microRNA sensors

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Abstract We have developed a technology for the profiling of miRNA expression in intact cells. The approach is based on sensor oligonucleotides, which upon entering the cell, bind specific miRNA targets, are cleaved as a result of this binding, and produce a fluorescent signal that is proportional to the abundance of the miRNA target. Specifically, the sensor oligonucleotides are completely complementary to a target miRNA species, are non-stabilized around the seed region (the region cleaved by the miRNA-RISC), and are labeled with a fluorescent dye and a quencher at their 5'- and 3'- end respectively. Upon entering the cell, these oligonucleotides engage the target miRNA by complementary base pairing. This leads to recruitment of the RNA induced silencing complex (RISC) to the duplex. The complex cleaves the sensor oligonucleotide and the miRNA is free to "catalyze" subsequent clevage reactions. The cleavage of the sensor oligo leads to separation between the dye and the quencher, and a resultant fluorescent enhancement that can be measured.
We have demonstrated the feasibility of this method for the sensing of the pro-metastatic miRNA-10b in cell-free extracts and intact cells using human and murine breast adenocarcinoma cell lines.
The miRNA epigenome represents a fundamental molecular regulator of metastasis. Consequently, developing tools to understand metastatic changes at the miRNA level can lead to the mapping out of a comprehensive and systematic atlas of cancer progression. The described technology is potentially transformative because it addresses this important issue. Furthermore, the technology has broad implications and can be utilized in any model system or clinical scenario to answer questions related to microRNA function. Specifically, the technology can help distinguish, assess, and/or monitor cancer stages and progression; aid the elucidation of basic mechanisms underlying cancer initiation and progression; facilitate early cancer detection and/or cancer risk assessment; and facilitate/accelerate the process of drug discovery.

Keywords: cancer, diagnosis, miRNA, optical imaging, metastasis, fluorescence, dye, quencher

1. Introduction

During the past decade, over 25,000 papers deposited on PubMed have reported on various aspects of miRNA genomics, biogenesis, mechanisms of action, pathway involvement, phenotypes in experimental models and disease abnormalities. About 40% of these publications have focused on the role of miRNAs in cancer. These studies have shown that miRNAs are dysregulated in almost all types of human cancer and specific signatures of aberrantly expressed miRNAs harbour diagnostic, prognostic and theranostic implications, including early diagnosis and staging, discrimination between different types of cancer, the identification of the tissue of origin of poorly differentiated tumors, prediction of chemotherapeutic response, etc. These are all challenges that are still largely unresolved and the subject of intense clinical interest.

Overwhelming evidence in the literature indicates that miRNAs are excellent biomarkers for any of these processes (Ling et al., 2013). Consequently, developing tools to understand how miRNAs participate in key aspects of carcinogenesis can lead to the mapping out of a comprehensive and systematic atlas of cancer progression.

Cinically, we envision a variety of scenarios that could benefit from the ability to profile miRNA exression. For example, it is now known that miR-10b is upregulated in the tumors of patients who harbor

metastatic disease, indicating that miR-10b could be used to diagnose the presence of metastasis or even to forecast invasive disease (Baffa et al., 2009; Chen et al., 2013; Iyevleva et al., 2012). The ability to detect miR-10b upregulation in the primary tumors by noninvasive or laparoscopic methods that specifically target a suspected lesion would be very clinically important. miR-10b and -155 are also part of a panel of miRNAs that demonstrate aberrant expression in tissue from patients with triple negative breast cancer that displays chemoresistance (Ouyang et al., 2014). In this context, noninvasive or laparoscopic imaging can help guide therapeutic decisions.

With the goal of making such diagnostics possible, we have developed technology for the profiling of tumor miRNAs in intact cells. The technology is based on sensors capable of reporting on miRNA expression. It is described in detail in (Yoo et al., 2014). In our studies, we have focused on a miRNA involved in the early stages of breast cancer metastasis. However, the developed methodology can be applied broadly to study miRNA regulation of any process related to carcinogenesis and response to therapy.

The technology is significant because it allows one to profile tumor miRNA signatures in intact cells. This is important because it is only through such studies that one can capture the true dynamics of molecular processes. Since the method is noninvasive to the cell, it will ultimately be possible to collect time-course data in genuine cellular environments and thus gain a better understanding of long-term trends.

In a laboratory setting, this new capability holds potential to:

1. help distinguish, assess, and/or monitor cancer stages and progression;

2. aid the elucidation of basic mechanisms underlying cancer initiation and progression.

The technology also has clinical relevance. The sensors can be delivered in vivo by conjugating them to a carrier. Consequently, a derivative of the developed technology may ultimately become applicable in a clinical setting and as such has the potential of significantly advancing our ability to diagnose and treat breast cancer. The clinical feasibility of the method is underscored further by the fact that optical imaging methods have already been applied for clinical breast imaging (Manohar et al., 2007; Poellinger et al., 2008; Soliman et al.; van de Ven et al., 2009). Alternatively, one can envision a utility for our method using laparoscopic or intraoperative diagnostics.

In a clinical setting, the technology could:

1. facilitate early cancer detection and/or cancer risk assessment; and

2. facilitate/accelerate the process of drug discovery.

2. The sensor can report on individual miRNA expression with high sensitivity and specificity (cell-free system) (Yoo et al., 2014).

To assess the feasibility of the proposed approach, we performed a study in which we designed a cleavable sensor oligo to detect miR-10b expression. MiR-10b has been implicated in epithelial to mesenchymal transition and breast cancer metastasis by multiple studies including by our own group (Yigit et al., 2013)' (Baffa et al., 2009; Ma et al., 2010; Ma et al., 2007). Considering that the focus of the application is to profile the expression of pro-metastatic miRNAs in breast tumor cells, we targeted miRNA-10b in our studies. Specifically, we designed an RNA sensor oligonucleotide that was completely complementary to the miRNA-10b seed region and was conjugated to a Cy5 dye at the 5' end and Iowa Black RQ quencher at the 3' end separated by 22 nucleotides (7.5 nm). Its sequence was: AC AAA UUC GGU UCU ACA GGG UA. First we determined the signal-to-background ratio by incubating 100nM of the sensor oligo with a 10μg/ml of nuclease. Cleavage of the oligo resulted in a 566% fluorescence enhancement after de-quenching compared to the noncleaved oligo incubated in RNase-free conditions.

To determine the sensitivity and specificity of detection, we designed a cell-free assay, based on Refs (Brown et al., 2005; Robb et al., 2005). Studies were performed in the metastatic human breast cancer cell line MDA-MB-231-luc-D3H2LN (Caliper Life Sciences, Hopkinton, MA). Briefly, the cells were lysed and cytoplasmic extracts prepared, as described in (Brown et al., 2005; Robb et al., 2005) Half of the extracts were incubated with a 10-fold excess of anti-miR10b locked nucleic acid (LNA) antisense oligonucleotides to miR-10b (ASO, Exiqon, Woburn, MA). LNA modification prevents these ASO from cleavage by miRNA and instead leads to inhibition of miRNA function (termed miR-10b-depleted control). The second half of the cell extracts was incubated with irrelevant oligo (SCR ASO), which does not bind miR-10b or any other of the annotated miRNAs and leaves miR-10b active.

Following, the miR-10b sensor oligo was titrated into the miR-10b-depleted and miR-10b-active extracts Fluorescence from the sensor oligo was then recorded using epifluorescence optical imaging (Ivis Spectrum, 649 nm excitation; 670nm emission).

There was distinctive fluorescence enhancement with increased concentration of the sensor oligo in the extracts in which miR-10b was active after treatment with irrelevant oligo (SCR ASO). On the contrary, the extracts in which miRNA-10b was depleted (inhibited) by incubation with miR-10b ASO the signal was significantly lower. Using linear regression analysis, we calculated a detection limit of 13.4nM.

Finally, we confirmed cleavage of the sensor oligo by miR-10b-RISC using gel electrophoresis. This indicated that the observed fluorescence enhancement is the result of cleavage of the sensor oligo and confirmed that miR-10b can mediate cleavage of the substrate oligo as designed, despite the fact that none of its known mRNA targets are regulated by cleavage. Electrophoresis was performed as described in (Brown et al., 2005; Robb et al., 2005).

3. The sensor can report on miRNA expression in intact cells (Yoo et al., 2014).

To test the hypothesis that the sensor as designed will be capable of reporting on miRNA expression in intact cells, we incubated MDA-MB-231-luc-D3H2LN cells with miR-10b-specific sensor at concentrations ranging from 25 to 1000nM. MiR-10b depleted cells obtained by inhibiting miR-10b with ASO (using lipofectamine transfection) served as control. There was a significant difference in fluorescence intensity between the cells treated with the sensor specific to miR-10b and the miR-10b-depleted control cells ($p<0.05$). These differences were independent of sensor concentration above 250nM.

To estimate the rate of sensor "turn-on" we performed a time course study at fixed sensor concentration of 125 nM. Maximum fluorescence intensity was reached within 16 hrs. In control cells depleted of miR-10b, the rate of sensor cleavage (non specific) was low and, even after 48 hrs of incubation, the fluorescence intensity did not reach maximum.

Fluorescence microscopy revealed that cells could easily be delineated based on fluorescence from the sensor. Finally, as an illustration of a related application, flow cytometry was used to compare cells treated with sensor alone or pretreated with the ASO. That experiment confirmed our observations using epifluorescence that the approach could quantitatively reflect differences in miRNA expression. Irrespective of the method used (flow cytometry or epifluorescence), the miRNA expression as measured by our approach was accurate because it fell within one standard deviation of that measured by the gold-standard qRT-PCR.

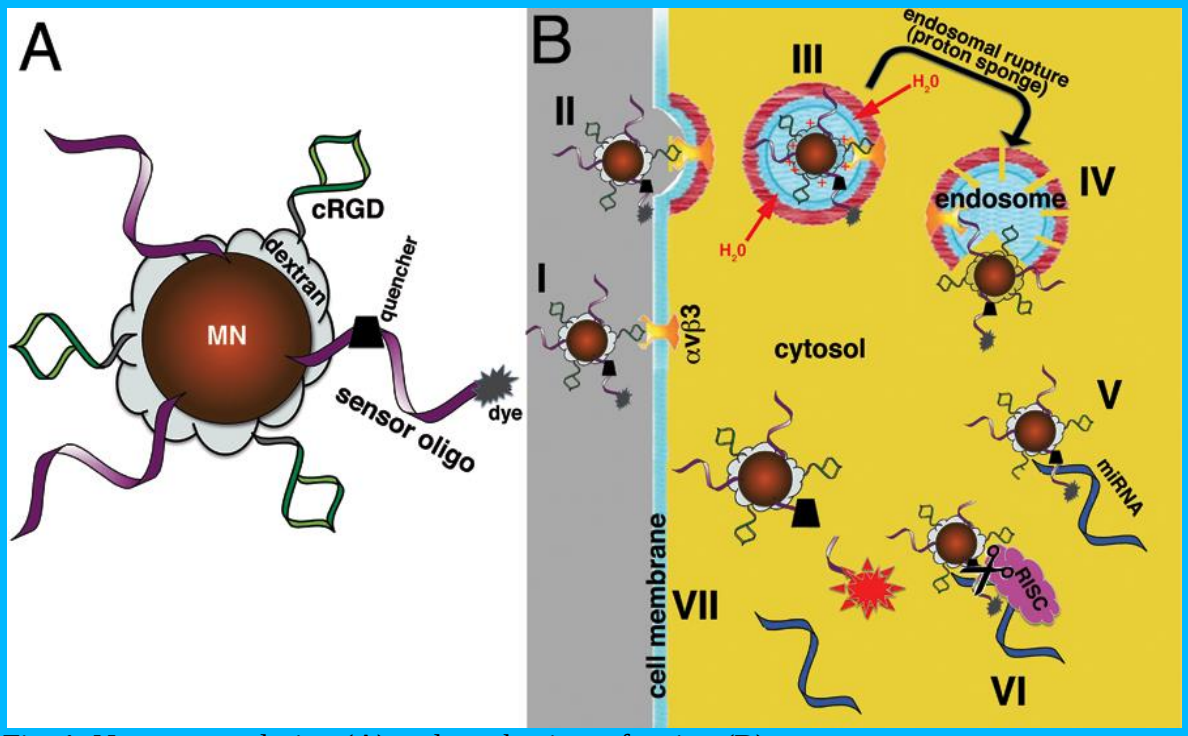

Fig. **1**. Nanosensor design (A) and mechanism of action (B).

4. In vivo application

The in vivo applicability of the sensor derives from a nanodelivery vector described extensively in our prior publications [Kumar, 2011 #30; Medarova, 2007 #29; Yigit, 2013 #34]. Specifically, we have developed nanoparticles that accumulate in tumor cells, following intravenous injection. Upon internalization of the nanoparticles by the cell, the nanoparticles efficiently engage the endogenous cytosolic RNA interference apparatus in a sequence-specific way.

The nanoparticles consist of dextran-coated iron oxide crystals (MN, 25-30 nm in diameter), conjugated to sensor oligos that are complementary to endogenous miRNA species (Fig. 1A). These sensor oligonucleotides are composed of RNA bases, are cleavable (non-stabilized by chemical modification) around the seed region (the conserved region within which the microRNA engages the RNA substrate), and are labeled with a fluorescent dye-quencher pair, so that upon cleavage of the oligonucleotide by the microRNA-RISC, there is fluorescence enhancement (Fig. 1A).

The proposed mechanism is described in Fig. 1B. Namely, the nanoparticles are endocytosed (Fig. 1BI and BII) by the tumor cells and localize to endosomes (Fig. 1BIII). Inside endosomes, the functionalized nanoparticles rich in unsaturated amines mediate the proton sponge effect by sequestering protons that are supplied by the v-ATPase (proton pump). This process keeps the pump functioning and leads to the retention of one Cl- ion and one water molecule per proton. Subsequent endosomal swelling and rupture leads to particle deposition in the cytoplasm (Fig. 1BIV) 31.

In the cytosol, the nanoparticles, which carry a sensor oligo complementary to an endogenous microRNA species, bind the microRNA (Fig. 1BV), leading to the recruitment to the duplex of the endogenous RNA induced silencing complex (RISC) and cleavage of the oligo at a specific position in the seed region (Fig. 1BVI). This cleavage results in separation between the quencher and dye located at the ends of the sensor oligo, and fluorescent turn-on. The microRNA is released from the complex and

is free to catalyze subsequent cleavage reactions (Fig. 1BVII).

The described mechanism exploits the endogenous process of RNA interference and takes advantage of powerful signal amplification, since millions of nanoparticles/oligos are delivered per cell, using our delivery method.

The in vitro studies are described in detail in our recent publication (Yoo et al., 2014). They indicate that the nanosensor design for in vivo application is feasible and lay the groundwork for in vivo pre-clinical and clinical experiments.

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