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Creating a flexible multiple microRNA expression vector by linking precursor microRNAs

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ABSTRACT

MicroRNAs (miRNAs) are ~22 nt non-coding RNA molecules that usually function as endogenous repressors of target genes. Many biological processes depend on faithful miRNA expression and miRNA profiling has revealed dysregulation of many miRNAs in neurological, and cardiovascular diseases, and in cancer. Despite this finding, most studies have focused on the function of single miRNAs or miRNA clusters. To better address physiologically relevant collaborative miRNA interactions, we developed a simple and flexible platform which expresses several miRNAs that have different genomic locations from a single transcript using endogenous pre-miRNA sequences. As a proof of principle we cloned the miR-34 tumor suppressor family and showed that the miR-34a/34b/34c vector expresses each miRNA at similar levels to individual miRNA containing vectors. Moreover, the miR-34a/34b/34c vector suppressed cell growth more than the individual miRNA vectors. We expect that this platform will be invaluable as a tool to study the complex and synergistic interactions of aberrantly expressed miRNAs in human diseases and may have applications for use in gene therapy.

1. Introduction

MicroRNAs (miRNAs) are ~22 nt non-coding RNA molecules that typically function as endogenous repressors of target genes [1]. A miRNA is usually transcribed by RNA Pol II as a primary transcript (pri-miRNA) which may be many kilobases long. The pri-miRNA is processed in the nucleus by the RNAse III enzyme Drosha, which uses the stem–loop secondary structure in the pri-miRNA to direct cleavage [2]. The Drosha product is a ~70 nt stem–loop precursor miRNA (pre-miRNA) that is exported to the cytoplasm. There, the RNAse III enzyme Dicer cleaves the pre-miRNA to ultimately yield the mature miRNA [3]. In animals, miRNAs can bind with imperfect complementarity to the 3′ untranslated region (3′UTR) of the target mRNA via the RNA-induced silencing complex. The resulting gene repression occurs by multiple mechanisms including enhanced mRNA degradation and translational repression [1,4].

Due to the promiscuity of miRNA binding to target mRNAs, each miRNA may control numerous genes and each mRNA may be controlled by many miRNAs [5]. Developmental timing, cell death, proliferation, hematopoiesis, insulin secretion, and the immune response are just a few examples of critical biological events that depend on faithful miRNA expression [6]. In agreement with their role in crucial biological processes, miRNA profiling has revealed extensive miRNA dysregulation in neurological, and cardiovascular diseases and in cancer [7–10].

The analysis of miRNA expression profiles in cancer has revealed that many tumor suppressor miRNAs (miRNAs that target oncogenes) are down-regulated in cancer and can potentially become therapeutic targets [9]. Manipulating miRNA expression to inhibit cancer progression may be clinically relevant just as RNAi is being used in some approaches to gene therapy [11]. A few studies have shown the potential utility of miRNA-based therapies in cancer [11]. These include the induction of apoptosis by the miR-34 family in colon cancer cell lines [12] and by miR-15a/16-1 in CLL [13], growth inhibition of cancer cells by let-7 [2,14,15], reduced metastasis by miR-126 in breast cancer [16], and regression of murine lung tumors by the let-7 [17].

Currently there are no reported studies using miRNAs for *in vivo* anti-cancer therapy. However, the development of methods for *in vivo* delivery of siRNA and shRNA) to silence single target genes has established technical approaches that could translate into miRNA therapy [18]. Gene therapies based on systemic delivery of siRNA/shRNA in preclinical models have made use of viral vectors, liposomes, and nanoparticles [19–22], but the same challenges encountered with delivering antisense and siRNA into cells will be faced with miRNA-based therapies. The primary obstacle is that introducing a charged, linear polymer across the membrane of a cell is exceedingly difficult. The clear advantage that a miRNA-based gene therapy will have over siRNAs, shRNAs, and antisense

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oligonucleotides is that multiple miRNAs can be co-transcribed and each miRNA has multiple targets. For instance, let-7 has been shown to down-regulates RAS, MYC, and HMGA2 oncogenes [14,15].

The general miRNA hairpin structure has been used to develop shRNA vectors for gene knockdown experiments or antagomir vectors to study miRNA knockdown [23,24] and show much promise for both functional studies and gene therapy [25]. Although miRNA profiling of disease states indicates that many miRNAs are either up- or down-regulated, most studies have focused on the function of single miRNAs or miRNA clusters. In addition, a recent report indicates that the miRNA-mediated repression of a target mRNA is additive with respect to the number of miRNA binding sites in the 3′UTR (irrespective of whether they are for the same miRNA or multiple miRNAs), while this effect is synergistic when the sites are within 40 bp of each other [26]. These reports suggest that thorough functional investigations of miRNAs should take into consideration the combinatorial effects of multiple miRNAs on target genes and pathways. Indeed, several groups have developed methods used which can express multiple microRNAs or shRNAs through a single transcript but the methods are either complicated or cannot ensure expression of mature microRNAs [24,27,28].

We developed a simple and flexible platform that can express multiple miRNAs from a single transcript using endogenous pre-miRNA sequences. We show here that the miRNA processing machinery can generate multiple mature miRNAs from a transcript made of inserts that include ~60 bp flanking the pre-miRNAs sequences. This platform will be invaluable as a tool to study the complex and synergistic interactions of aberrantly expressed miRNAs in human diseases and may have applications in gene therapy.

2. Materials and methods

2.1. Cell lines

T24, HCT116, and PC3 cells were obtained from the American Type Culture Collection. T24 and HCT116 cells were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS). PC3 cells were cultured in RPMI supplemented with 10% FBS.

2.2. Expression vector construction and transfections

Single miRNA expression vectors for miR-34a, miR-34b and miR-34c were made by cloning ~60 bp 5′ and 3′ of the pre-miRNA into the multiple cloning site for pcDNA3.1(+) (Invitrogen). The multiple miRNA expression vector was constructed by sequentially cloning the miR-34b and miR-34c inserts into the miR-34a expression vector. The primer sequences and restriction sites were:

5′-miR-34a-KpnI TAATGGTACCAGGCAGGACAGGCCT,
3′-miR-34a-BamH1 TGAAGGATCCATCTCTCGCTTCATCTTC,
5′-miR-34b-EcoR1 TGTGGAATTCTCGTCCGGGAGCTGCA,
3′-miR-34b-EcoRV ATAGGATATCTCAGGCATCTTCTCTCGA,
5′-miR-34c-EcoRV TGCAGATATCCAACTTGAGACTGGAAT,
3′-miR-34c-NotI TATAGCGGCCGCTGCACAGGCAGCTCAT.

Cells were seeded in 6-well dishes so that 24 h later they were 90% confluent. Cells were transfected using 10 μL Lipofectamine 2000 (Invitrogen) and 4 μg plasmid according to the manufacturer's protocol.

2.3. Reverse transcription and Taqman qPCR

RNA was extracted 48 h post transfection using Trizol (Invitrogen) according to the manufacturer's protocol. All reagents for miRNA Taqman assays were purchased from Applied Biosystems and used according to the manufacturer's protocol. All reactions were done in duplicate and the primer sequences for the U6 internal control were:

forward – CTCGCTTCGGCAGCACA,
reverse – AACGCTTCACGAATTTGCGT,
probe – FAM-AGATTAGCATGGCCCCTGCGCAA-BHQ.

2.4. Northern blot

Northern blots were done as described previously [29]. Briefly, 10 μg of total RNA was loaded onto a denaturing gel and transferred to a nylon membrane. The Star-Fire radiolabeled probes (Integrated DNA Technologies) were prepared by incorporation of [α-^{32}P] dATP 6000 Ci/mmol according to the manufacturer's protocol. The probe sequences were:

miR-34a-ACAACCAGCTAAGACACTGCCA,
miR-34b-CAATCAGCTAATGACACTGCC,
miR-34c-CAATCAGCTAACTACACTGCCT,
U6-GCAGGGGCCATGCTAATCTTCTCTGTATCG.

Prehybridization and hybridization were carried out using ExpressHyb Hybridization Solution (Clontech) following manufacturer's instructions.

2.5. Cell proliferation assay

Cell proliferation assays were conducted in triplicate as described previously [30]. Briefly, cells were transfected as described above. Cells in each well were trypsinized 48 h after transfection and equal cell numbers plated in 10 cm dishes with medium containing G418 (Sigma) (T24 400 μg/mL, HCT116 600 μg/mL and PC3 750 μg/mL). Medium was changed every 3–4 days and total cell numbers were counted after 13 days using the Z1 Coulter Particle Counter. Significance was determined by p-value <0.01 using a paired t-test.

3. Results

The key steps for the miRNA processing machinery to produce mature miRNAs is the recognition of both the hairpin structure and the junction between the single- and double-region of the pri-miRNA (SD junction). This implies that the sequence requirement for mature miRNA expression from an expression vector could be as little as a few base pairs in either direction of the pre-miRNA sequence [31,32]. Recent studies suggest that the minimal length for efficient pri-miRNA processed should be 110 nt [31]. Due to the small size of the pre-miRNA genes, it is technically feasible to clone many pre-miRNA genes into the same expression vector. Therefore, it is possible to clone multiple tumor suppressor miRNAs, which affect many different genes and/or pathways involved in tumorigenesis into one vector, creating a powerful miRNA-based cancer therapy.

As a proof of concept, we cloned the miR-34 tumor suppressor family (miR-34a, miR-34b and miR-34c) into a single expression vector, under the hypothesis that the combinatorial inhibitory action of these miRNAs on cancer cell proliferation will be stronger than that of each individual miRNA. miR-34a is located at chromosome 1p36, while miR-34b and miR-34c are located at chromosome 11q23, 418 bp apart (Fig. 1A). Previous studies have shown that restoring the expression of individual miRNA from the miR-34 family can induce apoptosis in cancer cell lines and inhibit cell growth in cancer stem cells [33,34]. miR-34a, miR-34b, and