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RESEARCH PAPER

The origin of exosomal miR-1246 in human cancer cells

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ABSTRACT

miR-1246 is considered an oncomiR in various cancer types. However, the origin and biogenesis of miR-1246 remain controversial which often leads to misinterpretation of its detection and biological function, and inevitably masking its mechanisms of action. Using next generation small RNA sequencing, CRISPR-Cas9 knockout, siRNA knockdown and the poly-A tailing SYBR qRT-PCR, we examined the biogenesis of exosomal miR-1246 in human cancer cell model systems. We found that miR-1246 is highly enriched in exosomes derived from human cancer cells and that it originates from RNU2-1, a small nuclear RNA and essential component of the U2 complex of the spliceosome. Knockdown of Drosha and Dicer did not reduce exosomal miR-1246 levels, indicating that exosomal miR-1246 is generated in a Drosha- and Dicer-independent manner. Direct digestion of cellular lysate by RNase A and knockdown of the RNU2-1 binding protein SmB/B′ demonstrated that exosomal miR-1246 is a RNU2-1 degradation product. Furthermore, the GCAG motif present in the RUN2-1 transcript was shown to mediate miR-1246 enrichment in cancer exosomes. We conclude that exosome miR-1246 is derived from RNU2-1 degradation through a non-canonical microRNA biogenesis process. These findings reveal the origin of an oncomiR in human cancer cells, providing guidance in understanding miR-1246 detection and biological function.

Abbreviations: CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; miRNA, microRNA; PDAC, pancreatic ductal adenocarcinoma; RNU2-1, U2 small nuclear RNA; RT-PCR, Reverse transcription polymerase chain reaction; sgRNA, single-guide RNA.

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Exosome; microRNA; RNU2-1; biogenesis; cancer

Introduction

miR-1246 is a microRNA (miRNA) that was identified in 2008 by sequencing of small RNAs in human embryonic stem cells [1]. The gene encoding miR-1246, *MIR1246*, was mapped to human chromosome *2q31.1* and its expression was found to be regulated by p53 [2]. Several miR-1246 target transcripts have since then been experimentally identified, such as SLC12A2 [3], RTKN2 [4], CCNG2 [5] and DENND2D [6]. In the context of cancer, miR-1246 was found to act as an oncomiR to promote tumor angiogenesis [7], growth and metastasis [8], migration and invasion [9], and cancer stemness in various malignancies [10–12], including pancreatic ductal adenocarcinoma (PDAC) [5]. We recently reported that miR-1246 is highly enriched in PDAC exosomes and that plasma exosome miR-1246, along with miR-196a, is elevated in patients with localized PDAC [13]. These previous findings indicate the biological significance of miR-1246 in human cancer, and the need for a better understanding of this miRNA species in cancer biology.

Intriguingly, the mature miR-1246 sequence fully overlaps with the central region of the RNU2-1 transcript, a small nuclear RNA serving as a scaffold for the U2 complex in the spliceosome [14]. This RNU2-1 fragment was detected in patient plasmas and described as a potential diagnostic biomarker for pancreatic and colorectal adenocarcinoma [15]. However, the concept of miR-1246 being derived from RNU2-1 has not been widely accepted and recent reports continue to reveal new insight of miR-1246 biology in various model systems without distinguishing miR-1246 and RNU2-1 detection and function [6,12,16–20]. This controversial scenario is primarily due to two reasons: first, there has been no decisive evidence to show that cellular miR-1246 is derived from RNU2-1. No knockdown or knockout experiments have been performed on either RNU2-1 or miR-1246 to prove that miR-1246 is derived from RNU2-1. Second, the methods used for miRNA detection have compounded this issue. Current miRNA detection mainly relies on two qRT-PCR strategies: the stem-loop TaqMan method and the poly-A tailing SYBR method [21–23] (Figure 1). To our knowledge, most recent studies on miR-1246 detection applied the stem-loop TaqMan method, which would theoretically detect both miR-1246 and RNU2-1 with the same primer set as depicted in Figure 1. On the other hand, the poly-A tailing SYBR method, by design, could differentiate miR-1246 and RNU2-1 based on the length of the detected nucleotide fragments indicated by their meting curves.

Considering the fact that both miR-1246 [5,7,9–12,24] and RNU2-1 [25–27] are significant small RNA molecules in cancer biology, we sought to clarify the issue by applying the

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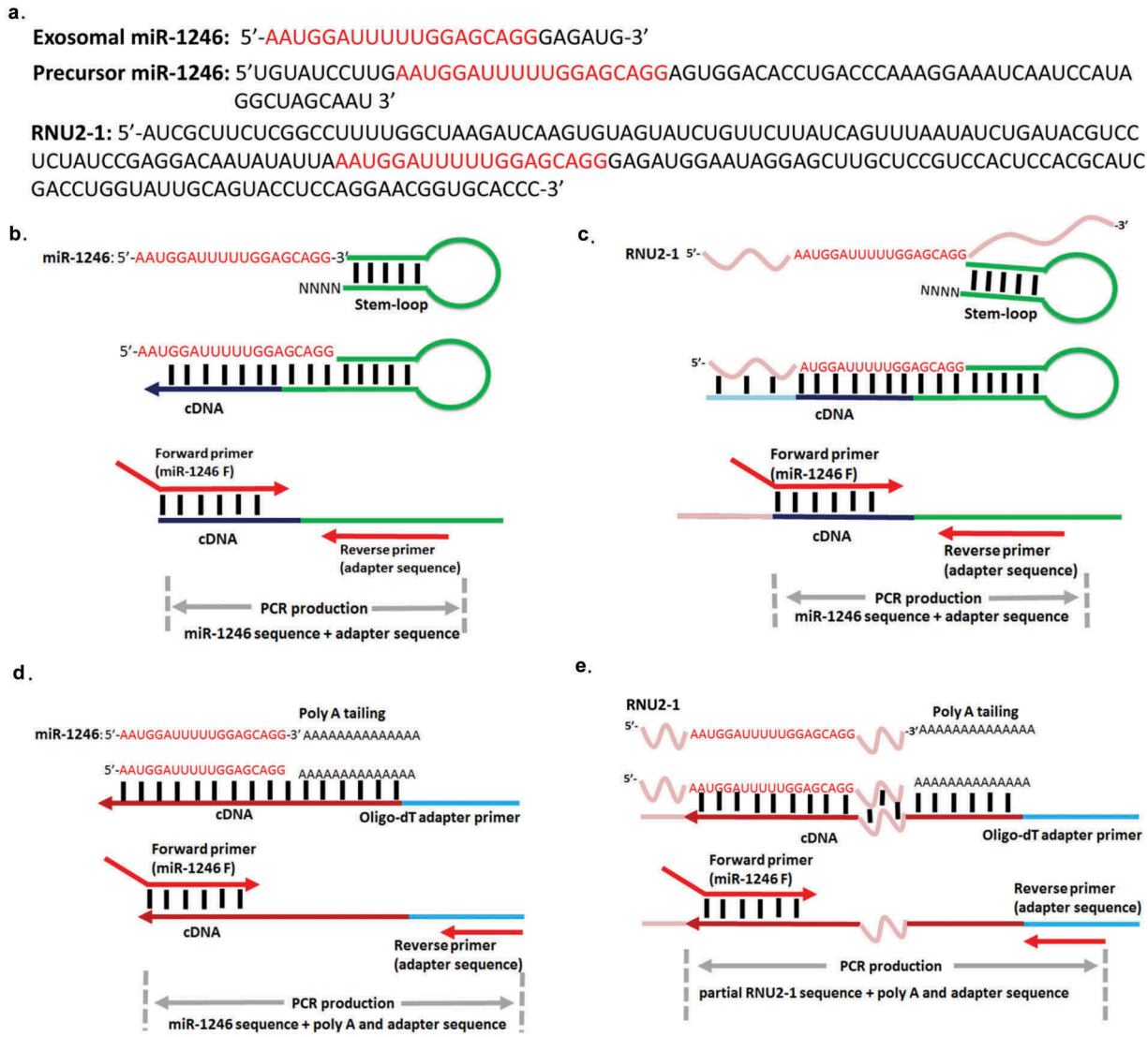


Figure 1. The sequence overlap of the precursor miR-1246, RNU2-1 and exosomal miR-1246, and comparison of two qRT-PCR detection strategies. (a). Exosomal miR-1246, precursor miR-1246, and RNU2-1 sequences. Mature miR-1246 as registered in miRBase (miRBase.org) is red colored. Schematic illustration of miR-1246 and RNU2-1 detection using the stem-loop qRT-PCR method (b and c). This method cannot qualitatively differentiate the detection of miR-1246 and RNU2-1. Schematic illustration of miR-1246 and RNU2-1 detection using the poly A tailing qRT-PCR method (d and e). This method can differentiate the detection of miR-1246 and RNU2-1.

poly-A tailing SYBR method to analyze miR-1246 expression in wild type and *MIR*1246 knockout PDAC cells and their exosomes. We found that a variant of the mature miR-1246 is detected in PDAC cell-derived exosomes (Figure 1(a)) that is originated from cellular RNU2-1 in a Drosha- and Dicer-independent manner. Our results provide guidance on understanding of miR-1246 detection and biological function in human cancer cells.

Results

miR-1246 is highly enriched in exosomes derived from PANC-1 cells

Next generation small RNA sequencing was performed using RNA from PANC-1 cells and exosomes, as we described [13]. We found that a great number of reads mapped to miR-1246, but frequently contained additional nucleotides at the 3′ end. The addition of GAGA to the miR-1246 sequence read would indicate that the origin of the sequence was from RNU2-1, while the addition of AGTG would indicate that the origin of the sequence was from the pre-miR-1246 (Figure 1(a)). Majority of the miR-1246 sequences detected in both PANC-1 cells and PANC-1 exosomes contained the GAGA, and none of them contain AGTG, indicating that the miR-1246 sequence detected is derived from RNU2-1 and not from the precursor miR-1246 (Table 1). qRT-PCR using the poly-A tailing SYBR method was then used to amplify miR-1246 from cellular and exosome RNA of PANC-1, MIA-PaCa-2, and hTERT-HPNE cells (Figure 2((a and b)). Melting curve analysis showed that, with the same primer set, the miR-1246 amplicon from cellular RNA melted at a higher temperature when compared to the miR-1246 amplicon from exosome RNA in all cell lines analyzed (Figure 2(a and b)), indicating that different fragments are amplified in cellular RNA versus exosome RNA by the qRT-PCR technology. The amplified fragments were then ligated into a cloning vector and

Table 1. Analysis of the detected miR-1246 sequences in PANC-1 cells and exosomes. miR-1246 sequence+GAGA resembles the sequence of RNU2-1, while the miR-1246 sequence+AGTG resembles the sequence of precursor miR-1246.

Raw sequence from NGS	Percentage of miR-1246-like sequences detected
miR-1246 sequence+GAGA in PANC-1 cells	75.4
miR-1246 sequence+GAGA in PANC-1 exosomes	64.6
miR-1246 sequence+AGTG in PANC-1 cells	0
miR-1246 sequence+AGTG in PANC-1 exosomes	0

sequenced. The sequencing results, from three individual clones, confirmed the amplification of an RNU2-1 fragment from cellular RNA and that of a miR-1246 variant sequence from exosomal RNA (Figure 2(c)). The results thus show that the miR-1246 variant sequences are enriched in PANC-1 exosomes but undetectable in cellular RNA preparations by the poly-A tailing SYBR qRT-PCR method. Instead the same primers amplified RNU2-1 from cellular RNA. As a control, when miR-1246 mimics were pre-mixed with the PANC-1 cell lysate, the miR-1246 sequence was detected by the poly-A tailing SYBR qRT-PCR (Supplement Figure 1). We also performed qRT-PCR using the stem-loop TaqMan method, which shows no difference between the amplicons from cellular and exosome RNAs (supplement Figure 2). Sequencing of the cellular amplicon by this method confirmed that the stem-loop TaqMan method cannot distinguish between miR-1246 and RNU2-1 sequence detection (data not shown). To determine whether the detected exosomal miR-1246 variant sequence is functional, a miR-1246 reporter gene plasmid was constructed and introduced to PANC-1 cells. As shown in Figure 2(d), mimics of both mature miR-1246 and the miR-1246 variant significantly suppressed luciferase activity in our model system, indicating that the highly enriched exosomal miR-1246 variant sequence is biologically active. We also performed northern blot analysis using cellular RNAs from different human cancer cell lines (Figure 2(e)). It turned out that in all cell lines tested, there was no miR-1246 being detected. Instead, a prominent band was detected at the size of RNU2-1.

Exosome miR-1246 is derived from RNU2-1

The exosome miR-1246 qRT-PCR amplicon sequence contains six extra nucleotides that match to the RNU2-1 sequence (Figure 2(c)), supporting the notion that it originates from RNU2-1. To definitively clarify the origin of exosomal miR-1246 in our model system, a knockout or knockdown approach was applied. First, we used the CRISPR-Cas9 HDR method [28], as shown in Figure 3(a), to replace the precursor sequence of miR-1246 on 2q31.1 with GFP in PANC-1 cells. GFP-positive genetically engineered cells were sorted by flow cytometry; and verified by fluorescence microscopy (Figure 3(b)). Homozygous replacement of the precursor miR-1246 sequence by the GFP-HDR fragment was verified by PCR on cellular DNA using miR-1246-up-F and miR-1246-down-R, covering upstream and downstream sequences of the precursor miR-1246 (Figure 3(c)). As shown in Figure 3(c), only the GFP-HDR fragment, and not the precursor miR-1246 sequence, was detected in the *MIR1246*KO cells. This was further confirmed by DNA sequencing of the PCR product (Supplement-sequence). Knockout of *MIR1246* significantly slowed down cell growth, as evidenced by the MTS assay (Supplement Figure 3). However, knockout of *MIR1246* did not alter PANC-1 exosomal miR-1246 levels (Figure 3(d)) as detected by poly-A tailing SYBR qRT-PCR, strongly indicating that exosomal miR-1246 is not derived from the precursor miR-1246. Stem-loop TaqMan qRT-PCR analysis of miR-1246 expression in exosomes derived from wild type and *MIR1246*KO PANC-1 cells confirmed that no differences of exosomal miR-1246 expression were detected between the two groups (Supplemental Figure 4). Next, since RNU2-1 is a component of the U2 complex in the spliceosomes and regarded as an essential small nuclear RNA in eukaryotic cells [29], we performed siRNA knockdown of RNU2-1 in PANC-1 cells. Transfection of PANC-1 cells with siRNAs targeting RNU2-1 (Supplement Table 1) did not reduce cellular RNU2-1 levels (Figure 4(a)), however a dramatic increase in exosomal miR-1246 expression was observed (Figure 4(b)), suggesting that exosomal miR-1246 is originated from RNU2-1 degradation. It is known that mature U2 snRNA is abundant and stable in eukaryotic cells, and that its levels often do not reflect rates of production [30,31]. The inability of the siRNA to significantly reduce cellular RNU2-1 levels is consistent with previous reports showing that RNU2-1 levels cannot be altered by siRNA likely due to its essentiality to cell viability and its well-balanced rate of turnover and production [29,32]. It is conceivable that RNU2-1 is being rapidly produced to offset siRNA-induced degradation, therefore cellular RNU2-1 level remains unchanged yet exosomal miR-1246 expression is enhanced due to siRNA-induced RNU2-1 degradation. Taken together, these results indicate that exosomal miR-1246 is derived from RNU2-1.

We also applied a forced expression approach to address the origin of exosomal miR-1246 in PANC-1 cells. The precursor miR-1246 was cloned into the pcDNA3.1 vector and transfected to PANC-1 cells. Interestingly, the precursor miR-1246 expression was only detected in the transfected PANC-1 cells and not in wild type PANC-1 cells (Figure 5(a)). In addition, no mature miR-1246 sequence was detected in the precursor miR-1246 transfected PANC-1 cells (Figure 5(b)). No significant difference of miR-1246 expression was detected in exosomes derived from the precursor miR-1246 transfected PANC-1 cells versus those from the empty vector transfected PANC-1 cells (Figure 5(c)), suggesting that the precursor miR-1246 is not processed to generate the mature miR-1246 in PANC-1 cells. In contrast, when RNU2-1 was cloned into pcDNA3.1 vector and transfected to PANC-1 cells, miR-1246 expression was dramatically increased in exosomes derived from the RNU2-1 transfected PANC-1 cells compared to those derived from the empty vector transfected PANC-1 cells (Figure 5(e)), further indicating that exosomal miR-1246 is derived from RNU2-1. Consistent with the siRNA knockdown experiment and previous reports [29–32], we did not

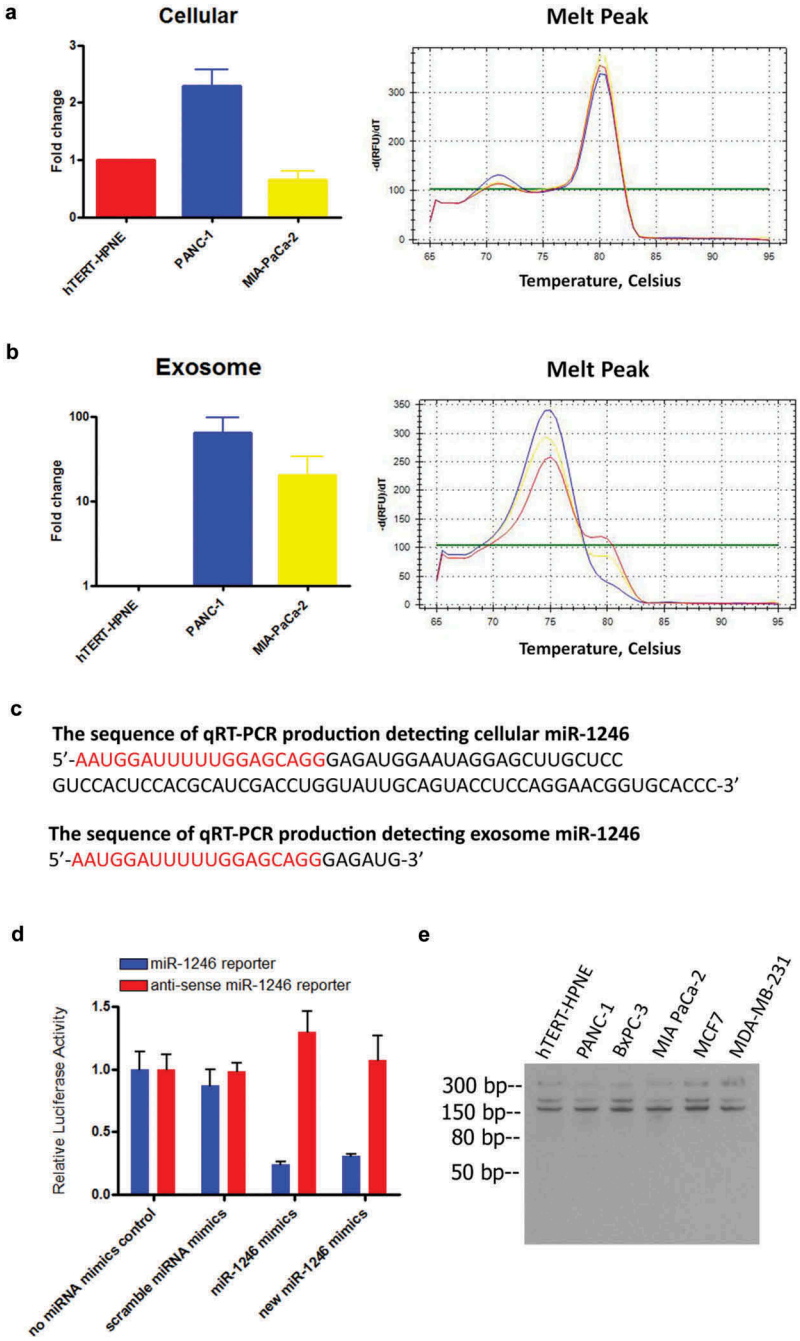


Figure 2. miR-1246-like sequences are highly enriched in PANC-1 exosomes. (a and b), qRT-PCR analysis using the poly A tailing method on miR-1246 expression in hTERT-HPNE, PANC-1 and MIA-PaCa-2 cells and their exosomes (means ± SEM, n = 3). The melting curves showed detection of different fragments in cells versus exosomes with the same set of primers. (c), Representative sequencing results from three individual clones of the detected exosomal miR-1246 and cellular RUN2-1. (d), miR-1246 target gene reporter assay. Mimics of mature miR-1246 and the exosomal miR-1246 variant suppress reporter gene activity as compared to scramble mimics (means ± SEM, n = 3). * $p < 0.05$, one-way ANOVA, followed by Dunnett analysis. (e), Northern blot analysis using a labeled miR-1246 probe against RNAs from several human cancer cell lines. There was no precursor miR-1246 and mature miR-1246 being detected and the primary band migrating at a size of RNU2-1 in all cell lines examined.

detect the increase in cellular RNU2-1 levels under the forced expression (Figure 5(d)).

Exosomal miR-1246 is generated through a non-canonical miRNA biogenesis process

It is well-established that the majority of human mature miRNAs are processed through the canonical miRNA biogenesis pathway, involving processing of the preliminary miRNA transcripts to the precursor miRNAs in the nucleus by Drosha and further processing of the precursor miRNAs to mature miRNAs by Dicer in the cytoplasm [33]. To determine whether exosomal miR-1246 is processed through the canonical miRNA biogenesis pathway, we knocked down expression of Drosha and Dicer in PANC-1 cells using shRNA technology. Transfection of the shRNA constructs to PANC-1 cells significantly reduced Drosha

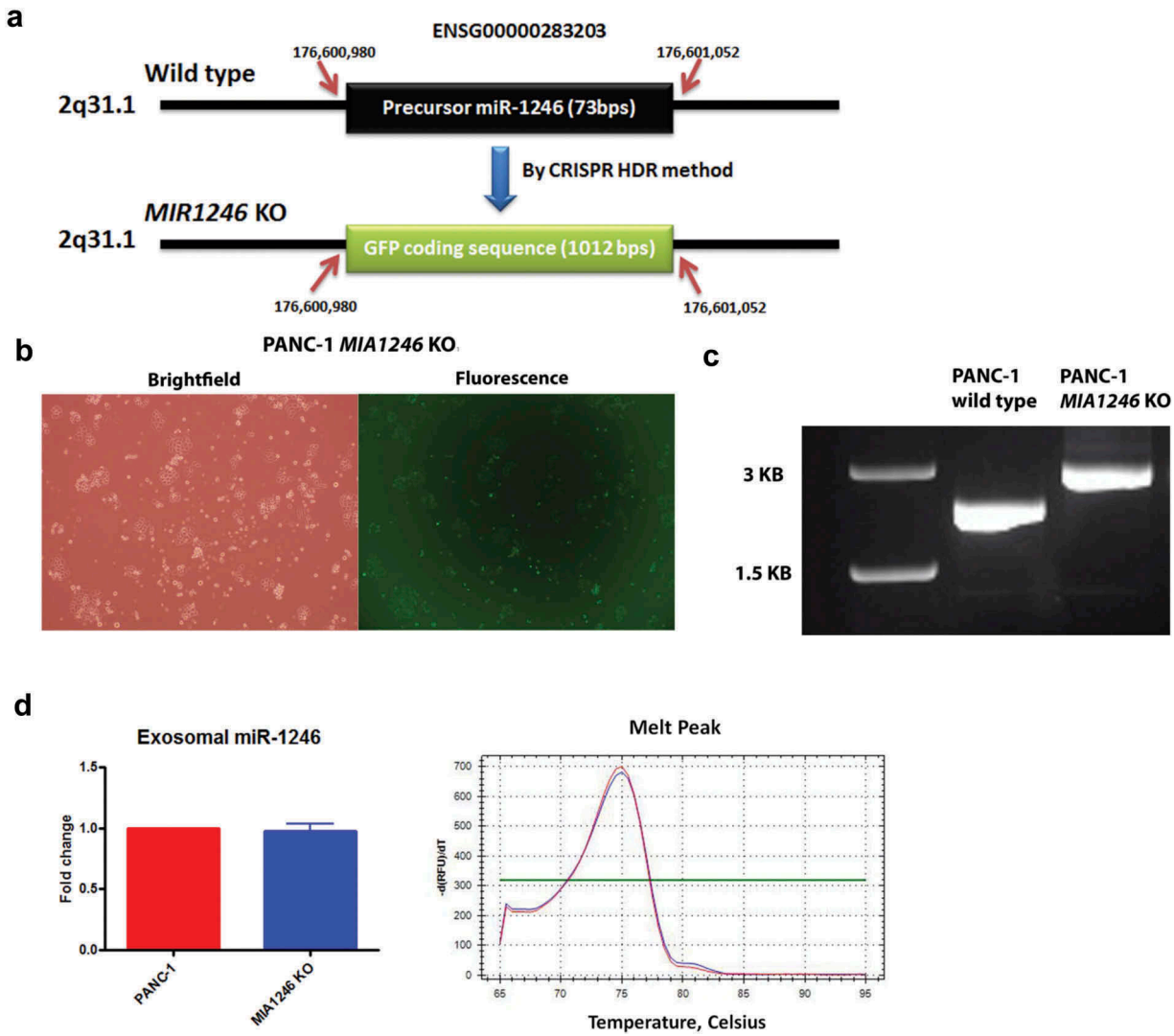


Figure 3. Effects of the *MIR1246* gene knockout on exosomal miR-1246 expression. (a), The strategy of CRISPR-Cas9 HDR knockout of the *MIR1246* gene. Ensembl gene ID and the precise sequence location are indicated. (b), GFP detection of the homozygous *MIR1246* knockout PANC-1 cells by fluorescence microscopy. (c), Agarose gel electrophoresis of the PCR products of DNA from wild type PANC-1 and the *MIR1246* knockout PANC-1 cells, using primers covering the DNA regions upstream and downstream of the *MIR1246* gene. (d), qRT-PCR analysis using the poly A tailing method detecting miR-1246 in exosomes derived from wild type and *MIR1246* knockout PANC-1 cells (means ± SEM, n = 3).

and Dicer expression as evidenced by western blot analysis (Figure 6(a)). Knockdown of Drosha or Dicer slightly reduced cellular RNU2-1 levels (Figure 6(b)). However, the knockdowns did not reduce exosomal miR-1246 expression; and in fact, knockdown of Drosha even increased exosome miR-1246 levels (Figure 6(c)), suggesting that exosome miR-1246 is not generated through the canonical miRNA biogenesis pathway, and a non-canonical miRNA biogenesis process, likely a RNU2-1 degradation pathway, is involved in this process. In a control experiment, knockdown of Drosha or Dicer significantly reduced miR-21 expression (Supplement Figure 5).

Exosomal miR-1246 is generated through RNU2-1 transcript degradation

To determine whether exosomal miR-1246 is generated from RNU2-1 transcript degradation, RNase A (12.5ug/mL, 50ug/mL or 250ug/mL) was incubated with PANC-1 cell lysate for 3 min, and the digestion was stopped by adding TRIzol reagent for RNA isolation. The Bioanalyzer analysis confirmed that RNase A treatment effectively degrades total cellular RNA (Figure 7(a and b)), and qRT-PCR analysis showed that miR-1246 is undetectable in control lysate but detected in RNase A-treated lysate in a concentration-dependent manner (Figure 7(c)). These observations strongly indicated that exosomal miR-1246 is derived from RNU2-1 transcript degradation. To further understand how the miR1246 sequence fragment was preserved during RNU2-1 degradation, we knocked down the expression of SmB/B', a protein known to bind to the miR-1246 fragment in the RNU2-1 transcript [34], in PANC-1 cells. As shown in Figure 7(d and e), knockdown of SmB/B' expression dramatically reduced exosomal miR-1246 levels (Figure 7(d and e)), indicating that the SmB/B' protein binding protects the miR-1246 sequence during RNU2-1 degradation.

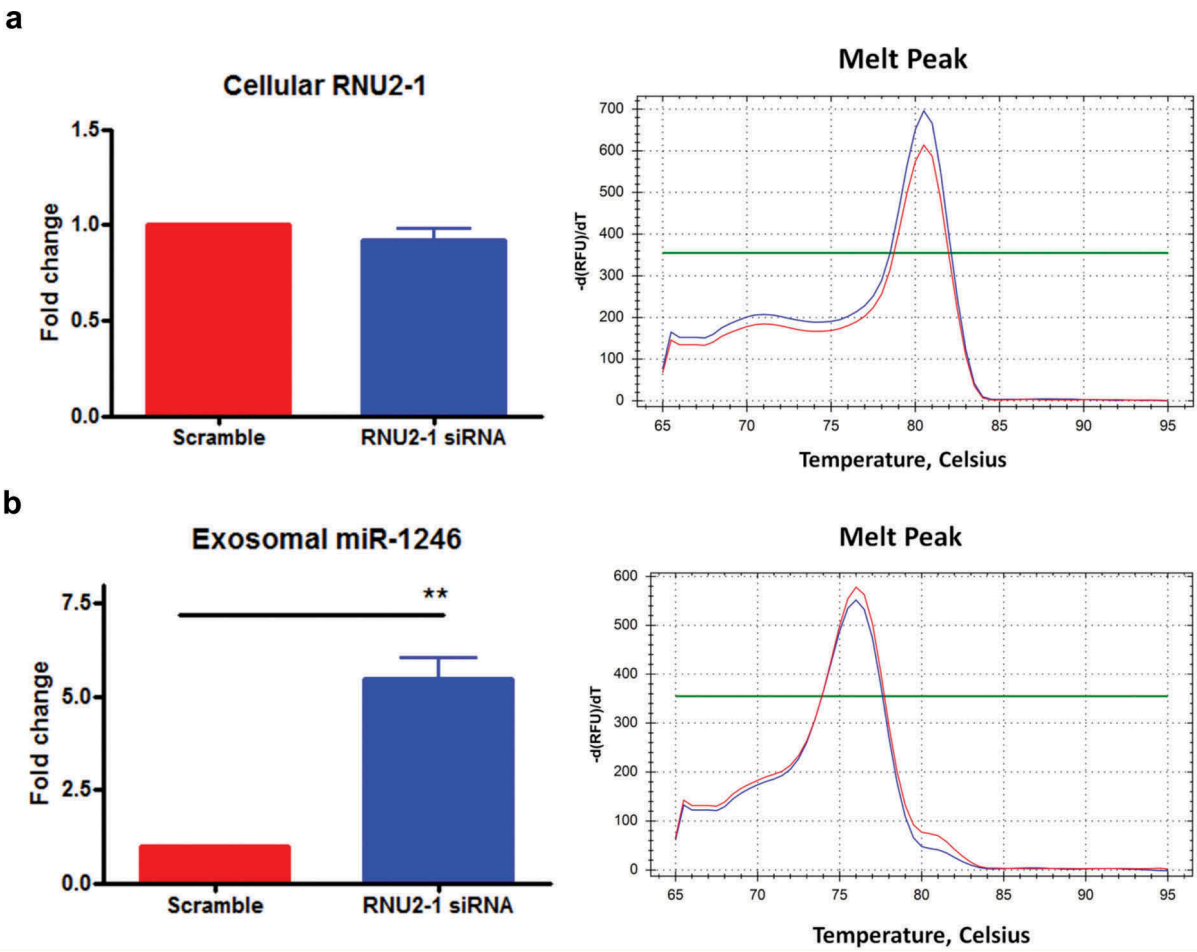


Figure 4. Effects of RNU2-1 knockdown on exosomal miR-1246 expression. (a), qRT-PCR analysis of RNU2-1 expression in scramble siRNA or RNU2-1 siRNA transfected PANC-1 cells. (b), qRT-PCR analysis of miR-1246 expression in exosomes derived from scramble siRNA or RNU2-1 siRNA transfected PANC-1 cells (means ± SEM, n = 3). ** $p < 0.01$, Student t-test, performed using ΔCT values.

The GCAG motif mediates miR-1246 enrichment in PANC-1 exosomes

Both RNU2-1 and RNU2-2 are expressed in eukaryotic cells [35,36]. The RNU2-2 sequence is highly similar to that of RNU2-1, with a major difference in the 5′ primary end of the transcripts. With regard to the miR-1246 fragment, the two snRNAs differ in 4 nucleotides at the 3′ end of the miR-1246 fragment of RNU2-1 (GCAG in RNU2-1 and AAUA in RNU2-2, Figure 8(a)). However, next generation sequencing analysis showed that only the miR-1246 fragment generated from RNU2-1 is highly enriched in PANC-1 exosomes (Figure 8(b)). We therefore hypothesized that the GCAG motif in RNU2-1 mediates exosomal miR-1246 enrichment in PANC-1 cells. To test this hypothesis, several mutants were generated using the pcDNA3.1-RNU2-1 construct and transfected to PANC-1 cells (Figure 8(c)). The qRT-PCR analysis of the exosomes isolated from the transfected cells showed that miR-1246 expression in exosomes derived from the GCAG to AATA mutant-transfected cells was significantly lower than that in exosomes derived from RNU2-1 wild type and other mutants transfected PANC-1 cells (Figure 8(c)), demonstrating that the GCAG motif mediates exosomal miR-1246 enrichment in PANC-1 cells.

Discussion

The origin and biogenesis of miR-1246 in eukaryotic cells remains controversial [15,37]. Using the human PANC-1 cell line as a model system, the present study provided evidence demonstrating that exosomal miR-1246 is derived from RNU2-1. The same was true in other human cells, including MIA-PaCa-2, and hTERT-HPNE lines. We further revealed that exosomal miR-1246 is processed via RUN2-1 transcript degradation in a Drosha- and Dicer-independent manner, and that the GCAG motif present in the miR-1246 fragment of the RNU2-1 transcript mediates exosomal miR-1246 enrichment in PANC-1 cells.

The evidence supporting our conclusion that exosomal miR-1246 originates from RNU2-1 includes the following: first, our small RNA next generation sequencing analysis of a cDNA library made from PANC-1 cells and their exosomes indicates that the majority of the miR-1246 reads detected are identical to fragments of the RNU2-1 transcript (Table 1), but not the precursor miR-1246 transcript, similar to a previous report [15]. The detection of various miR-1246 variant sequences in cells and exosomes suggest that miR-1246 is likely generated during RNU2-1 degradation. Second, the qRT-PCR analysis using the poly-A tailing SYBR method detected different fragments in cellular RNA versus exosomal

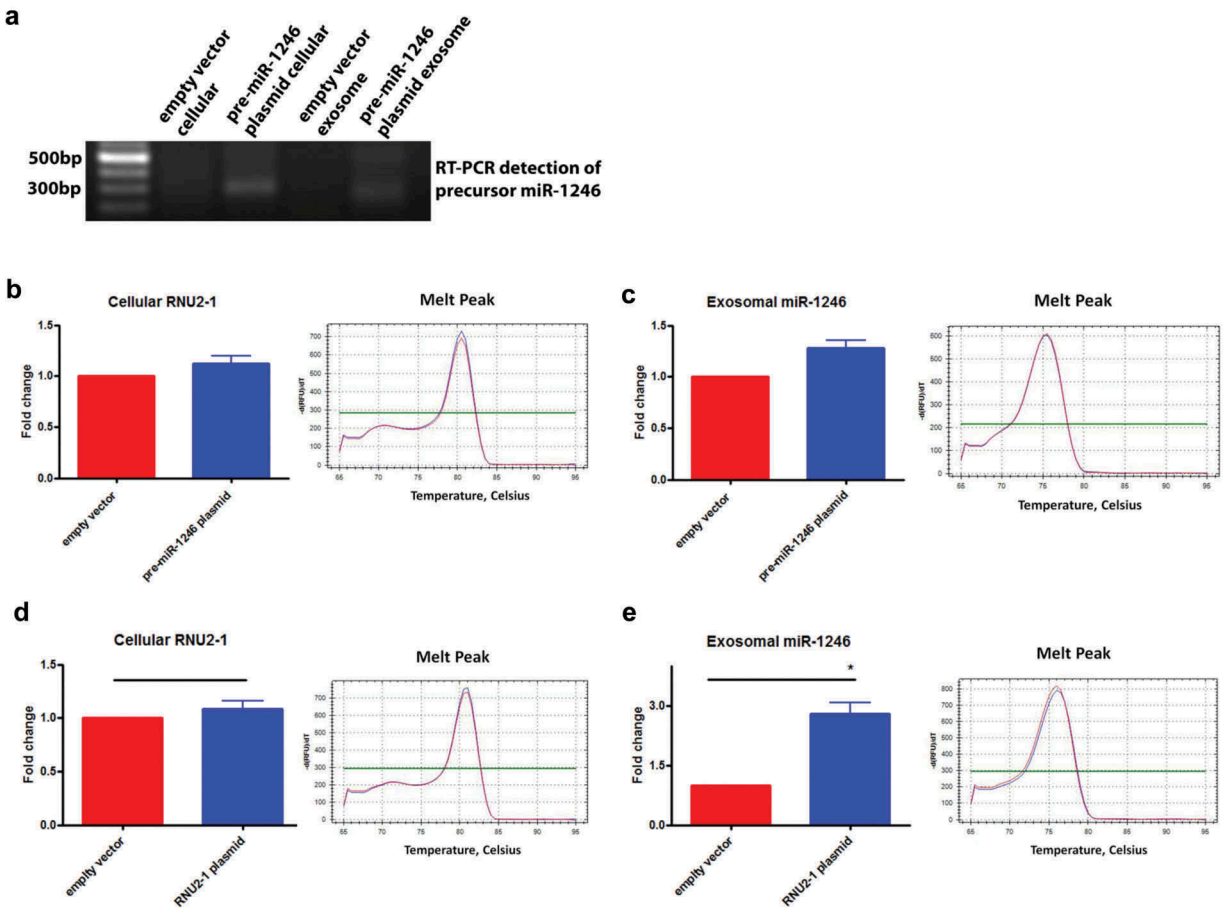


Figure 5. Effects of forced expression of the precursor miR-1246 or RNU2-1 on exosomal miR-1246 expression. (a), Agarose gel electrophoresis of the RT-PCR products detecting the precursor miR-1246 in PANC-1 exosomes and cells transfected with the precursor miR-1246 expression plasmid. (b), qRT-PCR detection of RNU2-1 in wild type and the precursor miR-1246 expression plasmid transfected PANC-1 cells. (c), qRT-PCR detection of miR-1246 in exosomes derived from wild type and the precursor miR-1246 expression plasmid transfected PANC-1 cells. (d), qRT-PCR detection of RNU2-1 in wild type and the RNU2-1 expression plasmid transfected PANC-1 cells. **e**, qRT-PCR detection of miR-1246 in exosomes derived from wild type and RNU2-1 expression plasmid transfected PANC-1 cells (n = 3, means ± SEM, for b. c, d, and e). * $p < 0.05$, Student t-test, performed using ΔCT values.

RNA, and sequencing of the fragments confirmed that the RNU2-1 sequence is detected in PANC-1 cells and a variant of miR-1246 resembling a RNU2-1 fragment was detected in exosomes. The inability to detect cellular miR-1246-like sequences by the poly-A tailing SYBR qRT-PCR is likely due to a very low copy number of this sequence in the cells or that the miR-1246-like sequences are rapidly exported via exosome secretion. Our northern blot analysis indicated that there is no mature miR-1246 being detected in several human cancer cell lines, supporting our observations from the poly A+ tailing SYBR qRT-PCR analysis. The miR-1246-like sequences are highly enriched in exosomes compared to its cellular content in other types of cancer cells as well [38]. Third, the most convincing evidence to demonstrate the origin of exosome miR-1246 was obtained from the *MIR1246* knockout cells. Knockout of the precursor miR-1246 sequence did not alter exosomal miR-1246 levels, assayed by both the poly-A tailing SYBR qRT-PCR and the Stem-loop TaqMan qRT-PCR, clearly showing that exosomal miR-1246 is not derived from the precursor miR-1246. In contrast, transfection of siRNAs targeting RNU2-1 to PANC-1 cells led to a significant increase in exosomal miR-1246 expression, indicating that the degradation of RNU2-1 produces miR-1246-like sequences that are rapidly encapsulated by and secreted through exosomes. This was further supported by the observations that direct digestion of PANC-1 lysate with RNase A led to a concentration-dependent production of miR-1246 and that knockdown of the RNU2-1 binding protein SmB/B' dramatically reduced exosomal miR-1246 levels in PANC-1 cells. The specific endogenous nuclease that degrades RNU2-1 transcript merits further investigation.

Our findings support the concept that cellular miR-1246 is not processed from the precursor miR-1246, and is rarely detectable in cellular RNAs. Forced expression of the precursor miR-1246 in PANC-1 cells led to the detection of the precursor transcript, but not the mature miR-1246, indicating a lack of a cellular mechanism to process the precursor miR-1246 into the mature form. Moreover, we were unable to detect the precursor miR-1246 in wild type PANC-1 cells and their exosomes by next generation sequencing or by qRT-PCR using the poly-A tailing SYBR method, bringing into question whether the miR-1246 gene is transcribed and its transcript processed. These findings are significant as new studies have demonstrated the

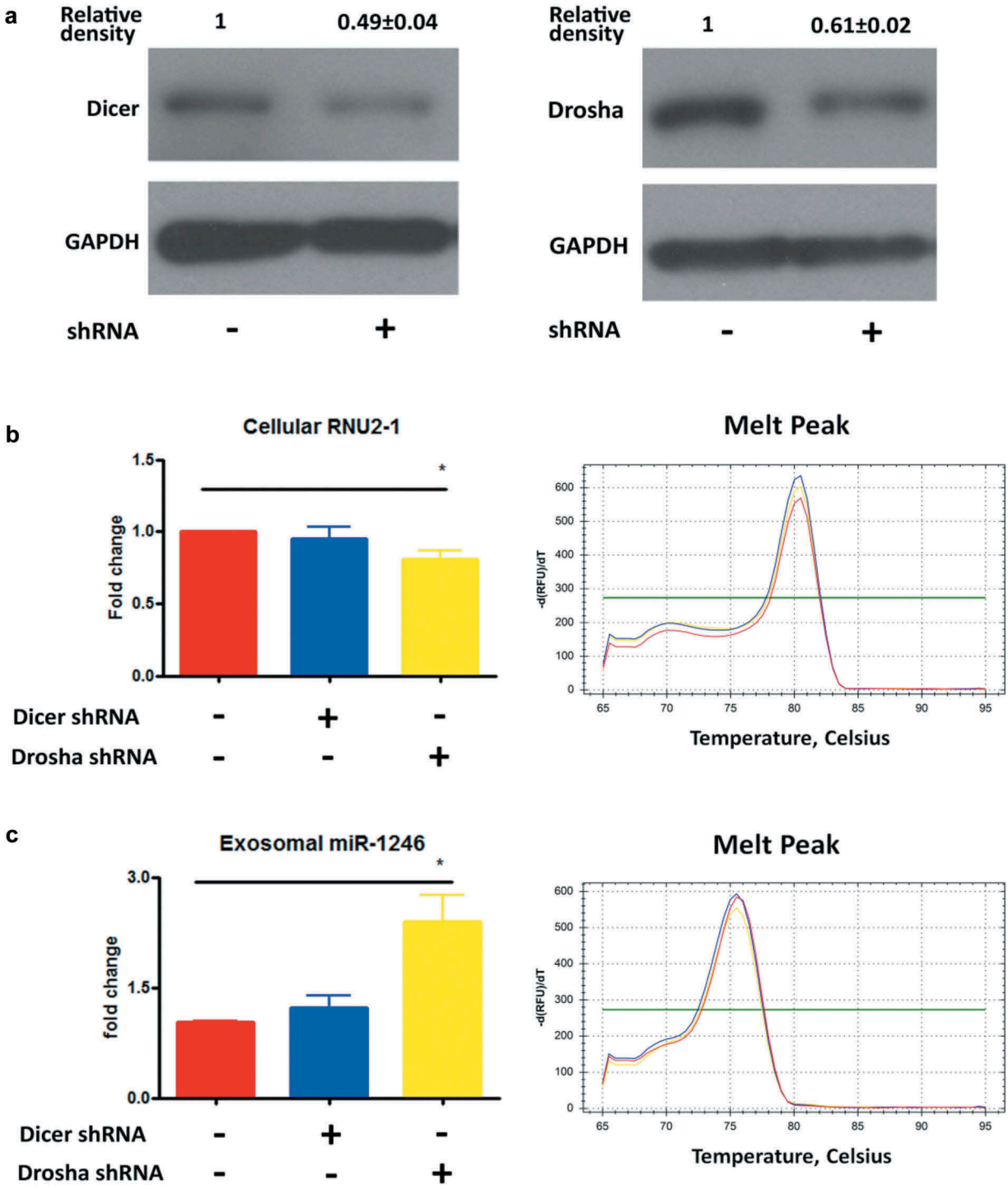


Figure 6. Effects of knockdown of Drosha and Dicer in PANC-1 cells on exosomal miR-1246 expression. (a), Western blot analysis confirming Dicer (left) and Drosha (right) knockdown in PANC-1 cells. (b), qRT-PCR analysis of RNU2-1 expression in Dicer and Drosha knockdown PANC-1 cells. (c), qRT-PCR analysis of miR-1246 expression in exosomes derived from Dicer and Drosha knockdown PANC-1 cells (means ± SEM, n = 3).

importance of miR-1246 in cancer biology [4,6,11,12,24,39], yet in these studies miR-1246 has been detected primarily by qRT-PCR using the stem-loop TaqMan method, which cannot qualitatively distinguish the detection of miR-1246 from RNU2-1. Given the fact that RNU2-1 is an essential component of the U2 complex of the spliceosome [14], which has been recognized to play significant roles in cancer biology [40–42], the inability to distinguish the detection of miR-1246 from RNU2-1 will likely lead to misinterpretation of experimental results, and impede the advancement of cancer biology involving in RNU2-1 and miR-1246.

The identification of the GCAG motif present in the RNU2-1 transcript that mediates exosome enrichment of miR-1246 sequences supports the concept that RNA sequence motifs are significant determinants of selective sorting of cellular RNAs into exosomes. This is consistent with recent reports showing the importance of certain RNA

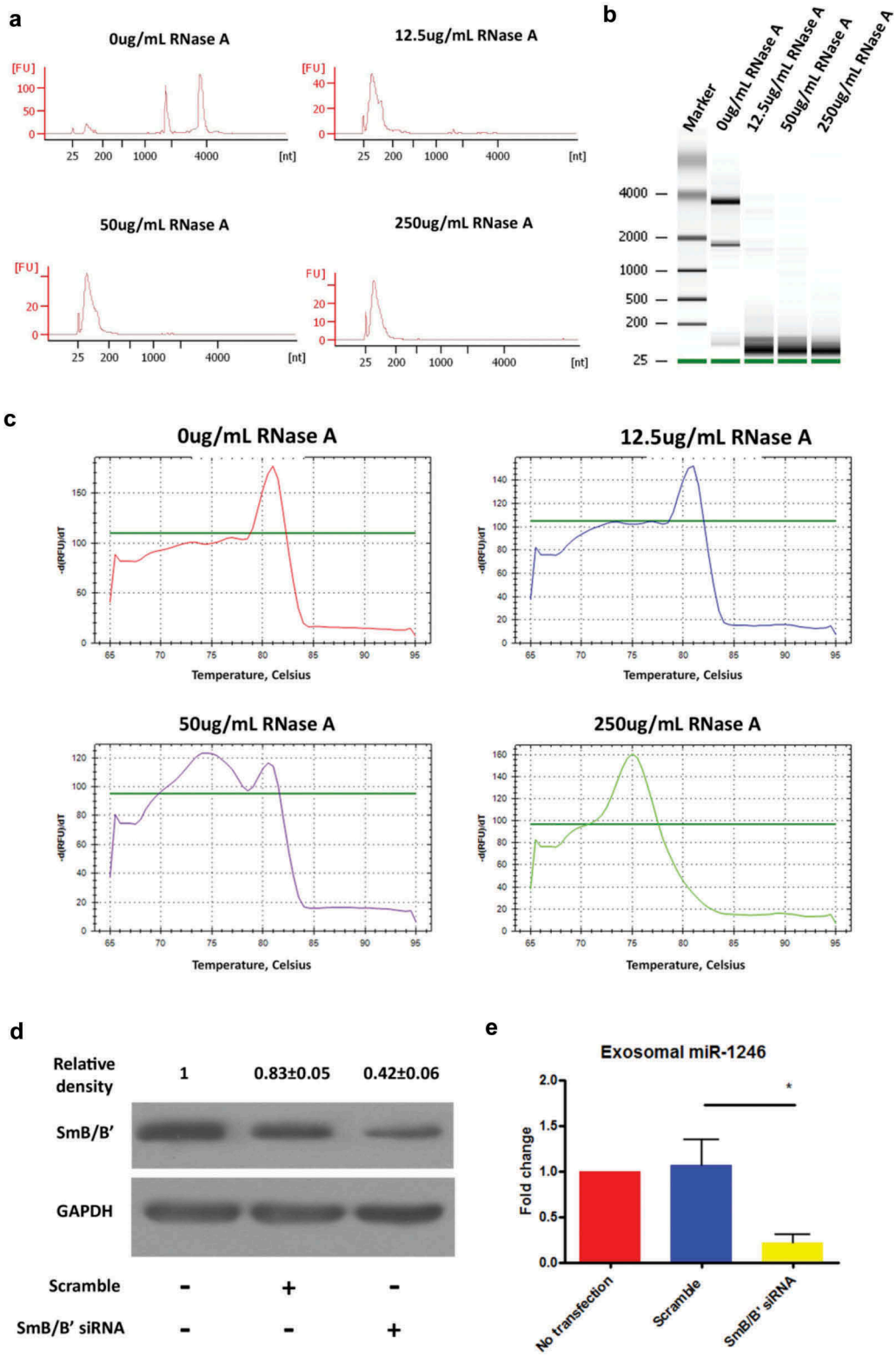


Figure 7. Exosomal miR-1246 is generated through RNU2-1 transcript degradation. (a and b), PANC-1 cell lysate was digested with RNase A at various concentrations for 3 min and the RNA isolated from the lysate was analyzed using Agilent 2100 Bioanalyzer. (c), The melting curves of qRT-PCR analysis showing the detection of RNU2-1 in control and 12.5 µg/ml RNase A-treated lysate, detection of miR-1246 in 250 µg/ml RNase A-treated lysate, and detection of both RNU2-1 and miR-1246 in 50 µg/ml RNase A-treated lysate. (d), Western blot analysis confirming SmB/B′ knockdown in PANC-1 cells. (e), qRT-PCR analysis of miR-1246 expression in exosomes derived from control and SmB/B′ knockdown PANC-1 cells (means ± SEM, n = 3). * $p < 0.05$, one-way ANOVA, followed by Dunnett analysis.

motifs present in selectively encapsulated exosomal miRNAs [43,44]. In addition, our findings that exosomal miR-1246 is derived from RNU2-1 through a non-canonical miRNA biogenesis process is also in line with recent reports showing that several miRNAs are derived from small nucleolar RNAs [45–47], and that expression of some miRNAs are independent of the canonical miRNA biogenesis pathway [48]. Furthermore, the conclusion that

a.

miR-1246: 5-'AAUGGAUUUUUGGAGCAGG-3'

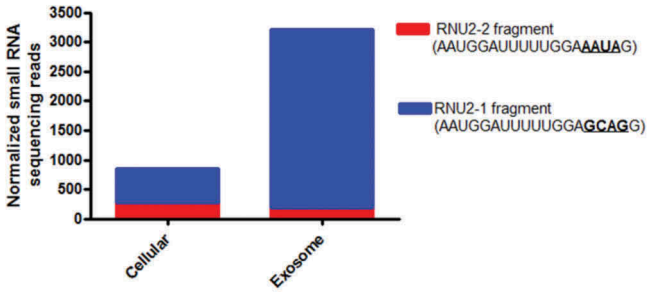
RNU2-1:

5-'AUCGCUUCUCGGCCUUUUGGCUAAGAUCAAGUGUAGUAUCUGUUCUUAUCAGUUUAAUAUCUGAUACGUCCUCUAUCCGAGGACAAUAUAUUA
AAUGGAUUUUUGGAGCAGGGAGAUGGAAUAGGAGCUUGCUCCGUCCACUCCACGCAUCGACCUGGUAUUGCAGUACCUCCAGGAACGGUGCACCC-3'

RNU2-2 :

5-'UCAGUUUAAUAUCUGAUACGUCCUCUAUCCGAGGACAAUAUAUUAAAUGGAUUUUUGGAAAUAGGAGAUGGAAUAGGAGCUUGCUCCGUCCA
CUCCACGCAUCGACCUGGUAUUGCAGUACUUCCAGGAACGGUGCACU-3'

b.



c.

pcDNA3.1-RNU2-1	Mutations made
RNU2-1 wild type:	AATGGATTTTTGGAGCAGG
GCAG to AATA RNU2-1:	AATGGATTTTTGGA**AATA**G
AATG to AATA RNU2-1:	**AATA**GATTTTTGGAGCAGG
ATTT to AATA RNU2-1:	AATGG**AATA**TTGGAGCAGG

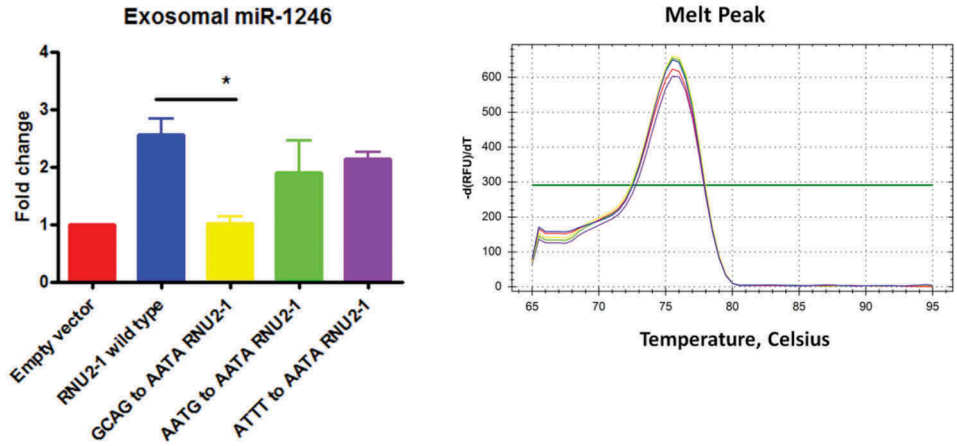


Figure 8. The GCAG motif in the RNU2-1 transcript is responsible for miR-1246 enrichment in PANC-1 exosomes. (a), Sequences of RNU2-1 and RNU2-2 with homology (green), miR-1246 fragment (red), and differences (black) highlighted. (b), small RNA sequencing reads match to RNU2-1 and RNU2-2 in PANC-1 cells and exosomes. (c), RNA motif mutants constructed using RNU2-1 expression plasmid. (d), qRT-PCR analysis of miR-1246 expression in exosomes derived from wild type and RUN2-1 mutants transfected PANC-1 cells (means ± SEM, n = 3). ** $p < 0.05$, one-way ANOVA, followed by Dunnett analysis.

exosomal miR-1246 is derived from RNU2-1 transcript may have a significant impact on improving our understanding of the biology of RNU2-1. It is well known that RNU2-1 is stably expressed and can serve as endogenous control for RNA detection [49], and as such RNU2-1 expression levels are difficult to alter in eukaryotic cells [29]. Thus, exosomal miR-1246 may serve as an indicator of RNU2-1 turnover or degradation in eukaryotic cells.

In conclusion, we have demonstrated that exosomal miR-1246 originates from RNU2-1 transcript degradation in human PDAC cells, and that the GCAG motif mediates miR-1246 enrichment in PDAC exosomes. These findings reveal the origin of an oncomiR in human cancer cells and suggest that current reports regarding miR-1246 detection or biological function in cancer cells should be interpreted with caution.

Methods

Cell culture

The human pancreatic epithelial cell line hTERT-HPNE, pancreatic cancer cell lines PANC-1, MIA-PaCa-2 and BxPC-3, and breast cancer cell lines MCF7 and MDA-MB-231 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured according to

ATCC's instructions except that exosome-depleted fetal bovine serum (FBS) and horse serum were used when appropriate. Exosome-depleted FBS and horse serum were prepared by pelleting the serum exosomes by ultracentrifugation at 100,000 × g for 2 hours at 4°C. Cells were routinely maintained in a humidified chamber at 37°C and 5% CO_2.

Exosome isolation

Exosomes were isolated from the culture medium utilizing a combination of centrifugation, ultracentrifugation, and filtration as we recently described [38,50].

Western blot analysis

Total protein was prepared by resuspending cells in RIPA Buffer (50mM Tris-HCl pH 7.4, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, and 0.1% sodium dodecyl sulfate) containing 1 mM phenlymethylsulfonyl fluoride, 5μg/ml leupeptin, 2μg/ml aprotinin, and 1μg/ml pepstatin A [38]. Approximately 30-40μg of protein from each sample was separated under reducing conditions on a 10% SDS-PAGE gel, transferred to a polyvinylidene fluoride membrane, blotted with a primary antibody against Dicer (Cell Signaling Technology, 3363S), Drosha (Cell Signaling Technology, 3364S), SmB/B' (Santa Cruz Biotechnology, sc-130670), and GAPDH (ProMab, 20035).

Small RNA library preparation and next generation sequencing

Total RNA was extracted from cells and exosome pellets using the TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, California) following the manufacturer's protocol [38]. RNA concentration was quantitated using the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Small RNA libraries were constructed using the New England Biolabs (NEB) NEBNext Multiplex Small RNA Library Prep Set for Illumina sequencers and the NEB standard protocol [38]. Each library was indexed to multiplex four samples per sequencing run on the Illumina MiSeq platform using MiSeq 50 cycle Reagent Kits v2. A minimum of 17 million 50-bp sequencing reads were collected from each sample and data were analyzed using the Genesifter software (formerly Geospiza, PerkinElmer, Santa Clara, CA, USA). The raw data of next generation small RNA sequencing was analyzed using TxetWrangler software (BARE BONES, North Chelmsford, MA, USA) to identify miR-1246 sequence.

Quantitative real-time reverse transcription polymerase chain reaction

For quantitative miRNA expression analysis, cDNA was synthesized from 80 ng of total RNA using the qScript miRNA cDNA Synthesis Kit (Quanta BioSciences, Inc., Beverly, MA, USA). An aliquot of cDNA, equivalent of 4 ng of the original RNA, was mixed with Perfecta SYBR Green SuperMix, Quanta Universal PCR Reverse Primer (Quanta, lot#015003), and a sequence specific forward primer (Table 2) or positive control forward primer (Quanta, Lot# 015644) in 20 μl PCR reactions. A synthetic *Caenorhabditis elegans* miR-54 (cel-miR-54) RNA oligonucleotide (Integrated DNA Technologies, Coralville, IA, USA) was spiked-into RNA samples as a control [51]. PCR reactions were run on a Bio-Rad CFX 96 Real-Time PCR (Bio-Rad, Hercules, CA, USA) instrument under following conditions: 95°C for 2 min, 40 cycles of 95°C for 5 sec, 60°C for 15 sec and 70°C for 15 sec. Changes in gene expression were calculated using the $\Delta\Delta C_T$ method: $\Delta C_T = C_T$ (target RNA) − C_T (PerfeCTa Human Positive Control for cell or cel-miR-54 for exosome); $\Delta\Delta C_T = \Delta C_T$ (experimental group)- ΔC_T (control group); the fold change = $2^{-\Delta\Delta CT}$. The specific primers used for RT-PCR reactions were listed in Table 2.

Sanger sequencing

cDNA was prepared from PANC-1 cellular and exosome RNA using the qScript miRNA cDNA Synthesis Kit (Quanta BioSciences, Inc., Beverly, MA, USA). The cDNA was amplified using the miR-1246-F (Table 2) and the Quanta Universal

Table 2. List of primers used in this study.

Primer name	Primer sequence (all 5′ to 3′)
miR-1246-F	GCGCGATGGATTTTTGGAGCAG
miR1246-up-F	TGCGTAACAGCTCCCTTTTT
miR1246-up-R	AAGTCCCGTTGATTTTGGTGTCAAAGGGCTATTGCAAACA
GFP-F	TGTTTGCAATAGCCCTTTGACACCAAAATCAACGGGACTT
GFP-R	GAGACATTGCATTTGGAGGCGGGAGGTGTGGGAGGTTTT
miR-1246-down-F	AAAACCTCCCACACCTCCCGCCTCCAAATGCAATGTCTC
miR-1246-down-R	GAGGACAGCAACGTCACAGA
pre-miR-1246-clone-F	CCGCTCGAGTGTATCCTTGAATGGATTTTTGGAGC
pre-miR-1246-clone-R	CCGCTCGAGATTGCTAGCCTATGGATTGATTTCC
miR-1246-precursor-F	GTGGACACCTGACCCAAAGGAAA
RNU2-1-clone-F	CCGCTCGAGATCGCTTCTCGGCCTTTTGG
RNU2-1-clone-R	CCGCTCGAGGGGTGCACCGTTCCTGGA
GCAG-AATA-L	GCAAGCTCCTATTCCATCTCCTATTTCCAAAAATCCATTTAATATATTG TCCTCGGATAGA
GCAG-AATA-R	TCTATCCGAGGACAATATATTAAATGGATTTTTGGAAATAGGAGATGG AATAGGAGCTTGC
AATG-AATA-L	TCCCTGCTCCAAAAATCTATTTAATATATTGTCCTCGGATAGAGG
AATG-AATA-R	CCTCTATCCGAGGACAATATATTAAATAGATTTTTGGAGCAGGGA
ATTT-AATA-L	TCCGAGGACAATATATTAAATGGAATATTGGAGCAGGGAGATGGAAT
ATTT-AATA-R	ATTCCATCTCCCTGCTCCAATATTCCATTTAATATATTGTCCTCGGA

PCR Reverse Primer (Quanta, lot#015003) in a standard PCR reaction. The PCR products were separated on a 1.5% agarose gel, and purified using the DNA gel purification kit (EZNA, D2500-01). The purified PCR products were cloned to the pCR4-TOPO vector (Invitrogen, lot#1734930) using the TOPO TA Cloning Kit (Invitrogen, lot#1747966). The cloned constructs were transformed to NEB® 5-alpha Competent *E. coli* cells (C2987I) and the cells were selected by an ampicillin containing LB agar plate at 37°C. Three random individual colonies were further amplified in ampicillin containing LB broth, and the plasmid DNA were prepared and sequenced.

Northern blot analysis

Northern blot was performed following a previous protocol [52] with a synthesized DIG labeled LNA probe for miR-1245. 15 μg total RNA were separated in a SequaGel (National diagnostics, cat. no. EC-833) and transferred to positively charged nylon membranes (Roche, cat. no. 11 209 299 001). The membranes were blocked, hybridized to the miR-1246 probe (QIAGEN, miRCURY LNA™ miRNA Detection Probe), and washed [52]. After incubating with anti-Digoxigenin-AP, Fab fragments (Roche, cat. no. 11 093 274 910) and CSPD (Roche, cat. no. 11 655 884 001), the membranes were exposed to X-ray film. The film was scanned and images cropped using Photoshop software.

Luciferase reporter gene assay

A luciferase reporter plasmid was constructed using the pGL3-Promoter vector (Promega, Madison, WI, USA) by inserting the miR-1246 target sequence (synthesized by IDT) at the XbaI site according to a well-established strategy for miRNA target gene study (Promega, pmirGLO Dual-Luciferase miRNA Target Expression). The antisense of the target sequence was also inserted into the XbaI site serving as a control plasmid. Mimics of mature miR-1246 and our newly identified miR-1246 like sequence (Figure 2(c)) were synthesized by Ambion (Life Technologies, Carlsbad, CA, USA). Cells were transfected using Lipofectamine 3000 (Invitrogen) with 1 μg of each constructed vector and 300 nM of the miR-1246, miR-1246 variant mimics or scramble controls in each well of the 96-well plate. In each transfection, 100ng of pRL-TK (Promega, Madison, WI) was included to serve as transfection controls. 72 hours after transfection, luciferase activity was analyzed using the Dual-Luciferase Reporter reagent (Promega, Madison, WI) with the Perkin Elmer EnVision® Multilabel Reader.

Knockout of MiR1246 in PANC-1 cells

The gene encoding miR-1246 (*MIR1246*) on 2q31.1 was knocked out using the CRISPR-Cas-9 Homology Directed Repair (HDR) method [28]. Guide RNA (gRNA) was designed using a software program from the http://crispr.mit.edu website, and the gRNA oligonucleotides (CACCGGCCTATGGATTGATTTCCTT, AAACAAGGAAATCAATCCATAGGCC) were ordered from IDT. The oligonucleotides were annealed and subcloned into the BsmBI sites of the lentiviral vector (Lenti CRISPR V2 from addgene, plasmid # 52961). The GFP-HDR fragment was constructed by connecting the DNA sequences upstream and downstream of *MIR1246* and the GFP coding sequence using overlap PCR. Briefly, the Phusion® High-Fidelity DNA Polymerase (NEB M0530S) was used to amplify the upstream and downstream sequences from PANC-1 chromosome DNA and the GFP coding sequence from the pEGFP-C1 vector, using respective primers (miR1246-up-F, miR1246-up-R, GFP-F, GFP-R, miR1246-down-F, miR1246-down-R) as shown in Table 2. An overlap PCR was performed to connect the three DNA fragments using a protocol previously described [53]. The Lenti CRISPR V2 vector and the HDR fragment were co-transfected to PANC-1 cells using the FuGENE® HD Transfection Reagent (E2311, Promega, Madison, WI, USA). Forty-eight hours post transfection, cells were selected by puromycin (1ug/ml) treatment for six days. The GFP positive cells were then sorted using the FACSJazz Cell Sorter (BD Bioscience, Sparks, MD, USA). The homozygous knockout of *MIR1246* was verified by PCR amplification of the genomic DNA and DNA sequencing of the amplified DNA production confirming the insertion of the GFP-HDR fragment. The selected *MIR1246* knockout cells were used for the downstream experiments.

Construction of precursor miR-1246 and RNU2-1 expression plasmids

The precursor miR-1246 and RNU2-1 fragments were PCR amplified using sequence specific primers (pre-miR-1246-clone-F/R, RNU2-1-clone F/R, see Table 2) from PANC-1 DNA samples. The purified PCR fragments and the pcDNA3.1(+) (Invitrogen) vector were digested with Xho1 (NEB, R0146L) respectively and purified. The cut pcDNA3.1(+) vector was dephosphorylated and ligated to the precursor miR-1246 or RNU2-1 fragments. The ligation was transformed to DH5α competent cell (NEB C2987H) and single colonies were obtained under ampicillin selection. The selected colonies were amplified, the plasmids were extracted and DNA sequencing was performed to verify a successful cloning. Based on the pcDNA3-RNU2-1 plasmid, the mutant RNU2-1 expression plasmids were made using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, 210518), using primers (GCAG-AATA-L/R, AATG-AATA-L/R, ATTT-AATA-L/R) listed in Table 2. Plasmid transfection of PANC-1 cells was performed using FuGENE® HD Transfection Reagent according to the manufacturer's protocol (Promega, E2311).

siRNA targeting RNU2-1 and SmB/B'

siRNAs targeting RNU2-1 sequence (shown in Supplemental Table 1) (IDT, catalog number: hs.Ri.RNU2-1.13.1, hs.Ri.RNU2-1.13.2) or SmB/B' mRNA (Santa Cruz, sc-36503) were transfected to PANC-1 cells (100nM, final concentration) using the FuGENE® HD Transfection Reagent (Promega, E2311). Forty-eight hours post transfection the cells were harvested and exosomes were isolated from the medium.

Dicer or drosha knockdown

The pSicoR human Dicer1 and pSicoR human Drosha1 shRNA plasmids were a gift from Tyler Jacks (Addgene plasmids # 14763 and # 14766). Plasmid DNA was transfected to PANC-1 cells using the FuGENE® HD Transfection Reagent. Seventy-two hours post transfection the cells were harvested and exosomes were isolated. Dicer and Drosha1 knockdown was confirmed by western blot analysis.

RNase a treatment of PANC-1 lysate

PANC-1 cell lysate was prepared using the passive lysis buffer (Promega, Madison, WI). The lysate was treated with various concentrations of RNase A (Omega Bio-tek, Norcross, GA) for 3 minutes at room temperature. Total RNA was extracted using the TRIzol reagent following the manufacturer's protocol. The quality of isolated RNA was analyzed by Agilent 2100 Bioanalyzer.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, Inc. La Jolla, CA, USA). Student's t test or one-way ANOVA was applied to determine significant differences among control and experimental groups.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Ethics approval and consent to participate

NA

Consent for publication

All authors have read this manuscript and approved for the submission.

Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional file.

Authors' contributions

XY carried out the study and drafted the manuscript. BNH supervised the experiments and helped drafting the manuscript. UK and AG assisted with cellular assays. WQD conceived of the study and participated in its design and coordination and finalized the manuscript. All authors read and approved the final manuscript.

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