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(54) **MIRNAS USEFUL FOR IDENTIFYING TARGETS ASSOCIATED WITH CANCER**

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(71) Applicant: **Research Foundation of the City University of New York**, New York, NY (US)

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(72) Inventors: **Olorunseun O. Ogunwobi**, Yonkers, NY (US); **Dibash K. Das**, Brooklyn, NY (US)

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(57) **ABSTRACT**

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Several miRNAs are described that are useful in diagnosing and treating prostate cancer, or pancreatic cancer. The miRNAs bind with targets that are associated with prostate cancer or pancreatic cancer. This permits the identification of those targets as well as a treatment methodology for prostate cancer.

(62) Division of application No. 15/338,704, filed on Oct. 31, 2016, now Pat. No. 10,138,481.

(60) Provisional application No. 62/350,277, filed on Jun. 15, 2016, provisional application No. 62/301,692,

**Specification includes a Sequence Listing.**

	Position																											
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
<b>miR-1207-3p</b>																												
<b>SEQ ID NO: 1</b>	5'	---	U	C	A	G	C	U	G	C	C	C	U	C	A	U	U	U	C	X <sub>1</sub>	X <sub>2</sub>						3'	
<b>SEQ ID NO: 2</b>	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>U</u>	C	G	A	C	C	G	G	A	<u>C</u>	U	A	A	A	G								5'	
<b>mir-198</b>																												
<b>SEQ ID NO: 3</b>	5'	---	G	G	U	C	C	A	G	A	G	G	G	A	G	A	U	A	G	G	U	U	C	X <sub>1</sub>	X <sub>2</sub>		3'	
<b>SEQ ID NO: 4</b>	3'	X <sub>1</sub>	X <sub>2</sub>	C	<u>U</u>	A	G	G	U	C	C	A	C	<u>A</u>	U	C	A	U	C	C	A	A	G				5'	
<b>mir-24-3p</b>																												
<b>SEQ ID NO: 5</b>	5'	---	U	G	G	C	U	C	A	G	U	U	C	A	G	C	A	G	G	A	A	C	A	G	X <sub>1</sub>	X <sub>2</sub>		3'
<b>SEQ ID NO: 6</b>	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>U</u>	C	G	A	G	U	C	A	A	G	U	C	C	U	C	G	U	C					5'	
<b>mir-1205</b>																												
<b>SEQ ID NO: 7</b>	5'	---	U	C	U	G	C	A	G	G	G	U	U	U	G	C	U	U	G	A	G	X <sub>1</sub>	X <sub>2</sub>				3'	
<b>SEQ ID NO: 8</b>	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>A</u>	A	C	G	U	C	C	C	A	A	A	C	G	A	<u>G</u>	A	C	U	C				5'	
<b>mir-1304-5p</b>																												
<b>SEQ ID NO: 9</b>	5'	---	U	U	U	G	A	G	G	C	U	A	C	A	G	U	G	A	G	A	U	G	U	G	X <sub>1</sub>	X <sub>2</sub>		3'
<b>SEQ ID NO: 10</b>	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>G</u>	A	C	U	C	C	G	A	U	G	U	C	A	C	U	C	U	<u>G</u>	C	A	C		5'	

**FIG. 1**

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	
	Position																										
Mutant miR-1207-3p																											
SEQ ID NO: 13	5'	---	U	C	G	C	A	U	G	G	C	C	U	C	A	U	U	U	C	X <sub>1</sub>	X <sub>2</sub>						
SEQ ID NO: 14	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>U</u>	C	G	U	A	C	C	G	G	A	<u>C</u>	U	A	A	A	G							
Mutant miR-198																											
SEQ ID NO: 15	5'	---	G	G	G	U	A	A	G	A	G	G	A	G	A	U	A	G	G	U	U	C	X <sub>1</sub>	X <sub>2</sub>			
SEQ ID NO: 16	3'	X <sub>1</sub>	X <sub>2</sub>	C	<u>U</u>	C	A	U	U	C	C	A	C	U	C	U	A	U	C	C	A	A	G				
Mutant miR-24-3p																											
SEQ ID NO: 17	5'	---	U	G	U	A	G	C	A	G	U	U	C	A	G	C	A	G	G	A	A	C	A	G	X <sub>1</sub>	X <sub>2</sub>	
SEQ ID NO: 18	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>U</u>	A	U	C	G	U	C	A	A	G	U	C	G	U	C	C	U	<u>C</u>	G	U	C		
Mutant miR-1205																											
SEQ ID NO: 19	5'	---	U	C	C	U	G	A	G	G	G	U	U	U	G	C	U	U	U	G	A	G	X <sub>1</sub>	X <sub>2</sub>			
SEQ ID NO: 20	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>A</u>	G	A	C	U	C	C	C	A	A	C	G	A	<u>G</u>	A	C	U	C					
Mutant miR-1304-5p																											
SEQ ID NO: 21	5'	---	U	U	A	G	U	G	G	C	U	A	C	A	G	U	G	A	G	A	U	G	U	G	X <sub>1</sub>	X <sub>2</sub>	
SEQ ID NO: 22	3'	X <sub>1</sub>	X <sub>2</sub>	A	<u>G</u>	U	C	A	C	C	G	A	U	G	U	C	A	C	U	C	U	<u>G</u>	C	A	C		

FIG. 2

## MIRNAS USEFUL FOR IDENTIFYING TARGETS ASSOCIATED WITH CANCER

### CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to, and is a divisional of, U.S. patent application Ser. No. 15/338,704 (filed Oct. 31, 2016) which is a non-provisional of, U.S. provisional patent applications 62/247,788 (filed Oct. 29, 2015); 62/301,692 (filed Mar. 1, 2016) and 62/350,277 (filed Jun. 15, 2016) the entirety of which are incorporated herein by reference.

### STATEMENT OF FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made with government support under grant number MD007599 awarded by the National Institute of Minority Health and Health Disparities, National Institute of Health (NIMHD/NIH). The government has certain rights in the invention.

### REFERENCE TO A SEQUENCE LISTING

[0003] This application refers to a "Sequence Listing" listed below, which is provided as an electronic document entitled "Sequence.txt" (10 kb created on Oct. 24, 2016) which is incorporated herein by reference in its entirety.

### BACKGROUND OF THE INVENTION

[0004] MicroRNAs (miRNAs) are regulators of gene expression that often suppress the translation of protein coding mRNAs through a RNA-induced silencing complex (RISC). Although some methods of identifying miRNA targets are available (e.g. luciferase assays) these methods are laborious, expensive and imperfect.

[0005] Methods of identifying miRNA targets is described in articles entitled "MicroRNA-10a Binds the 5'UTR of Ribosomal Protein mRNAs and Enhances Their Translation" by Orom et al. (Mol. Cell 30, 460-71, 2008) and "Isolation of microRNA targets using biotinylated synthetic microRNAs" by Orom et al. (Methods 43, 162-5, 2007). Also see an article entitled "Profiling Direct mRNA-microRNA interactions using synthetic biotinylated microRNA-duplexes" by Wani et al. (printed online at BioRxIV, May 22, 2014). These techniques are referred to as RNA pull-down assays. Briefly, a miRNA of interest is synthesized with a 3'biotin group. The miRNA binds to its mRNA targets in cells. The resulting complex is separated from other cellular materials by, for example, streptavidin beads, purified and subjected to PCR, microarray or sequencing analysis to identify the molecular targets of the miRNA.

[0006] The discussion above is merely provided for general background information and is not intended to be used as an aid in determining the scope of the claimed subject matter.

### BRIEF DESCRIPTION OF THE INVENTION

[0007] Several miRNAs are described that are useful in diagnosing and treating certain cancers. The miRNAs bind to molecular targets that are associated with specific cancers. This permits the identification of those targets as well as a treatment methodology for cancer. A method for the quantification of the miRNAs is also provided.

[0008] This brief description of the invention is intended only to provide a brief overview of subject matter disclosed herein according to one or more illustrative embodiments, and does not serve as a guide to interpreting the claims or to define or limit the scope of the invention, which is defined only by the appended claims. This brief description is provided to introduce an illustrative selection of concepts in a simplified form that are further described below in the detailed description. This brief description is not intended to identify key features or essential features of the claimed subject matter, nor is it intended to be used as an aid in determining the scope of the claimed subject matter. The claimed subject matter is not limited to implementations that solve any or all disadvantages noted in the background.

### BRIEF DESCRIPTION OF THE DRAWINGS

[0009] So that the manner in which the features of the invention can be understood, a detailed description of the invention may be had by reference to certain embodiments, some of which are illustrated in the accompanying drawings. It is to be noted, however, that the drawings illustrate only certain embodiments of this invention and are therefore not to be considered limiting of its scope, for the scope of the invention encompasses other equally effective embodiments. The drawings are not necessarily to scale, emphasis generally being placed upon illustrating the features of certain embodiments of the invention. In the drawings, like numerals are used to indicate like parts throughout the various views. Thus, for further understanding of the invention, reference can be made to the following detailed description, read in connection with the drawings in which:

[0010] FIG. 1 is a depiction of certain miRNA duplexes showing their relative alignments and overhangs; and

[0011] FIG. 2 is a depiction of certain mutant miRNA duplexes showing their relative alignments and overhangs.

### DETAILED DESCRIPTION OF THE INVENTION

[0012] This disclosure pertains to the discovery that certain miRNAs have an altered expression in some prostate cancer (PCa) cell lines, pancreatic cancer cell lines or gastric cancer cell lines in comparison to corresponding non-tumorigenic cell lines. These miRNAs are summarized in FIG. 1 and are referred to as miR-1207-3p (SEQ ID NO: 1, under-expressed in prostate cancer cell lines); miR-198 (SEQ ID NO: 3, under-expressed in pancreatic cancer cell lines); miR-24-3p (SEQ ID NO: 5, under-expressed in gastric cancer cell lines); miR-1205 (SEQ ID NO: 7, under-expressed in prostate cancer cell lines) and miR-1304-5p (SEQ ID NO: 9, over-expressed in prostate cancer). Furthermore, in ongoing clinical studies, miR-1207-3p has been found to be a prognostic biomarker in prostate cancer. However, there is currently no commercially available biotinylated mimic for use in the discovery of corresponding molecular targets. The ability to identify molecular targets of these miRNAs is important to understanding the molecular mechanisms of action of these miRNAs.

[0013] A series of miRNA sequences are disclosed. One synthetic biotinylated miR-1207-3p duplex comprises a twenty nucleotide sequence of miR-1207-3p (UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 1) wherein X<sub>1</sub>X<sub>2</sub> provide a two nucleotide overhang that forms a mature strand. In one embodiment, X<sub>1</sub>X<sub>2</sub> are UG. Biotin is

connected to the 3' hydroxyl group (OH) via a linker, such as a C6 linker. A passenger strand (GAAAU CAGGGCCAGCUUAX<sub>2</sub>X<sub>1</sub>, SEQ ID NO: 2) is provided. In one embodiment X<sub>2</sub>X<sub>1</sub> are UG. For simplicity of illustration the passenger strands in the figures are depicted from 3' to 5' to show their alignment with the corresponding mature strand. The passenger strand is complementary to the mature strand with the following exceptions: 1) there is a mismatch with position two of the mature strand, 2) other mismatches between the strands help improve disassociation of the two strands. This disassociation allows for the incorporation of the mature strand into the RNA-induced silencing complex (RISC). In one embodiment, the passenger strand has between twenty and thirty residues including a second overhang of between two and seven residues. In one embodiment, there are fewer than twenty-five residues. The passenger strand is hydrogen bonded to the mature strand and is at least 80% complementary, but less than 100% complementary with respect to the mature strand such that there is at least one mismatch. Mismatches are bolded. In one embodiment the mature strand is at least 90% complementary. In one embodiment, there is at least one mismatch between the mature strand and the passenger strand. In another embodiment there are at least two mismatches between the mature strand and the passenger strand.

**[0014]** Also, both strands (mature and passenger) have a 5' phosphate group and a 3' hydroxyl group. Both strands end in a two nucleotide arbitrary overhang on the 3' end.

**[0015]** Another synthetic biotinylated miR-198 duplex comprises a twenty-four nucleotide sequence of miR-198 (GGUCCAGAGGGGAGAUAGGUUCCUX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 3) wherein X<sub>1</sub>X<sub>2</sub> provide a two nucleotide overhang that forms a mature strand. In one embodiment, X<sub>1</sub>X<sub>2</sub> are CU. Biotin is connected to the 3' hydroxyl group (OH) via a linker, such as a C6 linker. A passenger strand (GAACCUAUCUCACCUCUGGAUCX<sub>2</sub>X<sub>1</sub>, SEQ ID NO: 4) is provided. In one embodiment, X<sub>2</sub>X<sub>1</sub> are AG.

**[0016]** Another synthetic biotinylated miR-24-3p twenty-four nucleotide sequence of miR-24-3p (UGGCUCAGUUCAGCAGGAACAGX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 5) wherein X<sub>1</sub>X<sub>2</sub> provide a two nucleotide overhang that forms a mature strand. In one embodiment, X<sub>1</sub>X<sub>2</sub> are UC. Biotin is connected to the 3' hydroxyl group (OH) via a linker, such as a C6 linker. A passenger strand (CUC CUCUGCUGAACUGAGCUAX<sub>2</sub>X<sub>1</sub>, SEQ ID NO: 6) is provided. In one embodiment, X<sub>2</sub>X<sub>1</sub> are GA.

**[0017]** Another synthetic biotinylated miR-1205 twenty-four nucleotide sequence of miR-1205 (UCUGCAGGGUUUGCUUUGAGX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 7) wherein X<sub>1</sub>X<sub>2</sub> provide a two nucleotide overhang that forms a mature strand. In one embodiment, X<sub>1</sub>X<sub>2</sub> are AC. Biotin is connected to the 3' hydroxyl group (OH) via a linker, such as a C6 linker. A passenger strand (CUCA GAGCAAACCCUGCAAAX<sub>2</sub>X<sub>1</sub>, SEQ ID NO: 8) is provided. In one embodiment, X<sub>2</sub>X<sub>1</sub> are CU.

**[0018]** Another synthetic biotinylated miR-1304-5p twenty-four nucleotide sequence of miR-1304-5p (UUUGAGGCUACAGUGAGAUGUGX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 9) wherein X<sub>1</sub>X<sub>2</sub> provide a two nucleotide overhang that forms a mature strand. In one embodiment, X<sub>1</sub>X<sub>2</sub> is CA. Biotin is connected to the 3' hydroxyl group (OH) via a linker, such as a C6 linker. A passenger strand (CAC GUCUCACUGUAGCCUCAGAX<sub>2</sub>X<sub>1</sub>, SEQ ID NO: 10) is provided. In one embodiment, X<sub>2</sub>X<sub>1</sub> are UG.

**[0019]** In one embodiment the above biotinylated microRNA-1207-3p duplex is used as a tool in RNA pull-down assays to discover all of the mRNA molecular targets of microRNA-1207-3p (miR-1207-3p). These targets can then be further studied for a much better understanding of miR-1207-3p-mRNA interactions and biology. The biotinylated scrambled oligonucleotide duplex serves as a negative control for the biotinylated miR-1207-3p duplex. There is no interaction with any known mRNAs. Nearly 20% of miRNA-mediated repression of target mRNAs occur without the canonical base pairing to the seed sequence, but rather by imperfect binding to the center of the miRNA sequence. Therefore, using this synthetic biotinylated duplex, an RNA pulldown method permits one to definitively discover any molecular targets of the mature strand. After the molecular targets are identified, there are many biological assays that can be performed to determine the functions and mechanisms of actions of the miRNA-mRNA interactions.

**[0020]** In addition, based on recent findings from in vitro studies of miR-1207-3p function, this synthetic biotinylated miR-1207-3p may have therapeutic applications. It may also have applications as a component tool of a highly quantitative clinical-grade diagnostic assay for prostate cancer. The disclosed synthetic biotinylated miRNA compositions also have applications as components of highly quantitative clinical-grade diagnostic assays for various diseases including cancers, and they may also have applications as bioimaging tools. Furthermore, the disclosed duplexes can be cloned into an expression plasmid, then used in establishing stable cell lines to evaluate the role of miR-1207-3p in in vivo prostate tumorigenesis. The disclosed synthetic microRNA duplexes can be used in non-viral methods of RNA delivery such as via nanoparticles to protect the miRNAs from degradation as well as increase their half-life in circulation. Thus, these microRNA duplexes via targeted delivery have the potential to produce even more pronounced therapeutic effects on cancers and other diseases.

#### Negative Control

**[0021]** The design of the negative control-scrambled oligonucleotide with biotin is made using the same approach as the miR-1207-3p biotinylated duplex with one added consideration. The mature strand, which comprises a scrambled sequence, was based on the same nucleotide composition as the miR-1207-3p sequence. The scrambled sequence (GUUCCACCGUCCUCUGUAX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 11) was then verified by the NCBI BLAST computer algorithm to confirm that it did not have a match for any known miRNA SEED recognition sequence. In one embodiment, X<sub>1</sub>X<sub>2</sub> is UG. Furthermore, the scrambled sequence did not have any 100% match with any mRNA of the human database. The corresponding passenger strand is given by UACAGGGGACGGUGGACCX<sub>2</sub>X<sub>1</sub> (SEQ ID NO: 12). In one embodiment, X<sub>2</sub>X<sub>1</sub> is UG.

#### Mutated Sequences as Negative Controls

**[0022]** In some embodiments, mutated forms of one of the duplexes described above are used as a negative control. They have been verified to have no match for any known miRNA SEED recognition sequence nor do they have any 100% match with any documented human mRNA. The designs of the mutant microRNA duplexes were made using

the same approach as the duplexes with the following added consideration: microRNA seed site positions 2-7 are used for direct miRNA-mRNA target recognition in all major target site types. Disrupting base pairing between miRNA positions 2-7 and the mRNA target site by mutating multiple nucleotides inhibits direct molecular mRNA targeting. Nucleotides 3 to 5 from the 5' end of the mature strand of each microRNA were arbitrarily changed. The changed sequence was based on the same nucleotide composition of the original microRNA sequence. The mutated sequences were then verified by the NCBI BLAST computer algorithm to confirm that they did not have a match for any known miRNA SEED recognition sequence. Furthermore, the mutated sequence did not have any 100% match with any mRNA of the human database.

**[0023]** The mutants alter three sequential nucleotides beginning at the third position in the mature strand with corresponding mutations in the passenger strand. In this fashion, SEQ ID NO: 13 (UC X<sub>3</sub>X<sub>4</sub>X<sub>5</sub>UGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub>) is a mutated form of SEQ ID NO: 1. The corresponding passenger strand is given by SEQ ID NO: 14 (GAAAUCAGGGCCA X<sub>5</sub>X<sub>4</sub>X<sub>3</sub>UAX<sub>2</sub>X<sub>1</sub>). See FIG. 2 where the passenger sequences are provided 3' to 5' to illustrate alignment with the mature strand. A summary is provided below with an example of the arbitrarily chosen mutations.

Original SEQ.	Mutated SEQ.	Primary Structure of Mature Strand
SEQ ID NO: 1	SEQ ID NO: 13	UCX <sub>3</sub> X <sub>4</sub> X <sub>5</sub> UGGCCCUCAUUUCX <sub>1</sub> X <sub>2</sub> e.g. X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> = GCA
SEQ ID NO: 2	SEQ ID NO: 14	GAAAUCAGGGCCAX <sub>5</sub> X <sub>4</sub> X <sub>3</sub> UAX <sub>2</sub> X <sub>1</sub> e.g. X <sub>5</sub> X <sub>4</sub> X <sub>3</sub> = UGC
SEQ ID NO: 3	SEQ ID NO: 15	GGX <sub>3</sub> X <sub>4</sub> X <sub>5</sub> AGAGGGGAGAUAGGUUCX <sub>1</sub> X <sub>2</sub> e.g. X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> = GUA
SEQ ID NO: 4	SEQ ID NO: 16	GAACCUAUCUCACCUCUX <sub>5</sub> X <sub>4</sub> X <sub>3</sub> UCX <sub>2</sub> X <sub>1</sub> e.g. X <sub>5</sub> X <sub>4</sub> X <sub>3</sub> = UAC
SEQ ID NO: 5	SEQ ID NO: 17	UGX <sub>3</sub> X <sub>4</sub> X <sub>5</sub> CAGUUCAGCAGGAACAGX <sub>1</sub> X <sub>2</sub> e.g. X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> = UAG
SEQ ID NO: 6	SEQ ID NO: 18	CUGCUCUGCUGAACUX <sub>5</sub> X <sub>4</sub> X <sub>3</sub> UAX <sub>2</sub> X <sub>1</sub> e.g. X <sub>5</sub> X <sub>4</sub> X <sub>3</sub> = CUA
SEQ ID NO: 7	SEQ ID NO: 19	UCX <sub>3</sub> X <sub>4</sub> X <sub>5</sub> AGGGUUUGCUUUGAGX <sub>1</sub> X <sub>2</sub> e.g. X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> = CUG
SEQ ID NO: 8	SEQ ID NO: 20	CUCAGAGCAAACCCUX <sub>5</sub> X <sub>4</sub> X <sub>3</sub> AAX <sub>2</sub> X <sub>1</sub> e.g. X <sub>5</sub> X <sub>4</sub> X <sub>3</sub> = CAG
SEQ ID NO: 9	SEQ ID NO: 21	UUX <sub>3</sub> X <sub>4</sub> X <sub>5</sub> GGCUACAGUGAGAUGUGX <sub>1</sub> X <sub>2</sub> e.g. X <sub>3</sub> X <sub>4</sub> X <sub>5</sub> = AGU
SEQ ID NO: 10	SEQ ID NO: 22	CACGUCUCACUGUAGCCX <sub>5</sub> X <sub>4</sub> X <sub>3</sub> GAX <sub>2</sub> X <sub>1</sub> e.g. X <sub>5</sub> X <sub>4</sub> X <sub>3</sub> = ACU

**[0024]** The design of another negative control-scrambled oligonucleotide is made using the same approach as the microRNA biotinylated duplexes with one added consideration. The mature strand, which consists of a scrambled sequence (GUUCCACCGUCCUCUGUAX<sub>1</sub>X<sub>2</sub>, SEQ ID NO: 23) which was verified by the NCBI BLAST computer algorithm to confirm that it did not have a match for any known miRNA SEED recognition sequence. Furthermore, the scrambled sequence did not have any 100% match with

any mRNA of the human database. The corresponding passenger strand is given by UACAGGGGACGGUGGACCX<sub>2</sub>X<sub>1</sub>, SEQ ID NO: 24.

Non-Biotinylated miRNAs

**[0025]** In one embodiment, a non-biotinylated miRNA is provided that is useful as a therapeutic. The miRNA can be administered to a patient as a treatment for prostate cancer. These non-biotinylated miRNAs are substantially identical to SEQ ID NOS. 1-10 except in that the arbitrary overhangs are omitted.

Original SEQ.	Core SEQ.	Primary Structure
SEQ ID NO: 1	SEQ ID NO: 25	UCAGCUGGCCCUCAUUUC
SEQ ID NO: 2	SEQ ID NO: 26	GAAAUCAGGGCCAGCUUA
SEQ ID NO: 3	SEQ ID NO: 27	GGUCCAGAGGGGAGAUAGGUUC
SEQ ID NO: 4	SEQ ID NO: 28	GAACCUAUCUCACCUCUGGAUC
SEQ ID NO: 5	SEQ ID NO: 29	UGGCUCAGUUCAGCAGGAACAG
SEQ ID NO: 6	SEQ ID NO: 30	CUGCUCUGCUGAACUCAGGCUA
SEQ ID NO: 7	SEQ ID NO: 31	UCUGCAGGGUUGCUUUGAG
SEQ ID NO: 8	SEQ ID NO: 32	CUCAGAGCAAACCCUGCAA
SEQ ID NO: 9	SEQ ID NO: 33	UUUGAGGCUACAGUGAGAUGUG
SEQ ID NO: 10	SEQ ID NO: 34	CACGUCUCACUGUAGCCUCAG

Reporter System

**[0026]** The establishment of reporter systems for the disclosed synthetic miR-duplex will be useful tools for monitoring the tissue and cellular localization, and the molecular interactions of the synthetic duplex. These tools are desirable for the real-time monitoring of the duplex in vitro and in vivo. In one embodiment, the reporter systems comprise one of the disclosed miRNA sequences covalently coupled to Green Fluorescent Protein (GFP) or D-Luciferin. In both of these synthetic duplex reporter systems, the recognition site (e.g. miR-1207-3p) is cloned into the 3' untranslated region (UTR) of either GFP or D-Luciferin's cDNA. The recognition site is the antisense sequence of miR-1207-3p GAAAUGAGGGCCAGCUGA (SEQ ID NO: 35). This area of the 3' UTR is known as the multicloning site (MCS) of the reporter cDNA and it is downstream of the reporter gene's open reading frame (ORF). The disclosed reporter imaging system directly incorporates the anti-sense strand specific to the miRNA nucleotide sequence for one of the disclosed miRNAs.

**[0027]** One application of the synthetic miRNA duplex-GFP and the miRNA duplex-D-Luciferin reporter systems is

that they allow, for visual detection and monitoring of the miRNA duplex in vitro and in vivo. Moreover, these reporter systems make it possible to perform real-time monitoring and visualization of the molecular interactions of the duplex, thus enabling understanding of its functions. The method of visualizing the protein for the identification of prostate cancer introduces a miRNA composition with a structure given by one of SEQ ID NO: 1 to 11 to a biological sample. The sample may be an in vitro sample or an in vivo sample. A corresponding passenger strand is utilized that has at least one mismatch. A reporter molecule is then introduced to the biological sample. The reporter molecule comprises a visual indicator such as include green fluorescent protein and D-Luciferin.

**[0028]** In one embodiment, a method for absolute quantification of a miRNA using polymerase chain reaction (PCR) is provided. The method creates a standard curve using a miRNA composition with a structure given by one of SEQ ID NO: 1 to 11. A corresponding passenger strand is utilized that has at least one mismatch. An output signal is obtained from a polymerase chain reaction (PCR) that was performed on a sample such that the PCR replicates the miRNA. The output signal is compared to the standard curve to quantify the concentration of the miRNA. Method for obtaining output signals in quantitative PCR (qPCR) are known to those skilled in the art. See, for example, U.S. Pat. No. 5,972,602.

#### Example of Method for Finding a Molecular Target

**[0029]** The molecular targets of miR-1207-3p were investigated. Potential targets were initially screened using two miRNA molecular target prediction algorithm tools (miR-Base and miRDB) which identified fibronectin type III domain containing 1 (FNDC1) as a putative molecular target of miR-1207-3p. FNDC1 contains the conserved 'Fibronectin type III domain' of fibronectin (FN1). FNDC1 protein expression was analyzed in several prostate epithelial cell lines (see Table 1). FNDC1 protein expression was found to be consistently higher in all the prostate cancer cell lines compared to the non-tumorigenic prostate cell line, RWPE-1. RWPE-1 had very low FNDC1 protein expression. Fur-

ther, overexpression of miR-1207-3p significantly inhibited the protein expression of FNDC1 by about 75%.

TABLE 1

Cell line	Description
RWPE-1	non-tumorigenic prostate epithelial cell line from Caucasian male
MDA PCa 2b	aggressive, androgen-dependent, from Black male
PC-3	aggressive, androgen-independent, from Caucasian male
E006AA	indolent, androgen-independent, from Black male
E006AA-hT	derived from E006AA, aggressive, androgen-independent, from Black male
LNCaP	aggressive, androgen-dependent, from Caucasian male
C4-2B	derived from LNCaP, aggressive, androgen-independent, from Caucasian male
WPE1-NA22	derived from RWPE-1, indolent, androgen-dependent, from Caucasian male

**[0030]** A dual-luciferase reporter assay was performed using the LUC-PAIR™ Duo-Luciferase assay system to determine if miR-1207-3p binds to the 3' untranslated region (UTR) of the FNDC1 mRNA. Prostate cancer (PCa) cell lines were used that model various characteristics of prostate cancer. Because of the significantly low level of endogenous expression of miR-1207-3p in the PC-3 and MDA PCa 2b PCa cell lines and their widespread use, these cell lines were used as cellular models for this assay. PC-3 and MDA PCa 2b cells were co-transfected with both the plasmid containing the sequence of the FNDC1 3'UTR and miR-1207-3p 50 nM mimic. Cells were transfected with 3'UTR clones of FNDC1 with a synthetic non-targeting oligonucleotide negative control as the control. A direct and specific interaction was observed between exogenous miR-1207-3p and the FNDC1 3'UTR. Overexpression of miR-1207-3p led to the suppression of activity of the luciferase reporter gene fused to the FNDC1 3'UTR by about 40% in PC-3 cells and about 60% in MDA PCa 2b cells compared to the cells transfected with the non-targeting 50 nM oligonucleotide negative control.

**[0031]** The miR-1207-3p was confirmed to directly bind to FNDC1 by performing RNA pulldown using our synthetic biotinylated miR-1207-3p duplex. This approach

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What is claimed is:

**1.** A method for absolute quantification of a miRNA using polymerase chain reaction (PCR), the method comprising steps of:

- creating a standard curve using a duplex micro-ribonucleic acid (microRNA) composition comprising:
  - a mature strand comprising a primarily structure selected from the group consisting of:

UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 1)

GGUCCAGAGGGGAGAUAGGUUCCUX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 3)

UGGCUCAGUUCAGCAGGAACAGUCX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 5)

UCUGCAGGGUUUGCUUUGAGX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 7)

UUUGAGGCUCACUGAGAGUGGACX<sub>1</sub>X<sub>2</sub>; (SEQ ID NO: 9)

wherein a X<sub>1</sub> and X<sub>2</sub> are each independently selected from A, G, C, or T to define a first overhang with at least two residues that include X<sub>1</sub> and X<sub>2</sub> and less than five residues, wherein the mature strand has fewer than thirty residues, the mature strand having a 3' end;

a passenger strand having between twenty and twenty-five residues including a second overhang of between two and seven residues, the passenger strand being hydrogen bonded to the mature strand, the passenger strand being at least 80% complementary, but less than 100% complementary with respect to the mature strand such that there is at least one mismatch; and biotin bound to the 3' end of the mature strand;

obtaining an output signal from a polymerase chain reaction (PCR) that was performed on a sample such that the PCR replicates an endogenous miRNA that corresponds to the mature strand;

comparing the output signal to the standard curve to quantify the concentration of the duplex micro-ribonucleic acid (microRNA).

**2.** The method as recited in claim 1, wherein the mature strand comprises UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub> (SEQ ID NO: 1).

**3.** The method as recited in claim 1, wherein the mature strand consists of UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub> (SEQ ID NO: 1).

**4.** The method as recited in claim 3, wherein the passenger strand consists of GAAUCAGGGCCAGCUUAX<sub>2</sub>X<sub>1</sub> (SEQ ID NO: 2).

5. A duplex micro-ribonucleic acid (miRNA) composition comprising:

a mature strand comprising a primary structure selected from the group consisting of:

UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 1)

GGUCCAGAGGGAGAUAGGUCCUX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 3)

UGGCUCAGUUCAGCAGGAACAGUCX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 5)

UCUGCAGGGUUUGCUUUGAGX<sub>1</sub>X<sub>2</sub>, (SEQ ID NO: 7)

UUUGAGGCUACAGUGAGAUGGCAX<sub>1</sub>X<sub>2</sub>; (SEQ ID NO: 9)

wherein a X<sub>1</sub> and X<sub>2</sub> are each independently selected from A, G, C, or T to define a first overhang with at least two residues that include X<sub>1</sub> and X<sub>2</sub> and less than five residues, wherein the mature strand has fewer than thirty residues, the mature strand having a 3' end;

a passenger strand having fewer than thirty residues including a second overhang of between two and seven residues, the passenger strand being hydrogen bonded

to the mature strand, the passenger strand being at least 80% complementary, but less than 100% complementary with respect to the mature strand such that there is at least one mismatch.

6. The duplex micro-ribonucleic acid (miRNA) composition as recited in claim 5, further comprising biotin bound to the 3' end of the mature strand.

7. The duplex micro-ribonucleic acid (miRNA) composition as recited in claim 5, wherein the mature strand comprises UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub> (SEQ ID NO: 1).

8. The duplex micro-ribonucleic acid (miRNA) composition as recited in claim 5, wherein the mature strand consists UCAGCUGGCCCUCAUUUCX<sub>1</sub>X<sub>2</sub> (SEQ ID NO: 1).

9. The duplex micro-ribonucleic acid (miRNA) composition as recited in claim 8, further comprising biotin bound to the 3' end of the mature strand.

10. The duplex micro-ribonucleic acid (miRNA) composition as recited in claim 5, wherein the first overhang consists of two residues that are X<sub>1</sub> and X<sub>2</sub> and the second overhang consists of two residues.

11. The duplex micro-ribonucleic acid (miRNA) composition as recited in claim 5, wherein the passenger strand and the mature strand each have fewer than twenty-five residues.

\* \* \* \* \*