Reports

A simple high-throughput technology enables gain-of-function screening of human microRNAs

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MicroRNAs (miRs) regulate cellular processes by modulating gene expression. Although transcriptomic studies have identified numerous miRs differentially expressed in diseased versus normal cells, expression analysis alone cannot distinguish miRs driving a disease phenotype from those merely associated with the disease. To address this limitation, we developed miR-HTS, a method for unbiased high-throughput screening of the miRNome to identify functionally relevant miRs. Herein, we applied miR-HTS to simultaneously analyze the effects of 578 lentivirally transduced human miRs or miR clusters on growth of the IMR90 human lung fibroblast cell line. Growth-regulatory miRs were identified by quantitating the representation (i.e., relative abundance) of cells overexpressing each miR over a one-month culture of IMR90, using a panel of custom-designed quantitative real-time PCR (qPCR) assays specific for each transduced miR expression cassette. The miR-HTS identified 4 miRs previously reported to inhibit the growth of human lung-derived cell lines and 55 novel growth-inhibitory miR candidates. Nine of 12 (75%) selected candidate miRs were validated and shown to inhibit IMR90 cell growth. Thus, this novel lentiviral library- and qPCR-based miR-HTS technology provides a sensitive platform for functional screening that is straightforward and relatively inexpensive.

MicroRNAs (miRs) are small non-coding RNAs that regulate gene expression at multiple levels (1–3). MiRs appear to regulate most coding genes and thereby participate in essentially all biological processes including cell survival, proliferation and differentiation (4–6). Consequently, alterations in miR expression and function play pathophysiological roles in cancers and other human diseases (6–8). For example, expression of the *miR-34* family is down-regulated in various cancers, and has been shown to be directly transactivated by p53, serving as a potent mediator of p53-dependent tumor suppressive mechanisms, including apoptosis, cell cycle arrest, and senescence (reviewed in Reference 9). Delivery of *miR-34a* mimics inhibited growth of lung cancers with low *miR-34a* expression in mouse models (10,11). A striking observation in this and other preclinical and clinical studies is the lack of toxicity of

miR-based therapeutics to normal cells and tissues, suggesting a large therapeutic window for miR-based therapies (8,12).

Substantial transcriptomic studies have identified many miRs differentially expressed between diseased and normal cells, as well as between different cell lineages and differentiation stages throughout developmental processes (e.g., during hematopoiesis) (6,7,13). However, differential expression of a particular miR does not guarantee a physiological or pathophysiological role for that miR. Changes in miR expression may be the result of the diseased state rather than the cause, and the function of a miR can be altered without changes in its expression level [e.g., due to context-dependent availability of auxiliary factors required for miR function (14,15)] – such miRs might be excellent biomarkers, but would not be operative therapeutic targets. High-throughput functional screens could distinguish “driver”

miRs from “passenger” miRs and thus speed the identification of therapeutically relevant miRs (16).

Unfortunately, current approaches for genome-wide functional/phenotypic screening of the human miRNome require equipment or expertise not readily available in many laboratories and are often prohibitively expensive. Approaches and examples of miR functional screens have been comprehensively reviewed in (16). One approach utilizes transfection of synthetic miR mimic/inhibitor libraries, in which synthetic miRs are arrayed individually in multiple 96- or 384-well plates. Such a miR mimic library approach was used successfully to identify miRs involved in G1 arrest of DGCR8 knockout ES cells (17). Nevertheless, in this approach, screening is limited by (i) the need for cells that can be transfected efficiently and (ii) the use of transient assays, which is disadvanta-

Method summary:

We introduce miR-HTS, a novel functional screening method for the human microRNA-ome (miRNome) that is high-throughput and sensitive. Using a lentiviral miR library and quantitative PCR, this method provides a sensitive platform for functional screening of the miRNome that is straightforward and cost-effective.

geous since synthetic miRs are diluted with every cell division. These limitations can be overcome by infection of cells with miR overexpression lentivirus (lenti-miR) libraries, with the individual lenti-miRs virus arrayed in 96-well plates. This approach has been used successfully to identify miRs suppressing growth (18) or metastasis (19) of cancer cell lines. The advantage of this format with one miR (mimic, inhibitor, or virus) per well is that the candidate miRs can be attributed very simply to enhance or decrease a specific function. However, arrayed format screens require assays adaptable to microplates and often require robotics to minimize well-to-well variation and other error-prone screening problems. These requirements and associated costs prevent broad application of arrayed miR functional screens.

An alternative screening strategy is to employ pooled lenti-miR or retroviral miR libraries, in which each virus encodes a unique miR or miR cluster (20–25). This screening strategy requires an assay for a phenotype that results from over- or under-representation of cells overexpressing particular miRs. Because these screens simultaneously assay all the miRs in a pool, they depend on downstream methods to distinguish the candidate miRs. Taking advantage of viral integration into the host genomic DNA, the unique miR transgene sequence of each miR virus can serve as the “barcode” for quantitating the number of cells overexpressing a particular miR. Thus, the under- or overrepresented barcode enables identification of each miR whose overexpression results in a decrease or increase in the number of cells infected with that particular miR in the functional screen. For example, the barcode of a miR whose overexpression inhibits cell growth would decrease over time; Izumiya et al. identified candidate pancreatic cancer suppressive miRs on this basis (22). Conversely, by determining barcodes enriched in migrated cells versus the starting cell population, candidate miRs that promoted invasiveness of cancer cell lines were identified (23,26). Although these studies successfully employed pooled viral miR libraries in functional screens, their viral miR barcode quantification methods required custom-made microarrays (22,24), bead-based detection systems (20,21), or high-throughput sequencing (23,26). Those approaches require access to costly high-throughput infrastructure and sophisticated informatics analyses, thereby restricting the accessibility of these assays.

We report here a simple barcode quantification strategy based on real-time quantitative PCR (qPCR) as an effective alternative to the aforementioned identification methods, allowing any laboratory with access to a real-time PCR machine to conduct functional screening of pooled miR libraries. Using a pooled human lenti-miR library and custom-designed qPCR assays to quantify lenti-miR barcodes, we established miR-HTS, a novel method that enables depletion- and enrichment-based functional screening of the human miRNome. As proof-

Figure 1. Design of nested PCR strategy and miR-HTS. (A) Illustration of SBI's miR lentiviral integrants in the host genome (double helices) between the two viral long-terminal repeats (LTRs). Positions of the primers and probes used in this study are depicted relative to the features of lentiviral integrant. CMV, cytomegalovirus promoter; miR, miR cassette; EF1 α , human elongation factor 1 α promoter; GFP, green fluorescence protein. (B) Experimental design of the miR-HTS conducted in this study. Each green circle is a cell transduced with 1 miR lentivirus. The letter in a green circle denotes the miR being overexpressed in the transduced cell. Open circles represent untransduced cells. Green circle with dashed outline depicts a transduced cell disappearing from the culture (e.g., miR-X). The abundance of miR-infected cells at each time point is determined by GRE-qPCR assays, normalized by GFP loading control qPCR assay, and compared with its starting representation at t_0 to calculate fold change in abundance.

of-principle, we used miR-HTS to screen the IMR90 human lung fibroblast cell line for miRs capable of regulating cell growth. We identified four known growth-inhibitory miRs, plus 55 miRs not previously known to regulate growth of IMR90 cells. Nine of 12 (75%) selected candidate miRs were independently validated and confirmed to inhibit IMR90 cell growth, demonstrating the miR-HTS as a straightforward and sensitive alternative to reported approaches.

Materials and methods

Cell culture

The IMR90 cell line was purchased from ATCC (Manassas, VA, USA), and cultured in Minimum Essential Medium (Mediatech, Manassas, VA, USA) containing 10% fetal bovine serum (Gemini, Sacramento, CA, USA), 1 mM sodium pyruvate (Lonza, Walkersville, MD, USA), 1 $\times$ GlutaMAX (Life Technologies, Grand Island, NY, USA), 1 $\times$ penicillin-streptomycin solution (Mediatech).

Design of genomic DNA representation qPCR (GRE-qPCR) assays and the GFP loading control assay

To design genomic DNA representation qPCR (GRE-qPCR) assays, the miR (plus flanking) insert sequences of each lenti-miR precursor construct were obtained from System Biosciences (SBI, Mountain View, CA, USA) under a mutual nondisclosure agreement between SBI and the University of Maryland Baltimore. GRE-qPCR assays were designed using Primer Express software from Applied Biosystems (ABI, Foster City, CA, USA) or were custom-designed by ABI and purchased. The internal miR-specific forward primers for each GRE-qPCR assay were custom-plated by ABI at 0.018 nmole per well in 384-well plates. The internal common reverse primer, GFP primers, and external common forward and reverse primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA). The GFP probe and internal common probe were synthesized by ABI. Sequences of all primers and TaqMan probes used in this study

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are listed in Supplementary Table S1. During the design of GRE-qPCR assays, care was taken to minimize potential nonspecific binding of internal miR-specific forward primers to unintended targets [due to sequence similarities between lenti-miRs (e.g., paralogous miRs)], by designing primers within the more variable miR flanking sequences. Nevertheless, six of the 578 internal miR-specific primers may fail to distinguish paralogous miRs (see comments in Supplementary Table S1).

miR-HTS procedure and data analysis

In each miR-HTS, 2.7 million IMR90 cells were infected at a multiplicity of infection (MOI) of 0.2 with the human lenti-miR pooled virus library (Cat. no. PMIRHPLVA-1, SBI) to achieve ~20% transduced cells. Eight μ g/mL polybrene (Sigma-Aldrich, St. Louis, MO, USA) was used as the infection vehicle (27). On days 3 (t_0), 11 (t_1), 19 (t_2) and 27 (t_3) after infection, a fraction of the infected culture (≥ 2 million cells) was harvested and genomic DNA isolated using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). The external common forward and reverse primers (Figure 1A) were used to amplify lentiviral integrants; these amplifications used 1.5 μ g genomic DNA (per 100 μ L PCR reaction) from each time point, 3% DMSO, 0.4 μ M external common forward and reverse primers, 0.2 mM dNTPs, 1 $\times$ Phusion HF buffer and 2 units Phusion High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA). PCR was performed with the following program: 98°C for 2 min, 25 cycles of 98°C for 10 s, 56.4°C for 30 s, 72°C for 90 s, and a final extension at 72°C for 5 min. PCR products were purified using the QIAquick PCR purification kit (Qiagen). 0.2 ng purified PCR products were used as templates for all GRE-qPCR assays; 0.002 ng template was used for the GFP loading control qPCR assay. In addition to the templates, each 20- μ L qPCR reaction contained 1 $\times$ TaqMan Universal Master Mix II (ABI), 900 nM forward and 900 nM reverse qPCR primers, and 250 nM TaqMan probes. qPCR was performed on the ABI 7900HT Fast Real-Time PCR System with the following program: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The qPCR results were analyzed using Sequence Detection System software (ABI); see Supplementary Methods for a detailed miR-HTS data-analysis protocol.

Cell growth assay

One day prior to transfection, IMR90 cells were seeded into 96-well plates (3000 cells per well). MiR mimics (5 or 10 nM, Dharmacon, Lafayette, CO, USA) were transfected using 0.15 μ L DharmaFECT2 (Dharmacon) per well in triplicates. On day 5 post-transfection, 10 μ L alamarBlue (Life Technologies) was added to each well and incubated for 4 h. The alamarBlue fluorescence signal was read on a VictorX3 plate reader (PerkinElmer, Waltham, MA, USA) using 530/578 nm excitation/emission filters.

Caspase activity

Cells were transfected using the same protocol as for the cell growth assay. Caspase activity was measured 4 days post-transfection using the Apo-ONE homogenous caspase 3/7 assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Results and discussion

To interrogate the roles of the human miRNome in regulating cellular processes, we developed a functional high-throughput screening (HTS) method that combines a pooled miR overexpression lentiviral (lenti-miR) library with a novel panel of qPCR assays to quantitate each lenti-miR in the library. Lentiviral vectors allow screening in either dividing or quiescent cell types (e.g., stem cells). Infections with the lenti-miR library as a pool make it a scalable screening procedure. In addition, qPCR is widely practiced with standard data-analysis protocols, in contrast to more complex data analyses necessary for custom microarray or high-throughput sequencing. The lenti-miR library used in this study contains 578 lentiviruses encoding 539 individual miRs and 39 miR clusters. Each lentivector contains a miR cassette consisting of the native human miR hairpin, plus a stretch

of 200–400 bp of genomic flanking sequences to allow for endogenous miR processing. The expression of each miR cassette is controlled by a CMV promoter; an EF1 α promoter-driven GFP is located downstream of the miR cassette (Figure 1A). Transduced cells co-express the miR and GFP, allowing flow cytometric quantification of transduced cells.

As a proof-of-concept evaluation of the miR-HTS strategy, we screened for miRs capable of regulating cell “growth” (i.e., proliferation and/or survival) of the IMR90 primary human lung fibroblast cell line (Figure 1B). We chose IMR90 for this proof-of-principle study for three reasons. First, *miR-34a* was already known to potentially inhibit IMR90 growth (28), providing a positive control. Second, performing the functional screen in this nonimmortalized cell line might generate a more comprehensive set of human miRs involved in growth than would be obtained using a transformed cell line with deregulated growth control. Third, defective miR processing has been observed in many cancer cell lines (29–31). Approximately 3 million IMR90 cells were transduced with the pooled lenti-miR library at ~20% efficiency (i.e., MOI = 0.2) and then cultured for 1 month. This MOI should result in <5% of infected cells containing >1 lentiviral insertion (32), thus avoiding combinatorial effects due to multiple transduced miRs in a single cell (33,34). Infecting 20% of ~3 million cells predicts that each miR lentivirus will integrate into ~1000 cells. Each cell transduced should have an independent genomic integration site (35), reducing the chance of detecting a lentiviral insertion site–specific effect (e.g., host gene disruption). In addition, the many different integration sites for each miR lentivirus should average positional effects on miR expression (e.g., clonal silencing of miR expression) (36).

Identifying the subset of miRs whose overexpression enhanced or reduced cell growth via the miR-HTS requires reliable detection of the changes in representation (i.e., number, abundance or relative frequency) of the cells containing each lenti-miR transgene within the entire transduced population over time in culture. Therefore, we developed the GRE-qPCR assay to quantify the abundance of cells infected with each lenti-miR by using the unique miR cassette sequence as a barcode. Each GRE-qPCR assay has three components (Figure 1A): (i) an internal miR-specific forward primer to identify each miR cassette, (ii) an internal common reverse primer, and (iii) an internal common probe. The internal common reverse primer and probe detect every lenti-miR transgene in the library, but not the endogenous miR locus of the host genome. For high-throughput analysis, each of the 578 different internal miR-specific forward primers for the GRE-qPCR assays was plated in a well of a 384-well plate.

Before conducting the actual miR-HTS, we determined the dynamic range and sensitivity of GRE-qPCR assays, and the amount

Figure 2. Dynamic range of GRE-qPCR assays. (A,B) IMR90 cells separately infected with either *miR-155* or *miR-222* lentivirus at 20% transduction efficiency were mixed at different ratios as described in the text. Genomic DNA samples were harvested from each mixture and used as templates for each GRE-qPCR assay for either *miR-155* (A) or *miR-222* (B), to measure the abundance of each lenti-miR barcode. Number of cells infected with either virus (x axis) was plotted against corrected Ct values (y axis; Ct corrected for template amounts). The numbers of cells infected with each virus are labeled above or below each symbol through panels A–D. Regression lines were fitted for cell numbers ≥ 50 for *miR-155* virus–infected cells, but for all cell numbers for *miR-222* virus infected cells. (C,D) Using the same genomic DNA samples as in panels A and B, the external common forward and reverse primers (illustrated in Figure 1) were used to amplify the miR cassettes from infected cells. Then, purified PCR amplicons were used as templates for each GRE-qPCR assay for either *miR-155* (C) or *miR-222* (D), to measure the abundance of each lenti-miR barcode. Similar to panels A and B, the number of cells infected with each virus (x axis, 25–10⁴ cells) was plotted against corrected Ct values (y axis). Regression lines were fitted to all cell numbers in panels C and D. The r^2 value of the fitted regression line is provided at the lower right corner of each panel.

of genomic DNA required for the GRE-qPCR assay to quantitate cell number accurately. First, three cultures of IMR90 cells were separately infected with *miR-155*, *miR-222*, or vector-only lentivirus at an MOI = 0.2, to mimic the miR-HTS conditions. Specific numbers of *miR-155*- and *miR-222*-infected cells were mixed at desired ratios to provide a 4-log range of different cell numbers infected with either of these lenti-miRs (i.e., 10–10⁵ infected cells). As there were 578 different miR cassettes in the library, the expected relative frequency of each specific miR cassette was 1 per 578 transduced cells. To mimic this expected frequency of each miR cassette/barcode in the library and the potential changes in frequency (under- or overrepresentation) during the 1 month miR-HTS culture, we added vector only–transduced cells at numbers ~20- to 1800-fold higher than that of lenti-miR virus infected cells (see Supplementary Table S2 for details of the different cell mixtures prepared). Genomic DNA was extracted from each mixture and subjected to GRE-qPCR assays for *miR-155* and *miR-222*, as well as qPCR assay for GFP (loading control present in every lentiviral integrant). The corrected cycle of

threshold (Ct, corrected for template amounts) values of qPCR assays were plotted against the numbers of cells infected with each lenti-miR, in mixtures containing 10–10⁵ cells transduced with the given miR cassette (Figure 2A, B). As expected, we saw a log-linear inverse correlation between the number of infected cells and the correct Ct values. The fitted regression line of the GRE-qPCR assay for *miR-155* lost linearity below 50 cells, indicating a detection limit of 50 cells (Figure 2A; regression lines fitted to values with 50–10⁵ cells or 10–10⁵ cells had $r^2 = 0.94$ or 0.84, respectively). The fitted regression line of *miR-222* maintained linearity to as low as 10 cells (Figure 2B; regression line fitted to values with 10–10⁵ cells had $r^2 = 0.98$). We determined that 1 or 1.5 μ g genomic DNA template per GRE-qPCR assay was required to accurately quantify the numbers of lenti-miR infected cells over a dynamic range of 50–10⁵ infected cells, when 20% of the cell population was transduced in these model experiments.

Given that IMR90 yields ~0.002 ng genomic DNA per cell, >250 million IMR90 cells would be needed to produce the 578 μ g genomic DNA needed to perform 578 GRE-qPCR assays (250

million IMR90 cells would require ~62 confluent T175 flasks). To reduce cell-number requirements for the miR-HTS, we utilized a nested PCR strategy to produce sufficient DNA from fewer cells. Integrated lentiviral DNA segments containing the miR cassette and GFP transgene were first amplified from genomic DNA samples, using external common forward and reverse primers designed to minimize PCR amplification bias favoring shorter amplicons (Figure 1A). Approximately 70% of the miR cassettes were 500–600 bp, with a global size range of 182–951 bp (Supplementary Figure S1A). The external common primers included a total of 1850 bp flanking each miR cassette, which reduced the relative length ratio of the longest to the shortest amplicon from 5.2-fold to 1.4-fold, as compared with the miR cassette alone (Supplementary Figure S1B). GRE-qPCR assays and GFP loading control qPCR assays were all nested within the amplicon defined by the external common forward and reverse primers (Figure 1A).

After removal of the external common primers to prevent their unwanted further amplification, purified PCR amplicons were used as templates for GRE-qPCR assays to determine the relative abundance of each miR cassette (Figure 1B). Using PCR amplicons (obtained by 25 cycles of amplification of the same initial genomic DNA samples used in Figures 2A,B), GRE-qPCR assays accurately quantified the number of cells infected with either lenti-miR virus, ranging from 25 to 10^4 cells (Figure 2C,D). The fitted regression line showed a strong log-linear inverse relationship between corrected Ct values and numbers of cells ($r^2 = 0.99$ for either *miR-155* or *miR-222*, with only 0.2 ng PCR amplicon used per GRE-qPCR assay; Figure 2C,D). We could not reproducibly detect a signal when the infected cell number was 10. Similar detection sensitivity and accuracy was observed when either 1.5 or 1 μ g genomic DNA was used to generate PCR amplicons, whereas the accuracy and sensitivity in cell number quantification became unreliable when only 0.5 μ g genomic DNA sample was used (not shown). We found that 10–30 cycles of PCR amplification generated similar sensitivity and accuracy in cell number quantification (not shown). We opted to use 25 cycles in the miR-HTS to minimize cost; this required only 0.2 ng purified PCR amplicons per GRE-qPCR assay, instead of the 20 ng purified PCR amplicons, which would be required using 20 cycles of amplification. The yield of PCR amplicons was ~300 ng per 1.5 μ g genomic DNA at 25 cycles of amplification, generating more than sufficient templates for all 578 different GRE-qPCR assays. While 1 or 1.5 μ g genomic DNA was optimal to maintain accuracy and sensitivity using this nested PCR strategy for IMR90 cells, optimization should be performed as in Figure 2 if the genomic DNA yield per cell of a given cell line is significantly different from that of IMR90.

Once the GRE-qPCR assays were shown to accurately quantify the abundance of lenti-miR-

infected cells, we performed the miR-HTS twice in IMR90 cells, as illustrated in Figure 1B. Genomic DNA was harvested from the lenti-miR library-infected cultures at the reference time point (t_0 , 3 days post-infection, to allow virus integration and GFP expression) and at three experimental time points (t_1 , t_2 , t_3 ; days 11, 19 and 27 post-infection, respectively, corresponding to every 2 passages; 4–5 population doublings of IMR90 cells per passage). To determine fold changes in abundance of each lenti-miR over time, the abundance of each lenti-miR at each experimental time point was quantified and normalized to its abundance at the reference time point (i.e., t_N/t_0). Cells overexpressing a miR with no effect on growth would be expected to maintain their starting representation over time ($t_N/t_0 = 1$; e.g., *miR-Z*). In contrast, cells overexpressing a miR promoting growth should become overrepresented ($t_N/t_0 > 1$; e.g., *miR-Y*), and cells overexpressing a miR inhibiting growth should become under-represented ($t_N/t_0 < 1$; e.g., *miR-X*).

Technical replicates of GRE-qPCR assays were highly reproducible; 2 independent GRE-qPCR assays performed using the same templates (i.e., assaying a single batch of PCR amplicons) were nearly identical ($r^2 \sim 1$; Figure 3A). Furthermore, when 2 batches of purified PCR amplicons were generated independently and then each used separately as templates for GRE-qPCR assays, only 4% of the GRE-qPCR assays had differences of ≥ 3 cycles in corrected Ct (Figure 3B). A 3-cycle difference in corrected Ct (i.e., $\Delta\Delta Ct = 3$) is equivalent to a 8-fold change in abundance (37). The 4% frequency of unexpected, ≥ 8 -fold differences observed between the replicates might be explained by the Monte Carlo effect occurring during independent PCR amplifications (38); this further suggested that any observed change less than 8-fold might be due to variations in PCR amplification instead of a true difference in abundance. Therefore, we set our candidate selection threshold at ≥ 8 -fold change from t_0 ; that is, only miRs that showed a ≥ 8 -fold change in abundance in miR-HTS were considered as growth-inhibitory or growth-promoting candidates (e.g., an 8-fold increase would be $t_N/t_0 = 8$, and an 8-fold decrease would be $t_N/t_0 = 0.125$ in Figure 4, Supplementary Table S3, and Supplementary Table S4). As we gain additional experience with this assay, we should be able to estimate the variance of the method more precisely, and we may be able to reduce the 8-fold threshold by reducing the Monte Carlo effect, for example, by pooling multiple independent PCR amplifications prior to the GRE-qPCR.

For the two independent miR-HTS experiments, 115 and 116 candidates fulfilled this criterion in the first and second replicate screens, respectively (Figure 4A, B). Fifty-nine candidates were identified in both miR-HTS replicates; 58 candidate growth-inhibitory individual miRs, and 1 candidate growth-inhibitory miR

Figure 3. Reproducibility of GRE-qPCR assays. (A) The same batch of PCR amplicons (generated using external common primers) was subjected to 304 miR-specific GRE-qPCR assays, in duplicate. The resulting corrected Ct values from each replicate were plotted against each other to examine technical reproducibility of the GRE-qPCR step. Each circle is a different lenti-miR. (B) Two batches of PCR amplicons generated separately were each subjected to 304 GRE-qPCR assays (same assays used in panel A). The resulting corrected Ct values from each batch were plotted against each other to examine the reproducibility of the amplification step using the external common primers. The circles outside of or on the solid lines are GRE-qPCR assays with ≥ 3 cycles of difference between the two batches of templates. The r^2 value of fitted regression line (not shown) on the lower right corner of each panel.

cluster (see Supplementary Table S4 for the average fold changes of the two replicate screens and Supplementary Table S3 for fold changes of each miR-HTS). No growth-promoting miR candidates were reproducibly identified in both miR-HTS replicate screens (Figure 4B). This observation suggested that none of the miRs in the library strongly stimulated IMR90 cell growth under the screening conditions employed, since we detected both under- and overrepresented lenti-miRs during initial evaluation of GRE-qPCR assay performance (Figure 2). As expected, *miR-34a* was among the growth-inhibitory miRs identified. Cells containing the lenti-*miR-34a* cassette had a ≥ 8 -fold decrease in abundance at both of the last two time points (t_2 and t_3). At t_1 , the representation of *miR-34a* virus-infected cells was 0.7% of that at t_0 , a >140 -fold decrease in abundance (Supplementary Table S4 and Figure 4C). We did not observe a further decrease in barcode abundance of *miR-34a* from

t_2 to t_3 , because the GRE-qPCR assay specific for *miR-34a* did not detect any *miR-34a*-infected cells at t_2 (or at t_3). Because the detection limit of a 20- μ L qPCR reaction is $C_t = 35$ for detecting a single molecule, qPCR results of $C_t > 35$ were unreliable (39,40). Thus, we assigned $C_t = 35$ when the raw C_t measurements were >35 ; this allowed us to calculate a minimal fold change when input templates were below our detection limit [as is commonly practiced in the literature (41,42)].

We observed different growth-inhibitory kinetics among the 59 growth-inhibitory candidates (Figure 4C, Supplementary Table S4). Twenty-six of the 59 candidates caused ≥ 8 -fold decreased in abundance at that final time point (i.e., candidates with $t_3/t_0 \leq 0.125$ in Supplementary Table S4), illustrated by the *miR-449a~449b* cluster. Fifteen of the 59 candidates caused ≥ 8 -fold decreased abundance at both of the last two time points (i.e., candidates with t_2/t_0 and t_3/t_0 both ≤ 0.125 in Supplementary Table S4), illustrated by *miR-34a*. Eight of the 59 candidates caused ≥ 8 -fold decreased abundance at all three experimental time points (i.e., candidates with $t_{1,2,3}/t_0$ all ≤ 0.125 in Supplementary Table S4), illustrated by *miR-550a-2*. We also identified 10 growth-inhibitory candidates that were undetected at all four time points (i.e., candidates with $t_{0,1,2,3}/t_0 = 0$ in Supplementary Table S4), illustrated by *miR-892b*. The presence of these 10 lenti-miR viruses in the initial library was verified by infecting REH, a human acute leukemia cell line, using the same lot of lenti-miR library (not shown). Therefore, failure to detect these 10 lenti-miRs in IMR90 cells during miR-HTS was not due to a technical problem with this lentivector or GRE-qPCR assay, because REH cells infected with these 10 lenti-miRs were easily detected at t_0 when a miR-HTS was conducted in the REH.

We selected 12 candidate miRs with different growth inhibitory kinetics to test individually for validation. For each selected miR, IMR90 cells were transfected with mature miR mimic, and cell growth was monitored (Figure 5A). *Caenorhabditis elegans miR-67* (*cel-miR-67*), which has minimal sequence homology to any human miR, was used as a negative control. *miR-937*, which was found not to inhibit IMR90 growth in the miR-HTS assays, provided an additional predicted negative control. Nine of 12 (75%) growth-inhibitory miR candidates tested suppressed IMR90 growth as compared with *cel-miR-67* mimic, whereas *miR-937* mimic did not affect IMR90 growth (Figure 5A, Supplementary Figure S2). Transient overexpression of candidate miR mimics demonstrated their direct growth-inhibitory capacity, and ruled out the possibility that the effects observed in the miR-HTS were due either to serendipitous mutations that may have arisen during the month-long miR-HTS culture period or due to lentiviral insertional disruption of the host genome. Except for *miR-513a*, for which we assayed both mature miR strands that could be

Figure 4. Results of miR-HTS conducted in IMR90. (A) Venn diagram of all growth-regulatory miR candidates identified from two independent miR-HTS replicates, including both growth-inhibitory and growth-promoting candidates. The number of candidates identified in the first replicate only (left), the second replicate only (right), and both replicates (middle) were indicated. (B) The average fold changes in abundance of the two miR-HTS replicates were log₂-transformed, sorted from low to high for each experimental time point. Growth-regulatory miR candidates are located in the shaded area above or below the dashed lines at log₂ = 3 (i.e., $t_N/t_0 = 8$) or log₂ = -3 (i.e., $t_N/t_0 = 0.125$). For legibility, data are only shown for miR-HTS replicates whose fold changes are consistent in direction. t_1 , t_2 , and t_3 were days 11, 19, and 27 post-infection, respectively, corresponding to every two passages. (C) Example miR candidates of different growth-inhibitory kinetics were summarized from 2 miR-HTS replicates (mean $\pm$ SD). The dashed horizontal line marks $Y = 0.125$; equivalent to an 8-fold decrease in abundance. *miR-892b* illustrated the candidates that were never detected at any time points examined during the miR-HTS in IMR90.

expressed from a single miR hairpin precursor, we assayed only the mature strand, which is currently thought to be expressed predominantly [most mimics used only had one strand commercially available; strand expression data was obtained from miRBase, www.mirbase.org (43,44)]. Interestingly, both *miR-513a-5p* and *miR-513a-3p* mimics suppressed IMR90 growth (Figure 5A), suggesting that both strands contributed to the *miR-513a*-mediated growth inhibition observed in the miR-HTS. Since both mature miR strands are expected to have been overexpressed during the miR-HTS, it remains to be determined whether the mature strand mimics not assayed in this study could suppress IMR90 growth, especially for the three tested miRs (i.e., *miR-150*, *miR-381*, and *miR-606*) that appeared to be false-positive candidates. Thus, at least 75% (i.e., 9 of 12) of the candidate miRs examined were verified to inhibit IMR90 growth (Figure 5, Supplementary Figure S2).

For *miR-34a*, *miR-298*, and *miR-892b*, we observed cell phenotypes (such as cell retraction and detachment) suggestive of cell death.

Therefore, we directly tested the ability of several candidate miRs to induce apoptotic signaling. Indeed, *miR-34a*, *miR-298*, and *miR-892b* overexpression activated caspase 3/7 by ~ 4 - to 7-fold, as compared with the mock-transfected control (Figure 5B). The data suggested that these miRs suppress growth in part by inducing apoptotic cell death. The miRs that did not induce caspase activation may instead repress cell proliferation or promote caspase-independent cell death.

This proof-of-principle use of our miR-HTS identified a set of 59 miR candidates that inhibited growth of the IMR90 human fetal lung fibroblast cell line in our functional screen. Approximately 35% of the growth-inhibitory miR candidates identified herein have been implicated previously in growth suppression in IMR90 and/or other lung cancer cell lines, including reports that directly evaluated cellular mechanism experimentally (i.e., cell cycle, apoptosis, senescence assays), and reports that observed expression changes associated with growth-inhibitory function (i.e., upregulated expression in senescent cells versus young cells, or downregulated expression in lung

Figure 5. Validation and characterization of selected growth-inhibitory miR candidates. (A) IMR90 cells were transfected with 10 nM miR mimics as indicated. On day 5 post-transfection, cell growth was assessed by the alamarBlue proliferation assay and normalized to the mock (no miR mimic)-transfected sample (i.e., mock as 100%). The *cel-miR-67* mimic and *miR-937* were negative controls (hatched bars). miR candidates were plotted using four different bar patterns (black, white, gray, checker board) matching respective growth-inhibitory kinetics illustrated in Figure 4C. Mean $\pm$ SEM are presented for three independent experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$, each miR mimic versus *cel-miR-67*. Some of these candidates showed significant growth inhibition as early as day 3 post-transfection; some showed significant growth inhibition with 5 nM miR mimics (see Supplementary Figure S2). (B) IMR90 cells were transfected with 10 nM miR mimics. On day 4 post-transfection, caspase-3/7 activity was measured and fold increase in caspase activity was normalized to mock-transfected sample (i.e., mock as 1). Mean $\pm$ SEM are presented for three independent experiments. *, $p < 0.05$, each miR mimic vs. *cel-miR-67*.

cancer samples versus normal counterpart cells; summarized and referenced in Supplementary Table S4). Fifteen of these 59 candidate miRs are known to be upregulated in senescent IMR90 cells, including *miR-449a/b* (45). Ten of these 59 candidate miRs, including four upregulated in senescent IMR90 cells, are known to be downregulated in lung cancers, including *miR-7* (46). Finally, four of the candidate miRs have been shown to promote apoptosis, promote senescence, or inhibit cell cycling in lung cancer cell lines (Supplementary Table S4). It will be interesting to examine whether any of the 11 novel growth-inhibitory miR candidates identified from IMR90 (i.e., the 11 miRs noted with "D" in the column labeled "Known Expression Pattern" of Supplementary Table S4) have tumor-suppressive activity in lung cancers that have downregulated expression of these specific miRs.

Intriguingly, the miR-HTS co-identified three pairs of candidates that are paralogs encoding the same miR hairpin sequence. Having exactly the same mature miR sequence, these paralogous miRs recognize the same targets and thus would be predicted to have the same effect on cell growth. These paralogous candidates include the *miR-550a-1* and *miR-550a-2* paralogs (*miR-550a-3* was not included in the library used), *miR-513a-1* and *miR-513a-2* paralogs, and the *miR-128-1* and *miR-128-2* paralogs (Supplementary Table S4). In addition, multiple members of three additional miR families (i.e., beyond the above three sets of paralogs) were identified in the set of growth-inhibitory candidates [e.g., *miR-888*, *miR-892a*, and *miR-892b* of the *miR-743* family (Supplementary Table S4)]. As a result of the miR hairpin sequence homology within each of these miR families (47), each miR family member may recognize many of the same targets and have similar functions. Moreover, the 59 growth-inhibitory candidates include *miR-34a*, *miR-34c*, and the *miR-449a~449b* cluster, which belong to two different miR families, but share identical

seed sequence. We were surprised to find that *miR-34b*, which is also a member of the *miR-34* family, was not among the candidates identified by the miR-HTS. Overexpression of *miR-34b* caused ≥ 8 -fold decrease in abundance at the last time point in only one of the two miR-HTS replicate screens.

In summary, we have established miR-HTS as a novel methodology to conduct unbiased high-throughput miR functional screens. The 75% validation rate among the 12 selected candidates of a proof-of-principle screen in the IMR90 cell line demonstrated the high fidelity of this approach. It should be possible to apply this miR-HTS technology to assess the roles of miRs in cellular processes other than growth-inhibition, targeting any depletion- or enrichment-based phenotype (e.g., one could identify the set of miRs that enhance differentiation using stage/lineage-specific surface markers to enrich the differentiated fraction from the entire cell population). The estimated costs per sample for miR-HTS is less than half that of other miR barcode quantification methods (see comparison in Supplementary Table S5). In addition, as the lenti-miR library expands, we can readily add more GRE-qPCR assays. Thus, this novel lenti-miR library-based and qPCR-based miR-HTS strategy provides a flexible and reliable platform for miR functional screening, with lower complexity and costs compared with published methods.

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Competing interests

The authors declare no competing interests.

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