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Cover letter

BRIGHAM AND WOMEN'S HOSPITAL

HARVARD MEDICAL SCHOOL

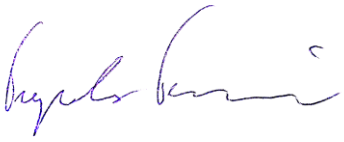
Department of Neurosurgery

Boston June 14th, 2019

Dear Editor of Journal of Visualized Experiments,

We are submitting a revised version of our manuscript, previously titled **Investigating microRNA synergism in brain tumor biology**" which takes into account the comments provided by the editor and reviewers.
A point-by-point response to the reviewers is also attached.
We have provided two additional Supplementary Figures pertaining the workflow with the computer programs for the execution of the protocol. If more of those images are needed, please do not hesitate to contact me.

Sincerely



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TITLE:

Characterization of Functionally Associated miRNAs in Glioblastoma and their Engineering into Artificial Clusters for Gene Therapy

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KEYWORDS:

miRNAs, clusters, in silico, gene therapy, synergism, glioblastoma

SUMMARY:

Described here is a protocol for characterizing modules of biologically synergistic miRNAs and their assembly into short transgenes, which allows simultaneous overexpression for gene therapy applications.

ABSTRACT:

The biological relevance of microRNAs (miRNAs) in health and disease significantly relies on specific combinations of many simultaneously deregulated miRNAs rather than the action of a single miRNA. The characterization of these specific miRNAs modules is a fundamental step in maximizing their use in therapy. This is extremely relevant because their combinatorial attributes can be practically exploited. Described here is a method to define a specific miRNA signature relevant to the control of oncogenic chromatin repressors in glioblastoma. The approach first defines a general group of miRNAs that are deregulated in tumors in comparison to normal tissue. The analysis is further refined by differential culture conditions, underscoring a subgroup of miRNAs that are co-expressed simultaneously during specific cellular states. Finally, the miRNAs that satisfy these filters are combined into an artificial polycistronic transgenes, which is based on a scaffold of naturally existing miRNA clusters genes, then used for overexpression of these miRNA modules into receiving cells.

INTRODUCTION:

miRNAs offer an unmatched opportunity for the development of a broad gene therapy approach to many diseases[1-3], including cancer[4,5]. This is based on several unique features of these biological molecules, including their small size[6], simple biogenesis[7], and natural tendency to function in association[8]. Many diseases are characterized by specific miRNA expression patterns,

which often converge on the regulation of complex biological functions[9]. The purpose of this method is first to define a strategy to identify groups of miRNAs that are synergistically relevant for specific cellular functions. Consequently, it provides a strategy for the re-establishment of such miRNA combinations in downstream studies and applications.

This method allows for functional analysis of multiple miRNAs at once, leveraging on their simultaneous targeting of a large number of mRNAs, thus recapitulating the complex landscapes of diseases. This approach has been recently employed to define a group of three miRNAs that 1) are simultaneously downregulated in brain cancer and 2) show a strong co-expression pattern during neural differentiation as well as in response to genotoxic stress by radiation or a DNA alkylating agent. The combinatorial re-expression of this module of three miRNAs by the clustering method described below results in profound interference with the biology of cancer cells and can be easily used as a gene therapy strategy for preclinical studies[10]. This protocol may be of particular interest to those involved in miRNA research and its translational applications.

PROTOCOL:

1. Characterization of functionally associated miRNAs in glioblastoma

1.1. **Analysis of broad differential miRNA expression in glioblastoma vs. brain**

1.1.1. First, determine the most significantly deregulated miRNAs in the tumor. This can be achieved using at least three different methods:

1.1.1.1. Mine the Cancer Genome Atlas, found at <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>, for sequencing data[11].

1.1.1.2. Perform microarray analysis from a fresh operative specimen[12].

1.1.1.3. Use previously published datasets[13].

NOTE: Whichever method is chosen, the output provides a bulk set of miRNAs whose expression is statistically correlated (either directly or inversely) with tumor biology. This initial group constitutes the pool of miRNAs that are further analyzed for the identification of a subset of miRNAs displaying the most stringent functional association by performing a more dynamic analysis of expression changes, as discussed below.

1.2. **Condition-specific miRNA expression analysis**

1.2.1. Induction of cell differentiation:

1.2.1.1. Use pre-coated poly-D-lysine (PDL) plastic dishes to favor the formation of a monolayer culture. For dish coating, dissolve PDL in water at a concentration of 100 µg/mL. Use 1 mL of solution per 25 cm^2 surface. Rinse 2x with PBS 6 h after application of the PDL solution.

1.2.1.2. Plate human neural stem cells in equal amounts (100,000 cells/5 mL) either in stem cell medium (neurobasal medium + B27 supplement + 20 ng/mL EGF/FGF), astrocytic differentiation medium (DMEM + 10% FBS), or neuronal differentiation medium (neurobasal medium + B27 supplement, + 2 µM retinoic acid)[14]. The protocol for oligodendrocyte differentiation requires addition of IGF-1 (200 ng/mL) after EGF/FGF removal[14,15].

1.2.1.3. After cell plating, place in incubator for 1 week at 37 °C, 5% CO_2.

1.2.2. RNA extraction:

1.2.2.1. After 7 days, remove the cells from the incubator and remove the medium.

1.2.2.2. Wash the cells with PBS (5 mL). Then, add 1 mL of lysis reagent (see **Table of Materials**) to lyse the cells and scrape the cells into 1.5 mL microfuge tube using a cell scraper. Keep the tubes containing the lysed cells at room temperature (RT) for 5 min.

1.2.2.3. Proceed to total RNA isolation following the manufacturer's instructions.

1.2.3. miRNA expression analysis:

1.2.3.1. Quantify the differential expression of specific miRNAs identified in step 1.1 among the three differentiation patterns by real-time PCR, using TaqMan probes and following the manufacturer's protocols for reverse transcription and PCR amplification (**Figure 1**).

1.3. **Validation of miRNA clusters by stress-specific challenges**

1.3.1. Culture G34 glioblastoma stem-like cells in neurobasal medium + B27 supplement + 20 ng/mL EGF/FGF. Start with 1 x 10^6 cells in 5 mL in a 25 cm^2 low attachment flask.

1.3.2. Starting 48 h after plating, add doubling concentrations of DNA alkylating agent temozolomide (TMZ) every 5 days, as follows:

1.3.2.1. Start with 5 µM for 5 days. After 5 days, remove the medium and replace with fresh medium without temozolomide, to allow the surviving cells to recover. After 48 h, add 10 µM TMZ and incubate for another 5 days.

1.3.2.2. Repeat step 1.3.2.1, doubling TMZ concentration each time, until a concentration of at least 100 µM is reached and cells become resistant to the drug[10].

1.3.3. In a parallel experiment, irradiate G34 cells as follows:

1.3.3.1. Starting 48 h after plating 1 x 10^6 cells in 5 mL in a 25 cm^2 low attachment flask, irradiate cells with 2 Gy of energy (any irradiator providing photon emission is acceptable). Return the cells

to the incubator and do not disturb. After 48 h, irradiate the cells again with additional 2 Gy of energy.

1.3.3.2. Repeat step 1.3.3.1 4x until a total of 10 Gy of energy is administered.

1.3.3.3. Return the cells to the incubator and let them recover in fresh medium for at least 5 days before downstream analysis.

1.3.4. After the induction of resistance, lyse cells using lysis reagent and extract total RNA according to the manufacturer's protocol.

1.3.5. Analyze the expression of the specific miRNAs identified in sections 1.1–1.2 as described in step 1.2.1 (**Figure 2**).

1.4. **Characterization of the functional convergence of clustered miRNAs**

1.4.1. Obtain the predicted targetome of each miRNAs defined in sections 1.2–1.3 obtained using miRNA targeting prediction tools.

NOTE: We generally resort to TargetScan, as its algorithm is periodically updated[16]. Additional prediction tools are miRanda[17], miRDB[18], and DIANA-miRPath[19]. All these programs are free, web-based applications.

1.4.1.1. From the front page of Targetscan, select the miRNA of interest from the pre-populated drop-down menu. Click the **Submit** button.

1.4.1.2. Download the resulting list of targets as a spreadsheet using the **Download table** link (**Supplementary Figure 1**).

1.4.1.3. To decrease the chances of false positive predictions, include only targets within the conserved sites column in downstream analyses. Also, further stringency can be obtained by using additional miRNA prediction programs (see above) and only including targets that are common to all algorithms.

1.4.2. Classify the resulting targetomes according to Gene Ontology categories using ToppGene Suite[20] to evaluate for the enrichment of pathways that are common to each miRNA.

1.4.2.1. Paste the list of targets obtained from step 1.4.1 in the "Training gene set" window, using HGNC symbol as the entry type. Click **Submit** > **Start**. The program will provide an output table showing the most significant "Go" categories for the entered list of genes (**Supplementary Figure 2**).

1.4.3. To finally establish the contribution of each miRNA to the regulation of a common pathway or cellular process (in this case, chromatin regulation), check each targetome obtained in step

1.4.1 against the full list of genes involved in the specific cellular process (i.e., chromatin regulation), using the Venn diagram function provided in the Bioinformatics and Evolutionary Genomics website <http://bioinformatics.psb.ugent.be/webtools/Venn>.

NOTE: This program allows the intersection of multiple groups at the same time (**Figure 3**). The specific unique targets provided by each miRNA are then annotated and selected for downstream functional experiments.

1.4.3.1. In the front page of the program, upload or copy/paste the list if target mRNAs for each miRNA or interest in the respective windows. Name each list with a unique identifier.

1.4.3.2. Click **Submit**. The program will provide a visual output of a Venn diagram with numbers of genes in each sector, as well as a full list of mRNAs for each subset and intersection combinations.

2. Assembly of miRNA modules into a polycistronic transgenic cluster

2.1. Preparation of transgenic scaffold based on miR-17-92 cluster locus

2.1.1. Obtain the nucleotide sequence of the miR-17-92 cluster from the Ensembl genome browser[21]. Select the ~800 base pair "core" sequence encompassing all six encoded miRNA hairpins of the locus and at least 200-nucleotide flanking sequences both upstream (5') and downstream (3') of the core sequence.

2.1.2. Paste the sequence above into any word editing program.

2.1.3. Define the sequence of each one of the six native hairpins by retrieving them in miRBase[22]. Mark each one of these sequences within the span of the previously identified "core" sequence. Any sequences between each hairpin represent spacer sequences, which are important for the correct processing of the transgene.

2.1.4. Remove the native hairpin sequences from the "core" sequence, with the exception of 3–5 nucleotides at both the 5' and 3' ends of each hairpin, which will serve as an acceptor for the new hairpins.

2.2. Building a new transgene encoding multiple miRNAs of choice

2.2.1. Obtain hairpin sequences of miRNAs intended to be overexpressed in the transgene from miRBase. Carefully note the specific nucleotides that are the sites of microprocessor cleavage.

2.2.2. Replace the removed hairpins (step 2.1.4) with the hairpin sequences of the desired miRNAs obtained in step 2.2.1.

2.2.3. Add desired restriction sequences at both flanking regions of the transgene to facilitate

subcloning into delivery vectors of choice. Verify that the chosen restriction sequences are not present within the sequence itself.

2.2.4. For negative controls, use 20-nucleotide scrambled sequences to replace the natural 20-nucleotide sequence of each mature miRNA. Generate these scrambled sequences using an online tool such as <https://genscript.com/tool/create-scrambled-sequence>.

2.3. **Verifying the two-dimensional structure of the transgene**

2.3.1. Copy the full transgenic sequence into the RNA structure prediction software program RNAweb Fold[23].

2.3.2. Using standard program settings, click **Proceed**.

2.3.3. Analyze the graphical output, particularly for the presence of well-defined hairpins and presence of double-stranded stem structures at least 11 nucleotides proximal to the microprocessor cleavage site. Also, look for the absence of branching points within the hairpin sequences (**Figure 4**).

3. Obtaining transgenes by DNA synthesis

3.1. After the design and in silico validation of the chimeric miRNA cluster, obtain the working sequence using DNA synthesis from commercial vendors[24] and proceed with downstream cloning into vectors and transgene delivery. We have used lentiviral vectors for in vitro delivery[10] and adeno-associated viruses (AAVs) for in vivo intracranial delivery[25].

REPRESENTATIVE RESULTS:

This method allowed characterization of a module of three miRNAs that are consistently downregulated in brain tumors, which are co-expressed specifically during neuronal differentiation (**Figure 1**) and involved in the tumor survival response after therapy (**Figure 2**). This is accomplished by regulating a complex oncogenic chromatin repressive pathway. This co-expression pattern suggested a strong synergistic activity among these three miRNAs (**Figure 3**). Consequently, taking advantage of the small size and simple biogenesis of miRNAs, the second part of this protocol was used to design a transgene (**Figure 4**) that could simultaneously recapitulate the expression of the three miRNAs in glioblastoma cells, both in vitro and in vivo, with significant interference in tumor biology and promising translational applicability (**Figure 5A,B,C**)[10]. Additionally, it was demonstrated that the transgenic cluster is also functional in a breast cancer model (**Figure 5D,E**).

FIGURE AND TABLE LEGENDS:

Figure 1: Characterization of a functional miRNA module in glioblastoma. **(A)** Volcano plot representing the differentially expressed miRNAs in human glioblastoma samples (n = 516) vs.

normal brain (n = 10) obtained from TCGA. In green are miRNAs with >4-fold difference. The red circle represents the 10 most significantly downregulated miRNAs in glioblastoma. **(B)** Relative expression of the 10 miRNAs selected in (A) during induction of different differentiation pathways in neural stem cells, showing clear upregulation of the miR-124, miR-128, and miR-137 modules during induction of neural differentiation. Mean ± SD from three biological replicates (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; Student's t-test, two-tailed). This figure has been modified with permission[10].

Figure 2: Confirmation of co-expression patterns of miRNA modules during genotoxic stress. Relative expression of the three miRNAs defined in **Figure 1** in multiple glioblastoma cells and cell lines (G62-mesenchymal; MGG4-proneural; U251, U87 pro-neural-like, and T98G mesenchymal-like glioblastoma cell lines) after induction of resistance to temozolomide (TMZ, pink bars) or ionizing radiation (RT, green bars). Reported are means with SD from two independent replicates. This figure has been modified with permission[10].

Figure 3: Analysis of functional convergence of the targetomes of co-expressed miRNAs. Venn diagram output from the Bioinformatics and Evolutionary Genomics website, simultaneously crossing the targetome of labelled miRNAs with the mRNAs constituting a GO category of interest (in this case, chromatin repressors). mRNAs uniquely targeted by single miRNAs were chosen for further downstream functional studies.

Figure 4: 2D structure of an engineered miRNA sequence encoding the three miRNAs cluster. Graphical output from the RNAweb Fold program. Note the presence of three well defined stem-loop structures which represent the hairpins of each respective miRNA (miR-124, miR-128, miR-137) encoded by the transgene.

Figure 5: Evidence of transgene processing and its downstream biological effect. (A) Relative quantification of miRNA expression after lentiviral-mediated transduction of G34 glioblastoma cells with the transgenic miRNA cluster (Cluster 3) or negative control (ctrl). Reported are means from three independent experiments ± SD. **(B)** Fluorescence microscope pictures of G34 glioblastoma spheres expressing negative control transgene vs. Cluster 3 transgene. Scale bar = 100 µm. **(C)** In vivo growth of intracranial human G34 cell xenografts expressing either control or Cluster 3 transgenes. Scale bar = 1 mm. This figure has been reproduced with permission[10]. **(D)** Relative quantification of miRNA expression after lentiviral-mediated transduction of MDA-MB-231 breast cancer cells with the transgenic miRNA cluster (Cluster 3) or negative control (ctrl). (**E**) Fluorescence microscope images of MDA-MB-231 breast cancer cells expressing negative control transgene vs. Cluster 3 transgene. Scale bar = 100 µm. All experiments were performed in triplicates (*p < 0.05; **p < 0.01; Student's t-test, two-tailed, multiple comparisons).

Supplementary Figure 1: TargetScan workflow. **(A)** Home page screenshot, showing selection options for miRNA search. **(B)** Representative search results for miR-137. List of target genes is in the left column. Red box denotes genes with conserved targeting sites (suggesting higher confidence of real targeting).

Supplementary Figure 2: ToppGene Suite workflow. (A) Home page screenshot showing the search box where the list of genes to be analyzed is inserted. **(B)** Representative search result for the miR-137 targetome, showing the most statistically significant Gene Ontology (GO) categories.

DISCUSSION:

This protocol is based on the notion that rather than functioning in isolation, miRNAs are biologically relevant by working in groups, and these groups are transcriptionally determined by specific cellular contexts[26]. To justify this approach from a translational perspective, a follow-up protocol that allows recreation of this multi-miRNA pattern in cells/tissues is introduced. This is possible by taking advantage of the relatively simple biogenesis of miRNAs, whereby the recognition of the characteristic miRNA hairpin by microprocessor is necessary and sufficient for correct miRNA processing[27]. At the same time, this minimal requirement allows use of the genetic scaffold of naturally occurring miRNA clusters as a backbone for the expression of desired miRNA modules, which are contained within short DNA sequences that can be fitted into any delivery vectors of choice. The major requirement of this protocol is maintaining the hairpin structure and sufficient length of the stem component to allow appropriate cleavage.

There are two major critical considerations regarding the execution of this protocol. The first point is the accurate determination of the optimal miRNA combinations. This is determined by careful analysis of not only the miRNA expression signature of cells or tissues compared to controls, but also of any simultaneous expression changes observed as the cells are experimentally manipulated. Once a set of miRNAs is defined, it is also fundamental to ascertain that the artificial modulation of each singularly does not modify expression of the others.

The second critical aspect concerns the construction of chimeric sequences to artificially recapitulate this functional miRNA clustering. It is fundamental to abide by the structural requirements of the miRNA processing machinery, which is the presence of a long enough stem sequence (measuring at least 11 nucleotides) at the origin of each miRNA hairpin[18], as well as maintenance of original spacing sequences of the native miRNA cluster scaffold. In our experience, fulfilling these two requirements has consistently yielded appropriate RNA folding (**Figure 4**) and resulted in successful multi-miRNA expression.

The major limitation of this technique is the finite number of miRNAs that can be clustered together into a functional transgene. We have successfully engineered sequences overexpressing up to six different miRNAs but observed some decrease in processing efficiency as the number of hairpins increases. So far the genetic structure of the miR-17-92 cluster has been used, because it is the one encoding the highest number of hairpin (six) within the shortest DNA sequence (~800 base pairs)[8]. As there are other natural miRNA clusters, it is anticipated that they could also be used for this purpose[28]. Finally, it has been observed that modification of spacing sequences among the hairpin decreases their processing, so there are some constraints regarding to what extent the native structure can be modified.

The most significant and advantageous aspect of this proposed method is that it allows the

engineering of transgenes that are able to simultaneously overexpress multiple desired miRNAs mainly using an in silico approach, limiting the need for tedious and time-consuming molecular cloning steps, as previously described[29-31]. The protocol described is easy to execute and does not require specialized equipment or skills.

In consideration of the recognized importance of miRNAs in molecular biology and their potential in therapeutic applications, this protocol is of interest to a large audience of researchers. This approach is expected to encourage future studies that focus on the combinatorial properties of miRNAs and will serve as a simple and robust tool for their execution. More importantly, these clustered transgenes represent ideal cargos for gene therapy vectors. Evidence of successful in vivo delivery of a 3-miRNA cluster has already been obtained via direct intratumoral intracranial injection of AAV vectors (unpublished data). It is thus anticipated that this technique will significantly boost the translational aspects of miRNA research.

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DISCLOSURES:

The authors report no conflicts of interest.

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Figure 1

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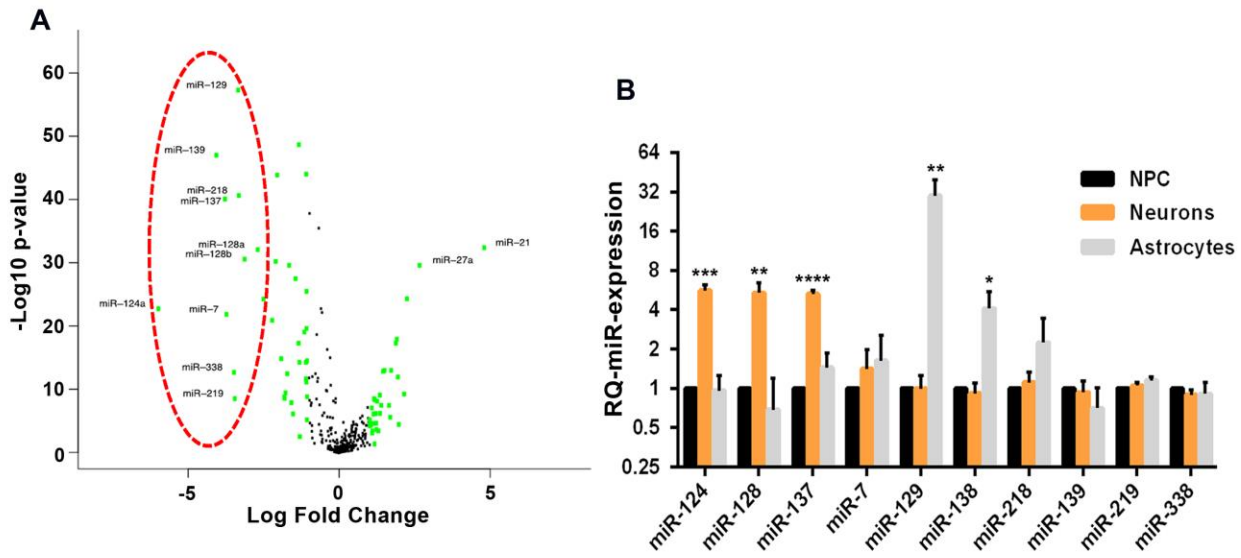

Figure 2

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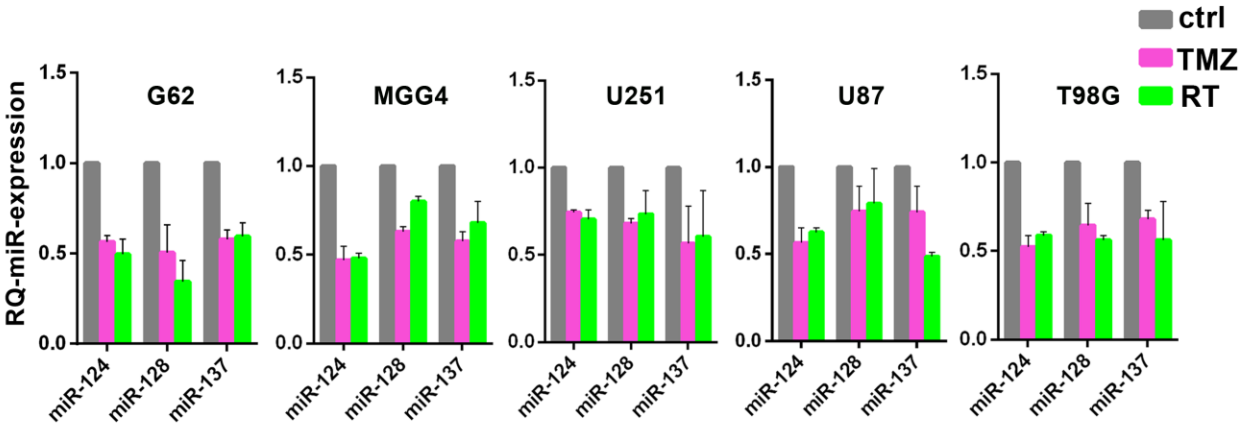

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Calculate and draw custom Venn diagrams

Bioinformatics Evolutionary Genomics

Input files:

List names	number of elements	number of unique elements
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miR-124	1097	1096
miR-128	906	904
miR-137	1065	1062
Overall number of unique elements		**2667**

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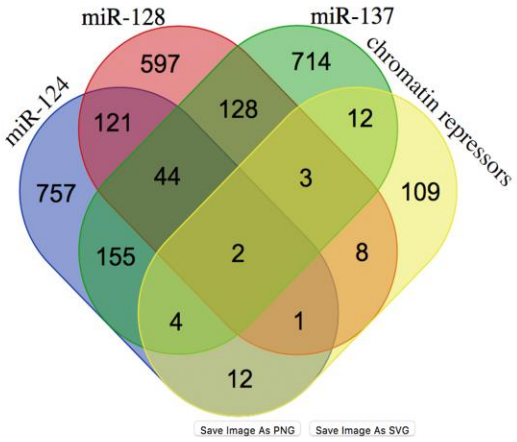

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Figure 4

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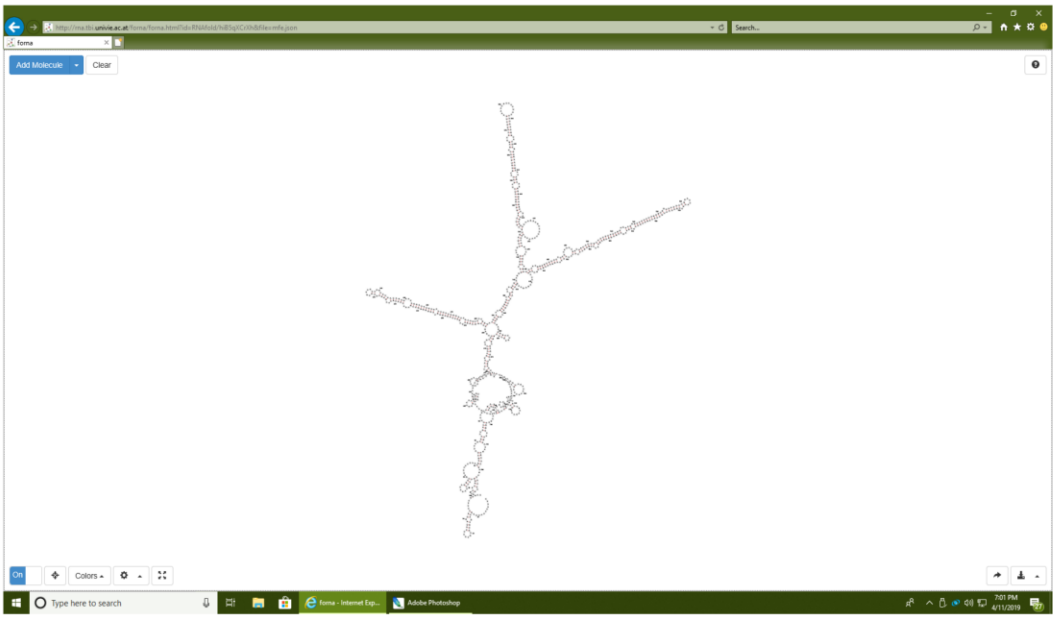

Figure 5

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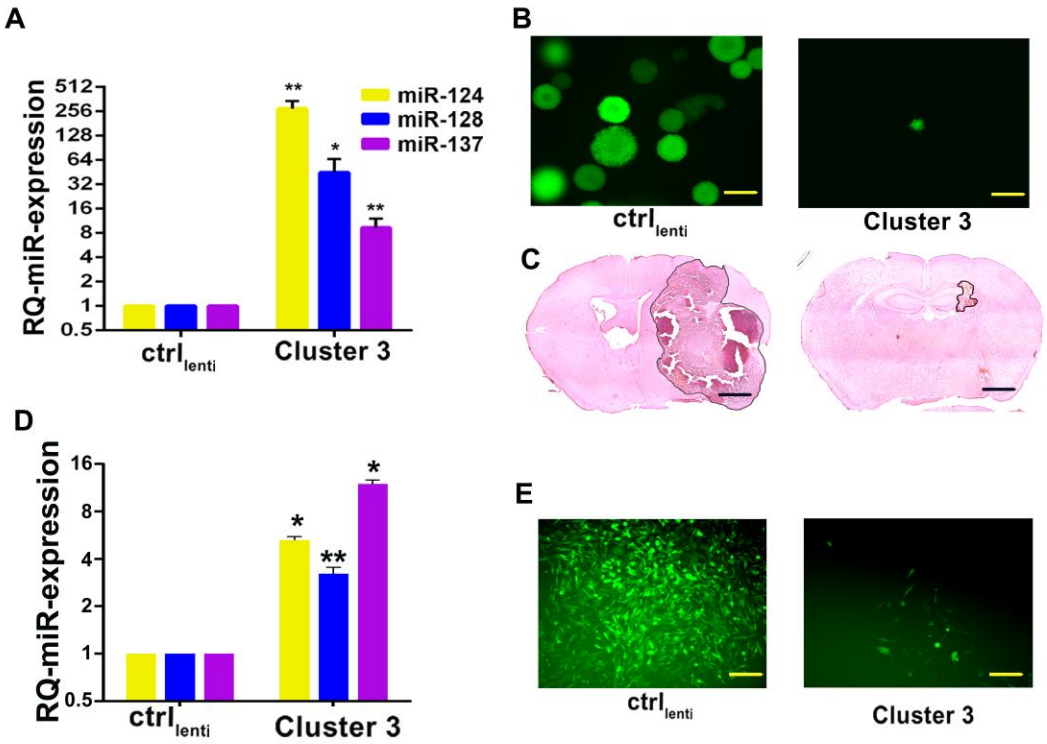

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/ Equipment

0.4% low melting temperature agarose
0.45 µM sterile filter unit
1.5-mL Microcentrifuge tube
6-Well plates
Athymic mice (FoxN1 nu/nu)
B-27 Supplement
Cell culture flask
Cell Scraper, 16cm
Cesium 137 irradiator
Chloroform
DMEM, high glucose, pyruvate
Dulbecco's phosphate-buffered saline
Eosin Y solution
Fetal Bovine Serum
Formalin solution
GlutaMAX Supplement
HEK-293
Hematoxylin solution
Human primary glioma stem-like cells (GBM62)

Human primary glioma stem-like cells (MGG4)
Lentiviral vector pCDH-CMV-MCS-EF1-copGFP
Lipofectamine 2000
Microcentrifuge refrigerated
Mounting medium

Nalgene High-Speed Polycarbonate Round Bottom Centrifuge Tubes
NanoDrop
Neural Progenitor cells (NPC)
Neurobasal Medium
Nikon eclipse Ti motorized fluorescent microscope system
Opti-MEM
PCR tubes
Penicillin-Streptomycin
Petri-Dishes 94/16
Poly-D-Lysine
Recombinant Human EGF
Recombinant Human FGF-basic
Retinoic acid
RNA Miniprep Kit
S1000 Thermal Cycler

Small Animal Image-Guided Micro Irradiator

Sorvall WX+ Ultracentrifuge
StemPro Accutase
StepOne Real-Time PCR System
SterilGARD biosafety cabinet
Sucrose
T98-G
TaqMan MicroRNA Reverse Transcription Kit
TaqMan Universal PCR Master Mix
Temozolomide
Tissue-Tek optimum cutting temperature
TRIzol Reagent
U251-MG
U87-MG
ViraPower Lentivector Expression system
Water, HPLC grade
Xylene

Company	Catalog Number
IBI Scientific	IB70058
Merck Millipore	SLH033RS
Eppendorf	22431081
Greiner Bio-One	657160
Envigo	069(nu)/070(nu/+)
Thermo Fisher Scientific	12587010
Greiner Bio-One	660175
Sarstedt	83.1832
JL Sheperd and Associates	Core Facility (Harvard Medical School)
Sigma-Aldrich	439142-4L
Thermo Fisher Scientific	11995040
Gibco	14190144
Sigma-Aldrich	E4009
Sigma-Aldrich	F9665
Sigma-Aldrich	HT501128
Thermo Fisher Scientific	35050061
American Type Culture Collecti	ATCC CRL-1573
Sigma-Aldrich	1051750500
System Biosciences	CD511B-1
Thermo Fisher Scientific	11668019
Eppendorf	model no. 5424 R, cat. no.5404000138
Thermo Fisher Scientific	4112APG
Thermo Fisher Scientific	3117-0380PK
Thermo Fisher Scientific	2000c
Thermo Fisher Scientific	21103049
Nikon, Japan	14314
Thermo Fisher Scientific	31985088
Sigma-Aldrich	CLS6571-960EA
Thermo Fisher Scientific	15140122
Greiner Bio-One	632180
Sigma- Aldrich	P4707
PeproTech	AF-100-15
PeproTech	AF-100-18B
Gibco	12587-010
Direct-zol	R2050
Bio-Rad	1852196
Xstrahal Life Sciences, UK	Core facility (Dana-Farber Cancer Institute, Boston, MA)

Thermo Fisher Scientific	75000100
Thermo Fisher Scientific	A1110501
Applied Biosystems	4376357
The Baker Company	SG403A-HE
Sigma-Aldrich	S9378
American Type Culture Collecti	ATCC CRL-1690
Thermo Fisher Scientific	4366596
Thermo Fisher Scientific	4324018
Tocris Bioscience	2706
Fisher Scientific	NC9636948
Thermo Fisher Scientific	15596026
American Type Culture Collecti	ATCC HTB-17
American Type Culture Collecti	ATCC HTB-14
Thermo Fisher Scientific	K4970-00
Fisher	W54
Sigma-Aldrich	534056

Comments/Description

Provided by Dr. E. A. Chiocca (Brigham and Women's Hospital, Boston, MA)

Provided by Dr. Hiroaki Wakimoto (Massachusetts General Hospital, Boston, MA)

Provided by Dr. Jakub Godlewski (Brigham and Women's Hospital, Boston, MA)

Lysis reagent

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Author(s): Vivek Bhaskaran and Pierpaolo Peruzzi

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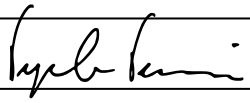
Signature:	
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Rebuttal Letter
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Response to Reviewers

We wish to thank each reviewer for her/his comments and suggestions. Please find below our point-by-point response.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
MicroRNAs do not affect gene expression in isolation. Several miRs can be over or under expressed at the same time and as such can affect a specific pathway. This method was developed in order to co-express a number of miRs that were found to be simultaneously deregulated in brain cancer and were shown to be co-expressed during neural differentiation. Using the method described in this article one can easily create a construct to co-express miRs to check their effect on cells of interest.

Major Concerns:
No major concerns.

Minor Concerns:
A lack in knowledge of Bioinformatics tools could be of concern when working on determining microRNAs that can work synergistically to regulate a specific pathway.

Thank you for your kind considerations. We just would like to emphasize how this protocol takes advantage of easily accessible bioinformatics tools and their basic applications, and does not require specific bioinformatics training to be executed.

Reviewer #2:

Manuscript Summary:
In Peruzzi P and Bhaskaran V, the authors present compelling evidence and detailed description on step-to-step approach in designing a module of microRNAs targeting brain tumor biology. Coupling with the video, the manuscript will be at great interest of JoVE readers. The manuscript is well written and only minor points need to be attended prior to acceptance for publication:

Major Concerns:
nil.

Minor Concerns:

Title : The title is still not entirely represent the content included in the text and video (coming soon). The manuscript is focusing on the systemic workflow in identifying a module of microRNAs Suggest to improve the title to highlight the type of study design.

Thank you for your consideration. The title has been changed accordingly.

PROTOCOL:

Line 66 - include webpage link of TCGA. This has been provided.

Line 73 - authors need to be consistent in using the terms of "miR" or "microRNAs" throughout the manuscript. We have kept the “microRNA” word throughout the manuscript.

Line 77 to line 80- the human stem cells are subsequently differentiated into astrocytes and neurones. It would be great if the authors can include information whether human stem cells can also be differentiated into oligodendrocytes? (if yes, author can briefly describe and cite some papers for this purpose). We have added in the manuscript the protocol and references for oligodendrocyte differentiation.

Line 94 to 100 - need to specify the glioblastoma stem-like cells used in the study and also the culture media (similar to as described in Line 77 to 80). This specification was provided.

Line 107 to 112 - author may suggest more microRNA prediction program (DIANA, miRDB and include their webpage link / inline equations). DIANA-miRPath and MIRDB have been added, including web links and citations.

Line 143 - why "encompassing all 6 encoded microRNA hairpins"? miR-17-92 cluster encodes 6 microRNA hairpins, thus the use of that sentence.

Line 179 - after this step, suggest the authors to include protocols to build transgene containing scramble sequences. The strategy for building scrambled sequences has been mentioned.

Line 189, italic "in vitro", "in vivo". Corrected.

Line 195 - Figure 1a - it is not very clear that the color of the circle is RED. Please amend. The color has been changed.

Line 200 - formatting issue on "Neural Stem Cells" ? Corrected

Line 205 to 210 - need to include abbreviation of TMZ and RT. For the TZ group, it is not clear that the bars are "violet" in color. Please amend. Explain the differences among G62, MGG4, U251, U87, T98G (are they proneural type? Mesenchymal? Different subtypes?) Subtypes have been clarified in the legend.

Line 228 - "in vivo" Corrected

Reviewer #3:

Manuscript Summary:

The authors developed a method to replicate the natural occurring synergistic co-expression of regulative microRNAs. Such method includes a) a strategy to identify MicroRNAs working synergistically and b) instructions how to re-establish such MicroRNAs combinations for further study and applications. They demonstrated the feasibility of the proposed method using a specific 31 microRNA signature (cluster) found relevant in glioblastoma, a brain cancer.

Main advantage indicated is the possibility to analyze multiple microRNAs at once, leveraging on their simultaneous targeting of a large number of mRNAs, and thus recapitulating more faithfully the complex landscapes of diseases. In order to replicate the biological conditions, microRNAs are combined together into an artificial polycistronic transgene which is based on the scaffold of naturally existing microRNA cluster gene, and is used for overexpression of these microRNA modules into receiving cells.

Major Concerns:
None

Minor Concerns:
Showing the efficacy of this protocol in another model could have been useful;
We have added Panel D and Panel E in Figure 5, showing the transduction of Cluster 3 in breast cancer cells MDA-MB-231 and its resulting antiproliferative effect.

This protocol requires a strong knowledge of Bioinformatics skills.
This protocol takes advantage of easily accessible bioinformatics tools and their basic applications, and does not require specific bioinformatics training to be executed.

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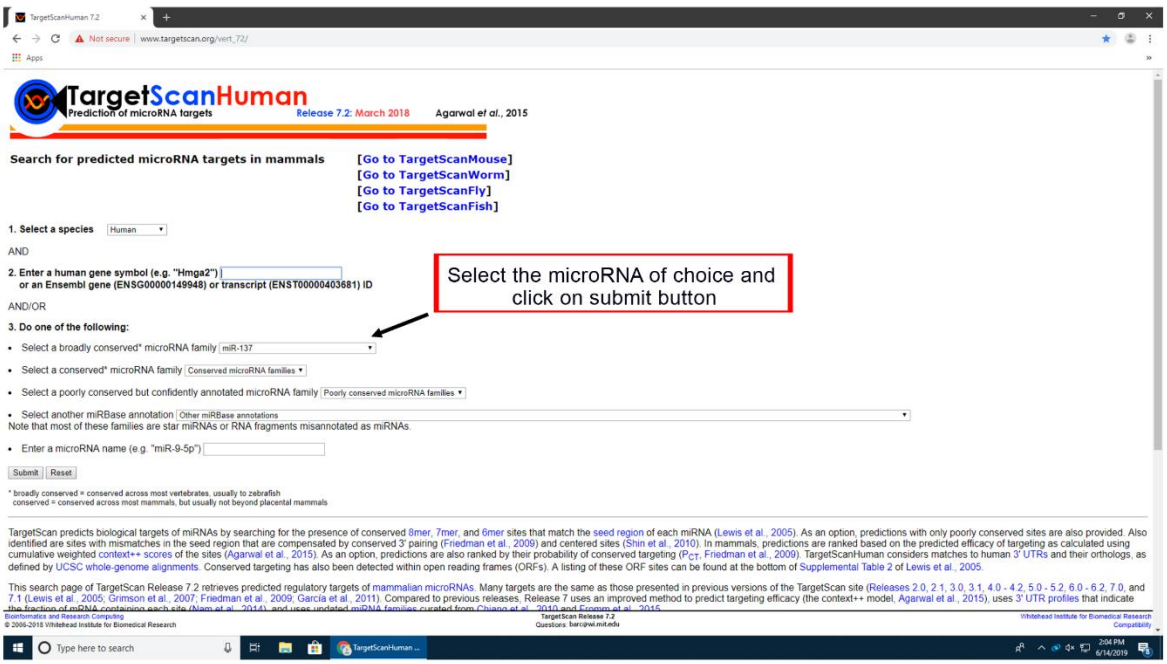
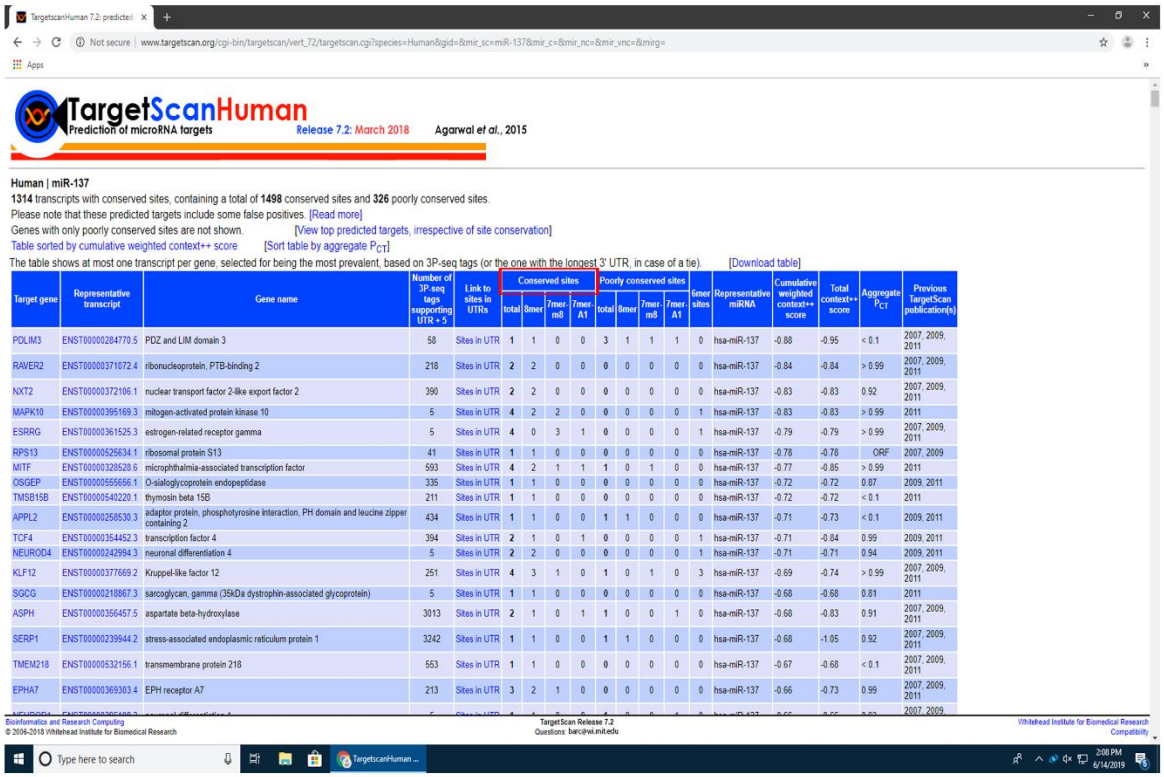
Supplementary Figure 1: TargetScan workflow

A



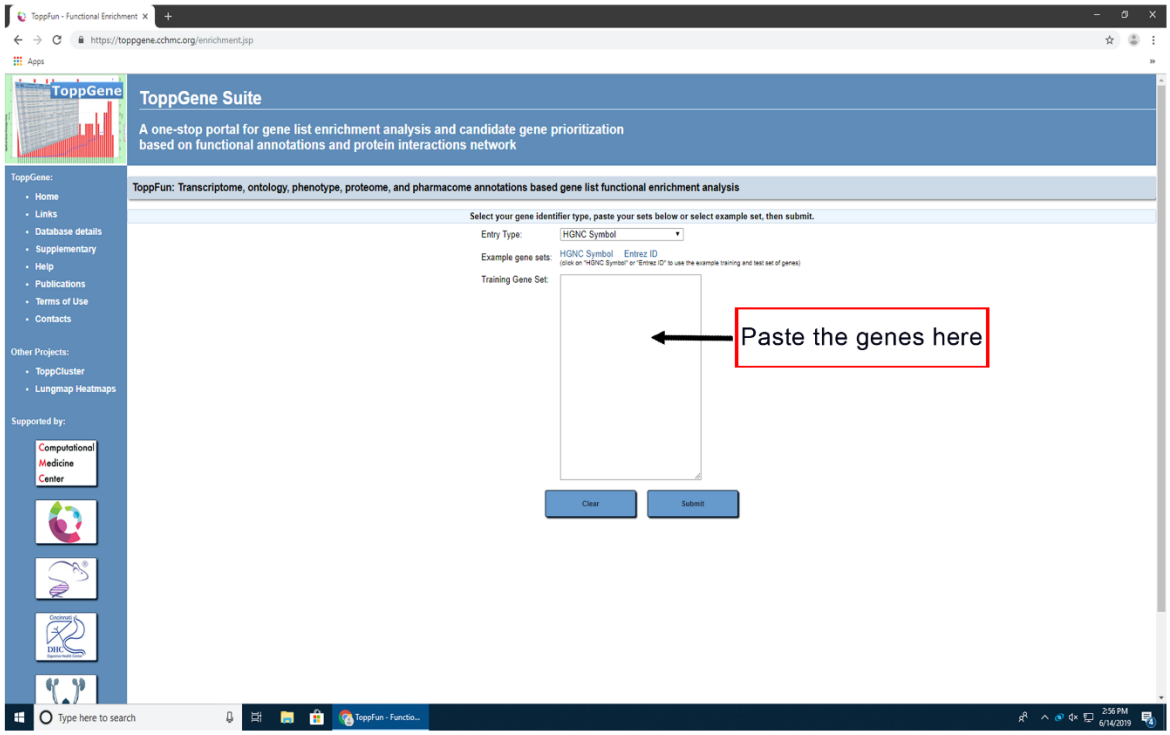
B



Target gene	Representative transcript	Gene name	Number of 3P-seq tags supporting UTR + 5	Link to sites in UTRs	Conserved sites				Poorly conserved sites				6mer sites	Representative miRNA	Cumulative weighted context++ score	Total context++ score	Aggregate P_{CT}	Previous TargetScan publication(s)
					total	8mer	7mer-m8	7mer-A1	total	8mer	7mer-m8	7mer-A1						
PDLIM3	ENST00000284770.5	PDZ and LIM domain 3	58	Sites in UTR	1	1	0	0	3	1	1	1	0	hsa-miR-137	-0.88	-0.95	< 0.1	2007, 2009, 2011
RAVER2	ENST00000371072.4	ribonucleoprotein, PTB-binding 2	218	Sites in UTR	2	2	0	0	0	0	0	0	0	hsa-miR-137	-0.84	-0.84	> 0.99	2007, 2009, 2011
NXT2	ENST00000372106.1	nuclear transport factor 2-like export factor 2	390	Sites in UTR	2	2	0	0	0	0	0	0	0	hsa-miR-137	-0.83	-0.83	0.92	2007, 2009, 2011
MAPK10	ENST00000395169.3	mitogen-activated protein kinase 10	5	Sites in UTR	4	2	2	0	0	0	0	1	0	hsa-miR-137	-0.83	-0.83	> 0.99	2011
ESRRG	ENST00000361525.3	estrogen-related receptor gamma	5	Sites in UTR	4	0	3	1	0	0	0	1	0	hsa-miR-137	-0.79	-0.79	> 0.99	2007, 2009, 2011
RPS13	ENST00000525634.1	ribosomal protein S13	41	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-137	-0.78	-0.78	ORF	2007, 2009
MITF	ENST00000328528.6	microphthalmia-associated transcription factor	593	Sites in UTR	4	2	1	1	1	0	1	0	0	hsa-miR-137	-0.77	-0.85	> 0.99	2011
OSGEP	ENST00000555656.1	O-sialoglycoprotein endopeptidase	335	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-137	-0.72	-0.72	0.87	2009, 2011
TMSB15B	ENST00000540220.1	thymosin beta 15B	211	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-137	-0.72	-0.72	< 0.1	2011
APPL2	ENST00000258530.3	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2	434	Sites in UTR	1	1	0	0	1	1	0	0	0	hsa-miR-137	-0.71	-0.73	< 0.1	2009, 2011
TCF4	ENST00000354452.3	transcription factor 4	394	Sites in UTR	2	1	0	1	0	0	0	0	1	hsa-miR-137	-0.71	-0.84	0.99	2009, 2011
NEUROD4	ENST00000242994.3	neuronal differentiation 4	5	Sites in UTR	2	2	0	0	0	0	0	0	1	hsa-miR-137	-0.71	-0.71	0.94	2009, 2011
KLF12	ENST00000377669.2	Kruppel-like factor 12	251	Sites in UTR	4	3	1	0	1	0	1	0	3	hsa-miR-137	-0.69	-0.74	> 0.99	2007, 2009, 2011
SGCG	ENST00000218867.3	sarcoglycan, gamma (35kDa dystrophin-associated glycoprotein)	5	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-137	-0.68	-0.68	0.81	2011
ASPH	ENST00000356457.5	aspartate beta-hydroxylase	3013	Sites in UTR	2	1	0	1	1	0	0	1	0	hsa-miR-137	-0.68	-0.83	0.91	2007, 2009, 2011
SERP1	ENST00000239944.2	stress-associated endoplasmic reticulum protein 1	3242	Sites in UTR	1	1	0	0	1	1	0	0	0	hsa-miR-137	-0.68	-1.05	0.92	2007, 2009, 2011
TMEM218	ENST00000532156.1	transmembrane protein 218	553	Sites in UTR	1	1	0	0	0	0	0	0	0	hsa-miR-137	-0.67	-0.68	< 0.1	2007, 2009, 2011
EPHA7	ENST00000369303.4	EPH receptor A7	213	Sites in UTR	3	2	1	0	0	0	0	0	0	hsa-miR-137	-0.66	-0.73	0.99	2007, 2009, 2011

Supplementary Figure 2: ToppGene Suite workflow

A



B