).

We also examined whether the overexpression of Dicer could alter senescence state in those transfected immortal LFS cells using the senescence-associated β-gal activity assay. Stable transfection of Dicer induced a 3-fold increase in senescence-associated β-gal staining relative to the vector control in MDAH087-N cells and a 4-fold reduction in the appearance of senescent cells after Dicer transfection of MDAH087-10 cells (Fig. 3G). In summary, we have identified opposite growth consequences on the 2 immortal LFS cells after Dicer transfection that appears to be associated with the functionality of the IFN signaling pathway.

**Transfection of Dicer activates IFN pathway in MDAH087-N cells as well as MDAH087PC cells, but not in MDAH087-10 cells**

Because Dicer overexpression is expected to raise the amount of double-strand miRNA intermediates, it is reasonable to speculate that double-strand, RNA-responsive IFN pathway is inducible by miRNA upregulation due to Dicer transfection into our LFS cells. The growth-repressive effects of Dicer overexpression exhibited in MDAH087-N cells are likely to be the result of activation of the IFN pathway.

To determine whether the kinetics of ISG expression was changed by Dicer upregulation, Dicer-transfected cells were harvested at different times after initial transient transfection (6, 24, and 48 hours) as well as after stable transfection.
Intriguingly, Q-RT-PCR revealed mild activation of several critical IFN response genes, including TLR3, IFNα/β, OAS1, and STAT1, by 48 hours after Dicer transfection in MDAH087-N but not MDAH087-10 cells (Table 2A). However, no significant change of ISGs was observed in either of the LFS cell lines stably transfected with Dicer (Table 1). A similar pattern of ISG induction was also observed in MDAH087PC cells although more rapid, persistent and to a greater magnitude (Table 2B). In fact, as an immediate response gene, IFNβ stimulation occurred as early as 6 hours after Dicer transfection in MDAH087PC cells, followed by production of all the other ISGs we tested at 24 hours that persisted until 48 hours of posttransfection (Table 2B).

Western blots of transfected LFS cells confirmed our observation of IFN pathway induction upon Dicer overexpression. Enhanced expression of both phosphorylated-STAT1 (at 24 and 48 hours) and IRF7 (at 6 and 24 hours) was detected after

Table 1. Q-RT-PCR analysis of Dicer and ISG induction in stable Dicer-transfected immortal LFS cells

<table>
<thead>
<tr>
<th>Gene</th>
<th>MDAH087-N Dicer/(-)</th>
<th>MDAH087-10 Dicer/(-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicer</td>
<td>17.15</td>
<td>16.41</td>
</tr>
<tr>
<td>TLR3</td>
<td>-1.17</td>
<td>-1.13</td>
</tr>
<tr>
<td>IFNα</td>
<td>-1.03</td>
<td>-1.27</td>
</tr>
<tr>
<td>IFNβ</td>
<td>-1.72</td>
<td>-1.54</td>
</tr>
<tr>
<td>IRF7</td>
<td>-1.12</td>
<td>-1.47</td>
</tr>
<tr>
<td>OAS1</td>
<td>-1.43</td>
<td>-1.61</td>
</tr>
<tr>
<td>STAT1</td>
<td>-1.21</td>
<td>-1.02</td>
</tr>
</tbody>
</table>

NOTE: (-), vector control cells.
Figure 3. Overexpression of Dicer promoted cell growth rate of MDAH087-10 cells whereas it induced growth arrest and senescence in MDAH087-N cells. Dicer and vector control stably transfected MDAH087 cells were plated in 24-well plates at either low (1,000 cells per well) or high density (2,000 cells per well). Cell growth rates were assessed by modified MTT cell-proliferation assay in MDAH087-10 cells (A) and MDAH087-N cells (B). Absorbance is directly proportional to the number of cells. Dicer-transfected MDAH087-10 (C) and MDAH087-N (D) cells were then cultured for over PD 35 (PD 0 started at day 14 after infection) to compare their cellular lifespan to vector control cells. E, colony formation assay. F, number counts of colony formation. One thousand cells were seeded in 100-mm plates following stable transfection; Giemsa stain was used to evaluate colony formation from Dicer- and vector control-transfected cells after 20 days of culture. G, senescence-associated β-gal assay was done on immortal MDAH087 fibroblasts after these cells were stably transfected with either Dicer or pcDNA3.1. β-gal-positive cells (%): percentage of cells which are positive for senescence-associated β-gal staining.

*, P < 0.03; **, P < 0.01.
Dicer transfection of MDAH087PC fibroblasts. Smaller elevation of those 2 proteins was observed in Dicer-transfected MDAH087-N cells although for a shorter period, but not in MDAH087-10 cells (Fig. 2A), which is consistent with our Q-RT-PCR data. Unlike the Q-RT-PCR data of stable Dicer-transfected LFS cells (Table 1), increasing levels of both phosphorylated-STAT1 and OAS1 protein were detected in MDAH087-N cells (Fig. 2B), whereas the MDAH087-10 cells showed little alteration in the protein levels of those ISGs. So far, the activation of the IFN signaling pathway by Dicer in MDAH087-N as well as its precrisis MDAH087 cells was consistent with our hypothesis that the adverse growth effects displayed upon Dicer overexpression were the result of IFN signaling pathway activation.

Manipulation of IFN pathway by siRNA disruption and overexpression of IRF7

To further verify that intact IFN signaling is both sufficient and necessary for cell mortality resulting from miRNA/Dicer upregulation, siRNA to IRF7 (siIRF7) was used to suppress IFN signaling because this transcription factor is essential to activate other ISGs and is inducible in MDAH087-N fibroblasts (Fig. 1). IRF7 induction after 24 hours of polyIC treatment was eliminated in MDAH087-N cells with siIRF7 in contrast to control siRNA (Fig. 4A). When Dicer was upregulated, no induction of ISGs was observed in MDAH087-N cells transfected with siIRF7. As expected, control siRNA did not affect Dicer’s ability to activate ISGs as both phosphorylated-STAT1 and IRF7 expression were upregulated at 6 and 24 hours with increased OAS1 expression at 48 hours (Fig. 4B). Consequently, disruption of IFN pathway signaling by siIRF7 rescued MDAH087-N fibroblasts from cell death caused by Dicer transfection (Fig. 4C).

We also stably overexpressed IRF7 to restore IFN signaling in MDAH087-10 cells. Dicer transfection promoted protein expression of phosphorylated-STAT1 (at 6 hours) and OAS1 (at 24 hours), whereas IRF7 remained at a higher expression level in IRF7-transfected MDAH087-10 cells (Fig. 5A). Cell viability was considerably decreased upon Dicer overexpression in those cells once ISGs were induced by IRF7 reactivation (Fig. 5B). All together, manipulation of the IFN pathway in immortal LFS fibroblasts through critical transcription factor IRF7 reversed their response to Dicer upregulation depending on whether their IFN pathway is functional and indicates a direct association between IFN pathway activation and miRNA/Dicer tolerance.

MiRNA contents in Dicer stably transfected immortal LFS fibroblasts

To confirm elevated expression levels of miRNA components in our immortal LFS cells after Dicer transfection, miRNA contents in those cells were quantified using the Agilent Small RNA Assay. Remarkably, Dicer-overexpressing MDAH087-N cells, which have a relatively normal IFN pathway, contained lower levels of miRNA (138 pg/μL) than vector control (421 pg/μL). In contrast, substantially higher miRNA quantity was observed in Dicer-transfected MDAH087-10 cells (20,544 pg/μL) compared with a much lower amount in vector-transfected cells (475 pg/μL; Table 3). MiRNA elevation was also observed in Dicer-overexpressing MDAH087-1 cells (data not shown). The absence of an increase in miRNA even with more miRNA biosynthesis appears to be the outcome of a negative selection resulting from a functional IFN pathway in MDAH087-N cells, such that cells could not harbor higher miRNA contents and survive. The higher miRNA tolerance in MDAH087-10 cells further supports the hypothesis that the growth-suppressive IFN signaling pathway has to be disrupted in order for miRNA upregulation to occur in early carcinogenesis.

Discussion

Here, we presented the first association between the disruption of IFN signaling and miRNA deregulation in cellular immortalization, a critical early mechanism leading to carcinogenesis. Immortal cells lacking normal innate immunity were able to harbor higher levels of miRNA generated by Dicer overexpression; whereas cells with a functional IFN response resisted miRNA expression most likely due to the stimulation of ISGs caused by increasing double-strand RNA
intermediates. Activation of this growth-suppressive IFN pathway resulted in cell death, growth inhibition, and senescence. Manipulating the activity of IFN pathway led to changed responses upon double-strand miRNA upregulation. Elevated Dicer expression in Dicer-transfected MDAH087-N cells is less and diminished during extended passage in culture. We explained this observation by a survival disadvantage for this cell line with higher ISGs stimulated by elevated miRNAs during growth selection and consistent with the growth-suppressive role of the IFN signaling pathway.

Recently, the expression levels of miRNA biogenesis enzymes have been reported to either positively or inversely correlate with malignant behavior of tumors, depending upon the cancer type. Interestingly, differential alterations of miRNA expression profiles due to changes in their biogenesis might be able to characterize and distinguish high- from low-grade cancers as Dicer is overexpressed in noninvasive precursor lesions of lung adenocarcinoma but downregulated in invasive adenocarcinoma (26). Similarly, an upregulation of Dicer, Drosha, and Exportin-5 was reported in the normal urothelium from bladder cancer patients, which was reversed once malignancy appeared (24). The transient global increase of miRNA resulting from enhanced miRNA machinery suggested that it might be a hallmark of premalignant lesions and miRNA upregulation may be a very early event during cancer development. Conversely, global repression of miRNA maturation due to miRNA processing impairment promoted cellular transformation and tumorigenesis (27). Moreover, suppression of Dicer expression has also been significantly associated with advanced tumor stage, metastasis, and poor prognosis (28, 29). The differential expression of Dicer in early versus late carcinogenesis has been attributed to its role in tissue development and differentiation, which is supported by the differentiation failure in Dicer-deficient mouse embryonic stem cells (30). Distinct patterns of miRNA/Dicer expression might not only be a signature of cancers with different tissue origins, but also encode the developmental history of cancers and therefore distinguish later stage tumors from more differentiated ones (8).

Although miRNA expression was differentially regulated in our immortal cell lines, we failed to detect any significant change in either mRNA or protein level of Dicer. This supported the hypothesis that the deregulation of miRNA expression observed in cancers may be caused by several mechanisms other than abnormality in miRNA biogenesis, including chromosome abnormalities, mutation, polymorphisms, and epigenetic regulation (13). A recent investigation of ovarian cancer supports this hypothesis that genomic and epigenetic alterations contributed to miRNA deregulation instead of changes in the miRNA biogenesis machinery (31).

Compared with other immortal MDAH087 cell lines, in MDAH087-N cells the IFN signaling pathway appears to be relatively normal based on the fact that polyIC treatment could induce an IFN response. However, MDAH087-N cells do have other defects when compared with its isogenic precriss MDAH087PC cells. We have previously found that the basal expression levels of several critical IFN pathway
genes in untreated MDAH087-N cells were repressed, although most of them can be restored following 5-aza-dC (5-aza-2'-deoxycytidine) treatment (18). We found that MDAH087-N cells failed to protect themselves against high-dose vesicular stomatitis virus (VSV) infection and under these conditions, MDAH087PC cells with fully functional innate immunity were not significantly killed (18). Although polyI:C treatment of MDAH087-N cells stimulated the expression of several critical ISGs including IRF7 and STAT1 at lower levels compared with its parent MDAH087PC cells, there was a lower basal expression and no induction of IRF5, an important transcriptional partner of IRF3 and IRF7.

Table 3. miRNA contents in Dicer stably transfected immortal LFS fibroblasts

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Total RNA, pg</th>
<th>miRNA, pg</th>
<th>miRNA/total RNA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDAH087-10 pcDNA3.1</td>
<td>100,000</td>
<td>475</td>
<td>0.48</td>
</tr>
<tr>
<td>MDAH087-10 Dicer</td>
<td>100,000</td>
<td>20,544</td>
<td>20.54</td>
</tr>
<tr>
<td>MDAH087-N pcDNA3.1</td>
<td>100,000</td>
<td>421</td>
<td>0.42</td>
</tr>
<tr>
<td>MDAH087-N Dicer</td>
<td>100,000</td>
<td>138</td>
<td>0.14</td>
</tr>
</tbody>
</table>
(Supplementary Table 2 and Fig. 1). In addition, we also noticed that the initial ISG stimulation in MDAH087-N cells occurred at 48 hours after Dicer transfection, whereas faster and stronger activation was evident at 6 hours in its parent MDAH087PC cells (Table 2). Taken together, these data indicate that, at least, partial deregulation of the antiviral signaling pathway had also taken place during immortalization in MDAH087-N cells. Distinct sets of ISGs were deregulated between the 2 immortal cell lines, implicating different mechanisms of immortalization, although the IFN pathway is consistently disrupted in these cells.

Loss of the IFN signaling pathway plays an essential role in the process of cancer development that is supported by its impairment not only in our immortal cell lines, but also in many human cancer cells (32). As an early event in immortalization, IFN pathway disruption is possibly coincident with transient upregulation of the miRNA machinery and miRNA overexpression in premalignant lesions. This IFN pathway appears to provide a checkpoint for the miRNA level tolerance and its abrogation leads to cellular immortalization and early events in tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. V. Narry Kim at Seoul National University in Korea for her kind gift of the pC3: Dicer-V5 vector, Dr. George Brush for helpful comments on the manuscript, Bin Ye for preparation of the microarrays for miRNA analysis, and the perinatology research branch (NICHID/NIH) at Wayne State University for their help with small RNA assay.

Grant Support

This work was supported by the Barbara and Fred Erb Endowed Chair in Cancer Genetics to M.A. Tainsky, funds from the Karmanos Cancer Institute, the State of Michigan Life Sciences Corridor to the Molecular Medicine, and Genetics Applied Genomics Technology Center at Wayne State University, and the Genomics and Biostatistics Core of the Karmanos Cancer Institute, P30CA023198.

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Received May 3, 2010; revised October 22, 2010; accepted November 4, 2010; published online January 3, 2011.

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Higher miRNA Tolerance in Immortal Li-Fraumeni Fibroblasts with Abrogated Interferon Signaling Pathway

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