Programmed Cell Death 4 (PDCD4) Is an Important Functional Target of the MicroRNA miR-21 in Breast Cancer Cells

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MicroRNAs are emerging as important regulators of cancer-related processes. The miR-21 microRNA is overexpressed in a wide variety of cancers and has been causally linked to cellular proliferation, apoptosis, and migration. Inhibition of miR-21 in MCF-7 breast cancer cells causes reduced cell growth. Using array expression analysis of MCF-7 cells depleted of miR-21, we have identified mRNA targets of miR-21 and have shown a link between miR-21 and the p53 tumor suppressor protein. We furthermore found that the tumor suppressor protein Programmed Cell Death 4 (PDCD4) is regulated by miR-21 and demonstrated that PDCD4 is a functionally important target for miR-21 in breast cancer cells.

Since their discovery (1–4), microRNAs (miRNAs) have emerged as integrated and important post-transcriptional regulators of gene expression in animals and plants (5, 6). In animals, miRNAs bind to partly complementary sequence motifs present predominantly within the 3′-untranslated regions (UTRs) and mediate translational repression, sometimes involving degradation of the target mRNA (7–9). miRNAs have been found implicated in a multitude of cellular processes including proliferation, differentiation, migration, and apoptosis (10–12). Accordingly, aberrant miRNA expression has been linked to diseases, including cancer (13–15). Evidence for the causal involvement of miRNAs in cancer comes from several sources. First, mapping of fragile sites and chromosomal loci with the localization of miRNA genes (16, 17), and detailed mapping studies have demonstrated loss of the miR-15/miR-16 cluster (18, 19). Reintroduction of these “tumor suppressor” miRNAs stalls the proliferation of cancer cells or induces apoptosis (18, 20–22). Second, genetic studies in model organisms have led to the identification of miRNAs with relevance for human cancer. This is exemplified by the analysis of let-7, which in Caenorhabditis elegans targets let-60/RAS and in humans is lost in some lung cancers, leading to overexpression of N-RAS (23). Third, forward genetic studies have demonstrated a role for the miR-17–92 cluster in lymphoma development in mice predisposed to cancer (24), and screenings of miRNA expression libraries in cell culture models of cancer have identified miR-372/373 and miR-221/222 as cancer-promoting miRNAs via repression of the tumor suppressor proteins LAT52 and p27, respectively (25, 26).

MicroRNA profiling studies of human tumors have shown cancer type-specific deregulation of miRNA expression and have identified a number of miRNAs with putative tumor suppressor or oncogenic functions (13, 15). Interestingly, miR-21 stands out as the miRNA most often found overexpressed in solid tumors (27), and increased levels of miR-21 have been found in very diverse cancer types including glioblastoma, breast, liver, and pancreatic cancers (11, 27–30). Furthermore, causal links between miR-21 expression and cancer-related processes such as proliferation, migration, apoptosis, and tumor growth have been demonstrated in human hepatocellular and breast cancer cells (11, 31).

Previously identified targets for miR-21 include the tumor suppressors tropomyosin 1 in breast cancer cells (32) and phosphatase and tensin homolog (PTEN) in hepatocellular carcinomas (11, 33). The widespread occurrence of miR-21 overexpression in cancer and the relatively few experimentally defined targets prompted us to perform expression array analyses of breast cancer cells transfected with miR-21 inhibitors to identify and functionally validate additional targets of miR-21.

EXPERIMENTAL PROCEDURES

Antibodies and Western Blot Analysis—MCF-7 cells were seeded in 6-well plates and transfected the following 2 days. The cells were harvested 5 days after the first transfection, washed once in phosphate-buffered saline, and lysed in radioimmuno precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8, 2 mM EDTA) containing 1 mM dithiothreitol, 1 mM Pefabloc (Roche Applied Science), 1 × Complete Mini protease inhibitor mixture (Roche Applied Science), 1 mM NaVO3, 10 mM NaF, 10 mM pyrophosphate, and 50 mM β-glycerophosphate. 15 μg of pro-
tein/lane was separated on a 4–12% NuPAGE Bis-Tris gel (Invitrogen) and transferred to a nitrocellulose membrane. The PDCD4 antibody was kindly provided by Dr. Iwata Ozaki, Japan. The p53 and CDK6 antibodies were purchased from Santa Cruz Biotechnology. The coflin 2 antibody was purchased from Cell Signaling and the vinculin antibody from Sigma-Aldrich.

miRNA Precursors, Anti-miRNA Oligonucleotides, and siRNAs—The locked nucleic acid (LNA)-modified oligonucleotide inhibitors used for miRNA knockdown were purchased from Exiqon. The miRNA precursor hairpins were purchased from Ambion. The PDCD4 SMARTpool siRNA was purchased from Dharmacon.

Cell Culture—HEK293 and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Biochorm), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) and incubated at 37 °C in 5% CO2. MCF-7 cells expressing an ectopic receptor were transduced with pRetroSuper-shp53 (34) or the empty pRetroSuper virus to obtain the MCF-7 shp53 and MCF-7 EV cell lines, respectively. For proliferation assays, MCF-7 cells were transferred to 1.0% fetal calf serum and triplicate independent transfections per treatment with miRNA inhibitors.

Vector Construction and Reporter Assays—A multiple cloning site was inserted into the pGL3 control vector (Invitrogen) at the XbaI site 3’ of the luciferase gene (hereafter pGL3). Six different fragments were PCR-amplified from human genomic DNA and cloned into pGL3. The primer sequences used for PCR amplification were as follows (restriction sites are underlined).

- SOCS5 FW, 5’-gggacgacagtctgcataatagttttt-3’; SOCS5 RV, 5’-gggatccgacactctattcataacctt-3’.
- IL6R FW, 5’-gggatccgacagtctgcaaaaggtt-3’; IL6R RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- BMPRII FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; BMPRII RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- CDK6 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; CDK6 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- BMPRII FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; BMPRII RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- CDK6 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; CDK6 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- PDCD4 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; PDCD4 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.

The QuikChange site-directed mutagenesis kit (Stratagene) was used to introduce one or two point mutations into the seed region of pGL3-PDCD4, giving pGL3-PDCD4MUT1 and pGL3-PDCD4MUT2, respectively. Mutagenesis primers used were as follows.

- MLI1 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; MLI1 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- MLI2 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; MLI2 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.

- MLI1 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; MLI1 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.
- MLI2 FW, 5’-gggatccgacagcagatgaatgcgtcga-3’; MLI2 RV, 5’-gggatccgacagcagatgaatgcgtcga-3’.

The pmir-21-loc reporter vector was constructed by inserting an oligo complementary to the mature miR-21 sequence into the pmIR-REPORT luciferase vector (Ambion).

HEK293 and MCF-7 cells were seeded in 96-well plates and transfected with 50 nM miRNA precursor or LNA, 100–150 ng of luciferase vector (pGL3 constructs), and 25 ng of Renilla vector (pRL-TK) using Lipofectamine 2000 (Invitrogen). 24 h after transfection, cells were harvested and luciferase activity was measured using the Dual-Glo luciferase assay (Promega).

Quantitative PCR Analysis—Total RNA from transfected cells was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Quantitative reverse transcription PCR was performed using the TaqMan reverse transcription kit (Applied Biosystems) and Sybr Green 2× quantitative PCR master mix (Applied Biosystems). PCR primers used were as follows: FAM3C FW, 5’-ctgagagcgccgagaaga-3’ and RV, 5’-tgagagcgccgagaagaaga-3’.

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**RESULTS**

**Suppression of MCF-7 Cell Growth by Inhibition of miR-21**—Several studies have demonstrated that miR-21 is an oncogenic miRNA with anti-apoptotic potential. Inhibition of miR-21 leads to growth suppression and apoptosis in glioblastoma and breast cancer cell lines, and loss of miR-21 can inhibit MCF-7 cell-derived tumor growth in vivo (30, 31, 33). MCF-7 cells express substantial amounts of miR-21 (supplemental Fig. S1), and consistent with previous findings, we observed a dose-dependent suppression of MCF-7 cell growth upon inhibition of miR-21 with an LNA-derived oligonucleotide inhibitor (Fig. 1A). Co-transfection of the LNA inhibitor with a luciferase reporter containing perfect complementarity to the mature miR-21 sequence (pmiR-21-luc) results in marked de-repression of luciferase activity, demonstrating a highly effective inhibition of endogenous miR-21 mediated by the LNA inhibitor (supplemental Fig. S2). The underlying mechanism of the role of miR-21 in tumorigenesis remains unclear, as only few targets for this miRNA have been experimentally verified (11, 32). Meng et al. (11) have recently shown that the tumor suppressor PTEN is a direct functional target of miR-21 in human hepatocellular cancer cell lines. Given the importance of PTEN in regulating the PI3K/AKT pathway and the frequency of PTEN mutations or silencing in a variety of cancers (38), this constitutes an appealing explanation for the overexpression of miR-21 observed in many cancer types (27, 28). To investigate the role of the PTEN-miR-21 interaction in breast cancer cells, we transfected MCF-7 cells with a miR-21 precursor, a miR-21 inhibitor, and appropriate controls. Interestingly, these treatments caused only subtle changes in PTEN protein levels (supplemental Fig. S3), suggesting that cell- and tissue type-specific differences may result in different functional miR-21 targets.

**Identification and Validation of miR-21 Targets by Microarray Analysis**—To identify targets of miR-21 that can explain the proliferation defect observed upon miR-21 inhibition in breast cancers cells, we performed microarray expression analyses using total RNA harvested from MCF-7 cells 24 h after transfection with either LNA-miR-21 or a scrambled control LNA not affecting cellular proliferation (Fig. 1B). We reasoned that cellular mRNAs subjected to increased degradation due to binding of endogenous miR-21 would be up-regulated upon miR-21 inhibition and that specific inhibition of the endoge-
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FIGURE 2. Identification of miR-21 targets. A, cluster heat map of Affymetrix microarray analyses of six independent biological samples showing the 402 transcripts significantly up-regulated by miR-21 inhibition in red and the 335 significantly down-regulated transcripts in blue. B, quantitative reverse transcription PCR validation of 18 up-regulated transcripts from an independent transfection experiment. CDKN1A, FAS, FAM3C, HIPK3, PRRG4, and ACTA2 do not contain matches to the miR-21 seed region, BTG2 and SESN1 contain a 6-mer seed match, and the remaining mRNAs harbor at least one 7-mer seed match. For each transcript the values are normalized to the LNA-scrambled control samples, and error bars represent ± S.D. of three replicates. C, the relative fraction of transcripts among the up-, down-, or non-regulated transcripts containing the indicated type of seed match. ***, one-tailed p values from Fisher’s exact test are: 6-mer up versus no-change, \( p < 1 \times 10^{-12} \); 7-mer-1A up versus no-change, \( p < 1.5 \times 10^{-12} \); 7-mer up versus no-change, \( p < 6.1 \times 10^{-16} \); 6-mer up versus down, \( p < 9.6 \times 10^{-10} \). D, frequency distribution of 7-mer seed matches for all miRNAs in miRBase showing a marked over-representation of the miR-21 seed match in the up- versus down-regulated genes. miR-590 and miR-21 have identical seed sequences.

miR-21 may cause fewer off-target effects than transfection with exogenous miRNA. Following normalization and statistical analysis we found 737 transcripts with significantly different expression between the LNA-miR-21-transfected cells and the controls, of which 402 (55%) were up-regulated and 335 (45%) down-regulated upon miR-21 inhibition (Fig. 2A and supplemental Table S1). To verify the array analysis, 18 up-regulated mRNAs were validated by quantitative reverse transcription PCR analysis of RNA from an independent experiment. Ten of the genes chosen for validation contained at least one miR-21 7-mer seed match, two contained a 6-mer, and six did not contain miR-21 seed matches in their 3′-UTR. All 18 genes showed, to varying degrees, increased mRNA expression levels upon miR-21 inhibition (Fig. 2B). It is unclear whether the mRNAs without matches to the miR-21 seed sequence are direct targets or whether their regulation is a result of secondary effects.

To get a qualitative assessment of the data, we analyzed for the presence of different types of miR-21 seed matches among the genes regulated by miR-21 inhibition. Notably, we found very significant over-representations of all miR-21 seed match categories among the genes up-regulated by miR-21 inhibition relative to gene sets that exhibited no change or down-regulated expression, strongly suggesting that inhibition of endogenous miRNAs can be used to identify bona fide targets (Fig. 2C). In effect, the motif complementary to a 7-mer miR-21 seed sequence (or miR-590, holding the exact same seed sequence) is by far the most prevalent motif when analyzing the 3′-UTRs of the up-regulated transcripts against all seed sequences present in miRBase (36) (Fig. 2D). In addition, an unbiased analysis for the frequency of all possible 7-mer sequence motifs, regardless of whether these match known miRNAs, shows that the miR-21 complementary motif is very highly enriched (\( p < 4 \times 10^{-12} \)) and represents the most frequently occurring sequence motif in the 3′-UTRs of the up-regulated versus the down-regulated transcripts. Hence, the data strongly suggest that expression array analysis following inhibition of endogenous miRNAs is a strong tool to identify miRNA targets subjected to increased mRNA degradation.

Potential Involvement of p53 in the miR-21 Pathway—Among the transcripts up-regulated upon miR-21 inhibition we noticed the presence of several mRNAs known to be regulated by the p53 tumor suppressor, including FAM3C, ACTA2, APAF1, BTG2, FAS, CDKN1A (p21), and SESN1. We validated the up-regulation of these p53-regulated mRNAs by quantitative reverse transcription PCR (Fig. 2B). A connection between miR-21 and p53 was further substantiated using the Ingenuity
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The empty pGL3 vector was not significantly affected by miR-21 inhibition (Fig. 5A). Although miR-21 caused only a slight regulation of pGL3-PDCD4MUT1, pGL3-PDCD4MUT2 remained unaffected by miR-21, suggesting a direct interaction between miR-21 and PDCD4 mediated through the seed region (Fig. 5B). Given the evidence presented above, depletion of PDCD4 by siRNA and assayed the effect of miR-21 inhibition led to a corresponding increase in endogenous protein levels relative to the effect of the scrambled control (Fig. 4C).

Depletion of PDCD4 Abrogates the LNA-miR-21-mediated Phenotype in MCF-7 Cells—PDCD4 is a tumor suppressor known to be up-regulated during apoptosis (40) and down-regulated in several cancer forms (41–43). The predicted interaction between the PDCD4 3′-UTR and miR-21 is illustrated in Fig. 5A. To further substantiate a direct regulation of pGL3-PDCD4 by miR-21 we introduced a single (pGL3-PDCD4MUT1) or double (pGL3-PDCD4MUT2) point mutation in the seed sequence of pGL3-PDCD4. Whereas miR-21 caused only a slight regulation of pGL3-PDCD4MUT1, pGL3-PDCD4MUT2 remained unaffected by miR-21, suggesting a direct interaction between miR-21 and PDCD4 mediated through the seed region (Fig. 5B). Given the evidence presented above, PDCD4 regulation by miR-21 at both the RNA and the protein levels and considering the reported tumor suppressor activity of PDCD4, we speculated that PDCD4 could be a functionally important target of miR-21. To investigate the biological importance of PDCD4 as a target of miR-21, we depleted MCF-7 cells of PDCD4 protein by siRNA and assayed the effect of miR-21 inhibition led to a corresponding increase in endogenous protein levels relative to the effect of the scrambled control (Fig. 4C).

DISCUSSION

Over the past few years the vast potential of miRNAs as regulators of cancer-related signaling pathways has fully emerged (10, 14). Understanding the connections between miRNAs deregulated in cancer and cellular signaling pathways involved in cancer has been hampered by our limited knowledge of miRNA target recognition. Although several studies have demonstrated a central role of the miRNA seed region in target binding (13, 37), additional binding requirements and constraints likely exist. In addition, studies have reported functional miRNA binding without perfect complementarity to the
Given the indications that miR-21 acts as an oncogene in a variety of tumor types, and the limited knowledge of miR-21 targets, our aim was to identify functionally relevant miR-21 targets in MCF-7 breast cancer cells. Bioinformatics analyses demonstrate a very significant over-representation of miR-21 complementary motifs among the transcripts up-regulated by miR-21 inhibition, demonstrating the validity of the experimental approach. We subsequently verified a direct responsiveness to miR-21 for a subset of the putative target mRNAs in heterologous reporter assays. Interestingly, among the six 3′-UTR sequences tested in luciferase assays, only the PDCD4 sequence responded to miR-21 inhibition.

The tumor suppressor PDCD4 was originally characterized as an inhibitor of cellular transformation in a mouse cell culture model (51). PDCD4 expression is down-regulated or lost in several tumor types (52, 53), and ectopic expression of Pdcd4 reduces tumor formation in a mouse skin cancer model (54). Consequently, PDCD4 has been indicated by several as a promising molecular target in cancer treatment (55–57). At the molecular level, PDCD4 binds and inhibits the translation initiation factor eukaryotic initiation factor 4a, thereby impacting on protein translation (58, 59). In addition, PDCD4 has been found to inhibit AP-1-mediated trans-activation (51) and to induce expression of the cyclin-dependent kinase inhibitor p21.
As a result, loss of PDCD4 confers growth advantages to the cells by several means and thereby facilitates the development of cancer. We demonstrate here that PDCD4 is directly regulated by the “oncomiR” miR-21. This is evident at the level of PDCD4 mRNA as well as protein where endogenous PDCD4 protein level is 3.5-fold up-regulated by miR-21 inhibition. Importantly, depletion of PDCD4 by siRNA transfection partly rescues the reduced cellular proliferation observed upon miR-21 inhibition in MCF-7 cells, demonstrating that PDCD4 is an important functional target of miR-21 in this model.

A recent report demonstrated that the stability of PDCD4 is controlled by the mTOR pathway since PDCD4 during mitogen stimulation is phosphorylated by the S6K1 kinase, which marks it for degradation by the proteasome (60). That miR-21 via repression of PDCD4 affects the PI3K/AKT/mTOR survival pathway in addition, knock down of the tumor suppressor protein p53 partly abrogated the proliferation decrease observed in MCF-7 cells following inhibition of miR-21. This suggests a functional link between miR-21, the miRNA most frequently found overexpressed in cancer (27), and the tumor suppressor pathway most often found mutated or otherwise obstructed in cancer (39). There is accumulating evidence of extensive cross-talk between the p53 and the PI3K/AKT/mTOR pathways (61), and our data may reflect such cross-coordination between important anti-cancer networks.

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REFERENCES
1. Lee, R. C., Feinbaum, R. L., and Ambros, V. (1993) Cell 75, 843–854
2. Lee, R. C., and Ambros, V. (2001) Science 294, 862–864
3. Lau, N. C., Lim, L. P., Weinstein, E. G., and Bartel, D. P. (2001) Science 294, 858–862

FIGURE 5. PDCD4 is an important functional target of miR-21. A, potential binding pattern of miR-21 to the 3’-UTR of PDCD4. B, firefly luciferase reporter assay with pGL3-PDCD4, pGL3-PDCD4MUT1, and pGL3-PDCD4MUT2 co-transfected into HEK cells with a Renilla luciferase transfection control plasmid, either alone or together with miR-21 or lin-4 precursor miRNAs. Shown are relative luciferase values normalized to transfections without miRNA. Data are shown as the mean ± S.D. of four replicates and are representative of two independent experiments. *** p < 0.001 using a two-tailed t test. C, verification of PDCD4 knock down in MCF-7 cells by Western analysis. D, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay in MCF-7 cells treated with PDCD4 or control siRNAs and transfected with inhibitors against miR-21 or a scrambled control. Cell number was quantified 5 days after transfection. Data are shown as the mean ± S.D. of three replicates and are representative of three independent experiments. The p value was calculated using a two-tailed t test.
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4. Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001) Science 294, 832–838
5. Pillai, R. S., Bhattacharya, S. N., and Filipowicz, W. (2007) Trends Cell Biol. 17, 118–126
6. Nilsen, T. W. (2007) Trends Genet. 23, 243–249
7. Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Euchas, R., and Pasquinel, A. E. (2005) Cell 122, 553–563
8. Jackson, R. J., and Standart, N. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4034–4039
9. Iorio, M. V., Ferracin, M., Liu, C. G., Veronese, A., Spizzo, R., Sabbioni, S., Chan, J. A., Krichevsky, A. M., and Kosik, K. S. (2005) Cancer Res. 65, 6029–6033
10. Pillai, R. S., Bhattacharya, S. N., and Filipowicz, W. (2007) Trends Cell Biol. 17, 118–126
11. Nilsen, T. W. (2007) Trends Genet. 23, 243–249
12. Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Euchas, R., and Pasquinel, A. E. (2005) Cell 122, 553–563
13. Jackson, R. J., and Standart, N. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 4034–4039
14. Iorio, M. V., Ferracin, M., Liu, C. G., Veronese, A., Spizzo, R., Sabbioni, S., Chan, J. A., Krichevsky, A. M., and Kosik, K. S. (2005) Cancer Res. 65, 6029–6033