Human polynucleotide phosphorylase selectively and preferentially degrades microRNA-221 in human melanoma cells

Swadesh K. Das^a, Upneet K. Sokhi^a, Sujit K. Bhutia^a, Belal Azab^a, Zhao-zhong Su^a, Devanand Sarkar^{a,b,c}, and Paul B. Fisher^{a,b,c,1}

^aDepartment of Human and Molecular Genetics, ^bVirginia Commonwealth University Institute of Molecular Medicine, and ^cVirginia Commonwealth University Massey Cancer Center, Virginia Commonwealth University School of Medicine, Richmond, VA 23298

Edited* by Carlo M. Croce, Ohio State University, Columbus, OH, and approved May 17, 2010 (received for review December 9, 2009)

MicroRNAs (miRNA), small noncoding RNAs, affect a broad range of biological processes, including tumorigenesis, by targeting gene products that directly regulate cell growth. Human polynucleotide phosphorylase (*hPNPase*^{old-35}), a type I IFN-inducible 3'-5' exoribonuclease, degrades specific mRNAs and small noncoding RNAs. The present study examined the effect of this enzyme on miRNA expression in human melanoma cells. miRNA microarray analysis of human melanoma cells infected with empty adenovirus or with an adenovirus expressing *hPNPase*^{old-35} identified miRNAs differentially and specifically regulated by *hPNPase*^{old-35}. One of these, miR-221, a regulator of the cyclin-dependent kinase inhibitor p27^{kip1}, displayed robust down-regulation with ensuing up-regulation of p27^{kip1} by expression of *hPNPase*^{old-35}, which also occurred in multiple human melanoma cells upon IFN- β treatment. Using both in vivo immunoprecipitation followed by Northern blotting and RNA degradation assays, we confirm that mature miR-221 is the target of *hPNPase*^{old-35}. Inhibition of *hPNPase*^{old-35} by shRNA or stable overexpression of miR-221 protected melanoma cells from IFN- β -mediated growth inhibition, accentuating the importance of *hPNPase*^{old-35} induction and miR-221 down-regulation in mediating IFN- β action. Moreover, we now uncover a mechanism of miRNA regulation involving selective enzymatic degradation. Targeted overexpression of *hPNPase*^{old-35} might provide an effective therapeutic strategy for miR-221-overexpressing and IFN-resistant tumors, such as melanoma.

hPNPase^{old-35} | microRNA degradation | miR-221 | miR-222 | p27Kip1

MicroRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs that regulate gene expression at the posttranscriptional level and play important roles in a multiplicity of biological functions, including cell differentiation, tumorigenesis, apoptosis, and metabolism (1). miRNA genes are initially transcribed principally by either RNA polymerase II or RNA polymerase III as long primary transcripts, which are further processed by the nuclear RNase Drosha and cytoplasmic RNase Dicer to produce precursor miRNAs and mature miRNAs, respectively (2). miRNAs recognize and bind to partially complementary sites in the 3' UTRs of target mRNAs, resulting in either translational repression or target degradation (3). The steady-state levels of miRNAs, crucial for its profound impact on a wide array of biological processes (4, 5), are presumably determined by the opposing activities of miRNA biogenesis and degradation. Although the framework of miRNA biogenesis is established, factors involved in miRNA dysregulation remain unknown. Recent work from Ramachandran and Chen (6) documented that an exoribonuclease encoded by *small rna degrading nuclease* (*sdn*) gene degrades mature miRNAs in *Arabidopsis*. Although in human cells the posttranscriptional control of miRNA is poorly defined, it can be hypothesized that enzymes involved in miRNA metabolism evolved from enzymes that process structural and/or catalytic RNAs, a view supported by the fact that a number of

known molecules involved in small RNA metabolism also function in the processing of rRNAs (7, 8).

Exosome, a multiprotein complex of exonucleases, is an important component of the RNA processing machinery in eukaryotes and is essential for the processing of noncoding small RNAs (9–11). Human polynucleotide phosphorylase (*hPNPase*^{old-35}), a highly evolutionarily conserved gene that catalyzes 3'-to-5' phosphorolysis as well as 5'-to-3' polymerization of RNA, acts as a trimer. Analysis of the expression profile of *hPNPase*^{old-35} revealed that it is predominantly a type I IFN-inducible gene and might play an essential role in IFN-induced growth arrest in human melanoma cells by executing its exonuclease activity on *c-myc* mRNA (12–15). Apart from *c-myc* mRNA degradation, *hPNPase*^{old-35} is also involved in regulating the levels of several small noncoding RNAs (16). Very recently, Wang et al. (17) reported that *hPNPase*^{old-35}, along with human mitochondrial SUV 3 (suppressor of Var1 3), degrades dsRNA in the 3'-to-5' direction. Considering the above findings, it seemed plausible that *hPNPase*^{old-35} would have an impact on the other small RNAs, like miRNAs.

The present study investigated the possibility that *hPNPase*^{old-35} might posttranscriptionally regulate miRNAs. We document that overexpression of *hPNPase*^{old-35} either by a replication-incompetent adenoviral vector or by IFN- β treatment resulted in robust and preferentially targeted down-regulation of miRNA-221 (miR-221) with consequent up-regulation of its suppressed target cyclin-dependent kinase inhibitor p27^{kip1}. Inhibition of *hPNPase*^{old-35} by shRNA or stable overexpression of miR-221 protected human melanoma cells from IFN- β -mediated growth inhibition, documenting the importance of these two molecules in mediating IFN- β antiproliferative function.

Results

***hPNPase*^{old-35} Selectively Down-Regulates Specific miRNAs.** To identify miRNA genes whose expression might be regulated by *hPNPase*^{old-35}, we initially compared miRNA expression profiles between empty adenovirus (Ad.vec) and adenovirus expressing *hPNPase*^{old-35} (Ad.*hPNPase*^{old-35})-infected HO-1 human melanoma cells by a DNA microarray containing oligonucleotide probes complementary to mature forms of miRNAs of human origin. The miRNA profiling identified 29 (12 not yet annotated in miRBase) miRNAs displaying differential expression by Ad.*hPNPase*^{old-35} compared with Ad.vec in a one-way ANOVA

Author contributions: S.K.D., D.S., and P.B.F. designed research; S.K.D., U.K.S., S.K.B., B.A., Z.-z.S., and D.S. performed research; S.K.D., U.K.S., S.K.B., and D.S. contributed new reagents/analytic tools; S.K.D., U.K.S., S.K.B., B.A., Z.-z.S., D.S., and P.B.F. analyzed data; and S.K.D., D.S., and P.B.F. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

¹To whom correspondence should be addressed. E-mail: pbfisher@vcu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.0914143107/-DCSupplemental.

(Table S1). Of interest, with the robust down-regulation of several miRNAs, a number of miRNAs were also not affected by *hPNPase*^{old-35}, suggesting specificity and selectivity of this enzyme-mediated miRNA down-regulation. To verify the microarray data and investigate whether down-regulation of specific miRNAs was mediated transcriptionally or posttranscriptionally, we measured the expression levels of primary precursor, precursor, and mature state of selective miRNAs by quantitative PCR (qPCR) and Northern blotting. We previously demonstrated in HO-1 cells (13, 14) that *hPNPase*^{old-35}-mediated up-regulation of p27^{Kip1}, a direct target of miR-221 and miR-222 (18), and p21^{mda-6/Waf1/CIP1} (19), a target of miR-106b. Considering these findings, we examined the expression of these three miRNAs by qPCR and Northern blotting. Additionally, to confirm *hPNPase*^{old-35} specificity in altering miRNA levels, qPCR/Northern blotting was also performed for miR-let7a, miR-184, and miR-25 (clusters with miR-106b), whose levels did not change in HO-1 cells when exposed to elevated levels of this enzyme. In agreement with the microarray analysis, this revealed a marked down-regulation of only the mature form of miR-221, miR-222, and miR-106b, without changing miR-25, miR-let7a, or miR-184 expression, after *Ad.hPNPase*^{old-35} infection of HO-1 cells. These findings demonstrate the selectivity of *hPNPase*^{old-35} in down-regulating specific miRNAs (Fig. 1*A* and *B*). In addition, because both primary and precursor miRNAs of selected mature miRNAs did not respond to *hPNPase*^{old-35}, it is assumed that down-regulation might be regulated at a posttranscriptional level and supports a hypothesis that *hPNPase*^{old-35}, through its enzymatic activity, can regulate the level of specific mature miRNAs.

hPNPase^{old-35} is an exoribonuclease, and to execute its activity it must bind to the miRNAs. To evaluate this possibility, *hPNPase*^{old-35} was immunoprecipitated from the *Ad.hPNPase*^{old-35}-infected cells

and the associated RNA extracted. Northern blotting analyses for different miRNAs demonstrated that only the mature form of specific miRNAs interacted with this protein (Fig. 1*C*). In contrast, miRNAs that were not down-regulated did not interact with *hPNPase*^{old-35}. An inverse correlation between these miRNAs and *hPNPase*^{old-35} in primary melanocytes and different melanoma cell lines supports the biological relevance of this protein in regulating specific miRNAs (Fig. S1).

Because *hPNPase*^{old-35} is a type I IFN-inducible gene (12), we hypothesized that IFN- β might also specifically reduce miR-221, miR-222, and miR-106b by induction of *hPNPase*^{old-35}. To explore this possibility, HO-1 cells were treated with 1,000 U/mL of IFN- β for 24 h, and the relative expression of all six miRNAs was analyzed by qPCR. As shown in Fig. 1*D*, a significant reduction in miR-221, miR-222, and miR-106b was observed after IFN- β treatment, whereas miR-25, miR-let7a, and miR-184 remained unaffected.

hPNPase^{old-35} Specifically and Selectively Degrades miR-221 in Vitro.

Because both *Ad.hPNPase*^{old-35} and IFN- β -mediated down-regulation were most robust and specific for miR-221, we next evaluated whether *hPNPase*^{old-35}-mediated down-regulation of miR-221 was greater than that of the other miRNAs. For this analysis, a C-terminal HA-tagged *hPNPase*^{old-35}-expressing construct (*hPNPase*^{old-35}-HA) (14) was used to prepare in vitro-translated *hPNPase*^{old-35} protein, and the authenticity of the translated product was confirmed by Western blotting analysis (Fig. 2*A*). Incubation with in vitro-translated *hPNPase*^{old-35} resulted in temporal degradation of different miRNAs with varied kinetics, whereas the level of miR-RNU44 remained unchanged. Among the miRNAs analyzed, miR-221 showed a statistically significant higher degradation (at 2 h, 49% vs. 31% and 39% for miR-222 and miR-106b, respectively) by *hPNPase*^{old-35} (*P* value was calculated by comparing miR-RNU44 and the different miRNAs) compared with other miRNAs (Fig. 2*B* and *C*, respectively).

Fig. 1. *hPNPase*^{old-35} regulates the expression of specific miRNAs. (A) HO-1 cells were either infected with *Ad.vec* or *Ad.hPNPase*^{old-35} at a multiplicity of infection of 5,000 viral particles per cell for 3 d, and expressions of indicated miRNAs were analyzed by qPCR using primary and mature miRNA-specific Taqman probes. (B) Northern blotting was performed to detect mature miRNAs and its precursor species (pre-) by using specific probes. (C) At 12 h (b) and 24 h (c) after infection with *Ad.hPNPase*^{old-35}, cell lysates were prepared as described in *Materials and Methods*, and *hPNPase*^{old-35} was immunoprecipitated by anti-*hPNPase*^{old-35} antibody. Immunoprecipitation with nonspecific IgG (a) was used as a negative control. Associated miRNAs were extracted, and expression analysis was performed by Northern blotting. (D) HO-1 cells were treated with IFN- β (1,000 U/mL) and subjected to real-time PCR for different miRNAs. Data are presented as mean normalized expression $\pm$ SD for triplicate determinations from three independent experiments.

Fig. 2. *hPNPase*^{old-35} degrades miRNAs in vitro. (A) The authenticity of the in vitro-translated protein, generated as described in *Materials and Methods*, was analyzed by Western blotting with anti-*hPNPase*^{old-35} antibody. (B) In vitro RNA degradation assays were performed as described in *Materials and Methods*. At different time points, the levels of indicated miRNAs were determined by qPCR. (C) Comparative degradation of miR-221, miR-222, and miR-106b at a 2-h time point using an in vitro degradation assay. The data represent mean $\pm$ SD of two independent experiments, each done in triplicate. (D) A direct interaction between targeted miRNAs with in vitro-translated *hPNPase*^{old-35} was confirmed by immunoprecipitation and Northern blotting.

Moreover, at 15 min, in vivo immunoprecipitation assays showed more mature miR-221 associated with *hPNPase*^{old-35} compared with the other miRNAs, and consistent with this finding, less mature miR-221 was detected in the input (Fig. 2D). All of these observations strongly suggest that miR-221 is the predominant regulated microRNA, at least among the miRNAs analyzed in the present study. We also confirmed that this effect was miRNA specific, because levels of miR-let7a and miR-184 did not change (Fig. 2B).

We confirmed and extended the findings obtained from HO-1 cells to additional human melanoma cells. Infection with Ad.*hPNPase*^{old-35} at a multiplicity of infection of 5,000 viral particles per cell for 3 d resulted in significant down-regulation of miR-221 (Fig. 3A) with a concomitant up-regulation of its direct target *p27*^{Kip1} (Fig. 3B). Similarly, miR-221 levels were also reduced upon treatment for 24 h with 1,000 U/mL of IFN- β (Fig. 3C). The expression levels of miR-let7a and miR-184 in melanocytes or different melanoma cell lines were not affected by infection with Ad.*hPNPase*^{old-35} or treatment with IFN- β , again suggesting specificity of the *hPNPase*^{old-35}/IFN- β effect. These findings establish functional and mechanistic links between *hPNPase*^{old-35} induction and miR-221 down-regulation by IFN- β . Compared with the metastatic melanoma cells, the miR-221 down-regulation was significantly less pronounced in normal FM-516 immortal melanocytes. Interestingly, infection with Ad.*hPNPase*^{old-35} or IFN- β treatment generated much less *hPNPase*^{old-35} mRNA, as determined by qPCR, in FM-516 cells compared with the melanoma cells, an intriguing observation that remains to be mechanistically explained (Fig. 3D).

IFN- β Fails to Down-Regulate miR-221 in *hPNPase*^{old-35}-shRNA-Expressing Cells. We next investigated whether knockdown of *hPNPase*^{old-35} would abrogate IFN- β -mediated down-regulation of miR-221. Infection of HO-1 cells with a lentivirus expressing *hPNPase*^{old-35} shRNA (*shhPNPase*^{old-35}) resulted in marked down-regulation of both basal and IFN- β -induced expression of *hPNPase*^{old-35} mRNA and protein compared with a lentivirus expressing control scrambled shRNA (shCon) (Fig. 4A). Cell

viability and colony formation assays revealed that *shhPNPase*^{old-35}-expressing cells were rendered resistant to IFN- β -induced growth inhibition compared with either control shRNA-infected cells or uninfected cells (Fig. S2). Expression of *shhPNPase*^{old-35} did not interfere with the ability of the HO-1 cells to respond to IFN- β , as evidenced by the induction of another IFN-inducible gene, melanoma differentiation associated gene-5 (*mda-5*) (20, 21) (Fig. 4B). On the contrary, *shhPNPase*^{old-35}-expressing cells displayed resistance to IFN- β -mediated miR-221 reduction (77% vs. 18% down-regulation in shCon and *shhPNPase*^{old-35} infected cells, respectively) (Fig. 4C, Left). As expected, the expression level of *p27*^{Kip1} was significantly lower in *shhPNPase*^{old-35}-expressing cells at 48 h after treatment with IFN- β (Fig. 4C, Right). These findings indicate that *hPNPase*^{old-35} mediates IFN- β -induced down-regulation of miR-221 and consequent up-regulation of *p27*^{Kip1}.

Overexpression of miR-221 in HO-1 Cells Confers Resistance to IFN- β -Mediated Growth Arrest.

To analyze the consequence of the IFN- β -mediated down-regulation of miR-221, two clones (Cl.9 and Cl.11) were selected on the basis of their higher miR-221 expression and maximum down-regulation of *p27*^{Kip1} compared with control and other engineered clones (Fig. 5A). The effect of IFN- β on growth of HO-1 control and miR-221-overexpressing clones was analyzed by trypan blue dye exclusion assays (Fig. 5B). Cells were treated with 500 or 1,000 U/mL of IFN- β for different times. After 3 d, a marked inhibition of cell growth (at 1,000 U/mL, >50%) was observed for the control HO-1 cells. In contrast, the viability of both miR-221-overexpressing clones was significantly higher than that of control cells (42.2% vs. 62.3% and 61.5% for Cl.9 and Cl.11, respectively), and at day 6 the resistance was more profound. As a corollary, fewer apoptotic cells were observed in both clones compared with parental HO-1 cells as determined by

Fig. 3. *hPNPase*^{old-35} down-regulates miR-221 expression in multiple melanoma cell lines. At 3 d after infection with either Ad.vec or Ad.*hPNPase*^{old-35} the specified cell type was analyzed for distinct miRNA expression (A) and *p27*^{Kip1} and EF-1 α proteins (B) as described in Materials and Methods. (C) Cell lines were treated with IFN- β for 24 h, and expression levels of indicated miRNAs were measured by qPCR. (D) Induction of mRNA for *hPNPase*^{old-35} in Ad.*hPNPase*^{old-35}-infected or IFN- β -treated cells was determined by qPCR and presented as fold-induction compared with FM-516 cells. The data represent mean $\pm$ SD of triplicate determinations for three independent experiments. Data points marked with asterisks indicate significant differences ($P < 0.01$) from the corresponding control data points.

Fig. 4. *hPNPase*^{old-35} shRNA confers resistance to IFN- β -mediated miR-221 down-regulation. HO-1 cells infected with a lentivirus expressing the indicated shRNA were treated with IFN- β (500 or 1,000 U/mL) for 24 h, and the expression of *hPNPase*^{old-35} (A) and *mda-5* (B) mRNA (Left) and protein (Right) were analyzed by qPCR and Western blotting, respectively. (C) HO-1 cells were treated as in A, and the expression of miR-221 was analyzed by qPCR (Right). HO-1 cells were treated as in A, except that IFN- β treatment was performed for 48 h and the expression of *p27*^{Kip1} analyzed by Western blotting (Right). The data represent mean $\pm$ SD of three independent experiments.

Fig. 5. HO-1 clones expressing miR-221 are resistant to IFN- β -mediated growth inhibition. (A) The stable miR-221-overexpressing HO-1 clones were analyzed for basal miR-221 level and its target p27^{kip1} by qPCR and Western blotting, respectively. (B) HO-1 cells either expressing empty vector (HO-1 Con) or miR-221 (Cl.9 and Cl.11) were treated with IFN- β at 500 or 1,000 U/mL. At the indicated times (d3 and d6) cells were trypsinized and counted as described in *Materials and Methods*. (C) Percentage of apoptotic cells in different clones was determined by Annexin-V binding assays after IFN- β (1,000 U/mL) treatment for the indicated times. (D) Colony formation assays using the indicated clones were performed as described in *Materials and Methods*. The data represent the mean $\pm$ SD of three independent experiments. *Significant difference ($P < 0.01$) vs. corresponding control points.

Annexin-V binding assays (Fig. 5C). The results obtained from cell viability assays were further substantiated by colony formation assays (Fig. 5D). In HO-1 cells, colony formation was significantly inhibited with 500 U/mL of IFN- β , and it was inhibited more than 90% with 1,000 U/mL of IFN- β . Consistent with the proliferation assay, miR-221-overexpressing clones showed significant resistance to IFN- β -induced inhibition of colony forming ability than in corresponding controls.

Because miR-222 shares the same target with miR-221 and both are overexpressed in different malignancies, we also established clones of HO-1 cells stably overexpressing miR-222. On the basis of expression level compared with the control clones, two clones (Cl.1 and Cl.5) were selected for further study (Fig. S3A). Because miR-222 also targets p27^{kip1}, we confirmed the down-regulation of this protein by Western blotting, which was down-regulated to a lesser extent than the miR-221 clones (Fig. S3B). Similarly, the survival and plating efficiency of these clones when exposed to IFN- β were also significantly lower than in miR-221-overexpressing clones, documenting that miR-221 can confer resistance to IFN- β -induced growth inhibition more robustly than miR-222 (Fig. S3C and D).

These interesting findings were corroborated by cell cycle analysis. Treatment with IFN- β in HO-1 control cells resulted in significant G₁ arrest at 24 h (47% at 0 h vs. 62% at 24 h; Fig. 6A). In contrast, both miR-221-overexpressing clones showed little increase in G₁ arrest after IFN- β treatment (48% at 0 h vs. 53% at 24 h), which was significantly less than the control HO-1 cells. Similar trends were observed at both 48 h and 72 h. As a corollary, both the basal and IFN- β -inducible levels of p27^{kip1} mRNA and protein were significantly lower in miR-221-overexpressing clones compared with the control HO-1 clone (Fig. 6B and C, respectively). Our findings suggest that IFN- β -induced *hPNPase*^{old-35} degrades miR-221, leading to an increase in p27^{kip1} that then elicits growth arrest (Fig. 6D).

Fig. 6. HO-1 clones expressing miR-221 are resistant to IFN- β -mediated cell cycle arrest. (A) The designated cell types were treated with the indicated doses of IFN- β for different times, and cell cycle was monitored by flow cytometry. Percentages of cell population in the G₁ phase. (B and C) Indicated cells were treated as in A, and p27^{kip1} mRNA (B) and protein (C) expression were determined by qPCR and Western blotting, respectively. The data represent mean $\pm$ SD of three independent experiments. (D) Schematic model for regulation of miR-221 by IFN- β . See text for details.

Discussion

The 3', 5' processing or degradation of RNA controls many cellular events, including the maturation of 5.8 S rRNA, the processing of many small RNAs, and the turnover of different types of mRNAs (22–25). *hPNPase*^{old-35} is a 3', 5' exonuclease that catalyzes the phosphorolysis of RNA, generating nucleoside diphosphates as cleavage products (26). We demonstrated previously that *hPNPase*^{old-35} could specifically degrade *c-myc* mRNA, resulting in growth arrest in human melanoma cells (16). Apart from our study, a number of additional potential substrates for *hPNPase*^{old-35} have been identified, including small RNA and noncoding RNA. Very recently, Wang et al. (17) described a model whereby mitochondrial *hPNPase*^{old-35} forms a heteropentameric complex with a helicase hSUV3 that functions in a coordinated manner to degrade dsRNA substrates in the presence of ATP. Mechanistically, helicase (hSUV3) unwinds the RNA substrate, and the exonuclease present in the same entity can efficiently degrade the structured RNA (18). *hPNPase*^{old-35} can also interact with RNase E in the degradosome, a multiprotein complex involved in RNA turnover (27), and these enzymes have been shown to interact functionally in the decay of some small RNAs such as RyhB, SgrS, and CrsB (28, 29). In eukaryotes, the exosome was shown to be essential in the maturation of noncoding RNAs (11). Consistent with these findings, in the present study we uncovered a role for *hPNPase*^{old-35} in mediating specific mature miRNA silencing in human melanoma cells, and we show targeted miRNA degradation in human cell lines. Although the comprehensive and detailed mechanisms of miRNA degradation by *hPNPase*^{old-35} is beyond the scope of this study, the simplest interpretation of our data is that *hPNPase*^{old-35} can directly recruit specific mature miRNAs and degrade these molecules through its exonuclease activity.

Our data document a reduction of specific miRNAs, including miR-221, miR-222, and miR-106b, after ectopic expression of *hPNPase*^{old-35}. Specificity of action was confirmed by analyzing miR-let7a and miR-184 levels, which remained unchanged by *hPNPase*^{old-35}. Interestingly, both of these miRNAs are considered to function as tumor suppressors, with miR-let7a and miR-184 showing reduced expression in lung cancer (30) and high-

grade astrocytomas (31), respectively. In contrast, the miRNAs regulated by *hPNPase^{old-35}* contribute to oncogenesis. miR-221 and miR-222 are highly homologous and coregulated miRNAs that share common predicted targets, and their role in oncogenesis is supported by the identification of p27^{Kip1} and p57^{Kip2} among their targets (18). Additionally, a number of miRNAs involved in oncogenesis, including miR-106b, miR-103, miR-107, miR-193, miR-29b, and miR-320 (19, 32–35), were also down-regulated by *hPNPase^{old-35}*. Compared with normal cells, *hPNPase^{old-35}* expression was down-regulated to a greater extent in their tumorigenic counterparts, suggesting a potential role for up-regulation of the oncogenic miRNAs in facilitating tumorigenesis. A detailed biochemical study is necessary to identify the molecular complex involving *hPNPase^{old-35}* that causes miRNA degradation and turnover. Further studies are also needed to define and comprehend the specific consequences of miRNA regulation by *hPNPase^{old-35}* and how these changes contribute to carcinogenesis.

Myc is an important regulator of cell proliferation and can regulate p27^{Kip1} levels either transcriptionally (36) or posttranscriptionally (37). We have previously shown that Ad.*hPNPase^{old-35}*-mediated or IFN- β -induced *hPNPase^{old-35}* protein selectively degrades *c-myc* mRNA and causes growth arrest at the G₁ to S phase in human melanoma cells (14, 15). Our present study reveals another mechanism of p27^{Kip1} regulation by *hPNPase^{old-35}* (i.e., via miR-221 degradation). This is indeed a very intriguing phenomenon, suggesting the importance of p27^{Kip1} in mediating the phenotypic effects induced by *hPNPase^{old-35}*. *hPNPase^{old-35}* is known to induce senescence, and one of the important components of senescence is G₁ cell cycle arrest that might be caused by p27^{Kip1}. We are currently investigating the role of the interactive network of *hPNPase^{old-35}*, *c-myc*, miR-221, and p27^{Kip1} in regulating the senescence process.

miR-221 is up-regulated in several human malignancies, including melanoma (38), breast carcinoma (39), hepatocellular (40), and malignant glioma (41). miR-221-overexpressing HO-1 clones were resistant to IFN- β when compared with control HO-1 cells, supporting the hypothesis that IFN- β -mediated growth inhibition is at least partially dependent on miR-221. Type I IFNs have been evaluated alone or in combination with other chemotherapeutics in the treatment of uveal (42) and cutaneous melanoma (43). Although some success has been reported using combination therapy, many tumors in patients display resistance to IFN- β (44). Overexpression of miR-221 might be one mechanism of IFN resistance, and strategies such as targeted overexpression of *hPNPase^{old-35}* might provide an effective means of overcoming this resistance.

In summary, the present study uncovers an innovative facet of *hPNPase^{old-35}* function in miRNA regulation, which has significant impact in understanding IFN- β action. We have recently demonstrated the efficacy of an adenovirus expressing *hPNPase^{old-35}* under the control of a cancer-specific PEG-3 promoter, suggesting that this strategy might be effectively used for cancer therapy (45, 46). The importance of miRNAs as potential oncogenes and the problem of IFN resistance in the clinical arena make targeted *hPNPase^{old-35}* expression a viable and potentially effective cancer therapeutic strategy.

Materials and Methods

Cell Lines and Adenoviruses. Normal immortal human melanocyte FM-516-SV (referred to as FM-516) and metastatic human melanoma HO-1, C8161, WM-278, and MeWo cell lines were cultured as previously described (14). The

construction and purification of an *hPNPase^{old-35}*-expressing replication-defective adenovirus was described previously (14).

Plasmid Construction and Establishing Stable Cell Lines. The genomic sequence of miR-221 was amplified by PCR using human genomic DNA as template and primers, sense: 5'-CCCAGCATTCTGACTGTTG-3' and antisense: 5'-TGTGA-GACCATTTGGGTGAA-3', and PCR product was cloned pcDNA3.1(+)/Hygro vector (Invitrogen). miExpress miRNA-222-Puro expression plasmid was purchased from GeneCopoeia, and these constructs were transfected into HO-1 cells using lipofectamine 2000 according to the manufacturer's protocol. Stable clones were established by selection in the corresponding antibiotics for 2 wk.

RNA Isolation, microRNA Microarray, and q-PCR. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen). For miRNA microarray, total RNA was isolated, and sample labeling, hybridization, and data analysis were performed using the miRCURY platform developed by Exiqon. qPCR was performed using the ABI 7900 Fast Real-Time PCR System and TaqMan MicroRNA and gene expression assays for individual miRNA and mRNA according to the manufacturer's protocol (Applied Biosystems).

Immunoprecipitation and Northern Blotting. For immunoprecipitation, an RNA-binding protein immunoprecipitation kit from Invitrogen was used according to the manufacturer's protocol. Oligonucleotides complementary to the different miRNAs were purchased from Exiqon, and Northern blotting was performed as previously described (14). Blots were stripped once and reprobed using an oligonucleotide complementary to U6 RNA.

Cell Proliferation and Colony Formation Assay. Trypan blue dye exclusion and Annexin-V binding assays were performed to determine viable and apoptotic cells, respectively. Colony formation assays were performed as previously described (14).

Generation of Lentiviruses Expressing shRNA for *hPNPase^{old-35}*. Lentiviruses expressing either control shRNA or shRNA for *hPNPase^{old-35}* were constructed using the Trans-Lentiviral Packaging System (Open Biosystems) according to the manufacturer's protocol. The lentiviruses were amplified and titered by standard plaque assay.

Protein Extraction and Western Blotting. Preparation of whole-cell lysates and Western blotting was performed as described previously (15). The primary antibodies included p27^{Kip1} and *hPNPase^{old-35}* from Santa Cruz Biotechnology and MDA-5 from Abcam. Blots were stripped and normalized by reprobing with anti-elongation factor-1 α (Upstate Biotechnology).

In Vitro Translation and in Vitro miRNA Degradation Assays. In vitro translation was performed using the TNT coupled reticulocyte lysate system (Promega) using the plasmids pcDNA3.1 as a control, and *hPNPase^{old-35}*-HA expression plasmid, according to the manufacturer's protocol. Ten micrograms of total RNA from HO-1 cells were incubated with 5 μ L of each in vitro-translated protein at 37 °C from 0.5 to 2 h. The RNA was repurified using the Qiagen miRNeasy mini kit, and qPCR for specific miRNAs was performed.

Cell Cycle Analysis. Cell cycle was analyzed using a FACSCalibur flow cytometer, and data were analyzed using Cell Quest software (Becton Dickinson).

Statistical Analysis. The data are reported as the mean $\pm$ SD of the values from three independent determinations, and statistical analysis was performed using Student's *t* test in comparison with corresponding controls. Probability values of <0.05 were considered statistically significant.

ACKNOWLEDGMENTS. This study was supported in part by National Institutes of Health Grants R01 CA097318, R01 CA134721, and P01 CA104177, and the Samuel Waxman Cancer Research Foundation (SWCRF). D.S. is the Harrison Endowed Scholar in Cancer Research. P.B.F. holds the Thelma Newmeyer Corman Chair in Cancer Research and is a SWCRF Investigator.

- Calin GA, Croce CM (2006) MicroRNA signatures in human cancers. *Nat Rev Cancer* 6: 857–866.
- Filipowicz W, Bhattacharyya SN, Sonenberg N (2008) Mechanisms of post-transcriptional regulation by microRNAs: Are the answers in sight? *Nat Rev Genet* 9:102–114.
- Tsuchiya S, Okuno Y, Tsujimoto G (2006) MicroRNA: Biogenetic and functional mechanisms and involvements in cell differentiation and cancer. *J Pharmacol Sci* 101:267–270.

- Pillai RS, Bhattacharyya SN, Filipowicz W (2007) Repression of protein synthesis by miRNAs: How many mechanisms? *Trends Cell Biol* 17:118–126.
- Plasterk RH (2006) Micro RNAs in animal development. *Cell* 124:877–881.
- Ramachandran V, Chen X (2008) Degradation of microRNAs by a family of exoribonucleases in *Arabidopsis*. *Science* 321:1490–1492.
- Ansel KM, et al. (2008) Mouse Eri1 interacts with the ribosome and catalyzes 5.8S rRNA processing. *Nat Struct Mol Biol* 15:523–530.