

# ***In vitro* quantification of specific microRNA using molecular beacons**

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## **ABSTRACT**

**MicroRNAs (miRNAs), a class of non-coding RNAs, have become a major focus of molecular biology research because of their diverse genomic origin and ability to regulate an array of cellular processes. Although the biological functions of miRNA are yet to be fully understood, tissue levels of specific miRNAs have been shown to correlate with pathological development of disease. Here, we demonstrate that molecular beacons can readily distinguish mature- and pre-miRNAs, and reliably quantify miRNA expression. We found that molecular beacons with DNA, RNA and combined locked nucleic acid (LNA)–DNA backbones can all detect miRNAs of low (<1 nM) concentrations *in vitro*, with RNA beacons having the highest detection sensitivity. Furthermore, we found that molecular beacons have the potential to distinguish miRNAs that have slight variations in their nucleotide sequence. These results suggest that the molecular beacon-based approach to assess miRNA expression and distinguish mature and precursor miRNA species is quite robust, and has the promise for assessing miRNA levels in biological samples.**

## **INTRODUCTION**

MicroRNAs (miRNAs) are a recently recognized class of short (19–25 nt), single-stranded, non-coding RNAs that regulate an array of cellular functions through the degradation and translational repression of mRNA targets. These targeted transcripts are typically involved in critical cellular processes, including differentiation, growth, proliferation and apoptosis (1). Thus, miRNAs are regarded as pivotal regulators of normal development and physiology, as well as disease. In human tissues, over

1400 miRNAs have been identified (2). miRNAs have been shown to be important in mammalian development (3) as well as various postnatal biological processes, including skeletal muscle proliferation (4), cancer (5), angiogenesis (6,7), cardiac hypertrophy and cardiac failure (8–10). Importantly, tissue levels of specific miRNAs have been shown to correlate with pathological development of disease (11). Thus, a rapid and efficient method of assessing miRNA expression would be useful for diagnosing disease and identifying novel therapeutic targets.

miRNAs in cells have three distinct forms: pri-miRNA, pre-miRNA and mature miRNA. Although only the mature form of the miRNA is involved in post-transcriptional regulation of gene expression, quantifying the relative expression levels of mature miRNA and its precursors is important for understanding miRNA transcription, localization and processing. Currently, the best methods for assessing precursor and mature populations of miRNA are northern analysis and qRT–PCR. Northern analysis requires the use of radioactive or hazardous agents and is labor intensive. Both reverse transcription (RT) and PCR reactions require the use of expensive enzymes and reagents, and a miRNA amplification step is required before signal can be detected. Northern analysis can simultaneously assess precursor and mature miRNAs by separating them based on size, but the approach to assessing precursor and mature miRNA levels via qRT–PCR usually requires RT and PCR reactions for each. For precursor miRNA, primers that are specific to the pri-miRNA or pre-miRNA forms are used (12,13). For assessing mature miRNA, a modification of the RT step that involves the addition of an oligonucleotide tail to the miRNA is usually required; however, this process may not allow for complete separation of signals from precursor and mature miRNAs. Distinguishing between precursor and mature miRNAs is important because their expression levels are not analogous; several studies have shown that precursor levels of

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miRNA may remain unchanged when the mature miRNA level varies widely (14–23).

Molecular beacons have been used for RNA sensitive detection both *in vitro* and in living cells (24). Molecular beacons are oligonucleotide (DNA or RNA) stem–loop hairpin probes containing an anti-sense hybridization sequence that is flanked by two short (usually 4–7 nt), self-complementary sequences (25). The termini of the probe are conjugated to a fluorescent dye and a suitable quencher for that dye. In the absence of complementary miRNA target, the probe forms a stem–loop structure that results in quenching of the fluorophore (Figure 1). Hybridization of the beacon to the complementary miRNA sequence, which is energetically more favorable, opens the hairpin probe, thus physically separating the fluorophore from the quencher, and allows the fluorophore to fluoresce upon excitation (26). In theory, the probe elicits a signal only upon direct hybridization to the complementary RNA sequence. Therefore, RNA assessment can be performed in one step without the need for RT or amplification steps, and excess probe does not have to be removed prior to measurement.

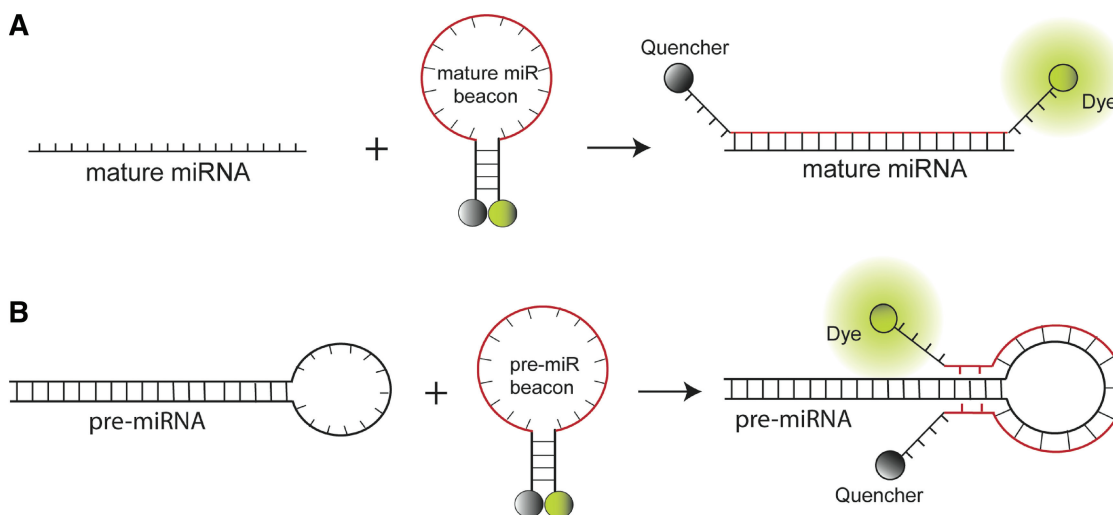
In this study, we describe the use of molecular beacons to target miR-21 (miRNA 21), a miRNA that has received extensive studies. For example, miR-21 has been found to be consistently upregulated in several cancerous tissues (27), and implicated to modulate cell survival. It has also been detected in quiescent cells, including endothelial cells (28,29), vascular smooth muscle cells (30), and cardiac myocytes (31). Our results suggest that molecular beacons can distinguish between mature and pre-miRNAs, and thus have the potential to become a powerful tool for assessing mature miRNAs and pre-miRNAs in clinical samples.

## MATERIALS AND METHODS

### Molecular beacon design

DNA or RNA molecular beacons complementary to either the mature nucleotide sequence of miR-21 or the hairpin loop region of the pre-miR-21 were designed. These beacons contained at least 22 complementary bases, a 6FAM fluorophore on the 5'-end, and a Black Hole Quencher 1 (BHQ1) or an Iowa Black quencher (IABlk) on the 3'-end. Similarly, a DNA–locked nucleic acid (LNA) molecular beacon complementary to the mature nucleotide sequence of miR-21 was designed with a 5'-end 6FAM fluorophore and 3'-end Iowa Black quencher (IABlk). For the LNA beacon, every other nucleotide of the complementary sequence was replaced with a LNA base (Table 1); the ribose moiety of the nucleic acids is modified with an extra bridge connecting the 2' and 4' carbons, which significantly enhances the stability of the beacon hybridizing to the miRNA. The secondary structure and melting temperature of each beacon were determined by the mfold (32) program before beacons were synthesized. The molecular beacons were synthesized by Sigma (DNA beacons) or Integrated DNA Technologies (DNA–LNA beacons, RNA beacons). Each beacon had a density of at least 3 OD and was purified by HPLC. Molecular beacons were suspended in nuclease-free water to a concentration of 1 µg/µl and stored in an opaque tube at –20°C.

DNA molecular beacons were designed to be complementary to the mature sequences of miR-24 or miR-27b. Each beacon consists of a 5' 6FAM fluorophore, a 3' BHQ1, and a hairpin loop containing nucleotides complementary to the 20–22 nt of the mature miRNA flanked by residues necessary to form a beacon stem. The molecular



**Figure 1.** Schematic illustration of molecular beacon hybridization assays. In the absence of complementary target, the molecular beacon designed for mature miRNA (mature beacon) or pre-miRNA (precursor beacon) forms a stem–loop structure that brings the quencher in close proximity to the fluorophore, thereby quenching the fluorescence emission. Hybridization of the beacon to its miRNA target opens the hairpin, which disrupts the stem of the beacon and physically separates the fluorophore from the quencher and allows fluorescence emission upon excitation. See Table 1 for beacon sequence that is complementary to miRNA target. For some beacons, but not all, there may be one or more nucleotides that overlap between the beacon stem and the mature miRNA sequence. (A) Hybridization of the mature beacon to the mature miRNA target. (B) Hybridization of the precursor beacon to the pre-miRNA target. This beacon hybridizes to the loop sequence of the pre-miRNA hairpin structure.

**Table 1.** Characteristics of miRNA molecular beacons

Probe	Sequence	Dye	Quencher	Probe length	Stem length	$T_m$ (°C)
Mature miR-21 DNA	<u>GCGCGTCAACATCAGTCTGATAAGCTACGCGC</u>	6FAM	BHQ1	32	6	64
Pre-miR-21 DNA	<u>GGTGTGGCCATGAGATTCAACAGTCAACACC</u>	6FAM	BHQ1	31	7	63
Mature miR-21 LNA <sup>a</sup>	<u><b>C</b>GCGT<b>C</b>AACAT<b>C</b>AGT<b>C</b>TGATAAGCT<b>C</b>ACG<b>C</b>G</u>	6FAM	IABk	30	5	73
Pre-miR-21 LNA	<u><b>C</b>CTGT<b>T</b>GCCAT<b>G</b>AGAT<b>T</b>CAACAG<b>T</b>CAACAG<b>G</b></u>	6FAM	IABk	31	7	67
Mature miR-21 RNA	<u><i>CCGTC</i>AACATCAGTCTGATAAGCTACGG</u>	6FAM	IABk	28	4	50
Pre-miR-21 RNA	<u><i>CGTTG</i>CCATGAGATTCAACAGTCAACG</u>	6FAM	IABk	27	5	61
Mature miR-24 DNA	<u>TGGCTGTTCTGCTGAACTGAGCCA</u>	6FAM	BHQ1	25	5	54
Mature miR-27b DNA	<u>GGCAGAACTTAGCCACTGTGAATCTGCC</u>	6FAM	BHQ1	28	6	58

<sup>a</sup>LNA = locked nucleic acid; LNA bases are shown by bold letters in the sequences. Bases complementary to the miRNA are shown in italics. Bases that comprise the beacon stem are underlined.

BHQ1 = Black Hole Quencher 1; IABk, Iowa Black quencher.

**Table 2.** miRNA sequences used for molecular beacon hybridization assays

Target	Sequence
Wild-type mature miR21	5' TAGCTTATCAGACTGATGTTGA 3'
Mutant mature miR21 m1	5' TA <sub>A</sub> CTTATCAGACTGATGTTGA 3'
Mutant mature miR21 m3	5' TA <sub>A</sub> CT <sub>C</sub> GTCAGACTGATGTTGA 3'
Mutant mature miR21 m5	5' TG <sub>A</sub> CT <sub>C</sub> GCCAGACTGATGTTGA 3'
Wild-type pre-miR21	5' TGTCCGGTAGCTTATCAGACTGATGTTGACTGTTGAATCTCATGGCAACACCAGTCGATGGGCTGTCTGACA 3'
Mutant pre-miR-21 p1	5' TGTCCGGTAGCTTATCAGACTGATGT <sub>C</sub> GACTGTTGAATCTCATGGCAACACCAGTCGATGGGCTGTCTGACA 3'
Mutant pre-miR-21 p3	5' TGTCCGGTAGCTTATCAGACTGATGT <sub>C</sub> GACTG <sub>C</sub> CGAATCTCATGGCAACACCAGTCGATGGGCTGTCTGACA 3'
Mutant pre-miR-21 p5	5' TGTCCGGTAGCTTATCAGACTGATG <sub>C</sub> CGACTG <sub>C</sub> CGAG <sub>T</sub> TCTCATGGCAACACCAGTCGATGGGCTGTCTGACA 3'
Mature miR-24	5' TGGCTCAGTTCAGCAGGAACAG 3'
Mature miR-27b	5' TTCACAGTGGCTAAGTTCTGC 3'
Mature miR-27a	5' TTCACAGTGGCTAAGTTCCGC 3'

beacons were synthesized by Integrated DNA Technologies. The beacons were reconstituted and stored just as the miR-21 beacons mentioned previously.

### Synthetic miRNAs used for beacon hybridization

To assess the sensitivity and specificity of the molecular beacons, the RNA sequences of mature miR-21, miR-155, miR-24, miR-27b and miR-27a were synthesized by Integrated DNA Technologies. In addition to the mature miR-21 (22 nt), pre-miR-21 (72 nt) was synthesized and used for miR-21 molecular beacon hybridization assays. Both DNA (Integrated DNA Technologies, IDT) and RNA forms of miR-21 (Sigma and Midland Certified Reagent Company) were used in these assays. As shown in Table 2, three mutants of the wild-type mature miR-21 and three mutants of the wild-type pre-miR-21 were also used in the hybridization assays. Specifically, for the mature miR-21 mutants, 1, 3, or 5 nt of the mature sequence were mutated; m1 had one nucleotide that was mutated, m3 had three mutated nucleotides and m5 had five mutated nucleotides. Similarly, for the mutant pre-miR-21 sequences, 1-, 3- or 5-nt mutations were introduced at random into the hairpin loop region of the precursor. The precursor mutants were labeled as p1, p3

and p5, respectively. For all sequences, mutations were designed so that G was replaced with A and C was replaced with T. All synthetic miRNAs were suspended in nuclease-free water to a concentration of 1 nmol/μl and stored at -20°C.

### Melting curves for beacon-miR-21 duplexes

To determine the melting temperature of each miR-21 beacon with the synthetic wild-type or mutant miRNAs, melting curves (curves of fluorescence intensity  $F$  as a function of temperature  $T$ ) for the miR-21 beacons incubated with wild-type or mutant miRNAs were generated. Beacon (0.1 μl) was added to PBS, pH 7.4, with or without the indicated concentration of miRNA or pre-miRNA to a final volume of 50 μl in a 0.2 ml optical tube. The final beacon concentration was 200 nM and the miRNA concentration was 500 nM. After an initial denaturation step (3 min at 95°C), melting curves were determined by measuring the fluorescence signal as the temperature of the reaction was lowered from 80°C to 25°C, using a Stratagene Mx3005P Real Time PCR System. The fluorescence intensity for each beacon-miRNA duplex was calculated and graphed versus the temperature. The point of the steepest slope on each

melting curve indicates the melting temperature of the duplex formed by the molecular beacon and miR-21.

### Beacon hybridization assay

Hybridization of beacon to miRNA *in vitro* was assessed at 37°C using a Tecan Safire plate reader to record fluorescence intensities. Molecular beacons and synthetic miRNA were added to PBS, pH 7.4, to a final volume of 50 µl and final beacon concentration of 200 nM. The reactions were incubated at 37°C for 20–60 min in a 384-well flat bottom tray before the intensity of the fluorescence was determined. The gain intensity of the reactions was determined for each experiment and the Tecan Safire generated relative fluorescence intensity values or relative fluorescence units (RFU).

To determine the sensitivity of the miR-21 molecular beacons in hybridizing to miR-21, melting curves were generated for each beacon and miRNA over a range of miRNA concentrations as described above. The background, i.e. fluorescence signal from the molecular beacon alone was subtracted from the fluorescence signal from the duplexes formed by the beacons and miRNA. The lowest concentration of miR-21 with positive fluorescence difference values indicates the lowest concentration of bound miR-21 detectable by the molecular beacon.

### RNA from endothelial cells

RNA was extracted from human umbilical vein endothelial cells (HUVECs) grown on 100-mm culture dishes in the presence of 0.1% gelatin at 37°C and 5% CO<sub>2</sub>. Cells were subjected to static conditions or 24 h of unidirectional shear stress at a force of 15 dynes/cm<sup>2</sup> using a cone-in-plate viscometer at 37°C and 5% CO<sub>2</sub>. After the 24-h shear conditions, the cells were lysed and removed from the culture dish. RNA was extracted using the MirVana miRNA Isolation kit from Ambion, Inc. Total RNA was collected and stored at –80°C.

### qRT-PCR assays

For qRT-PCR experiments, 50 ng of total RNA were used with the TaqMan MicroRNA Reverse Transcription kit to produce a cDNA product of each miRNA. The reaction mix was incubated at 16°C for 30 min, 42°C for 30 min, and 85°C for 5 min. The cDNA product was used as the template for the real-time PCR reaction, where specific primers and probes from the TaqMan MicroRNA Assays (Ambion) were used to quantify the expression of miRNAs. For the PCR, the samples were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s.

## RESULTS

### Molecular beacons can distinguish mature miR-21 and pre-miR-21

In living cells, miRNAs exist in two different forms outside the cell nucleus: (i) a pre-miRNA of ~100 nt that is folded into a hairpin with a long double-stranded portion and (ii) a short mature miRNA of ~20 nt that is

incorporated into the RISC complex and binds to target mRNA. It is believed that only the mature form of miRNA is active in targeting mRNA for degradation or transcriptional repression. Therefore, it is desirable that assays of miRNA expression are able to distinguish between mature miRNA and pre-miRNA.

In this work, we designed molecular beacons that hybridize to the mature miR-21 and pre-miR-21, respectively; the molecular beacon that hybridizes to pre-miR-21 has a hybridization sequence complementary to the loop region of the hairpin and nearby region of the pre-miRNA (Table 1 and Figure 1B). Because this loop sequence is part of both pre-miR-21 and pri-miR-21, the molecular beacon designed for pre-miR-21 detection could potentially hybridize to pri-miR-21 as well.

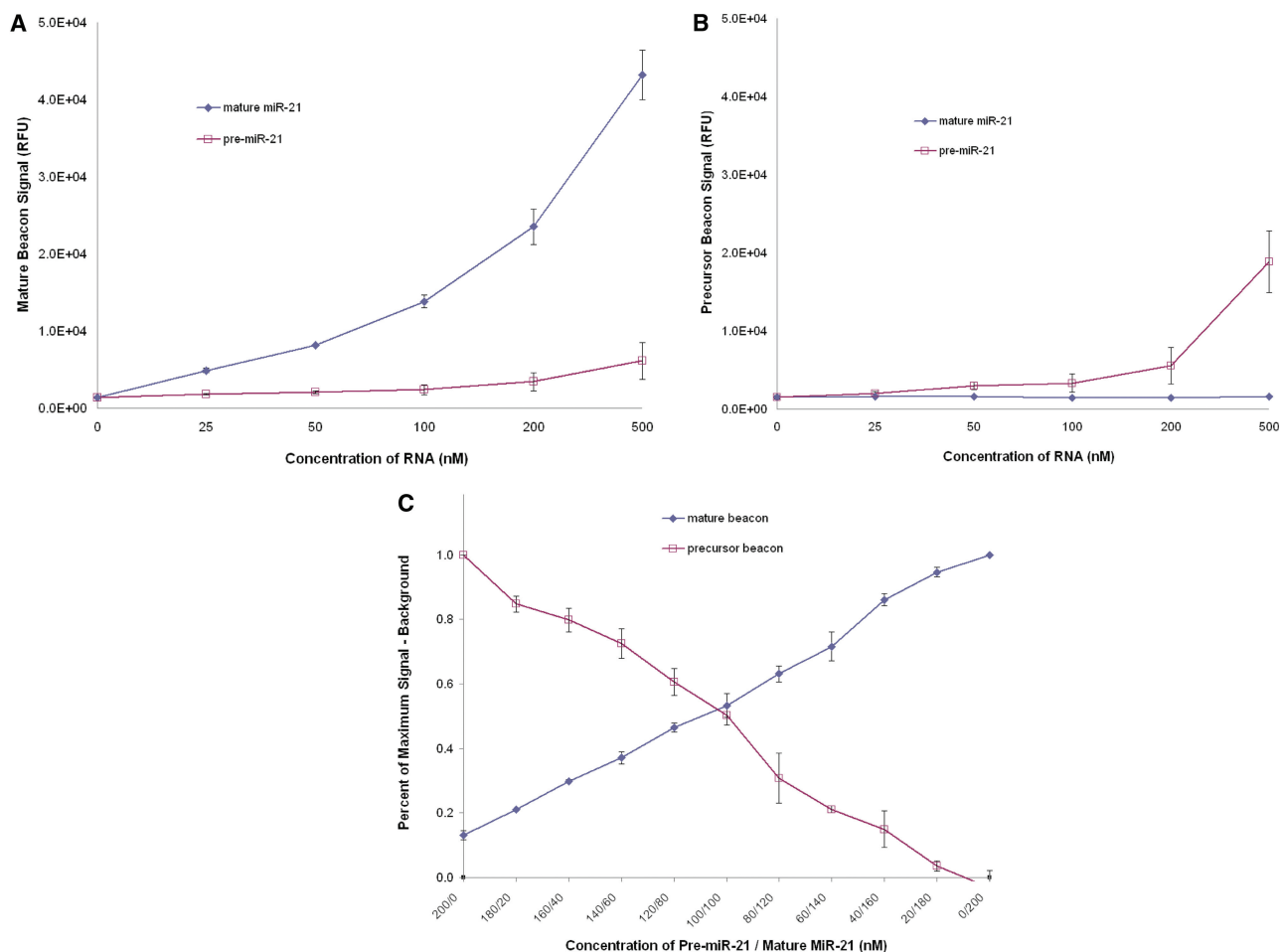
We found that, at 37°C, molecular beacons targeting the mature miR-21 (mature beacon) hybridized only to the mature form of miR-21 at concentrations <50 nM. Even at higher concentrations of pre-miR-21, the mature beacon predominantly hybridized to the mature miR-21 so that the two populations of miR-21 could be readily differentiated (Figure 2A and B).

To further demonstrate the specificity of the beacons for mature versus pre-miRNA, beacons hybridizing to either mature miR-21 or pre-miR-21 were incubated with mixed solutions of mature miR-21 and pre-miR-21 (Figure 2C). We found that the beacon designed to hybridize to mature miR-21 produced a very low level of fluorescence signal when incubated with solution containing only pre-miR-21 (of 200 nM concentration). However, this signal increased linearly as the concentration of mature miR-21 in the mixture was increased to 200 nM (Figure 2C). Conversely, the fluorescence signal produced by the beacon designed to hybridize to precursor miR-21 decreased linearly as the concentration of pre-miR-21 in the mixture was decreased (Figure 2C). These data clearly demonstrate that molecular beacons designed to bind to different forms of miR-21 can distinguish mature miR-21 and pre-miR-21.

### Molecular beacon specificity

To determine whether the molecular beacons designed for targeting mature miR-21 are specific for this miRNA, we incubated the mature miR-21 beacon with mature miR-155 RNA. As shown in Figure 3A, the mature miR-21 beacon hybridized only to mature miR-21, with very low fluorescence signal from mature miR-155 RNA of increasing concentration. Both conventional DNA beacons and LNA beacons showed a similar specificity (Figure 3B).

We assessed the ability of molecular beacons to detect other specific miRNAs. DNA beacons complementary to mature miR-24 or miR-27b were designed, synthesized and assessed in hybridization assays. We found that fluorescence signal from the mature miR-24 beacons increased linearly as the concentration of mature miR-24 increased. There was negligible signal from the mature miR-24 beacons in the presence of the mature miR-27b sequence (Figure 3C). Likewise, the mature miR-27b beacon demonstrated a linear increase in signal when incubated

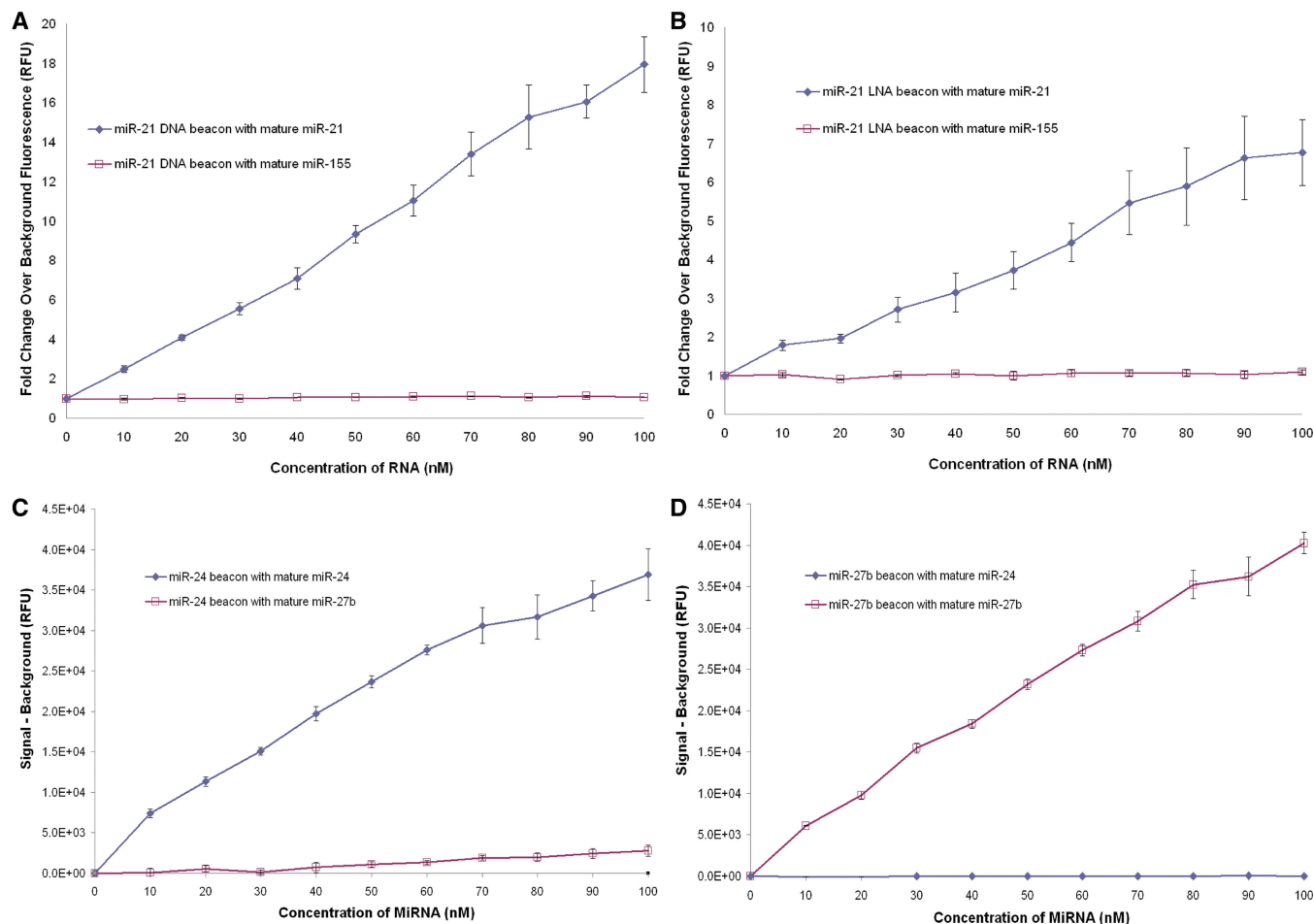


**Figure 2.** Hybridization of miR-21 molecular beacons to mature or precursor miR-21 RNA. The mature miR-21 DNA beacon (A, 200 nM) or precursor miR-21 DNA beacon (B, 200 nM) was incubated with the indicated concentrations of mature miR-21 RNA or pre-miR-21 RNA (60 min at 37°C). The relative fluorescence signal from each molecular beacon alone in PBS was used as a background measurement and subtracted from the sample signal. (C) Mature or pre-miR-21 beacon hybridized to mixtures of mature RNA and pre-miR-21 (0–200 nM). Each data point represents mean  $\pm$  SEM of three separate experiments.

with increasing concentrations of the mature miR-27b, and this beacon did not emit signal when incubated with mature miR-24 (Figure 3D). Interestingly, in humans, these two miRNAs are located within the same intronic sequence on chromosome 9. These results further demonstrated the specificity of molecular beacons in detecting miRNAs.

With proper design, molecular beacons have the potential to distinguish single base variations in target mRNA sequence (33), although having a high enough (>5) signal-to-background ratio in discriminating between closely related RNA species is still a significant challenge. To show the potential of molecular beacons in distinguishing small variations in miRNA sequence, melting curve experiments were performed with mutated mature miR-21 or pre-miR-21. The mature miR-21 DNA beacon hybridized to both the wild-type and mutant mature miR-21, but the melting temperature of the duplex formed by the mature beacon and mature miR-21 decreased as the number of point mutations increased (Figure 4A). In contrast, the mature miR-21

DNA beacon had a similar affinity for different mutants of pre-miR-21 (Figure 4B) because the mutations did not affect the beacon hybridization site. As expected, the DNA beacon complementary to pre-miR-21 did not hybridize to wild-type or mutant mature miR-21 (Figure 4C). The pre-miR-21 DNA beacon hybridized to wild-type and mutated pre-miR-21; however, the mutants of pre-miR-21 containing 3- or 5-point mutations showed decreased melting temperatures for their duplexes with the pre-miRNA beacon (Figure 4D). The melting temperature of the duplex formed by the mutant pre-miR-21 p1 (Table 2) and the pre-miR-21 DNA beacon was not altered from that of the wild-type pre-miRNA sequence and the precursor miR-21 DNA beacon (Figure 4D). The lack of change in the melting temperature between the wild-type pre-miRNA and the p1 mutant may be due to the location of the mutation. The single point mutation was located near the end of the precursor beacon hybridization region where the beacon may not have a strong interaction with the pre-miRNA. However, the pre-miRNA beacon showed differences with



**Figure 3.** Specific hybridization of mature miRNA molecular beacons. The mature miR-21 DNA molecular beacon (**A**, 200 nM) or the mature miR-21 LNA molecular beacon (**B**, 200 nM) was incubated (37°C for 60 min) with mature miR-21 RNA or mature miR-155 RNA. The mature miR-24 DNA molecular beacon (**C**, 200 nM) or the mature miR-27b DNA molecular beacon (**D**, 200 nM) was incubated with the mature miR-24 or mature miR-27b at 37°C for 60 min. Each data point represents mean  $\pm$  SEM of three separate experiments.

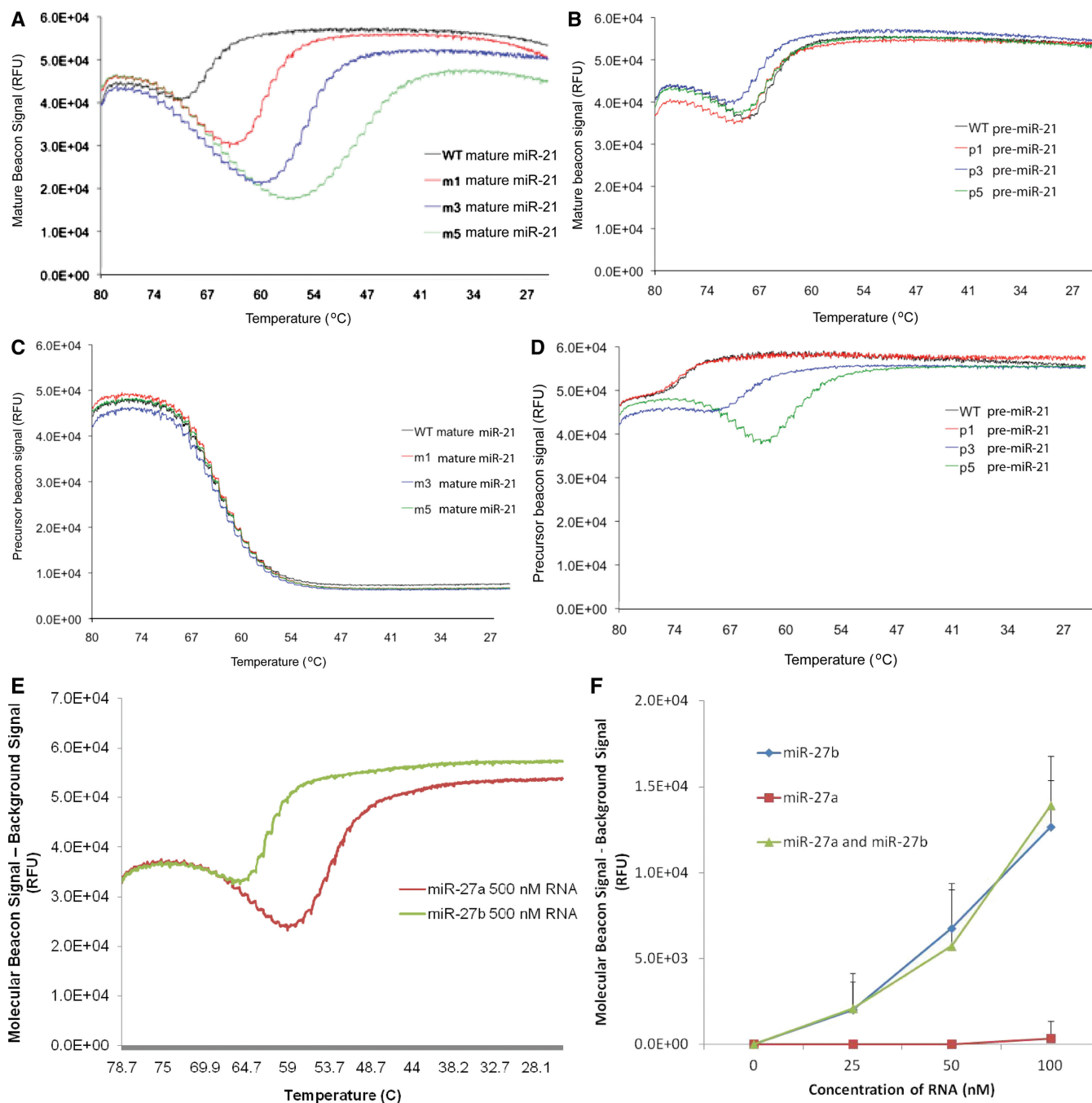
mutant targets containing point mutations in the middle of the beacon hybridization site. These findings indicate that the miRNA-targeting molecular beacons are specific, and have the potential to distinguish miRNAs with close sequence homology.

We further assessed the specificity of the mature miR-27b beacon by determining its ability to hybridize to miR-27b and miR-27a in a background of total RNA extracted from endothelial cells (Figure 4E and F). miR-27b and miR-27a are found in the same gene cluster and differ by only one nucleotide (Table 2). In this analysis, the temperatures at which miR-27b beacon dissociated from miR27b were higher than those at which it dissociated from miR-27a (Figure 4E, the low points of each curve), suggesting that the miR-27b molecular beacon has the potential to distinguish miR-27b from miR-27a in a background of total RNA extract if the hybridization assay was performed above the disassociation temperature of miR-27a. Indeed, in hybridization assays at 60°C where the miR-27b molecular beacon was incubated with respectively miR-27b, miR-27a, or an equal mixture of miR-27b and miR-27a at increasing

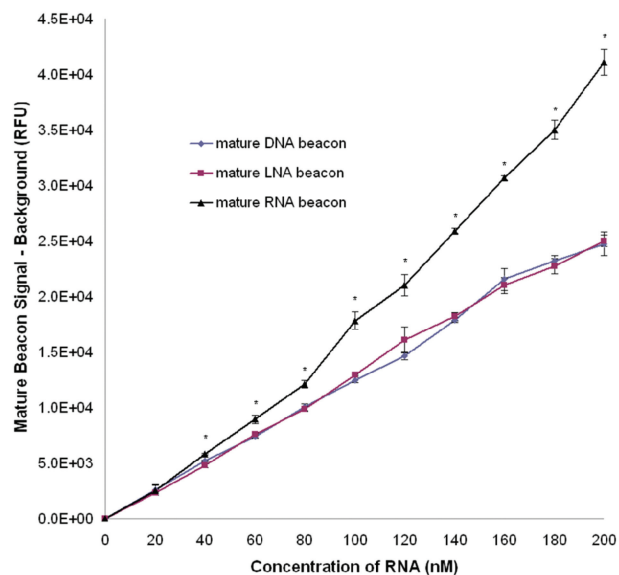
concentrations, the fluorescence signals obtained were essentially due to its hybridization to miR-27b (Figure 4F).

### The effect of molecular beacon backbone chemistry

Molecular beacons may be composed of DNA or RNA backbones. It is well established that RNA/RNA complexes have a higher affinity than DNA/RNA complexes. In a previous study we confirmed that molecular beacons with RNA (2'-O-methyl RNA) backbone have a higher affinity for RNA than that of molecular beacons with DNA backbone (34). In addition, there have been reports illustrating enhanced affinity of LNA bases for oligonucleotides in cells (35). Figure 5 shows the relative fluorescence intensity of mature miR-21 molecular beacons with different (DNA versus RNA versus LNA) backbones in the presence of increasing concentrations of mature miR-21. At mature miR-21 concentrations >10 nM, the RNA beacon showed up to 1.7-fold higher fluorescence intensity compared to that of the DNA or DNA-LNA beacon, whereas the signal intensities from DNA beacons and DNA-LNA beacons were similar.



**Figure 4.** Analysis of the DNA molecular beacons binding to miR-21 mutants or closely related miRNA family members. Mature miR-21 mutants, m1, m3 and m5, had 1-, 3-, or 5-point mutations in their sequences regions, respectively. The pre-miR-21 mutants, p1, p3 and p5, had 1-, 3-, or 5-point mutations in the hairpin loop region of the double-stranded structure, respectively. Molecular beacons (200 nM) were incubated with the mature miR-21 or pre-miR-21 sequences (500 nM) for 3 min at 95°C, cooled to 80°C, and then the temperature was lowered by 1°C/min. (A) Fluorescence of mature miR-21 DNA molecular beacon with wild-type and mutant mature sequences. (B) Fluorescence of mature molecular beacon incubated with wild-type and mutant pre-miR-21s. (C) Fluorescence of the pre-miR-21 DNA molecular beacon with wild-type and mutant mature sequences. (D) Fluorescence of pre-miR-21 molecular beacon with wild-type and mutant pre-miR-21. The melting temperature for each molecular beacon complex is shown as the steepest slope of the graph. Each melting curve is representative of three separate experiments. (E) Melting curves for mature miR-27b DNA molecular beacon and mature miR-27b or mature miR-27a in the background of total cellular RNA. The mature miR-27b molecular beacon (200 nM) was incubated with 0.5 µg total cellular RNA + 500 nM miR-27a or 0.5 µg total cellular RNA + 500 nM miR-27b. The hybridization mixtures were heated to 95°C for 3 min, cooled to 80°C, and then the temperature was decreased by 1°C per minute until the mixture reached 25°C. Fluorescence values were recorded continuously from 80°C to 25°C. (F) Binding of the mature miR-27b molecular beacon to miR-27b or miR-27a at 60°C. The mature miR-27b DNA molecular beacon (200 nM) was incubated with mature miR-27a RNA (red line), mature miR-27b RNA (blue line), or a 1:1 mixture of both (green line) at concentrations of 25 nM, 50 nM, and 100 nM. Each reaction was heated to 95°C for 3 min and cooled to 60°C. The fluorescence signal of each reaction was determined after a 10-min incubation at 60°C. Background fluorescence was subtracted from each data point. Each data point represents mean ± SEM of three separate experiments.



**Figure 5.** Relative fluorescence intensity of mature miR-21 molecular beacons with different backbone chemistries (DNA versus RNA versus LNA) hybridized to mature miR-21 RNA. The different molecular beacons (200 nM) were incubated with miR-21 in PBS at 37°C for 60 min prior to recording the fluorescence intensity. For each experiment, background fluorescence was subtracted from mature beacon signal and each data point represents mean  $\pm$  SEM of three separate experiments. \* $P < 0.005$ , RNA beacon versus LNA beacon.

At very low concentrations of mature miR-21 RNA, from 0 to 10 nM, the mature miR-21 RNA beacon had higher relative fluorescence intensity than the DNA or DNA–LNA beacon (Figure 6A). In the presence of pre-miR-21, the mature miR-21 RNA beacon also had a higher fluorescence signal at most concentrations, however the overall signal for each beacon was very low and not much above background fluorescence (Figure 6B). Likewise, the pre-miR-21 beacons did not hybridize to mature miR-21 (Figure 6C). However, the pre-miR-21 beacons hybridized to pre-miR-21 with a detectable fluorescence signal (Figure 6D). Interestingly, there was no significant difference in hybridization of the pre-miR-21 RNA beacon compared to hybridization of the pre-miR-21 DNA–LNA beacon or the pre-miR-21 DNA beacon, although the DNA–LNA beacon gave increased signal intensity compared to the other pre-miR-21 beacons. This may be due to the more rigid backbone of the DNA–LNA beacon and its ability to denature the hairpin of the pre-miR-21.

### Molecular beacon hybridization to miRNA in heterogeneous RNA sample

To demonstrate that molecular beacons have the sensitivity and specificity to detect mature miRNA and pre-miRNA in a heterogeneous RNA sample, we incubated the mature miR-21 DNA beacon or the pre-miR-21 DNA beacon with total RNA that had been isolated from human umbilical vein endothelial cells (HUVECs) subjected to unidirectional shear stress (15 dynes/cm<sup>2</sup>, 24 h) or static conditions. We have previously shown by

qRT–PCR that miR-21 levels were increased in HUVECs subjected to shear stress (36). Figure 7A shows the fluorescence signals from hybridization assays using the mature miR-21 DNA beacon. As the concentration of total RNA used in the assay was increased from 1 to 5  $\mu$ g, there was an increase in fluorescence signal from the mature miR-21 beacon. Furthermore, with the higher concentrations of total RNA in the solution, a 2- to 3-fold increase in beacon fluorescence was observed for RNA from sheared cells compared to RNA from control cells. These results indicate that the mature miR-21 molecular beacon has the sensitivity and specificity required to detect shear stress induced changes in miR-21 level in HUVECs.

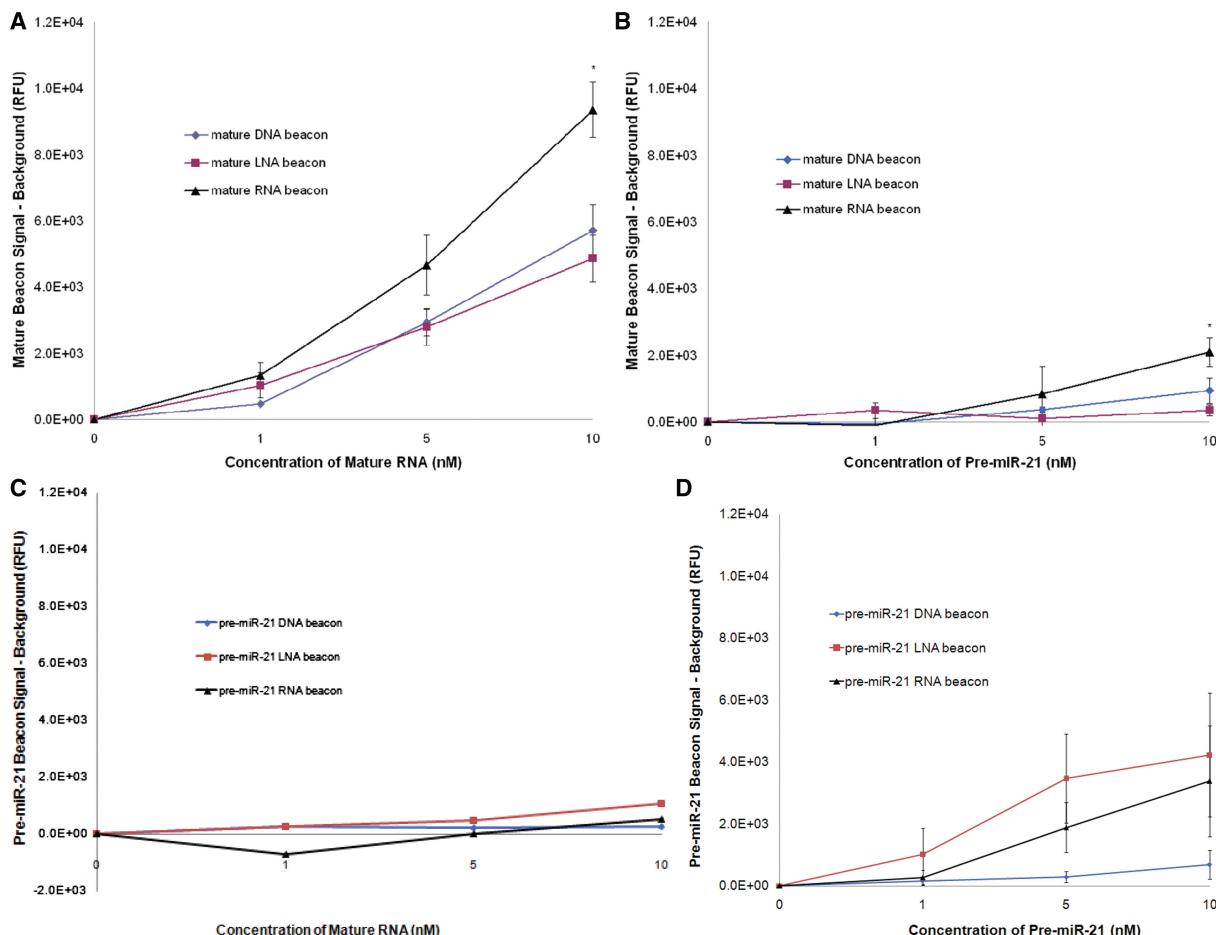
We performed hybridization assays using the pre-miR-21 DNA beacon with total RNA from HUVECs, and observed an increase in signal as the amount of total RNA was increased (Figure 7B). No fluorescence of the beacon was detected with 1  $\mu$ g of RNA, but the beacon did fluoresce at higher concentrations of total RNA. In fact, hybridization assays using total RNA from sheared cells had increased fluorescence compared to those using control RNA, suggesting that unidirectional shear stress increased pre-miR-21 levels. This is a very novel finding and is currently being investigated further.

qRT–PCR is the standard technique to assess miRNA expression, therefore we compared this method with the molecular beacon based method in their ability to assess shear stress induced changes in miR-21, miR-24 and miR-27b. As with miR-21, we have previously shown that miR-24 and miR-27b are increased in HUVECs subjected to unidirectional shear stress (36). Figure 8A shows that, for all these three miRNAs, the relative increase in miRNA expression induced by shear stress (3-fold for miR-21, 1.7-fold for miR-24, 1.5-fold for miR-27b) was similar using two different methods, qRT–PCR and molecular beacons (targeting mature miRNA). Figure 8B depicts the correlation of the results obtained using qRT–PCR and that using three molecular beacons described in this study. Molecular beacons were able to distinguish small fold changes in miRNA expression in the background of total RNA, similar to that of PCR. These findings support the validity of using the molecular beacon based method to assess miRNA expression in cells and tissues.

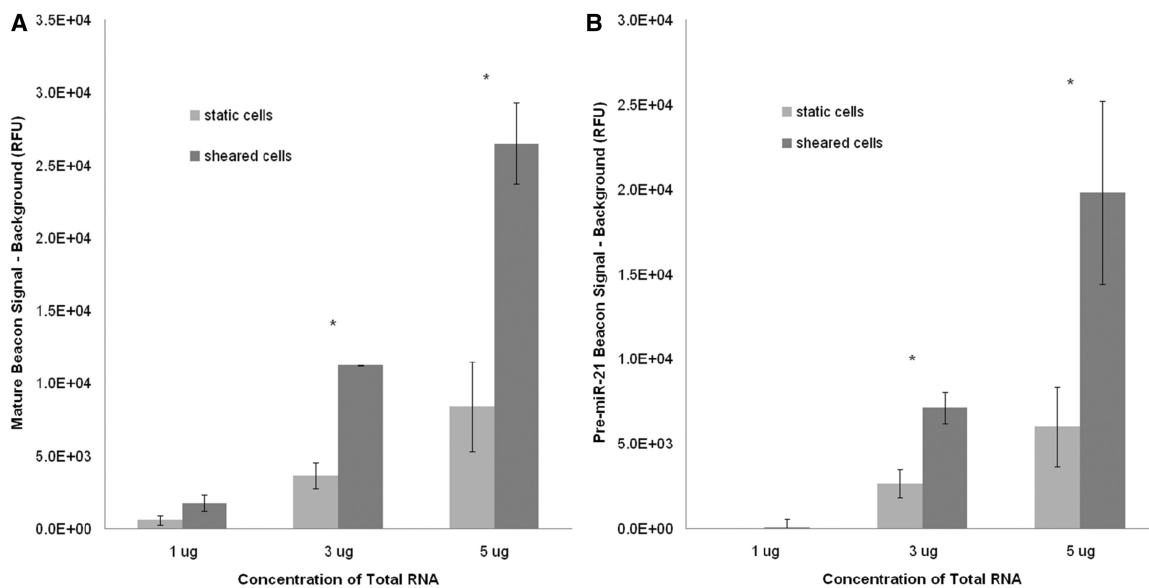
## DISCUSSION

In this study, we demonstrated that molecular beacons designed to detect a specific miRNA can effectively and efficiently assess miRNA levels as well as distinguish between mature miRNA and pre-miRNA species. Therefore, molecular beacons have the potential to be powerful tools for quantifying miRNA expression in biological specimens. Although most of this study was focused on miR-21, we showed that molecular beacons could be designed for other specific miRNAs as well. These results may have significant implications for expression profiling of many miRNAs. Specifically, we were able to show that the beacon-based method is effective for detecting miRNAs with a range of GC content (miR-21,

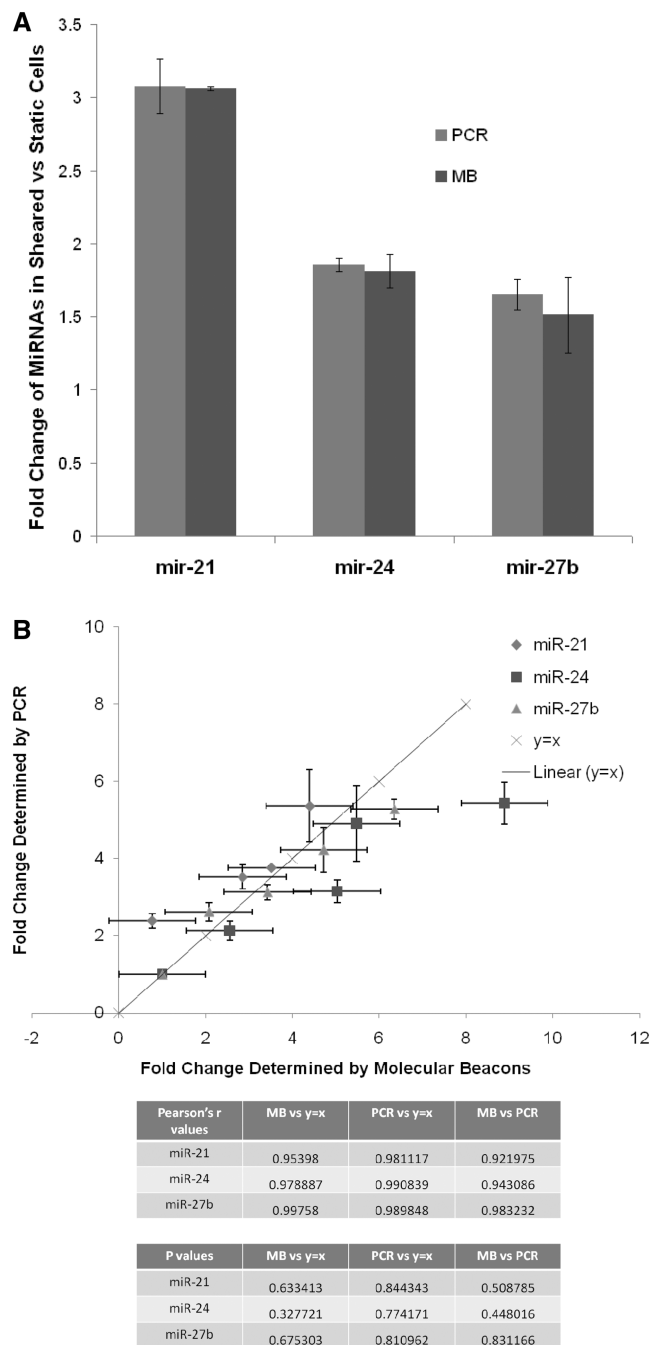




**Figure 6.** Differences in sensitivity among miR-21 beacons with varying backbone chemistries. Molecular beacons (200 nM) were incubated with miR-21 RNA (0–10 nM) in PBS at 37°C for 60 min prior to recording the fluorescence intensity. For each experiment, background fluorescence was subtracted from beacon signal and each data point represents mean ± SEM of three separate experiments. (A) The different mature miR-21 molecular beacons incubated with mature miR-21 RNA (0–10 nM), \**P* = 0.006 RNA beacon versus DNA beacon. (B) Mature miR-21 beacons incubated with pre-miR-21 (1–10 nM), \**P* = 0.004, RNA beacon versus DNA beacon. (C) The precursor miR-21 molecular beacons incubated with mature miR-21. (D) Precursor miR-21 molecular beacons incubated with pre-miR-21.



**Figure 7.** The use of molecular beacons to assess miR-21 expression in RNA isolated from HUVECs. Total RNA was extracted from HUVECs subjected to static conditions or 24 h of unidirectional shear (15 dynes/cm<sup>2</sup>). (A) Molecular beacon hybridization assay was performed using the mature miR-21 DNA beacon (200 nM) and different amounts of HUVEC total RNA. \**P* < 0.05 sheared cells versus static cells. (B) hybridization assay performed using pre-miR-21 DNA beacon (200 nM) and different amounts of HUVEC total RNA. \**P* < 0.05, sheared versus static cells. For each experiment, background fluorescence was subtracted from beacon signal and each data point represents mean ± SEM of three separate experiments.



**Figure 8.** Comparison of qRT-PCR with molecular beacons in assessing miRNA levels. (A) Mature miRNA levels in total RNA extracted from static and sheared (15 dynes/cm<sup>2</sup>) HUVECs. For each qRT-PCR reaction, 0.05  $\mu$ g of RNA was used and PCR was performed using TaqMan miRNA probes. Molecular beacon hybridization assays were performed with 200 nM beacon and 1  $\mu$ g of total RNA. For each experiment, background fluorescence was subtracted from beacon signal and each data point represents mean  $\pm$  SEM of three separate experiments. (B) Scatter plot of miRNA expression levels from qRT-PCR versus that of molecular beacon hybridization assays in total RNA from HUVECs. For each qRT-PCR reaction, 10–50 ng of RNA was used for the RT reaction and PCR was performed using TaqMan miRNA probes against miR-21, miR-24 and miR-27b. The PCR results were normalized to RNU48 expression levels. Molecular beacon hybridization assays were performed with 200 nM beacon and 50 ng of total RNA with 1–5 nM mature miRNA. For each experiment, background fluorescence was subtracted from beacon signal and each data point represents mean of three separate experiments. A Pearson's

36%; miR-24, 54.5%; miR-27b, 47.6%). This is important since beacon-miRNA hybridization affinity is enhanced with increased GC content.

When designing molecular beacons to detect specific messenger RNAs, a BLAST analysis is typically used to identify multiple sequences within the target transcript that are both unique and capable of hybridizing to a complementary probe sequence with high affinity. However, for molecular beacons designed to detect mature miRNAs, there is little flexibility in choosing the hybridization sequence of the beacon, since the length of miRNAs (21–25 nt) is similar to the probe length of a typical beacon. Furthermore, the melting temperature of the beacon-miRNA duplex is largely determined by the GC content of the miRNA, which ranges from 35% to 55% for most widely expressed miRNAs. These features of miRNA require that molecular beacon design be optimized for each mature miRNA and pre-miRNA.

The molecular beacons designed in this study have a stem length of 6 nt, and in the case of the mature miR-21 beacon, the first base of the stem was shared with the beacon loop hybridization sequence. We found that a stem length of 5–6 nt and beacon melting temperature of 55–65°C were optimal for beacon hybridization, and we expect that, in general, stem lengths for molecular beacons that bind to miRNAs could be 4–7 nt (24). Despite the limited ability to modify the melting temperatures of the beacon-miRNA duplex, the three miR-21-targeting molecular beacons with DNA, RNA and DNA-LNA backbones respectively, were sensitive and specific, as demonstrated by the ability to detect low levels of miR-21 (1 nM) and distinguish miRNAs with slight variations in nucleotide sequence.

Most miRNAs identified thus far belong to families that have miRNA sequences differing by <3 nt. Although miRNA family members may have the same or similar mRNA targets and miRNAs derived from a single pri-miRNA may be similarly regulated, studies have shown that this is not always the case (37,38). Thus, accurate profiling of miRNA expression levels requires a method that can distinguish individual members of a miRNA family. Currently, the most common assay for profiling miRNA expression levels involves microarray analysis. However, microarrays frequently have cross-hybridization problems in which the probes cannot distinguish miRNAs that differ by only a few nucleotides (38). Molecular beacons can be designed to hybridize to specific miRNAs that have some homology (e.g. in the same family), although cross-hybridization is still an issue. However, as we show for miR-27a/27b, molecular beacons can hybridize to different miRNAs in the same family with high specificity when the optimal hybridization temperatures are used. Molecular beacons also have

**Figure 8.** Continued

coefficient was calculated for each miRNA by comparing the PCR fold change values to  $y = x$ , the MB fold change values to  $y = x$ , and the PCR fold change values to the molecular beacon fold change values. *P*-values also were calculated for each data set comparison. MB = molecular beacon.

the potential as a useful tool to study single nucleotide polymorphisms (SNPs) in miRNA precursors by distinguishing single point mutations in pri-miRNA or the pre-miRNA forms. It has been shown that the presence of SNPs may have important pathophysiologic consequences because they affect miRNA processing or hybridization of mature miRNAs to target mRNA (39–41). However, in this case, molecular beacons have to be carefully designed and the hybridization temperatures optimized.

Molecular beacons have been used successfully to detect and quantify mRNA in living cells (42). However, it remains a challenge to use these probes to assess miRNA levels in living cells due to low abundance (typically <5 nM). Although for fixed cells this difficulty could be overcome by using a signal amplification method (43), the current design of molecular beacons with organic fluorophores suffers from low signal-to-background ratio, and imaging miRNA in living cells is currently not possible (data not shown). This limitation may be mitigated through the use of advanced fluorophores such as quantum dots (44), or an amplification strategy.

The most important features of the molecular beacon based method are: (i) it can be used to assess relative levels of mature miRNA and pre-miRNA in total RNAs extracted from cells or tissue; (ii) it can detect multiple miRNAs in the same sample, as demonstrated in this study; and (iii) once beacon design is optimized, the assessment of miRNA levels can be performed in 20–30 min. Clearly, in the current design, the amount of RNA needed for sensitive miRNA detection using molecular beacons is much higher than that for a PCR assay; however, molecular beacons have the potential to assess the expression of multiple miRNAs in a single sample. It is also quite feasible to perform a hybridization assay with mature and pre-miRNA molecular beacons that have fluorophores with non-overlapping spectra and determine the relative levels of these two forms of miRNA in the same RNA sample. Therefore, molecular beacon based miRNA detection may offer a hybridization assay format that is sensitive, specific, fast, easy to multiplex, and capable of distinguishing mature and pre-miRNAs.

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