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Cancer Genes and Genomics

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Reduced DICER1 Elicits an Interferon Response in Endometrial Cancer Cells

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Abstract

DICER1 is essential for the generation of mature miRNAs and other short noncoding RNAs. Several lines of investigation implicate DICER1 as a tumor suppressor. Reduced DICER1 levels and changes in miRNA abundance have been associated with aggressive tumor phenotypes. The global effects of reduced DICER1 on mRNA transcript abundance in tumor cells remain largely unknown. We used short hairpin RNA to stably knock down DICER1 in endometrial cancer cell lines to begin to determine how reduced DICER1 activity contributes to tumor phenotypes. DICER1 knockdown did not affect cell proliferation but caused enhanced cell migration and growth in soft agar. miRNA and mRNA profiling in KLE cells revealed overall decreases in miRNA levels and changes in the relative abundance of many mRNAs. One of the most striking changes in mRNA levels was the upregulation of IFN-stimulated genes (ISG), the majority of which lack known miRNA target sequences. IFNβ, a key upstream regulator of the IFN response, was significantly increased in DICER1 knockdowns in the AN3CA, Ishikawa, and KLE endometrial cancer cell lines and in the normal endometrial cell line EM-E6/E7/TERT. IFNβ secreted in media from KLE and EM-E6/E7/TERT shDcr cells was sufficient to activate an IFN response in HT29 cells. The reduced miRNA processing in DICER1 knockdowns was associated with increases in pre-miRNAs in the cytoplasm. Our findings suggest that elevated pre-miRNA levels trigger the IFN response to double-stranded RNA. We thus report a novel effect of reduced DICER1 function in cancer cells. *Mol Cancer Res; 10(3); 316–25. ©2012 AACR.*

Introduction

Endometrial cancer is the most common gynecologic malignancy in the United States and approximately 15% of patients suffer from recurrent disease (1, 2). Discovery of the molecular lesions contributing to endometrial tumorigenesis will provide opportunities for targeted therapies.

DICER1 is an RNASE III helicase necessary to process double-stranded RNA (dsRNA) in mammalian cells, the predominant form of which is miRNA. Primary miRNAs (pri-miRNAs) are cleaved by the enzyme DROSHA into pre-miRNAs. Pre-miRNAs are transported out of the nucleus by EXPORTIN-5 and processed in the cytoplasm by DICER1 and accessory proteins. Mature miRNAs go with AGO proteins to pair imperfectly with the 3′-untranslated regions (UTR) of target mRNAs and either impede translation or degrade the mRNAs (3). About 60% of human genes may be regulated posttranscriptionally by miRNAs (4, 5). Given the key role of miRNAs in gene regulation, it is not surprising that DICER1, DROSHA, and other RNA interference (RNAi) components have been implicated as "tumor suppressors" in solid tumors (6–10). Germline loss-of-function mutations in *DICER1* are associated with the pleuropulmonary blastoma tumor susceptibility syndrome (8). The penetrance of inherited *DICER1* mutations is, however, modest and it has been proposed that DICER1 is a haploinsufficient tumor suppressor (11). A recent report on somatic DICER1 mutations in nonepithelial ovarian tumors further supports the notion that DICER1 is a haploinsufficient tumor suppressor (12). *DICER1* is an essential gene. The Dicer1 homozygous knockout mouse is embryonic lethal (13, 14). Conditional deletion of Dicer1 in a mouse Kras lung cancer model caused homozygous knockout cells to die, but heterozygous tumors to be more aggressive than wild-type tumors (7), consistent with role of Dicer as a haploinsufficient tumor suppressor. Our group previously showed that lower DICER1 mRNA levels in endometrial cancer are associated with recurrence and accelerated disease progression (15).

The IFN response is a component of the innate immune response to pathogens such as RNA viruses. Viral dsRNA binding to Toll-like receptor 3 (TLR3) on the cell membrane or IFIH1 (MDA5), PKR, or RIG-1 in the cytoplasm triggers IRF3 and NF-κB translocation to the nucleus and

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transcription of early genes, specifically *IFNβ*. Secreted IFNβ activates cell surface receptors by autocrine and paracrine means to induce activation of STAT1 and expression of IFN-stimulated genes (ISG). Next, IFNα genes are transcribed leading to downstream effects including global inhibition of translation and apoptosis (16–19). Innate immunity and IFN responses in malignancies are context dependent and often paradoxical. An immune response may mediate tumor cell killing; IFNs have been used to treat a variety of human cancers (20, 21). However, inflammatory cytokines downstream of the IFN response have been linked to cellular transformation (22). Cellular senescence can trigger an IFN response (23), but increases in ISGs such as ISG15 and IFI44 are prognostic for breast and lung cancer recurrence, respectively (24, 25). The IFN response and how it impacts tumor behavior is likely determined by a complex and context-dependent interaction of tumor cell–specific effects and humoral responses.

Using short hairpin RNAs (shRNA) we reduced DICER1 levels in endometrial cancer and normal cell lines by greater than 50%. mRNA and miRNA profiling studies revealed global perturbations in RNA levels. The most striking change observed was an increase in transcription of IFNβ and ISGs characteristic of an IFN response. We show that the IFN response in endometrial cells with reduced DICER1 results from accumulation of pre-miRNAs in the cell cytoplasm.

Materials and Methods

Cell culture

Four endometrioid endometrial cancer cell lines were investigated. AN3CA and KLE were purchased from the American Type Culture Collection. The Ishikawa cell line was a gift from Dr. Stuart Adler (Washington University School of Medicine, Department of Internal Medicine, St. Louis, MO). The MFE296 cell line was kindly provided by Dr. Pamela Pollock (Queensland University of Technology, Brisbane, Queensland, Australia) and the HT29 cell line was kindly provided by Dr. Loren Michel (Washington University, St. Louis, MO). The EM-E6/E7/TERT cell line was originally reported by Mizumoto and colleagues (26) and kindly provided by Dr. Pamela Pollock. Cell lines were grown as previously described (27) and authenticated as reported in Dewdney and colleagues (28).

Lentiviral transduction to create stable knockdowns

DICER1 and GSK3β knockdowns were created in AN3CA, EM-E6/E7/TERT, Ishikawa, KLE, and MFE296 cell lines as previously described (29). Virus production and infections were carried out according to established methods (30). DROSHA knockdown was created with virus kindly provided by Michael Kuchenreuther in Dr. Jason Weber's laboratory (Washington University, St. Louis, MO).

The short hairpin sequences used were:

shDcrA 5′-GCTCGAAATCTTACGCAAATA-3′
shDcrC 5′-GCCAAGGAAATCAGCTAAATT-3′
shDro2 5′- CGAAGCTCTTTGGTGAATAAT-3′
shDro4 5′- CCAGCGTCCATTTGTACTATT-3′
shGSK3β 5′-AGCAAATCAGAGAAATGAAC-3′
shLuc 5′CCCTCTGAACATGAGCATCAA-3′
shRFP 5′-TGCTAAGGAGTTTGGAGACAA-3′ (31)

The shDcr3 hairpin construct was designed by Sigma-Aldrich.

Reverse transcription PCR

Total cellular RNA was extracted utilizing the TRIzol method (Invitrogen). Nuclear and cytoplasmic fractions were prepared using the Norgen Biotek Cytoplasmic and Nuclear Purification Kit, according to the manufacturer's instructions (Norgen Biotek). RNA concentration was determined with the NanoDrop machine and software (Thermo Fisher Scientific). cDNA was generated using 1 μg total RNA and the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative reverse transcription PCR (qRT-PCR) of pre-miRNAs and the DUSP6 control was done using SYBR Green (BioRad) methods. The primers used are listed in Table 1.

Expression of DICER1, DROSHA, IFI44, IFI44L, IFI6, IFIH1, IFNβ1, MX1, and OAS3 mRNAs, and LET7B, LET7D, MIR107, MIR183, MIR450A, MIR542 pri-miRNAs was assessed by qRT-PCR TaqMan assays (Applied Biosystems) and the Applied Biosystems 7500 Fast real-time PCR system and software. Human β-actin was used as the endogenous control as previously described (32). Expression of let-7c, miR-10a, miR-16, miR-29b, and miR-126b mature miRNAs was assessed by quantitative TaqMan microRNA assays (Applied Biosystems) and the Applied Biosystems 7500 Fast real-time PCR system and software. U6 was used as the endogenous control (9). Relative expression levels were calculated using the $\Delta\Delta C_t$ method (33).

All qPCR assays were carried out in triplicate and then repeated with new cDNA synthesis. Minus RT controls

Table 1. Primers used for qRT-PCR

Transcript	Forward	Reverse
PRELET7D	5′-TTTAGGGCAGGGATTTTGC-3′	5′-TAAGAAAGGCAGCAGGTCGT-3′
PREMIR183	5′-CGCAGAGTGTGACTCCTGTT-3′	5′-TCGTGGATCTGTCTCTGCTC-3′
PREMIR450A	5′-AAACTATTTTTGCGATGTGTTCC-3′	5′-TGCAAAATGTCCCCAATACA-3′
DUSP6	5′-CCCCTTCCAACCAGAATGTA-3′	5′-TGCCAAGAGAAACTGCTGAA-3′

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(reverse transcriptase negative cDNA synthesis reactions) were carried out for at least one sample per plate.

miRNA profiling

KLE and AN3CA cell lines were subjected to global miRNA profiling with Nanostring technology. A total of 749 miRNAs were evaluated using the nCounter Human miRNA Panel CodeSet.

RNA sequencing

PolyA+ RNA was purified from total RNA using the Dynabeads mRNA Purification Kit (Invitrogen). Each sample was resuspended in 2 μL of 100 mmol/L zinc acetate and heated at 60°C for 3 minutes to fragment the RNA by hydrolysis. The reaction was quenched by the addition of 2 μL volumes of 200 mmol/L EDTA and purified with an Illustra Microspin G25 column (GE Healthcare). First strand cDNA was made using hexameric random primers and SuperScript III Reverse Transcriptase (Invitrogen), and the product was treated with *E. coli* DNA ligase, DNA polymerase I, and RNase H to prepare double stranded cDNA using standard methods. cDNA libraries were end repaired with a Quick Blunting Kit (New England BioLabs) and A-tailed using Klenow exo- and dATP. Illumina adapters with 4 base barcodes were ligated to cDNA and fragments ranging from 150 to 250 bp were selected using gel electrophoresis. Libraries were enriched in a 10-cycle PCR with Phusion Hot Start II High-Fidelity DNA Polymerase (Thermo Fisher Scientific) and pooled in equimolar ratios for multiplex sequencing. Single read, 36-cycle runs were completed on the Illumina Genome Analyzer IIx.

Sequenced reads were aligned to the human reference sequence (hg19/NCBI Build 37.1) using Tophat (34). Reads that aligned uniquely to the reference sequence were considered for gene expression quantification with Cufflinks (35). Gene expression was normalized using the Cufflinks provided option for quartile normalization.

Western blots

Western blot analysis of DICER1 was done as previously described (27, 33). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. Antibodies used were as follows: rabbit anti-DICER1 H212 (sc-30226, 1:200; Santa Cruz Biotechnology, Inc.), goat anti-rabbit IgG-HRP (sc-2030, 1:2,500; Santa Cruz Biotechnology), rabbit anti-DROSHA (ab12286, 1:750; Abcam), mouse anti-GAPDH (NB615, 1:4,000; Novus Biologicals), goat anti-mouse IgG-HRP (sc-2005, 1:5,000; Santa Cruz Biotechnology, Inc.), rabbit polyclonal anti-STAT3 H-190 (sc-7179, 1:200; Santa Cruz Biotechnology), rabbit anti-phospho-STAT3 Ser727 (9134, 1:500; Cell Signaling Technology), rabbit anti-phospho-STAT3 Tyr705 EP2147Y (04-1059, 1:500; Millipore). Band intensities were quantified using the program ImageJ (NIH).

ELISA

ELISA was done with the *Verikine-HS* Human Interferon Beta Serum ELISA Kit (PBL Interferon Source).

Cell proliferation, wound healing, and colony formation assays

For cell proliferation assays 100,000 cells were plated in 6-well plates in triplicate. Cells were trypsinized and counted using trypan blue staining and a hemocytometer every 24 hours for 120 hours.

Wound healing assays were done using AN3CA and KLE cells. Cells were grown to confluency then scratched down the middle of the plate. Cells were photographed every 4 to 6 hours for up to 96 hours (GE Healthcare IN Cell Analyzer 2000). The area of the "scratch" (area not filled in) was determined for each time point.

Growth of endometrial cancer cell lines in soft agar was determined as follows: First a base layer of 0.5% agar was plated in media, then a top layer of 0.3% agar in media with 30,000 cells per well was plated in 6-well dishes. After 4 weeks, cells were stained with crystal violet and imaged. Colonies were counted.

IFN stimulation

PolyI:C (Invitrogen) was diluted into the media of cells or transfected using the Dharmafect reagent (Thermo Fisher Scientific).

Let-7 inhibition

Let-7 inhibition was done as previously described (36). The CHECK-2 vector with the let-7b target site cloned into the 3′-UTR was a kind gift from Annaleen Vermeulen (Thermo Fisher Scientific).

Results and Discussion

Stable knockdown of DICER1

We used shRNA and lentiviral infection to stably knockdown DICER1 in 4 endometrial cancer cell lines and a transformed normal endometrial epithelium cell line; AN3CA, Ishikawa, KLE, MFE296, and EM-E6/E7/TERT. Of 5 hairpins tested, 2 (shDcrA and shDcrC) resulted in substantial reductions in DICER1 protein levels (Fig. 1A). Knockdowns were generated with shDcrA and shDcrC hairpins and shLuc and shRFP controls. Stable knockdown of DICER1 (<50% of controls) persisted for up to 30 passages for all cell lines, with the exception of MFE296, for which knockdown was unstable (Fig. 1B and data not shown). In KLE, DICER1 was reduced to approximately 10% of controls, suggesting that sufficient shRNA processing can occur with substantially reduced DICER1 activity (Fig. 1B). An additional shRNA targeting the DICER1 3′-UTR (shDcr3) was used in KLE cells leading to greater than 50% reduction in DICER1 protein levels (Supplementary Fig. S1).

Cell doubling times were similar in DICER1 knockdowns and control cells (Supplementary Fig. S2A). Cell migration was increased in AN3CA shDcr cells (Fig. 1C), but no difference was seen in KLE shDcr cells. The Ishikawa and EM-E6/E7/TERT cells could not be evaluated in the cell migration assay because they did not grow as monolayers on glass slides (Supplementary Fig. S2A). In both KLE and

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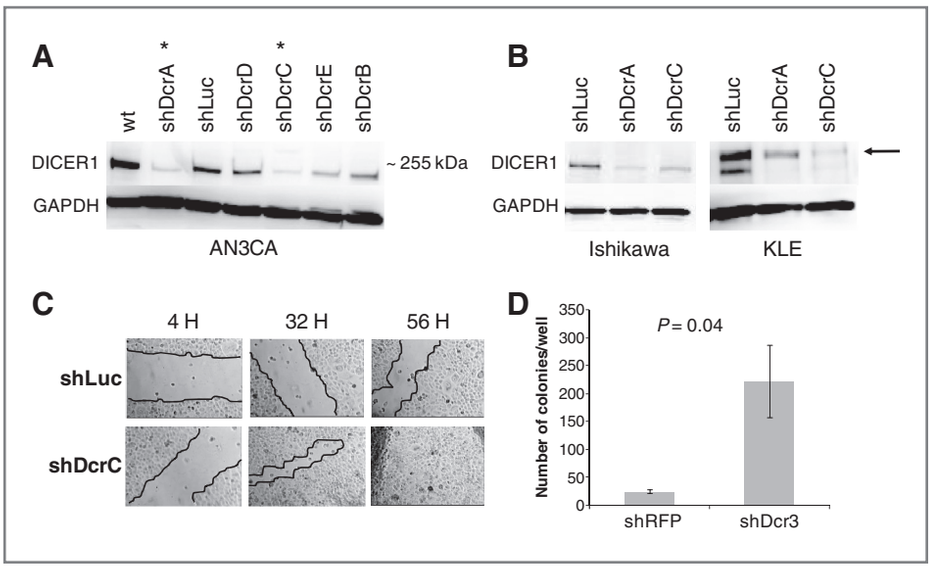

Figure 1. Knockdown of DICER1 in endometrial cell lines. A, Western blot analysis of DICER1 expression in AN3CA (passage 5) with shRNA A–E against DICER1 or Luciferase control. * denotes hairpins showing greatest degree of knockdown. B, Western blot analysis of representative knockdowns in additional cell lines, Ishikawa (passage 16) and KLE (passage 26). Arrow denotes nonspecific band of higher molecular weight above the approximately 255 kDa DICER1 band in KLE. C, representative wound healing assay in AN3CA cells. shLuc control and shDcrC knockdown at 4, 32, and 56 hours. Bold black lines denote wound outlines. D, increased soft agar colony formation in KLE cells with DICER1 knockdown. Results are one representative experiment of 2 conducted in triplicate. KLE cells were plated and growth in soft agar was assessed by staining with crystal violet after 30 days.

EM-E6/E7/TERT, shDcr cells formed more colonies in soft agar than control cells (Fig. 1D and Supplementary Fig. S2B). These *in vitro* assays for cancer-associated phenotypes suggest that reduced DICER1 in endometrial cancer cells can result in increased cell motility and anchorage independence. This increased cell motility was previously shown in breast cancer cell lines and attributed to a reduction in miR-200 and upregulation of genes involved in epithelial–mesenchymal transition (37).

We profiled miRNAs globally in shDcr cells to identify reductions in particular miRNAs that might contribute to cancer-associated phenotypes. Nanostring miRNA profiling studies in AN3CA cells as well as KLE knockdowns and controls revealed 133 of 749 miRNAs interrogated were expressed at appreciable levels. When the average levels of miRNA expression in the 2 KLE knockdowns were compared with the KLE shLuc control, 64% of the 133 miRNAs showed reduced levels in the knockdowns (Supplementary Table S1 and Fig. 2A). miR-200 was not expressed in endometrial cancer cell lines (Supplementary Table S1), so could not be responsible for the cancer-associated phenotypes mentioned above. We observed clear increases in a subset of miRNAs (Fig. 2A), as previously described, in colon cancer cells with reduced DICER1 protein (9). Similar effects on miRNA abundance were seen with both knockdowns in the KLE cell line; however, the magnitude of changes in miRNA levels seemed greater in the shDcr3 knockdown than in the shDcrA knockdown. For the shDcrA knockdown, 76 of 133 miRNAs were less than in shLuc control (average $\log_2$ fold change −0.502). With the shDcr3 knockdown, 95 of 133 miRNAs were less abundant than in the shLuc control with an average −0.828 fold change ($\log_2$). KLE shDcrA cells were evaluated at passage 15 and shDcr3 cells at passage 5. The more pronounced effect on miRNA levels seen with the shDcr3 knockdown could be attributable to more efficient targeting of DICER1 with the shDcr3 construct, greater reduction in DICER1 protein levels at earlier passages, or compensation for DICER1 as shDcrA cells were passaged (e.g., stabilization of miRNAs).

qRT-PCR of 5 miRNAs previously shown to be expressed in normal and cancerous endometrium (38) confirmed the relative abundance reported by Nanostring profiling in AN3CA and KLE cells. qRT-PCR in pooled endometrial cancers confirmed the rank order of 5 miRNAs reported by Nanostring (Supplementary Table S1 and Fig. 2B). miR-16 was the highest expressed of the 5 miRNAs by profiling and qRT-PCR. miR-29b was the lowest expressed by both profiling and qRT-PCR. qRT-PCR confirmed the Nanostring profiling and the functional reduction of DICER1 processing, as 5 mature miRNAs were significantly decreased in KLE shDcr cells (Fig. 2C). pri-miRNAs, the initial miRNA transcripts that are processed by DROSHA, were not significantly altered, showing that effects on mature miRNAs were due to a defect in miRNA processing, not transcription (Fig. 2D).

DICER1 knockdown effects on mRNA expression: upregulation of IFN response genes

To further assess the functional consequences of DICER1 knockdown, we profiled mRNA expression using

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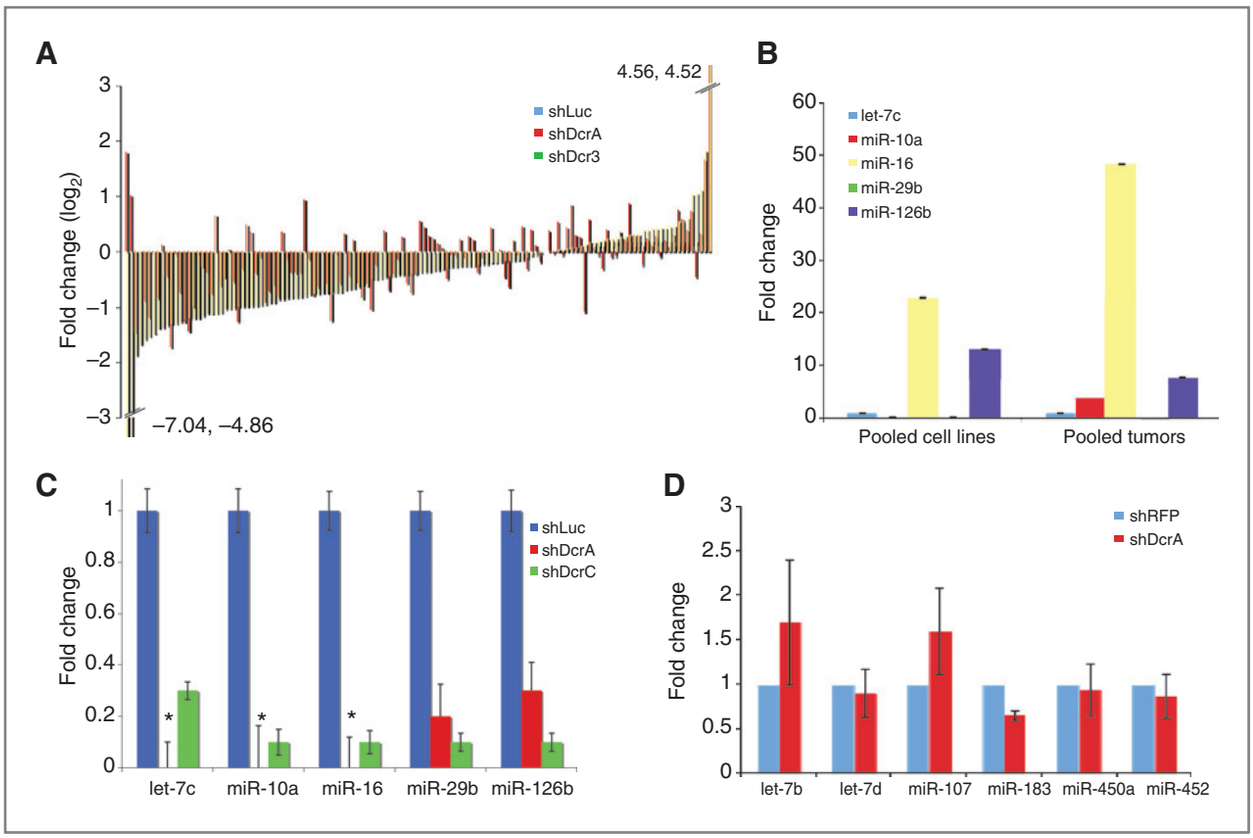

Figure 2. miRNA expression in DICER1 knockdown cells. A, Nanostring miRNA profiling of 133 expressed miRNAs in the KLE cell line. B, relative abundance of 5 miRNAs in endometrial cancer cell lines and tumors by TaqMan qRT-PCR assays, normalized to *U6* reference gene. RNA from 4 cell lines (AN3CA, KLE, Ishikawa, and MFE296) and 4 tumors was pooled and converted to cDNA. C, levels of 5 miRNAs in control and shDcr KLE cells by TaqMan qRT-PCR assays, normalized to *U6* reference gene. * denotes miRNAs that were undetectable in shDcrA cells. D, pri-miRNAs in KLE cells measured by TaqMan qRT-PCR and normalized to *β-actin* reference gene. Fold change shDcrA/shRFP is plotted on the y-axis. For all qRT-PCR experiments, error bars are SD (data shown is average of 2 experiments conducted in triplicate).

RNA-Sequencing (RNA-Seq) in KLE cells (35). Out of 9,935 genes expressed in KLE by RNA-Seq, 584 were upregulated more than 2-fold in shDcr cells (Supplementary Table S2). Gene Ontology analysis showed enrichment for functions associated with response to virus or other pathogens when the upregulated gene set was analyzed (Supplementary Table S3). A striking number of ISGs were upregulated (17 of the 28 present in the RNA-Seq data set; Fig. 3A). The probability of 17 genes at random being upregulated in this set is quite low ($P < 1.2^{*}$ 10E-14). qRT-PCR confirmed upregulation of 6 of 7 ISGs tested (Fig. 3B). Similar increases in 6 ISG transcripts were seen in independent knockdowns, providing biologic validation of the effect of reduced DICER1 in KLE cells (shDcrA and shDcr3, Fig. 3B; shDcrC, data not shown). To explore a possible mechanism for IFN response activation in shDcr cells, we evaluated mRNA levels of transcription factors that might target ISGs. No transcription factors predicted to bind upstream of the activated ISGs were overexpressed in shDcr cells by RNA-Seq (data not shown). Direct miRNA effects on ISG transcript levels were ruled out as the ISGs have no known targets in their 3′-UTRs for miRNAs expressed in KLE (Supplementary Table S4).

This increase in ISGs seemed to be a canonical IFN response (17, 18). To determine whether the upstream *IFNβ* gene was upregulated and activating ISGs, we assessed IFNβ mRNA and protein levels in DICER1 knockdowns. RNA-Seq did not detect expression of *IFNβ1* in any of the cell lines investigated, as would be expected for a low abundance transcript. *IFNβ1* transcript was, however, detectable using qRT-PCR. Two shDcr hairpins caused upregulated *IFNβ1* transcript compared with shLuc (Fig. 3C). The control shRFP hairpin did not significantly upregulate *IFNβ1*, whereas the shDcrC hairpin did (data not shown). shRNA alone does not trigger the IFN response (39, 40). We tested the possibility that knockdown of a cell-essential gene might activate the IFN response by measuring *IFNβ1* transcript levels in KLE shGSK3β cells. Because neither the shGSK3β nor the control shLuc and shRFP hairpins activate the IFN response, we concluded that the IFN response seen is a DICER1-specific effect. The *IFNβ1* transcript was upregulated at least 2-fold in DICER1 knockdowns in 2

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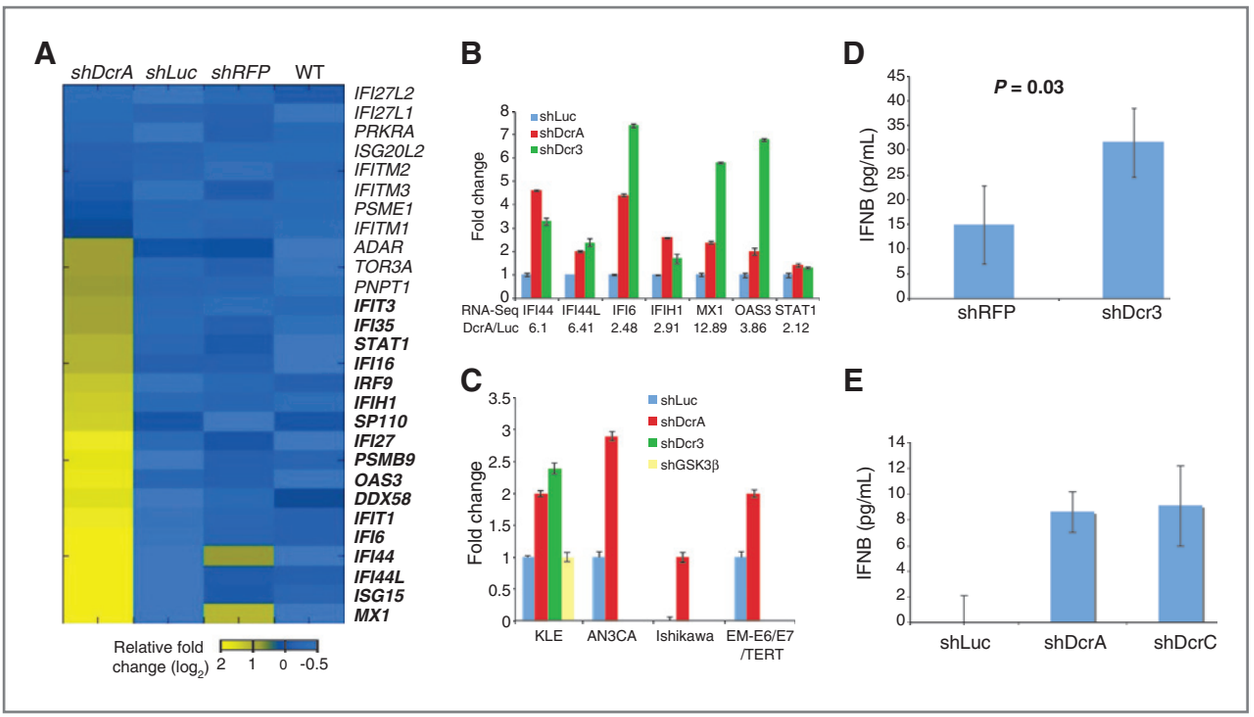

Figure 3. Upregulation of ISGs in shDcr cells. A, RNA-Seq heat map for ISGs. Genes indicated in bold revealed a fold change greater than 2 relative to the average expression of the 3 controls, shLuc, shRFP, and WT (untransfected KLE cells). B, qRT-PCR validation of ISGs upregulated in RNA-Seq. Fold change shDcr/shLuc is plotted on y-axis. C, *IFNβ1* transcript levels are increased in shDcr cells in 4 cell lines. shDcr3 and shGSK3β were only carried out in the KLE cell line. For all qRT-PCR experiments, mRNA levels were assessed using TaqMan qRT-PCR assays, normalized to *β-actin* reference gene. Error bars are SD (data shown is average of 2 experiments conducted in triplicate). IFNβ protein levels in cell culture media are increased in KLE (D) and EM-E6/E6/TERT (E) shDcr cells. IFNβ was assessed by ELISA. One representative experiment of 3 conducted in triplicate is shown. Error bars represent SEM. Significance was determined by *t* test.

additional endometrial cancer cell lines, AN3CA and Ishikawa, and an immortalized normal endometrial cell line, EM-E6/E7/TERT (Fig. 3C). The increase in *IFNβ1* transcript due to reduced DICER1 led to increased IFNβ protein levels in the media of KLE shDcr cells (Fig. 3D). A similar increase in IFNβ protein was observed in EM-E6/E7/TERT shDcr cell media (Fig. 3E), showing that reduced DICER1 leads to increased IFNβ expression in both normal and cancer endometrial cell lines.

DICER1 knockdown causes a canonical IFN response

As some cancer cell lines have abrogated IFN responses (19), we postulated that activation of the IFN response in KLE might be an artifact of a mutated IFN response pathway. However, the IFN response is intact in the KLE endometrial cancer cell line. Transfection with polyI:C, a dsRNA analog, activated the IFN response (Fig. 4). *IFNβ1* transcript levels rose rapidly and peaked at 6 hours, with concomitant increases in ISGs (Fig. 4B). In addition, a

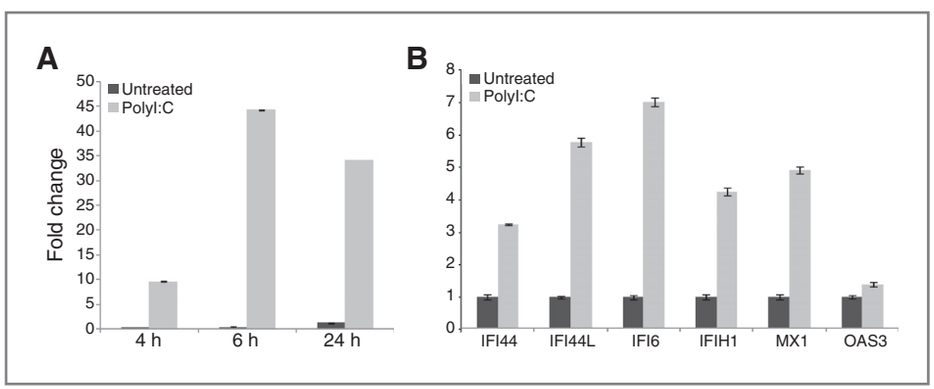

Figure 4. IFN response in the KLE cell line. A, upregulation of *IFNβ1* in polyI:C-treated KLE 4, 6, and 24 hours posttransfection. B, ISGs upregulation 24 hours post polyI:C transfection. Transcript abundance was measured by TaqMan qRT-PCR assays, normalized to *β-actin* reference gene. Error bars are SD (data shown is average of 2 experiments conducted in triplicate).

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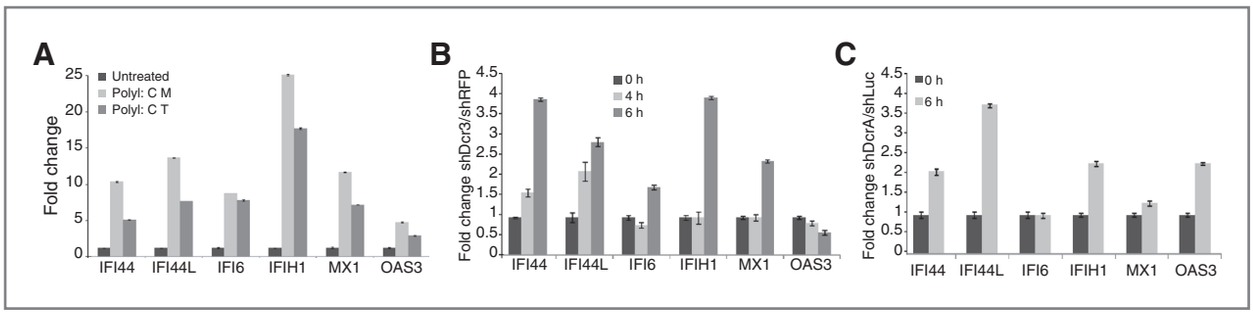

Figure 5. KLE and EM-E6/E7/TERT shDcr media stimulate an IFN response in HT29 cells. A, polyI:C stimulates a canonical IFN response in HT29 cells. PolyI:C was diluted into media (PolyI:C M) or transfected (PolyI:C T) into HT29 cells and RNA was isolated. B, shDcr3 but not shRFP media stimulates a canonical IFN response in HT29 cells. Media was transferred from KLE shRFP and shDcr3 cells to HT29s and RNA was isolated. Y-axis represents fold change of shDcr3/shRFP media. C, shDcrA but not shLuc media stimulates a canonical IFN response in HT29 cells. Media was transferred from EM-E6/E7/TERT shLuc and shDcrA cells to HT29s and RNA was isolated. Y-axis represents fold change of shDcrA/shLuc media. For all qRT-PCR experiments, transcript abundance was measure by TaqMan qRT-PCR assays, normalized to *β-actin* reference gene. Error bars are SD (data shown is average of 2 experiments conducted in triplicate).

cytoplasmic receptor sensing dsRNA (IFIH1) was overexpressed in KLE cells with low DICER1 (Fig. 3B). The IFN response in KLE shDcr cells upregulates the same genes as that in KLE cells transfected with polyI:C, albeit with a smaller magnitude (Fig. 3B, Fig. 4B).

We used media transfer to determine the biologic activity of secreted IFNβ protein in the media of shDcr cells. HT29 colon carcinoma cells exhibit a strong IFN response (40, 41), activating IFNβ and ISGs when polyI:C is either added to the cell culture media or transfected (Fig. 5A, Supplementary Fig. S3). Transfer of media from KLE shDcr3 cells to HT29 cells stimulated an IFN response, whereas shRFP cell media did not (Fig. 5B). Media from EM-E6/E7/TERT shDcr cells similarly stimulated 4/6 ISGs (Fig. 5C), indicating DICER1 knockdown causes an IFN response in both normal and cancerous endometrial cells. When media from KLE shDcr3 cells was transferred to KLE shRFP cells, no appreciable IFN response was seen (Supplementary Fig. S4). This difference could be due to the relative strength of IFN responses in KLE and HT29 cells (Fig. 4B, Fig. 5A). KLE shDcr3 cells in culture reflect long-term, continual IFNβ stimulation and KLE controls may not respond to a short stimulus with conditioned medium as HT29s do.

Pre-miRNAs build up in the cytoplasm and may cause an IFN response

To determine a mechanism for activation of the IFN response, we focused on a candidate miRNA. Members of the let-7 miRNA family, known for their tumor-suppressive roles (42, 43), were significantly reduced in shDcr cells (Fig. 2C and Supplementary Table S1). The let-7 family downregulates the cytokine interleukin-6, which when activated leads to phosphorylation of STAT3 by NF-κB, resulting in an inflammatory response linked to cellular transformation (22). To determine whether let-7 was responsible for the IFN response, we inhibited let-7 in KLE cells (Supplementary Fig. S5A). No increase in *IFNβ1* was observed when let-7 was inhibited (Supplementary Fig. S5B). Thus, let-7 alone was not responsible for the activation of the IFN response.

dsRNA (usually viral) activates the IFN response in mammalian cells. Our studies suggested a possible mechanism for IFN response upregulation by reduced DICER1. Mature miRNAs are too short (averaging 22 nt) to elicit the IFN response through viral dsRNA sensors (17, 44). However, their precursor molecules, pre-miRNAs, are larger (∼70 nt) and as such could be recognized by cytoplasmic dsRNA sensors IFIH1, PKR, or RIG-1 (45). We determined the subcellular location of pre-miRNAs in control and shDcr cells (Fig. 6A). Pre-let7d, pre-miR183, and pre-miR450a were increased in the cytoplasmic fraction of shDcr cells. The corresponding mature miRNAs were decreased in shDcr cells (Supplementary Table S5), reflecting reduced DICER1 processing that results in buildup of pre-miRNAs and reduction of processed, mature miRNAs. Buildup of pre-miRNAs in the cytoplasm may elicit the canonical IFN response. To determine the specificity of this effect, we knocked down DROSHA in the KLE cell line (Fig. 6B). Knockdown of DICER1 or DROSHA causes a reduction in mature miRNAs because of reduced processing. However, only DICER1 knockdown results in a buildup of pre-miRNAs. Lower levels of DROSHA did not trigger an IFN response as indicated by *IFNβ1* levels (Fig. 6C). Interestingly, DROSHA knockdown seemed to decrease *IFNβ1* transcript levels. This could point to a role for pre-miRNAs in modulating the IFN response. Because DROSHA knockdown results in fewer pre-miRNAs (due to reduced pri-miRNA processing), this finding showed that pre-miRNA buildup, rather than a decrease in mature miRNAs, causes the IFN response.

Our data point to the accumulation of pre-miRNAs in the cytoplasm as the trigger for the IFN response we observed in cells with reduced DICER1 activity (Fig. 7). This is unlikely to be an effect of the system used; whereas siRNAs may activate the IFN response in mammalian cells (46), shRNAs do not (40). The immune response to dsRNA is highly conserved, with organisms such as plants and fungi enacting an RNAi-based response to viral RNA (47). Prior studies showing that overexpression of pre-miRNAs can activate the

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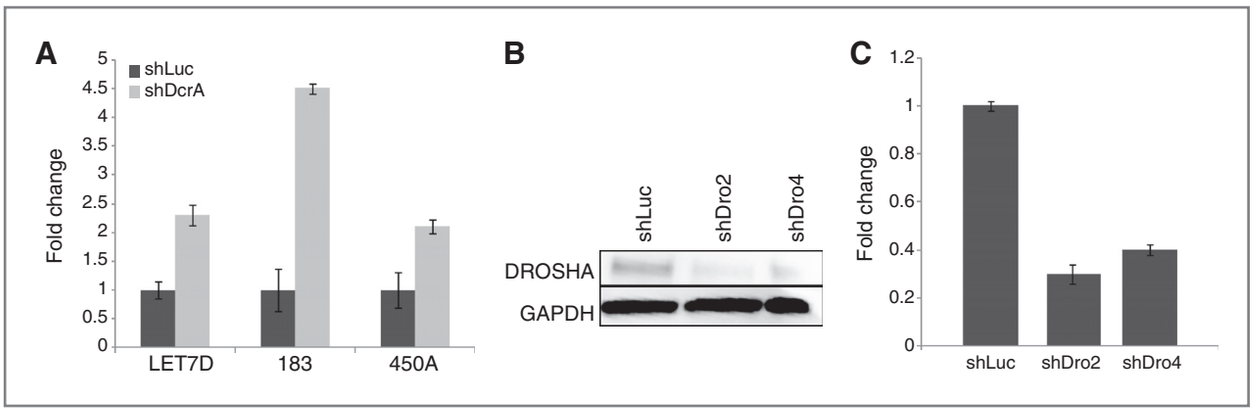

Figure 6. Pre-miRNAs build up in the cytoplasm of shDcr cells. A, pre-miRNAs are increased in shDcr cytoplasm. qRT-PCR was carried out on LET7D, MIR183, and MIR450A pre-miRNAs in shLuc and shDcrA cytoplasmic RNA, using DUSP6 mRNA as an endogenous control. B, KLE whole cell lysates were probed for DROSHA using *GAPDH* as a reference gene. One representative experiment (of 2). C, *IFNβ1* transcript is not increased in shDrosha cells. *IFNβ1* was measured by TaqMan qRT-PCR and normalized to *β-actin* reference gene. For all qRT-PCR experiments, error bars are SD (data shown is average of 2 experiments conducted in triplicate).

IFN response in zebrafish (48) and that so-called "long hairpin RNAs" similarly activate the innate immune response (49) are consistent with our findings that build-up of pre-miRNAs elicits an IFN response. It remains unclear whether or how the IFN response is related to cancer phenotypes such as the increased cell migration and growth in soft agar we observed in our DICER1 knockdown cells.

Although DICER1 homologs are required for the immune response in many eukaryotes including *D. melanogaster* (50), several lines of investigation have indicated that DICER1 may not be necessary for the IFN response in mammals (17). Li and Tainsky evaluated the effects of increased DICER1 in Li-Fraumeni fibroblasts with and without an intact IFN response and showed that overexpression of DICER1 can activate the IFN response (19). The difference in responses seen in fibroblasts in which DICER1 levels were increased and epithelial cells with reduced DICER1 could reflect cell type–specific differences or potentially opposing functional consequences of excess and deficient DICER1 activity in mammalian cells. DICER1

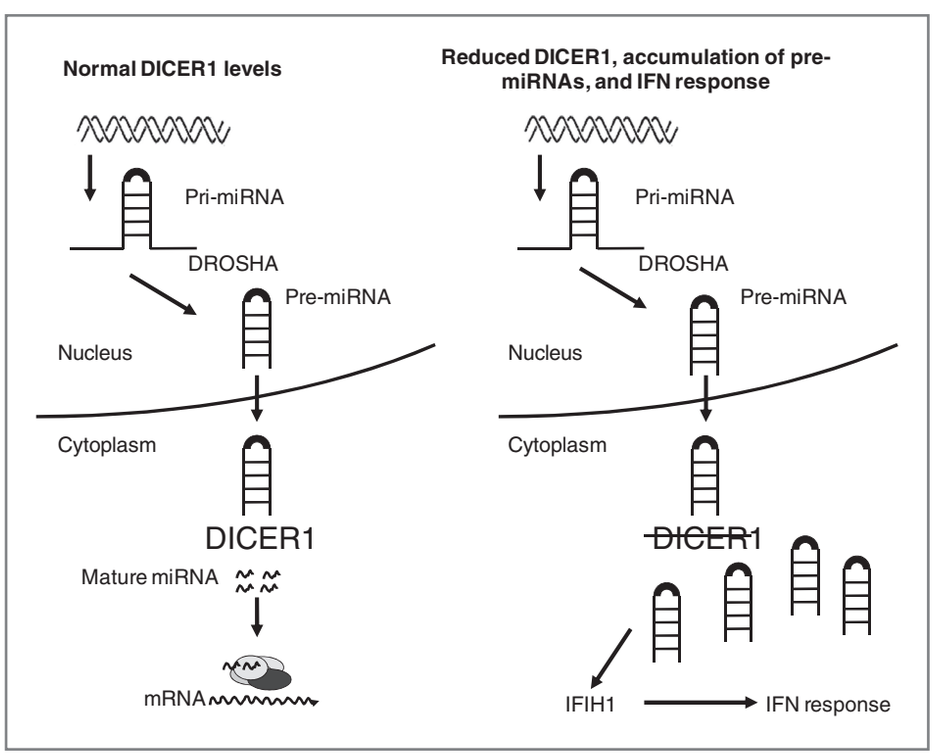

Figure 7. Model for pre-miRNA buildup and IFN stimulation. In cells with normal levels of DICER1 (left), pre-miRNAs are processed to mature miRNAs and mRNAs are targeted for silencing. In cells with reduced DICER1 (right), pre-miRNA processing is inhibited and pre-miRNAs build up in the cytoplasm. This dsRNA can be sensed by cytoplasmic sensors such as IFIH1 and activate the IFN response.

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knockdown cell lines have increased susceptibility to influenza virus infection, implying DICER1 is necessary for recognizing viral dsRNA (51). However, the cancer cell lines we studied were not challenged by virus. In the absence of viral infection, pre-miRNAs have a stimulatory effect on the IFN response. The relationship between alterations in the miRNA processing machinery and the mammalian IFN response may point to a previously unrecognized role for DICER1 in tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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