MicroDNAs (MIDs) and Transcriptional Regulation

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In this report we are pleased to be the first to introduce “microDNAs”, a new biopharmaceutical concept similar to the newly discovered microRNAs a mechanism by which cell regulates its genetic activities at post-transcriptional level. Synthetic microDNAs are designed to target non-coding region of the gene to interfere with transcription. This can provide a powerful tool to prevent massive production of mRNA by undesired gene activities. Therefore drugs are not required to interact with overwhelming number of mRNA and microRNA copies that may present unwanted side effects. Below we begin with a brief review of microRNAs mechanism and other related gene inhibitors (nucleotides) following with a report on our innovative method of regulating gene activities at transcriptional level.

MicroRNAs (MIRs) and post-transcriptional Regulation

At the level of transcriptional regulation, transcription-factors have expanded during the evolution of gene regulation. The genes are regulated perhaps by multiple transcription factors in higher organisms. At the level of post-transcriptional regulation, new mechanisms of gene regulation have emerged. One such mechanism is the growing class of ~22-nucleotide-long non-coding RNAs, known as microRNAs (miRNAs), which function as repressors in all known animal and plant genomes. Although transcription factors and miRNAs are two of the best studied gene regulatory mechanisms (figure 1), there are many other layers of gene regulation, including: cell signaling; mRNA splicing, polyadenylation and localization; chromatin modifications; and mechanisms of protein localization, modification and degradation.
Figure 1. **Gene regulation by transcription factors and microRNAs.** One or more transcription factors (TF) can bind to cis-regulatory sites in non-coding region of the gene to activate transcription. After transcription, one or more microRNAs bind to cis-regulatory sites of mRNA to prevent translation.

MiRNAs have stirred tremendous excitement in cancer research. In the human genome, close to 1000 mature miRNAs have been identified to this point and it may well increase above thousands in near future. The bioinformatics data indicates that each miRNA can control hundreds of gene targets and they can function as tumor suppressors and oncogenes.

Recent studies show that some miRNAs are directly involved in human cancers, including lung, breast, brain, liver, colon cancer, and leukemia. Overexpressed miRNAs in cancers, such as mir-17-92, may function as oncogenes and promote cancer development by negatively regulating tumor suppressor genes and/or genes that control cell differentiation or apoptosis. Under-expressed miRNAs in cancers, such as let-7, are known to function as tumor suppressor genes and may inhibit cancers by regulating oncogenes. MiRNA expression profiles could be used as biomarkers for cancer diagnostics and miRNA therapy might become useful for cancer prevention and therapeutics. Some miRNAs have been considered to have potential clinical applications as a novel biomarker for breast cancer diagnosis and prognostic factor. Exploitation of the therapeutic potential of RNA interference will be an important task and achieved through the further understanding of the mechanisms of gene regulation by miRNAs.

**RNA interference (RNAi)**

Similar to the repressor activities of endogenous miRNAs, synthetic double and single stranded RNAs have been able to down regulate gene activities at translational or post-transcriptional level. RNAi (RNA interference) refers to a double stranded RNA (dsRNA) to specifically target a gene's product, resulting in
destruction of its mRNA. The use of antisense RNA to interfere with a gene’s activity in *C. elegans* was first utilized by Su Guo and Ken Kemphues\(^\text{14}\) to study *par-1*; however, it was reported that control sense RNA also produced a *par-1* mutant phenotype. Subsequently, it was discovered by Fire et al.\(^\text{15}\) that it is the presence of dsRNA, formed from the annealing of sense and antisense strands present in the *in vitro* RNA prep, which is responsible for producing the interfering activity. Introduction of dsRNA into an adult worm results in the loss of the targeted endogenous mRNA from both the adult and its progeny. This phenomenon has been effectively harnessed to study an ever increasing number of maternal and zygotic genes in *C. elegans*.

Small inhibitory RNA compounds (siRNA) or RNAi have created enthusiasm and much excitement as therapeutic drugs against all gene causing diseases including human cancers. It has been reported (Zamore, P.D. et al.\(^\text{16}\) and Caplen, N.J. et al.\(^\text{17}\)) that synthetic siRNAs are more potent and efficient than traditional antisense compounds.

The striking difference between siRNA and traditional antisense compounds is that siRNA is highly effective without any chemical modifications. The absence of chemical modifications, specifically the phosphorothioate backbone results in a lower toxicity of siRNA compared with antisense oligomers. The longer duration of inhibition coupled with the reduced toxicity of siRNA enables the evaluation of gene function in complex and long-term phenotypic assays in cell culture models and may enable improved efficacy and dosing regimens for therapeutic applications.

Cells are triggered by RNAi to destroy pieces of RNA that match a particular target sequence before being translated into a protein. However some investigators have been worried that RNAi may have unwanted affect on ‘off-target’ genes. Study has found that RNAi could also change expression of RNAs that closely resembled a target RNA (Jackson AL. et al.\(^\text{18}\)). Investigators have also found that RNAi might alter the levels of proteins that are not related to the target RNA (Sccheri PC. et al.\(^\text{19}\)).

In vivo study by Matthias, J. et al.\(^\text{20}\) showed that effective target-gene silencing in the mouse and hamster liver was achieved by systemic administration of synthetic siRNA without any demonstrable effect on the endogenous microRNA (miRNA) level or activity.

**Synthetic MicroDNAs (MiDs) and transcriptional control**

Transcriptional activation or repression is an essential mechanism in the precise control of gene expression. Hypomethylation contributes to chromosomal instability and possibly to increased expression of some proto-oncogenes (Maldonado E et al.\(^\text{21}\)). The most direct mechanism by which DNA methylation can interfere with transcription is to prevent the binding of basal transcriptional
machinery or ubiquitous TF (Transcription Factors) that require contact with cytosine (C) in the major groove of the double helix (Curradi M et al.\textsuperscript{22}).

Here we introduce synthetic “microDNAs (MIDs)” a new class of ~ 20-25 nucleotide- long DNAs capable of repressing the activity of the target gene by mechanisms that have not been clarified yet. However they are designed to target non-coding regions of the cancer causing genes. Therefore the inhibition might be possible through the direct binding of MIDs to cis- regulatory sites and/or to some Transcription Factors (TF) that normally activate transcription. Here we suggest two possible models for their actions (figure 2).

**Model 1**

![Model 1 Diagram]

Figure 2.) Model 1 suggests that one or more microDNAs bind to cis-regulatory sites of the non-coding region of the target gene to prevent gene activation by transcription factors. Model 2 suggests that microDNAs bind to transcription factors to prevent them from activating gene transcription.

This technology provides highly specific tools that could be more efficient than RNAi and other competitors. MicroRNAs mechanism seems to be an expensive way for the cell trying to suppress the activity of a bad gene for the following reasons:

1) Transcription factors and transcriptional machinery are at work to make numerous copies of mRNA.
2) MicroRNA genes must constantly work to produce thousands of microRNA copies to overcome mRNA production and stop protein translation.
This seems to be an energy consuming process that might make sense for the regulation of good genes but may be a wasteful way to stop bad genes all the time.

MicroDNAs on the other hand, need to interact with only one or two copies of the target gene that are present in each cell. Thus the inhibition of a disease causing gene requires much less microDNA molecules compare to microRNAs or other similar gene inhibitors. This could also eliminate many non-specific interactions that might cause side effects. The similarities and differences between microRNAs and microDNAs are illustrated in table 1.

<table>
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<th>Table 1. Comparing microDNAs and microRNAs</th>
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<td><strong>Gene Regulator</strong></td>
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<td>Size</td>
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<td>Target</td>
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In vitro studies suggest that the inhibition of the target gene starts after the first round of DNA replication, usually 24 hours after treatment depending on cell doubling time (figure 3, 4). Cell growth suppression maximizes at day 6 or 7 and the inhibition effect is sustained for weeks (data not included). This alone makes microDNAs superior to its other competitors.
Figure 3. Bcl-2 is inhibited at transcriptional level by specific microDNAs in follicular small cleaved cell lymphoma (FSCCL). 2x10^5 cells were treated with HPLC purified anti-bcl-2 microDNAs directly without using any nanoparticles to facilitate transfection. Cells were counted using trypan blue and hemocytometer.

Apoptosis Induced in HL60 Leukemia Cells

Figure 4. Blocking of both bcl-2 and k-ras transcription by their specific microDNA Inhibitors induced apoptosis in HL60 leukemia cells. 1x10^4 cells per well were seeded in a 96 well plate and treated with 10 µM partially purified microDNAs (desalted) in media without serum. 3 hrs later serum was added to 10% final concentration and incubation continued at 37°C under 5% CO2. Fresh media was added after 24 hrs and the experiment terminated after 48 hrs and MTT test was performed.

Apoptosis Induced in HL60 Leukemia Cells
Figure 5. Ki-ras in inhibited by HPK (HPLC purified ki-ras microDNA inhibitor), HPKP (HPK nanoparticles), HPKPR (HPKP with rituximab 1:1 mole/mole), RX (rituximab) and CP (control microDNA nanoparticles). HL60 cells were treated with ki-ras inhibitor as in figure 4 experiment. Nanoparticles were formed simply by mixing DNA with a ployarginine peptide (1:2 mids/peptid). The nanoparticles were sized at 100 nm with a net positive charge of about 20. Control microDNA and peptide nanoparticles had little or no effect on cell viability. In this experiment 3.5 µg of microDNAs equaled the repressing effect of 145 µg rituximab.

The idea was first developed in (1994) before the discovery of microRNAs. The author was then a scientist at Michigan Cancer Foundation (now known as Karmanos Cancer Institute). The original idea was to design oligonucleotide template to promote DNA methylation at some specific regulatory sites and thus silence the target gene.

ProNAi Therapeutics Inc. in Kalamazoo, Michigan is pursuing this technology by raising million of dollars to commercialize these drugs. The company has several leading compounds and the preclinical efficacy and safety studies of PNT2258 have been completed successfully. FDA has approved its IND application to take PNT2258 to clinical trial.

References