

Systematic study of *cis*-antisense miRNAs in animal species reveals miR-3661 to target *PPP2CA* in human cells

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ABSTRACT

MicroRNAs (miRNAs) suppress targeting gene expression through blocking translation or triggering mRNA degradation and, in general, act in *trans*, through a partially complementary interaction with the 3' untranslated region (3' UTR) or coding regions of a target gene. Although it has been reported previously that some miRNAs suppress their target genes on the opposite strand with a fully complementary sequence (i.e., natural antisense miRNAs that act in *cis*), there is no report to systematically study such *cis*-antisense miRNAs in different animal species. Here we report that *cis*-antisense miRNAs do exist in different animal species: 48 in *Caenorhabditis elegans*, 17 in *Drosophila*, 36 in *Mus musculus*, and 52 in *Homo sapiens* using a systematical bioinformatics approach. We show that most of these *cis*-antisense miRNAs can efficiently reduce the expression levels of their target genes in human cells. We further investigate hsa-miR-3661, one of the predicted *cis*-antisense miRNAs, in detail and demonstrate that this miRNA directly targets the coding sequence of *PPP2CA* located on the opposite DNA strand and inhibits the *PPP2CA* expression. Taken together, these results indicate that *cis*-antisense miRNAs are conservative and functional in animal species including humans.

Keywords: miRNA; *cis*-antisense; gene expression; miR-3661; *PPP2CA*; human cells

INTRODUCTION

MicroRNAs (miRNAs) are a class of small RNA genes with 21–25 nt, which regulate their target genes in various species. Most animal miRNAs regulate the expression of other genes predominantly in *trans* through partially complementary interactions with 3' untranslated regions (3' UTRs) or transcript coding regions of the target genes. However, some animal miRNAs are discovered as transcribed antisense to exons of noncoding or protein-coding genes (Bartel 2004). Such antisense exonic miRNAs, together with the intronic miRNAs and the miRNAs in the sense strand of genes, are termed as intragenic miRNAs (Rodriguez et al. 2004; Hinske et al. 2010, 2014). The function of such antisense exonic miRNAs were investigated in mouse miR-126 and miR-136 that are transcribed antisense of a retrotransposon-like gene (*RTL1*) and a down-regulated *RTL1* mRNA level through a fully complementary sequence match with the target gene (Seitz et al. 2003). These *cis*-antisense exonic miRNAs are similar to the natural antisense miRNAs (nat-miRNAs) reported later in the plant (Lu et al. 2008), but with one main difference:

cis-antisense miRNAs are derived from precursors with a compact hairpin, whereas nat-miRNAs are derived from the precursors with large introns. To date, *cis*-antisense miRNAs were only reported in mouse; whether these *cis*-antisense miRNAs functionally exist in various animal species including humans has not been systematically studied.

To address this question, we performed a genome-wide analysis to identify miRNAs that are transcribed antisense to exons of known genes. As a result, we found that such *cis*-antisense miRNAs with a fully complementary antisense sequence against their target (protein-coding or noncoding) genes exist in different animal species: 48 in *C. elegans*, 17 in *Drosophila*, 36 in mouse, and 52 in the human genome (including miR-127/136 as reported [Seitz et al. 2003]). We then demonstrated that almost all of the *cis*-antisense miRNAs efficiently suppressed the expression levels of their target genes in human cells. We further studied in detail the effects of one *cis*-antisense miRNA, miR-3661, on its target gene, *PPP2CA*.

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Article published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.052894.115>.

PP2A α is the catalytic subunit of PP2A and encoded by the *PPP2CA* gene. PP2A is one of the major cellular serine–threonine phosphatases (Wera and Hemmings 1995). PP2A is a bona fide tumor suppressor consisting of multiple different subunits and targets a multitude of substrates (Janssens and Goris 2001; Sablina and Hahn 2008). PP2A α is highly conserved from yeast to human. Loss-of-function mutations in PP2A subunits or up-regulation of PP2A-specific inhibitors that contribute to transformation were found in a variety of cancers (Eichhorn et al. 2009). One of the most important dephosphorylating substrates for PP2A is AKT (Andjelković et al. 1996; Meier et al. 1998; Chen et al. 1999; Sato et al. 2000; Ivaska et al. 2002; Resjö et al. 2002). PP2A negatively regulates the AKT pathway by dephosphorylating AKT itself and GSK-3 β , an AKT-phosphorylated target (Shaw et al. 1997), which plays a critical role in cell proliferation, unlike the regulation of PP2A at the transcriptional level that has been reported in detail (Altiok et al. 1997; Chen et al. 2009; Liu et al. 2012). There are few reports on the regulation of *PPP2CA* at the post-transcriptional level, particularly via a miRNA-related regulation. Only recently, it was reported that miR-155 can negatively regulate PP2A (Lashine et al. 2015). Our results indicate that miR-3661 can regulate *PPP2CA* expression in human cells in a *cis*-antisense manner. These results demonstrate that the *cis*-antisense miRNAs conservatively and functionally exist in humans.

RESULTS

Fifty-two potential *cis*-antisense miRNAs and their antisense transcribed genes are identified in the human genome

We performed a genome-wide analysis to identify miRNAs that are transcribed antisense to known genes (Fig. 1A). In the human (*Homo sapiens*) genome, 432 miRNAs were found in the antisense orientation of 313 protein-coding/noncoding genes. Among the 432 miRNAs, 380 miRNAs that are in the opposite DNA strands at the genomic locus of introns of 280 genes were excluded from this study. Finally 52 miRNAs (including miR-127/136 as reported [Seitz et al. 2003]) were identified as the candidates for suppressing the expression of their 33 antisense target genes in a *cis*-antisense manner with fully complementary sequences (Table 1). Among the 52 miRNAs, 25 miRNAs target coding sequence (CDS) (including miR-127/136 as reported [Seitz et al. 2003]) and 27 miRNAs target noncoding sequence including exons, noncoding genes 3'/5' UTR of protein-coding genes (Table 1). To identify the *cis*-antisense miRNAs in other animal species, we applied the same analysis procedure to *Mus musculus* (*M. musculus*), *Drosophila melanogaster* (*D. melanogaster*), and *Caenorhabditis elegans* (*C. elegans*). As a result, we identified 235 miRNAs in *M. musculus*, 66 miRNAs in *D. melanogaster*, and 177 miRNAs in *C. elegans* genome, in the antisense orientation of protein-coding/noncoding genes, re-

spectively. After excluding the miRNA transcribed antisense to the introns of genes, finally 36 (*M. musculus*), 17 (*D. melanogaster*), and, 48 (*C. elegans*) *cis*-antisense miRNAs were obtained (Supplemental Tables 1–3).

Most of the identified *cis*-antisense miRNAs functionally suppress their targets in humans

Next, we were interested in determining whether these human miRNAs can biologically suppress their antisense target genes. Among the 52 miRNAs, 30 miRNAs are from 15 precursors (5p and 3p strands of a pre-miRNA) targeting the same gene (Table 1), and 22 miRNA target genes individually. Therefore, we tested the 37 miRNAs for functional measurement. We obtained 29 commercially available miRNA mimics and the TaqMan assays of their target genes. We transfected these miRNA mimics to 293FT cells and measured their target expressions using a real-time PCR approach. The results showed that most miRNA mimics can efficiently suppress the expression of their targets (Fig. 1B); only three miRNA mimics could not suppress their antisense targets: miR-4665-5p targeting the 5' UTR of *KIAA2026*, miR-136-5p targeting the CDS of *RTL1*, and miR-3665 targeting the CDS of *SLAN1* (Table 1; Fig. 1B). These results indicate that most of the *cis*-antisense miRNAs are functional.

MiR-3661 specifically targets *PPP2CA* in human cells

To further elucidate the functions of miRNAs acting in human cells, we chose miR-3661 because it targets *PPP2CA* that codes PP2A α , an important catalytic subunit of PP2A. MiR-3661 was first identified in 2010 (Hansen et al. 2010) and then further confirmed by another group (Persson et al. 2011); however, its function remains unclear. We found that miR-3661 is located on the opposite strand of *PPP2CA*, the PP2A α protein-coding region (Fig. 2A). A perfect complementary sequence exists between miR-3661 and the *PPP2CA* gene (Fig. 2B). The Northern blot data confirmed that miR-3661 is an expressed miRNA (Fig. 2C). To examine the relative expression pattern between miR-3661 and the *PPP2CA* gene, we compared the RNA levels of miR-3661 and *PPP2CA* in different human cell lines. The results showed a moderate positive correlation with high confidence between the expression of miR-3661 and the *PPP2CA* gene (Fig. 2D,E), suggesting that the miR-3661 and *PPP2CA* genes have a correlating expression profile.

Because *PPP2CA* has another isoform, *PPP2CA-iso* (Migueleti et al. 2012), and the two forms share the same miR-3661 target sequence, we then investigated whether *PPP2CA-iso* can also be efficiently targeted by miR-3661. For this purpose, we examined the effects of miR-3661 targeting *PPP2CA* and *PPP2CA-iso* in different cell lines: MRC5SV (transformed fibroblast), Beas2B (transformed lung epithelial cells), H1299 (lung tumor cells), and A549 (lung tumor cells). The results showed that similar to *PPP2CA*, *PPP2CA-iso* is also

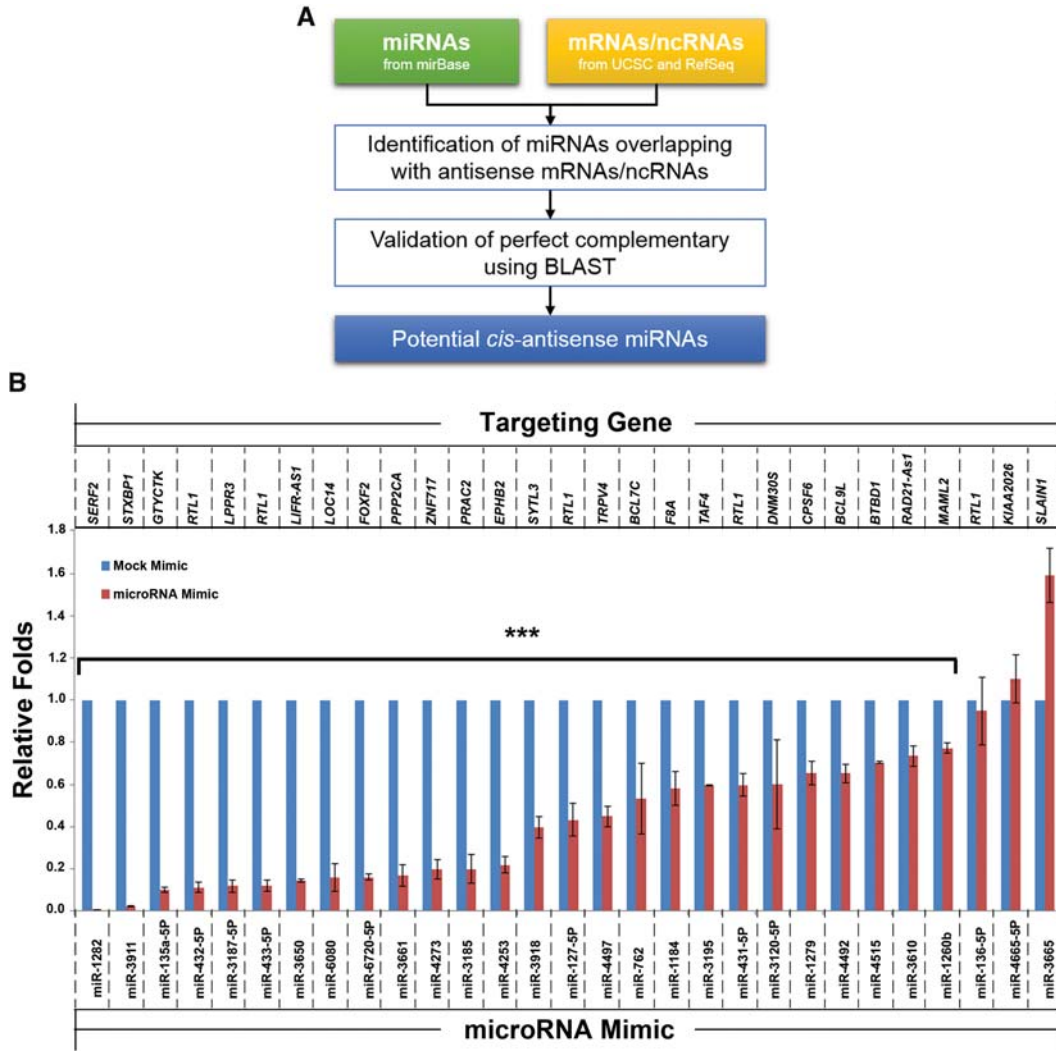


FIGURE 1. Identification of *cis*-antisense miRNAs. (A) Flowchart of genome-wide identification of potential *cis*-antisense miRNAs. (B) Most of the identified *cis*-antisense miRNAs functionally suppress their targets in human cells. RNA levels of the *cis*-targeting genes (their names are listed at the top of the figure) were measured using 293FT cells transfected with the control RNA (mock mimic) and the indicated miRNAs mimic (their names are listed at the bottom of the figure) at 30 nM final concentration. Each assay was repeated in at least three independent experiments. The statistical analysis was performed by comparing the mRNA levels of target genes from the cells treated with miRNA mimic to that treated with control RNA (mock mimic). (***) $P < 0.001$.

efficiently suppressed by miR-3661 (Fig. 3A), indicating that both *PPP2CA* and its isoform are good targets of miR-3661. In addition, the protein levels of PP2Ac α were efficiently suppressed by miR-3661 in different cell lines as well (Fig. 3B). To further study the specific effect of miR-3661 on the suppression of *PPP2CA*, we compared the effects of miR-3661 and different miRNAs (including miR-3133, miR-4282, miR-3607, miR-4703, and miR-4277) that are predicted (using miRanda [Betel et al. 2008] and TargetScan software [Lewis et al. 2005]) to have a potential for targeting 3' UTR of *PPP2CA* in 293FT cells. The results showed that only the miR-3661 mimic efficiently suppressed the PP2Ac α protein level, and all other miRNA mimics, similar to the control RNA, had no effect (Fig. 3C). Combining the transfection of the miR-3661 mimic and the miR-3661 inhibitor blocked

the suppression effect of the miR-3661 mimic alone on the PP2Ac α protein level (Fig. 3D). Next, we designed and constructed a vector encoding a Flag-tagged *PPP2CA* mutated at the targeting sequence by miR-3661 (Fig. 3E, left panel). The data showed that the mutant Flag-PP2Ac α protein level cannot be affected by the miR-3661 mimic although the wild-type Flag-PP2Ac α can be suppressed successfully (Fig. 3E, right panel). These results clearly demonstrate that the effect of the miR-3661 on suppressing the PP2Ac α protein level is specific.

Suppression of PP2Ac α by miR-3661 increased its target phosphorylation in human cells

Next, we wanted to know whether pri-miR-3661 can be processed to a mature miRNA in cells and whether the

TABLE 1. Genome-wide potential *cis*-antisense miRNAs and their antisense transcribed genes of *Homo sapiens*

No.	miRNA	Name of antisense gene	Type of antisense gene ^a	Complementary sites in the gene ^b	Chromosome
1	hsa-miR-4253	<i>EPHB2</i>	Protein coding	CDS	1
2	hsa-miR-3120-3p	<i>DNM3OS</i>	Noncoding	Exon	1
3	hsa-miR-3120-5p	<i>DNM3OS</i>	Noncoding	Exon	1
4	hsa-miR-7515	<i>MIR7515HG</i>	Noncoding	Exon	2
5	hsa-miR-7704	<i>HAGLR</i>	Noncoding	Exon	2
6	hsa-miR-135a-5p	<i>GLYCTK</i>	Protein coding/noncoding	3' UTR/exon	3
7	hsa-miR-135a-3p	<i>GLYCTK</i>	Protein coding/noncoding	3' UTR/exon	3
8	hsa-miR-4273	<i>ZNF717</i>	Protein coding	CDS	3
9	hsa-miR-3650	<i>LIFR-AS1</i>	Noncoding	Exon	5
10	hsa-miR-3661	<i>PPP2CA</i>	Protein coding	CDS	5
11	hsa-miR-6720-3p	<i>FOXF2</i>	Protein coding	CDS	6
12	hsa-miR-6720-5p	<i>FOXF2</i>	Protein coding	CDS	6
13	hsa-miR-3918	<i>SYTL3</i>	Protein coding	3' UTR	6
14	hsa-miR-196b-5p	<i>HOXA10-AS</i>	Noncoding	Exon	7
15	hsa-miR-196b-3p	<i>HOXA10-AS</i>	Noncoding	Exon	7
16	hsa-miR-3610	<i>RAD21-AS1</i>	Noncoding	Exon	8
17	hsa-miR-4665-5p	<i>KIAA2026</i>	Protein coding	5' UTR	9
18	hsa-miR-4665-3p	<i>KIAA2026</i>	Protein coding	5' UTR	9
19	hsa-miR-3911	<i>STXBP1</i>	Protein coding	Intron-3' UTR	9
20	hsa-miR-1260b	<i>MAML2</i>	Protein coding	CDS	11
21	hsa-miR-4492	<i>BCL9L</i>	Protein coding	5' UTR	11
22	hsa-miR-1279	<i>CPSF6</i>	Protein coding	3' UTR	12
23	hsa-miR-4497	<i>TRPV4</i>	Protein coding	5' UTR	12
24	hsa-miR-7106-5p	<i>CCDC42B</i>	Protein coding	3' UTR	12
25	hsa-miR-7106-3p	<i>CCDC42B</i>	Protein coding	3' UTR	12
26	hsa-miR-3665	<i>SLAIN1</i>	Protein coding	CDS	13
27	hsa-miR-1260a	<i>NGB</i>	Protein coding	3' UTR	14
28	hsa-miR-431-5p	<i>RTL1</i>	Protein coding	CDS	14
29	hsa-miR-431-3p	<i>RTL1</i>	Protein coding	CDS	14
30	hsa-miR-433-3p	<i>RTL1</i>	Protein coding	CDS	14
31	hsa-miR-433-5p	<i>RTL1</i>	Protein coding	CDS	14
32	hsa-miR-127-5p	<i>RTL1</i>	Protein coding	CDS	14
33	hsa-miR-127-3p	<i>RTL1</i>	Protein coding	CDS	14
34	hsa-miR-432-5p	<i>RTL1</i>	Protein coding	CDS	14
35	hsa-miR-432-3p	<i>RTL1</i>	Protein coding	CDS	14
36	hsa-miR-136-5p	<i>RTL1</i>	Protein coding	CDS	14
37	hsa-miR-136-3p	<i>RTL1</i>	Protein coding	CDS	14
38	hsa-miR-1282	<i>SERF2</i>	Protein coding/noncoding	CDS/exon	15
39	hsa-miR-4515	<i>BTBD1</i>	Protein coding	IGR-5' UTR	15
40	hsa-miR-762	<i>BCL7C</i>	Protein coding	5' UTR	16
41	hsa-miR-3185	<i>PRAC2</i>	Protein coding	CDS	17
42	hsa-miR-6080	<i>LOC146880</i>	Noncoding	Exon	17
43	hsa-miR-3187-3p	<i>LPPR3</i>	Protein coding	CDS	19
44	hsa-miR-3187-5p	<i>LPPR3</i>	Protein coding	CDS	19
45	hsa-miR-99b-5p	<i>SPACA6P-AS</i>	Noncoding	Intron-exon	19
46	hsa-miR-99b-3p	<i>SPACA6P-AS</i>	Noncoding	Exon	19
47	hsa-let-7e-5p	<i>SPACA6P-AS</i>	Noncoding	Exon	19
48	hsa-let-7e-3p	<i>SPACA6P-AS</i>	Noncoding	Exon	19
49	hsa-miR-125a-5p	<i>SPACA6P-AS</i>	Noncoding	Exon	19
50	hsa-miR-125a-3p	<i>SPACA6P-AS</i>	Noncoding	Exon	19
51	hsa-miR-3195	<i>TAF4</i>	Protein coding	CDS	20
52	hsa-miR-1184	<i>F8A1</i>	Protein coding	CDS	X
	hsa-miR-1184	<i>F8A2</i>	Protein coding	CDS	X
	hsa-miR-1184	<i>F8A3</i>	Protein coding	CDS	X

^aType of antisense gene includes protein-coding gene and noncoding gene. Some genes encode both protein-coding genes and noncoding genes in alternative isoforms. If a miRNA is in the opposing DNA strand of both protein-coding and noncoding isoforms, both two types will be listed.

^bComplementary sites in the gene denotes the sites in the gene that a miRNA might bind it with perfect matches. For protein-coding genes, five types of complementary sites—that is, 5' UTR, 3' UTR, CDS, Intron-3' UTR, and IGR-5' UTR—are involved. IGR stands for intergenic region. For noncoding genes, exon and intron-exon are involved. If a miRNA is in the opposing DNA strand of both protein-coding and non-coding isoforms, the complementary sites in the two isoforms will be listed correspondingly.

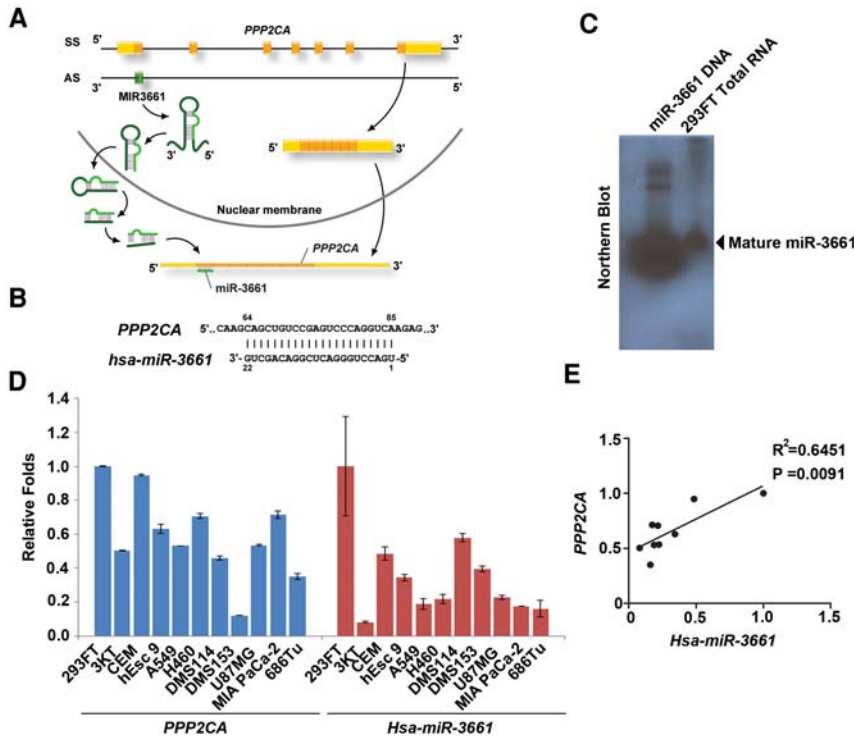


FIGURE 2. MiR-3661 is located on the opposite strand of the *PPP2CA* coding region in the human genome. (A) The genomic view of the *PPP2CA* and miR-3661 genes: coding sequence (CDS), untranslated region (UTR), and miRNA regions are represented as orange, yellow, and green bars, respectively. (AS) antisense strand, (SS) sense strand. The model for *cis*-antisense miRNA biosynthesis and function: the miRNA processing initiated with the splicing of pri-miRNA transcripts to yield pre-miRNA hairpins that are transported to cytoplasm. After Dicer cleavage, the mature miRNAs bind to target RNAs and direct the translation inhibition or cleavage of the sense transcripts of their targets. (B) The perfect complementary between *PPP2CA* and miR-3661. The binding positions are shown above (*PPP2CA*) and below (miR-3661) the sequences. (C) The mature miR-3661 was identified in 293FT cells using a Northern blot. The left lane was the synthetic DNA sequence of the miR-3661, which was as the positive control, and the right lane was the total RNA of 293FT cells. (D) RNA levels of *PPP2CA* and miR-3661 were measured from various human cell lines as indicated. (E) Scatterplots illustrating the correlation of gene expressions between *PPP2CA* and Hsa-miR-3661 from various human cell lines as indicated.

mature miR-3661 can functionally target PP2Ac. For this purpose, we first constructed the vectors encoding pri-miR-3661 (miR-3661-1 and miR-3661-2) with different lengths at the 5' and 3' flanking sequences (Fig. 4A). After transfecting the plasmid into 293FT cells, both pri-miR-3661 plasmids (miR-3661-1 and miR-3661-2) resulted in a significant increase in the mature miR-3661 levels (Fig. 4B), supporting that pri-miR-3661 can be processed well to mature miR-3661 in the cells. At the same time, both pri-miR-3661 plasmid transfections efficiently decreased the levels of *PPP2CA* mRNA and protein, and increased the phosphorylation levels of AKT or GSK-3 β (dephosphorylating substrates of PP2A) (Fig. 4C,D), supporting that pri-miR-3661 functionally targets PP2Ac. These results clearly indicate that the PP2A function is suppressed by miR-3661 to directly target *PPP2CA*.

3661 can efficiently suppress *PPP2CA*.

Among the 29 tested *cis*-antisense miRNAs, three (miR-4665-5p, miR-136-5p, and miR-3665) did not suppress their targets (Fig. 1B), which might be due to the following two reasons. One is that the three miRNAs may only inhibit the translation of the target genes, instead of triggering target degradation. The other is that the three miRNAs are all located near the 5' end of the target genes and this region may form a complex structure that hinders the interaction of these miRNAs and their target RNAs. Target site accessibility has been found to be very important for miRNA recognition and targeting effectiveness (Kertesz et al. 2007). Of course, these possibilities need to be verified in future studies. Our results, the positive data from 26 among 29 tested miRNAs, indicate that most of the *cis*-antisense miRNAs are functional. We also investigated whether there were other miRNAs that had the potential to target 3' UTR of *PPP2CA*. Using

DISCUSSION

Most miRNAs are found to suppress the expression of genes prevalently in *trans*. Some miRNAs are located within genomic regions of protein-coding genes or noncoding genes (Rodriguez et al. 2004; Hinske et al. 2010, 2014). These intragenic miRNAs were studied with their host genes and the results suggest that a complex transcription and regulation relationship exists between them (Monteys et al. 2010; Yan et al. 2011; Son et al. 2014). The subset of intragenic miRNAs, *cis*-antisense miRNAs that are transcribed antisense to exons of protein-coding or noncoding genes, is rarely studied. Although it has been reported that miR-127/136 in mouse cells is located on the opposite strand of *RTL1* (Seitz et al. 2003), there is no report until now to systematically study whether the *cis*-antisense miRNAs functionally exist in different animal species. In this study, by systematically analyzing genomes from different animal species, we identified 52 *cis*-antisense miRNAs in the human (*Homo sapiens*), 48 in the *C. elegans*, 17 in the *Drosophila*, and 36 in the mouse (*Mus musculus*) genome. Then, we performed biological studies and showed that most *cis*-antisense miRNAs can suppress their targets well in human cells. We further investigated in detail the relationship between one of our predicted *cis*-antisense miRNAs, miR-3661, and its target *PPP2CA* in human cells. Our results showed that miR-

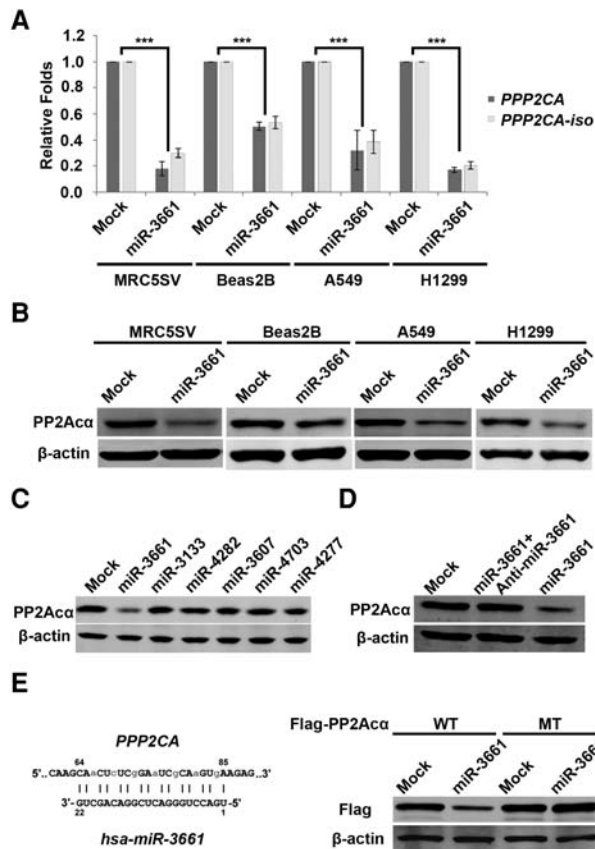


FIGURE 3. MiR-3661 specifically targets *PPP2CA* expression in human cells. (A) RNA levels of *PPP2CA* and *PPP2CA-iso* were measured from different human cell lines, MRC5SV, Beas2B, A549, and H1299, that were transfected with the control RNA (mock) or miR-3661 mimic at 30 nM final concentration. (B) The protein levels of PP2Aα were measured from MRC5SV, Beas2B, A549, and H1299 cells transfected with the control RNA (mock) or miR-3661 mimic at 30 nM final concentrations. β-actin was used as the internal loading control. (C) The protein levels of PP2Aα were measured from 293FT cells transfected with 30 nM of a control RNA or the predicted miRNAs as indicated. (D) The protein levels of PP2Aα were measured from 293FT cells transfected with the control RNA (mock), miR-3661 mimic alone or together with a miR-3661 inhibitor at 30 nM final concentration. (E) (Left panel) the top sequence is the mutant plasmid construction of *PPP2CA*, light gray; the mutated sites of the *PPP2CA* sequence targeted by Hsa-miR-3661 (bottom sequences). (Right panel) The protein levels of Flag-PP2Aα were measured from 293FT cells transfected with 30 nM of wild-type (WT) or mutant (MT) Flag-PP2Aα together with the mock or miR-3661.

miRanda (Betel et al. 2008) and TargetScan software (Lewis et al. 2005), we predicted several miRNAs that target 3' UTR of *PPP2CA*; however, none of them could efficiently suppress the *PPP2CA* expression as miR-3661 does (Fig. 3C). These results indicate that miR-3661 is the most efficient miRNA to target *PPP2CA* because of its perfect complementary to the target as compared with other 3' UTR-targeting miRNAs.

Our results indicate that *cis*-antisense miRNAs function as a natural post-transcriptional regulator for the antisense transcribed genes. Because *cis*-antisense miRNAs and their

antisense transcribed genes are on opposite strands, it is possible that they have their own transcriptional regulation. However, a correlational expression between miRNAs and their targets has been reported previously (Wang and Li 2009), although the underlying mechanism remains unclear. We also found a positive correlation between *PPP2CA* and miR-3661 ($R^2 = 0.6451$; Fig. 2E). We thought that miR-3661 and *PPP2CA* might share the same enhancers or silencers or be under the same locus control region (LCR) because of the opposite strand location. Again, the prediction needs to be verified in future studies. In addition, our data do not exclude the other regulations of PP2A for normal physiologic cellular function. When *PPP2CA* was dramatically decreased by miR-3661, cells may have compensatively up-regulated *PPP2CA* via other pathways to maintain its normal function. A moderate positive correlation ($R^2 = 0.6451$) between *PPP2CA* and miR-3661 also suggests the involvement of other regulation pathways. Thus, what we show in this study added miR-3661 as a new regulation factor to maintain the balance of *PPP2CA*, which contributes to the homeostasis of PP2A function.

In this study, we systematically investigated whether the antisense exonic miRNAs could regulate the target genes in *cis*. The results showed that the *cis*-

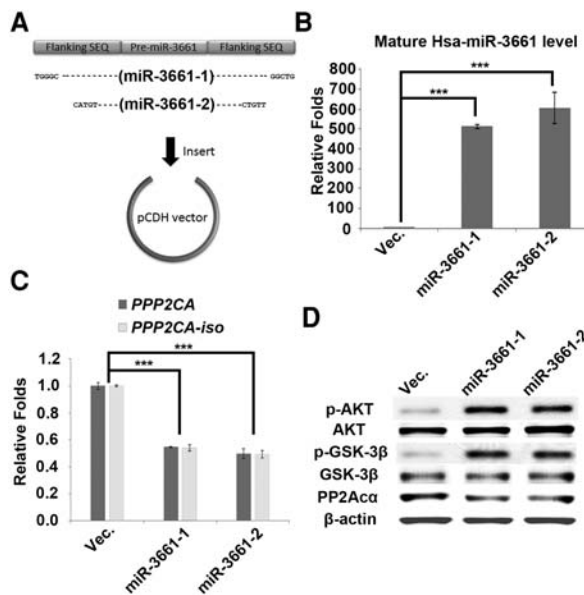


FIGURE 4. Suppression of PP2A by miR-3661 increases its target phosphorylation in human cells. (A) Schematic overview of pri-miR-3661 expression plasmids. (B) The RNA levels of Hsa-miR-3661 were measured from 293FT cells transfected with the control vector and two constructs of pri-miR-3661 expression plasmids. (C) The mRNA levels of *PPP2CA* and *PPP2CA-iso* were measured from 293FT cells transfected with the control vector and two constructs of pri-miR-3661 expression plasmids. (D) The protein levels of PP2Aα, phosphorylated AKT (p-AKT), AKT, phosphorylated GSK-3β (p-GSK-3β), and GSK-3β were measured from 293FT cells transfected with the control vector and two constructs of pri-miR-3661 expression plasmids.

antisense miRNAs effectively suppressed the target genes transcribed in the opposite strand. However, *cis*-antisense miRNAs may also have other targets regulated in *trans*. For example, hsa-miR-135a-5p is validated to suppress the expression levels of antisense transcribed gene *GLYCTK* in our study (Fig. 1B). It was also reported to regulate the expression of *JAK2*, *NR3C2*, *APC*, *HOXA10*, and *MYC* in *trans* from miRTarBase, which contains the largest amount of validated miRNA–target interactions (Supplemental Fig. 1; Kozomara and Griffiths-Jones 2014). Whether *cis*-antisense miRNAs regulate *cis*- and *trans*-targets simultaneously or separately needs further verification.

Taken together, we report for the first time that *cis*-antisense miRNAs are functional in different animal species, particularly in humans. Such conserved *cis*-antisense regulation for some miRNAs may play an important role in adjusting their targeting gene expressions.

MATERIALS AND METHODS

Genome-wide identification of potential *cis*-antisense miRNAs and their antisense transcribed genes

The sequence and genomic annotations of human miRNAs were obtained from a miRBase database release 21 (Kozomara and Griffiths-Jones 2014). The sequences of protein-coding and non-coding genes were downloaded from the RefSeq database (Pruitt et al. 2014) and the genomic annotations from the UCSC genome browser (Karolchik et al. 2014). The genomic annotations of miRNAs and RefSeq genes were both based on the human genome assembly GRCh38. By comparing the genomic loci of miRNAs and exons of RefSeq genes, all the miRNAs that overlapped with the antisense exons were obtained for sequence analysis. The identified miRNAs were further validated by aligning each miRNA to the antisense gene sequences with the parameters “-word_size 11 -perc_identity 100 -strand minus -ungapped” (Altschul et al. 1990). The miRNAs that had a perfect complementary to the antisense mRNAs/ncRNAs were obtained as potential *cis*-antisense miRNAs. The same analysis procedure was performed to identify potential *cis*-antisense miRNAs in the other three species: *M. musculus*, *D. melanogaster*, and *C. elegans*. All the analyses were performed in the R programming language (version 3.0.2).

Cell lines and culture

Human cell lines—293FT (transformed embryo kidney cells, purchased from Invitrogen), MRC5SV (transformed human fibroblasts, as described previously [Liu et al. 2015]), U87MG (brain tumor cells, purchased from ATCCs), MIA PaCa-2 (pancreas carcinoma cells, purchased from ATCC), and TU686 (oral squamous cells, obtained from Dr. Georgia Chen’s laboratory [Shin et al. 2013])—were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS). CEM cells (leukemic cells, purchased from ATCC) were grown in MEM supplemented with 10% FBS. 3KT cells (hTERT-immortalized bronchial epithelial cells, obtained from Dr. Shay’s laboratory [Ramirez et al. 2004]) were grown in keratinocyte-SFM medium (purchased from

Invitrogen). Beas2B cells (transformed human brachial epithelial cells, purchased from ATCC) were grown in Dulbecco’s Modified Eagle’s Medium/Nutrient Mixture F-12 Ham supplemented with 10% FBS. Small cell lung cancer cell lines, DMS114 and DMS153, were purchased from ATCC and grown in Waymouth’s MB 752/1 medium supplemented with 10% FBS. Non-small cell lung cancer (NSCLC) cell lines, A549, H1299, and H460, were purchased from ATCC and grown in RPMI 1640 medium supplemented with 10% FBS. hESC-H9 cells (human embryo stem cells) were purchased from WISC Stem Cell Bank and grown on BD Matrigel hESC-qualified matrix-coated plates (BD Biosciences) in mTeSR 1 medium (purchased from Stem Cell Technologies).

Northern blot

The total RNA was extracted from 293FT cells. Twenty micrograms of total RNA was separated in prepared 12% Denaturing Urea Polyacrylamide Gel. After transferring to a nylon membrane (GE Healthcare), the membrane was hybridized with a special probe, 5-CAGCTGTCCGAGTCCCAGGTCA-Dig-3, to measure the mature miR-3661. At the same time, the synthesized DNA sequence of mature miR-3661 was used as a positive control. The membrane was soaked in a solution containing the DIG antibody according to the manufacturer’s instructions (DIG Wash and Block Buffer Set; Roche). The signals were obtained by exposing the membrane to X-rays at room temperature as described previously (Hu et al. 2014).

Plasmid construction

The plasmid of encoding Hsa-miR-3661-1 and -2 was generated by inserting a PCR product from the human genomic library as a template with the primers (Forward1: 5-TGGGAATTCTGGGCG CCGGTCTCGGAGACTC-3; Reverse1: 5-TGCGG ATCCCAGCC TCAACAGCCGCCAGAAG-3; Forward2: 5-TGGGAATTCCATG TTGCAC CCTCCTCAC-3; and Reverse2: 5-TGCGGATCCAACA GCCGCCAGAAGTACAC-3) into the plasmid pCDH-CMV-MCS-EF1-Puro (SBI). The plasmid encoding Flag-human *PPP2CA* was generated by inserting a PCR product from a human cDNA library as the template with the primers (Forward: 5-TGAGAATTCAA TGGACGAGAAGGTGTTC-3 and Reverse: 5-GTG GGATCCTTA CAGGAAGTAGTCTGG-3) into the plasmid p3XFLAG-CMV (Sigma-Aldrich). Three rounds of PCR-mediated mutagenesis were done on p3XFlag-PPP2CA to generate Hsa-miR-3661 resistant *PPP2CA* expression plasmid with the primers (Forward: 5-CAaCTcTCgGAaTCgCAaGTgAAGAGCCTCTGCG AGAAG-3 and Reverse: 5-TCACTTGCgATTCCGAGAGTTGCTTGCCTCGTT CAGC-3), which is completely resistant to Hsa-miR-3661.

RNA isolation, RT-PCR, and real-time PCR

Total RNA was extracted from human cells using miRNeasy mini kits (QIAGEN). cDNA was synthesized using 500 ng of total RNA from each sample using the SuperScript VILO cDNA Synthesis Kit (Life Technologies), followed by triplicate qPCR reactions using TaqMan assays with the TaqMan Fast PCR Universal master mix on an Applied Biosystems 7500 Fast real-time PCR system. For miRNA, a TaqMan miRNA reverse transcription kit was used to prepare the products for the TaqMan miRNA assays as described previously (Liu et al. 2015). RNA levels of *PPP2CA* and its isoform were measured

using primers designed to detect either the standard (Forward1: 5-GAT CTT CTG TCT ACA TGG TGG TCT C-3 and Reverse1: 5-ACA CAT TGG ACC CTC ATG GGG AA-3) or the novel isoform (Forward1 and Reverse2: 5-CCA GTT ATA TCC CTC ATG GGG AAC-3). GAPDH was used as an internal control (forward primer: 5-TGC ACC ACC AAC TGC TTA GC-3; reverse primer: 5-GGC ATG GAC TGT GGT CAT GAG-3).

Western blot

Western blot was performed using standard techniques. The antibodies against human PP2A α and β -actin were purchased from Santa Cruz Biotechnology Inc. The antibodies against human phosphorylated AKT (Thr308), AKT, phosphorylated GSK-3 β (Ser9), and GSK-3 β were purchased from Cell Signaling Technology Inc.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank Dr. Georgia Chen for the cell lines and Ms. Doreen Theune for editing this manuscript. X.Y. acknowledges support from the China National High Technology Research and Development Program (2014AA020604), and Y.W. acknowledges support from the U.S. National Institutes of Health (CA186129, CA185882, and P30CA138292).

Received June 17, 2015; accepted October 3, 2015.

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