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Analysis of combinatorial miRNA treatment to regulate cell cycle and angiogenesis

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Phillip Steindel, Ph.D.
Review Editor
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RE: JoVE59460R1

Dear Dr. Steindel:

Thank you for evaluating the above referenced manuscript titled "Analysis of combinatorial miRNA treatment to regulate cell cycle and angiogenesis", which I am submitted for publication in JoVE on behalf of all the authors, following the appropriate revisions, as were requested by the editorial comments and our efforts to address all of the comments appropriately.

Attached please find:

1. A point-by-point summary of our response to the reviewers' comments
2. A clean copy of the revised manuscript.

We hope that you find this revised manuscript acceptable for publication.

Sincerely yours,

George Matthaiolampakis, Ph.D

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Title:
Analysis of Combinatorial miRNA Treatments to Regulate Cell Cycle and Angiogenesis

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KEYWORDS:
transfection, miRNA, lung cancer, cell cycle, apoptosis, angiogenesis, RNA sequencing

SUMMARY:
miRNA therapeutics have significant potential in regulating cancer progression. Demonstrated here are analytical approaches used for identification of the activity of a combinatorial miRNA treatment in halting cell cycle and angiogenesis.

ABSTRACT:
Lung cancer (LC) is the leading cause of cancer-related deaths worldwide. Similar to other cancer cells, a fundamental characteristic of LC cells is unregulated proliferation and cell division. Inhibition of proliferation by halting cell cycle progression has been shown to be a promising approach for cancer treatment, including LC.

miRNA therapeutics have emerged as important post-transcriptional gene regulators and are increasingly being studied for use in cancer treatment. In recent work, we utilized two miRNAs, miR-143 and miR-506, to regulate cell cycle progression. A549 non-small cell lung cancer (NSCLC)

cells were transfected, gene expression alterations were analyzed, and apoptotic activity due to the treatment was finally analyzed. Downregulation of cyclin-dependent kinases (CDKs) were detected (i.e., CDK1, CDK4 and CDK6), and cell cycle halted at the G1/S and G2/M phase transitions. Pathway analysis indicated potential antiangiogenic activity of the treatment, which endows the approach with multifaceted activity. Here, described are the methodologies used to identify miRNA activity regarding cell cycle inhibition, induction of apoptosis, and effects of treatment on endothelial cells by inhibition of angiogenesis. It is hoped that the methods presented here will support future research on miRNA therapeutics and corresponding activity and that the representative data will guide other researchers during experimental analyses.

INTRODUCTION:

The cell cycle is a combination of multiple regulatory events that allow duplication of DNA and cell proliferation through the mitotic process[1]. Cyclin-dependent kinases (CDKs) regulate and promote the cell cycle[2]. Among them, the mitotic CDK (CDK1) and interphase CDKs (CDK2, CDK4, and CDK6) have a pivotal role in cell cycle progression[3]. Retinoblastoma protein (Rb) is phosphorylated by the CDK4/CDK6 complex to allow cell cycle progression[4], and CDK1 activation is essential for successful cell division[5]. Numerous CDK inhibitors have been developed and evaluated in clinical trials over the last few decades, indicating the potential of targeting CDKs in cancer treatment. In fact, three CDK inhibitors have been approved for the treatment of breast cancer recently[6-10]. Thus, CDKs, and in particular, CDK1 and CDK4/6, are of great interest in regulating cancer cell progression.

miRNAs (miRs) are small, non-coding RNAs and post-transcriptional regulators of gene expression, regulating approximately 30% of all human genes[11]. Their activity is based on translational repression or degradation of messenger RNAs (mRNAs)[12]. Illustrative of their biological significance, more than 5,000 miRNAs have been identified and a single miRNA molecule can regulate multiple genes[11,13]. More importantly, miRNA expression has been associated with different diseases and disease statuses, including cancer[13]. In fact, miRNAs have been characterized as oncogenic or tumor suppressors, being capable to either promote or suppress tumor development and progression[14,15]. The relative expression of miRNAs in diseased tissues can regulate disease progression; thus, exogenous delivery of miRNAs has therapeutic potential.

Lung cancer is the leading cause of cancer-related deaths and greater than 60% of all lung malignancies are non-small cell lung cancers[16,17], with a 5-year survival rate of less than 20%[18]. The use of miR-143-3p and miR-506-3p was recently evaluated for targeting the cell cycles in lung cancer cells[11]. miR-143 and miR-506 have sequences that are complementarity to CDK1 and CDK4/CDK6, and the effects of these two miRs on A549 cells were analyzed. The experimental details are presented and discussed in this paper. Gene expression, cell cycle progression, and apoptosis were evaluated using different experimental designs and timepoints following transfection. We used real-time quantitative PCR (RT-qPCR) methods along with microarray analysis to measure specific gene expression, and next-generation RNA sequencing was used to determine global gene dysregulation[11]. The latter method identifies the relative abundance of

each gene's transcript with high sensitivity and reproducibility, while thousands of genes can be analyzed from a single experimental analysis. Additionally, apoptotic analysis due to miRNA treatment was performed and is described here. Bioinformatics supplemented the pathway analysis. Presented here are protocols used for analysis of the therapeutic potential of the combinatorial miR-143 and miR-506.

The main purpose of this protocol is to identify the effects of miRNAs in cells, with a focus on the cell cycle. The variety of techniques presented here span from gene expression analysis pre-translation (using qPCR) to elaborate and novel techniques for gene analysis at the protein level, such as microarray analysis. It is hoped that this report is helpful for researchers interested in working with miRNAs. Additionally, methodology for flow cytometric analysis of the cell cycle and apoptosis of cells is presented.

PROTOCOL:

1. miR-143 and miR-506 transfection

CAUTION: Use latex gloves, protective eyeglasses, and a laboratory coat while performing the described experiments. When required, use the biosafety cabinet with the cabinet fan on, without blocking the airways or disturbing the laminar airflow. Always set the protecting glass window to the appropriate height, as described by the manufacturer.

1.1. Seed NSCLC A549 cells in a T25 cm^2 flask/6/96 well plate in DMEM/F12K media supplemented with 10% FBS and 1% penicillin-streptomycin (culture media) in a tissue culture hood and incubate overnight at 37 °C with 5% CO_2 in a tissue culture incubator.

1.2. Suspend miR-143 and/or miR-506 mimics, or scramble siRNA with transfecting agent (2.4 µg of miR were mixed with 14 µL of transfecting agent; see **Table of Materials**) in 500 µL of transfection media and 1.5 mL of serum and antibiotic-free DMEM/F12K media at a final miRNA concentration of 100 µM. miRNA amount may require optimization on different cells and concentrations. Appropriate approaches include the transfection of cells with increasing concentrations of miRNA (i.e., 50–200 nM) and evaluation of expression downregulation of the genes of interest.

1.3. Remove culture media from flask/plate and wash once with 1x PBS.

1.4. Add miRNA/scramble-transfecting agent complexes and incubate at 37 °C with 5% CO_2 for 6 h (flask size defines the added incubation volume).

1.5. Replace the media with 4 mL of culture media and incubate cells for 24 h and/or 48 h.

1.6. Harvest transfected cells by trypsinization, by adding 1 mL of trypsin-EDTA in each T25 cm^2 flask, incubate for 5–10 min at 37 °C, and add 3 mL of culture media to harvest the cells. Place the contents of each flask into a separate, marked 15 mL tube. Work in a tissue culture hood.

1.7. Centrifuge at 751 x *g* for 5 min and remove the supernatant.

NOTE: Caution is required during supernatant removal, as agitation of the tube may cause loss of cells.

1.8. Add 2 mL of 1x PBS and centrifuge for 5 min at 751 x *g.*

1.9. Repeat steps 1.7 and 1.8 once to remove any traces of media and supernatant.

NOTE: At this stage, sample tubes can be stored at -80 °C or can be used immediately.

2. RNA extraction

2.1. Clean the work area by spraying with 70% isopropyl alcohol and RNAse decontamination solution.

2.2. RNA extraction should be performed using an appropriate RNA kit (see **Table of Materials**).

2.3. Remove the tubes from -80 °C and allow them to thaw. Add 300 µL of lysis buffer and pipette up and down to break the cell membrane.

2.4. Add an equal volume of 100% ultra-pure ethanol.

2.5. Mix well and place in separating column.

2.6. Centrifuge between 11 and 16 x *g* for 30 s and remove the flow-through.

2.7. Add 400 µL of washing buffer and centrifuge to remove the buffer.

2.8. Add 5 µL of DNAse I with 75 µL of DNA digestion buffer in each sample and incubate for 15 min.

2.9. Wash the sample with 400 µL of RNA prep buffer.

2.10. Wash 2x with RNA wash buffer.

2.11. Add nuclease-free water to the column, centrifuge, then collect the RNA.

2.12. Measure total RNA concentration with a UV spectrophotometer.

3. RT-qPCR

3.1. Prior to RT-qPCR, synthesize the cDNA. Subsequent to RNA concentration quantification, place 1 µg of RNA in a 20 µL final volume of reaction to prepare cDNA in a PCR tube. Always work on a clean bench.

3.2. All other necessary ingredients are included in **Table 1.**

[Place **Table 1** here]

3.3. Incubate in a thermal cycler with the following temperature conditions: 42 °C for 30 min; 95 °C for 2 min; 4 °C until collection of samples.

3.4. Use immediately in regular PCR or qPCR, or store cDNA at -20 °C.

3.5. Prepare forward and reverse primer solutions with DNase/RNase-free water at a concentration of 10 µM from stock primer solution.

3.6. Prepare separate master mixes for each gene to be detected, according to the number of samples. For each cDNA sample (qPCR well), the reaction quantity is prepared according to **Table 2**.

[Place **Table 2** here]

3.7. Place each sample in the respective wells. For each sample and analyzed gene, perform the reaction in triplicates, or at least in duplicates,

3.8. Design the sample layout for the 96 well qPCR plate. For each sample and analyzed gene, perform the reaction in triplicates, or at least in duplicates.

3.9. Seal the 96 well plate with an optically clear adhesive cover.

3.10. Quick-spin the plate to allow the reaction mixture to reach the bottom of each well.

3.11. Run RT-qPCR according to the following thermal gradient:
1) 50 °C for 2 min
2) 95 °C for 2 min
3) 95 °C for 15 s
4) Take reading
5) 60 °C for 1.5 min
6) Repeat step 3 for 39 times

3.12. Run the following thermal gradient in continuation of the above to determine melting curve which indicates single product amplification.
7) 65 °C for 0.31 min
8) +0.5 °C/cycle
9) Plate read
10) Repeat step 8 for 60 times until reaching 95 °C
11) 72 °C for 2 min

4. Agarose gel electrophoresis to confirm single gene amplification

4.1. Prepare 1% agarose gel in 1x TBE buffer.

4.2. Add ethidium bromide (EtBr) in warm (~50 °C) gel until achieving a final concentration of approximately 0.2–0.5 µg/mL. EtBR binds with DNA and allows it to be visualized under UV light in a gel imager.

4.3. Pour warm gel in a gel tray supplied with an electrophoresis gel box. Attach the provided comb tightly for uniform wells.

4.4. Allow the gel to rest and cool to room temperature (RT) for ~30 min.

4.5. When the gel is solidified, place the gel and tray in the gel box.

4.6. Fill the gel box with 1x TBE buffer until the gel is completely covered.

4.7. Measure the concentration of the DNA from PCR amplification process using a UV spectrometer.

4.8. Take ~15 ng of DNA in a small PCR tube, add 5 µL of dye, and add the required amount of nuclease-free water to achieve a 15 µL total volume.

4.9. Load the DNA ladder and samples into the wells.

4.10. Run the gel at 100 V until the dye line is approximately 75%–80% down the gel.

NOTE: Make sure the gel runs from a negative to positive charge.

4.11. Remove the gel and place it in the gel imager to visualize DNA.

5. Cell cycle analysis

5.1. Seed 5 x 10^5 cells for each sample in a T25 cm^2 flask and perform a transfection according to the protocol described in section 2.1, steps 2.1.1–2.1.5.

5.2. After 24 h and 48 h, then harvest the cells by trypsinization.

5.3. Transfer the cell suspensions to 15 mL sterile tubes and label them properly.

5.4. Centrifuge samples at 751 x *g* for 5 min and discard the supernatant.

5.5. Add 2 mL of ice-cold 1x PBS, vortex, and centrifuge at 751 x *g* for 5 min. Discard the supernatant.

5.6. Repeat the washing step with 1x PBS to remove residual media.

5.7. Resuspend and break the pellet by adding 200 µL of ice-cold 1x PBS by pipetting.

5.8. Fix the cells by adding 2 mL of 70% ice-cold ethanol dropwise to the tube while vortexing gently.

5.9. Incubate tubes for 30 min at RT and place the tubes at 4 °C for 1 h.

5.10. Remove tubes from 4 °C and centrifuge at 751 x *g* for 5 min.

5.11. Add 2 mL of ice-cold 1x PBS, vortex, and centrifuge at 751 x *g* for 5 min. Discard the supernatant.

5.12. Add 500 µL of 1x PBS with propidium iodide (50 µg/mL) and ribonuclease A (200 µg/mL)

5.13. Incubate for 30 min at RT while protecting the samples from light.

5.14. Acquire data on flow cytometer. Use forward vs. side scatter (FSC vs. SSC) to select the main population of cells, excluding debris at the bottom left corner of the FSC vs. SSC density plot and cell clusters at the top to top-right side of the FSC vs. SSC density plot.

5.15. Analyze data for identification of cell populations per cell cycle stage with appropriate software.

6. Apoptosis assay

6.1. Seed 5 x 10^5 cells for each sample in a T25 cm^2 flask and perform a transfection according to the protocol described in section 2.1, steps 2.1.1-2.1.5.

6.2. After 24 h and 48 h, harvest the cells by trypsinization.

6.3. Transfer the cell suspensions to 15 mL sterile tubes and label them properly.

6.4. Centrifuge at 751 x *g* for 5 min and discard the supernatant.

6.5. Add 2 mL of ice-cold 1x PBS, vortex, and centrifuge at 751 x *g* for 5 min. Discard the supernatant.

6.6. Repeat the washing step with 1x PBS to remove residual media.

6.7. Dilute 10x Annexin V binding buffer to 1x with ice-cold dH_2O.

6.8. Add 1 mL of 1x Annexin V binding buffer to each sample tube and resuspend gently.

6.9. Place 96 µL of cell suspension in a 1.5 mL microcentrifuge tube.

6.10. Add 1 µL of Annexin V-FITC conjugate and 12.5 µL of propidium iodide (PI) to the tube containing the cell suspension.

6.11. Incubate the cell suspension for 10 min on ice in the dark.

6.12. Add 250 µL ice-cold 1x Annexin V binding buffer to each sample tube to dilute.

6.13. Analyze samples with flow cytometer immediately.

7. Protein expression by antibody cell cycle microarray

7.1. Seed 5 x 10^5 cells for each sample in T25 cm^2 flasks and perform a transfection according to the protocol described in steps 2.1.1–2.1.5.

7.2. After 24 h and 48 h, harvest cells by trypsinization.

7.3. Transfer cell suspensions to 15 mL tubes and label them accordingly.

7.4. Centrifuge at 751 x *g* for 5 min and discard the supernatant.

7.5. Add 2 mL of ice-cold 1x PBS, vortex and centrifuge at 751 x *g* for 5 min. Discard the supernatant.

7.6. Repeat the washing step with 1x PBS.

7.7. Add 150 µL of lysis buffer supplemented with protease inhibitors. Pipet up and down gently to disrupt the cell membranes.

7.8. To prevent any lysis buffer interference, perform a buffer exchange to replace the lysis buffer with labeling buffer, using the manufacturer's solvent exchanging columns.

7.9. Quantify the total protein with a BCA assay.

7.10. Take 70 µg of protein sample and add labeling buffer to achieve a final volume of 75 µL.

7.11. Add 100 µL of dimethylformamide (DMF) to 1 mg of biotin reagent (biotin/DMF).

7.12. Add 3 µL of the biotin/DMF to each protein sample (biotinylated protein sample) and incubate for 2 h at RT.

7.13. Add 35 µL of stop reagent and mix by vortexing.

7.14. Incubate samples at RT for 30 min.

7.15. Remove the microarray slides from the refrigerator so that they warm to RT for 1 h before use.

7.16. Perform blocking for non-specific binding by incubating the slides with 3% dry milk solution (in blocking reagent provided by manufacturer) in a Petri dish with continuous shaking for 45 min at RT.

7.17. Wash the slides with ddH_2O water (unless otherwise specified, washing takes place with ddH_2O).

7.18. Repeat the washing step ~10x to completely remove the blocking solution from the slide surfaces. This is important to achieve a uniform and low background.

7.19. Remove excessive water from the slide surfaces and proceed to the next step without letting the slides dry.

7.20. Prepare coupling solution by dissolving 3% dry milk in a coupling reagent.

7.21. Add 6 mL of the coupling solution and to the full quantity of the previously prepared biotinylated protein sample from step 7.12.

7.22. Place one slide in one well of the coupling chamber provided by the vendor and add ~6 mL of protein coupling mix to it.

NOTE: Ensure that the slide is completely submerged in protein coupling mix solution.

7.23. Cover the coupling chamber and incubate for 2 h at RT with continuous agitation in an orbital shaker.

7.24. Transfer the slides to a Petri dish and add 30 mL of 1x washing buffer. Place the Petri dish in orbital shaker, shake for 10 min, and discard the solution.

7.25. Repeat step 7.24 2x.

7.26. Rinse the slides with ddH_2O water extensively as described in steps 7.17 and 7.18 and proceed to next step immediately to avoid drying.

7.27. Add 30 µL of Cy3-streptavidin (0.5 mg/mL) in 30 mL of detection buffer.

7.28. Place the slide in a Petri dish and add 30 mL of detection buffer containing Cy3-streptavidin.

7.29. Incubate in an orbital shaker for 20 min with continuous shaking protected from light.

NOTE: Cy-3 is a fluorescent dye. Cover with aluminum foil or operate under dark conditions to maintain fluorescence intensity.

7.30. Perform steps 7.24–7.26 and allow the slide to dry using a gentle stream of air or placing the slide in a 50 mL conical tube and centrifuge at 1300 x *g* for 5–10 min.

7.31. Place the slide in slide holder and cover with aluminum foil.

7.32. Scan the slide in a microarray scanner with the appropriate excitation and emission wavelengths. In the case of Cy3, the excitation wavelength peak is at ~550 nm and emission peak at ~570 nm.

7.33. Analyze the data (see **Table of Materials**) for software used.

8. RNA sequencing

8.1. Seed 5 x 10^5 cells for each sample in T25 cm^2 flasks and perform a transfection according to the protocol described in section 2.1, steps 2.1.1–2.1.5.

8.2. After 24 h and 48 h, harvest the cells by trypsinization.

8.3. Transfer the cell suspensions to 15 mL tubes and label them accordingly.

8.4. Extract RNA according to section 2.1, steps 2.1.1–2.1.6.

8.5. Check RNA quality as well as concentration with a bioanalyzer. An RNA integrity score (RIN) above eight and appropriate histograms are necessary to confirm RNA quality.

8.6. From the total RNA, use ~2 µg of the sample for RNA sequencing (messenger RNA from total RNA)

8.7. Sequence using a next-generation sequencer[11].

8.8. Run quality trimming and map with a reference genome from FASTQ files generated from the RNA sequencing machine[11].

8.9. Upload FASTQ files and raw read the count data to Genebank, following the instructions of the <https://www.ncbi.nlm.nih.gov/> website.

NOTE: See accession number SRP133420 for previous results.

9. Tube formation assay

9.1. Assess the angiogenic potential of miRNA-transfected human umbilical vein endothelial cells (HUVECs) 36 h post-transfection, as described in steps 2.1.1-2.1.5, using HUVEC cells instead of A549 cells.

9.2. Starve HUVECs with M199 starvation media for 4 h at 37 °C with 5% CO_2.

9.3. Remove reduced growth factor basement membrane matrix from -80 °C and store at 4 °C overnight, allowing gradual thawing to avoid bubble formation and polymerization.

9.4. Carefully and slowly coat wells of a 96 well plate with 0.04 mL of reduced growth factor basement membrane matrix, avoiding bubble formation. Perform the whole procedure under a laminar flow hood.

9.5. Fill adjacent wells of the 96 well plate with 0.1 mL of PBS to maintain humidity and preserve temperature.

9.6. Incubate the 96 well plate at 37 °C for at least 20 min so that polymerization of basement membrane extract is achieved. Do not incubate for more than 1 h.

9.7. Trypsinize transfected HUVECs from each group and resuspend in medium M199 at a concentration of 1 x 10^5 cells/mL.

9.8. Add 0.1 mL of each cell suspension to the wells containing polymerized basement membrane matrix in the 96 well plate.

9.9. Incubate the 96 well plate at 37 °C with 5% CO_2 while preparation of the growth factors takes place. Preparation of growth factors also takes place under the laminar flow hood.

9.10. Reconstitute growth factors (VEGF) in 2x the final desired concentration (4 ng/mL concentration for 2 ng/mL final concentration) and add 0.1 mL of growth factor-containing M199 starvation medium on top of the 0.1 mL of M199 starvation medium with the cells. For non-VEGF-treated wells, add 0.1 mL of M199 starvation medium on top of the 0.1 mL of M199 starvation medium with the cells.

9.11. Incubate the 96 well plate for 6 h at 37 °C with 5% CO_2.

9.12. At the end of the incubation period, obtain images of each well with 4x magnification, using a brightfield microscope connected with a digital camera.

9.13. Process images with software equipped with an "angiogenesis analyzer" plug-in[19]. Use three parameters, the number of nodes, number of junctions, and total sprout length, to compare the effects of miRNA treatment on angiogenesis.

REPRESENTATIVE RESULTS:

Gene expression analysis using RT-qPCR and gel electrophoresis

Differential gene expression analysis using RT-qPCR demonstrated significant downregulation of the targeted genes CDK1, CDK4, and CDK6. CDK1 and CDK4/6 were shown to be instrumental for the G2/M and G1/S transitions, respectively. The performed analysis allowed direct comparison between individual miRs and combinatorial miR activity. The use of scramble siRNA with the transfecting agent permitted evaluation of any interference from the procedure on detected gene downregulation, which was minimal. The data were statistically analyzed using a two-tailed student's *t*-test, and $p < 0.05$ was considered statistically significant (**Figure 1**). Prior to qPCR, the primer sequences were evaluated using primer-BLAST <https://www.ncbi.nlm.nih.gov/tools/primer-blast/> for single gene amplification. This was also confirmed by analyzing the amplification products through gel electrophoresis. A single band of DNA products was detected for each analyzed gene (**Figure 1D**), confirming single gene amplification. CDK6 single amplification was confirmed (data not shown).

[Place **Figure 1** here]

Cell cycle distribution using flow cytometry

Propidium iodide staining of cellular nucleic acids is a standard method to visualize cell population in different stages of the cell cycle by quantitation of DNA content. The combinatorial treatment of miR-143 and miR-506 halted the cell cycle at two checkpoints, G0/G1 and G2/M, as indicated through flow cytometric analysis (**Figure 2**).

[Place **Figure 2** here]

Annexin V/PI apoptosis assay by flow cytometry

Following transfection of A549 cells with miR-143 and miR-506, an apoptosis assay was performed using Annexin V and PI staining and flow cytometry. It was identified that the combinatorial treatment induced significant apoptosis at 24 h and 48 h timepoints. Compared to the negative controls, the fold-change of apoptotic cells was determined as detected by the Annexin V positive cells, due to the miR treatment as presented in **Figure 3**.

[Place **Figure 3** here]

Cell cycle antibody microarray

Mechanistic responses to treatment can be identified through changes in protein expression. Differential expression was evaluated at a protein level of genes associated with the cell cycle pathway using a pathway-specific antibody microarray. Protein extracts were used for analysis from cells transfected with miR-143/506. The microarray analysis allowed for semi-quantitative analysis of ~60 cell cycle-associated proteins, with six replicates for each specific antibody. The approach allows a broader perspective of mechanistic behavior within a specific pathway, identification of molecular targets for further evaluation, and performing of analysis at the post-translational level. Due to the semi-quantitative principle of the method, any results on specific genes need to be confirmed through western blotting. Indicatively, in this analysis, a decreased expression of proteins associated with cell cycle progression was detected. This included the targeted CDK1 and CDK4 at both 24 h and 48 h post-transfection (**Figure 4A**), as detected by qPCR.

[Place **Figure 4** here]

RNA sequencing and pathway analysis using pathway analysis software

Next-generation sequencing accurately analyzes gene expression at the RNA level. The method allows for identification of multiple gene changes through a single analysis (in this protocol, the analysis detected the expression of >18,000 genes). Due to the large number of detected genes, bioinformatics analysis was used for efficient determination of pathway behavior (**Figure 4B**). Software was then used (see **Table of Materials**) to predict G1/S and G2/M phase arrests and the downregulation of S phase initiation (**Figure 4C**). Furthermore, the RNA sequencing results can be compared to qPCR data. In this study, the RNA sequencing confirmed the findings from the qPCR analysis, indicating a downregulation of CDK1 (48%, $p < 0.001$, FDR < 0.001), CDK4 (68%, $p < 0.001$, FDR < 0.001), and CDK6 (71%, < 0.001, FDR < 0.001) due to combinatorial miR-143 and miR-506 activity. Statistical analysis was performed by the EdgeR software used for calculation of the relative gene expression, calculating *p* values using Negative Binomial[20,21]. Bioinformatics analysis can be performed for the functional evaluation of miRNA activity and prediction of potential molecular targets, as illustrated in **Figure 5**.

[Place **Figure 5** here]

Endothelial tube formation assay

The in vitro endothelial tube formation assay is widely used to study angiogenesis and is reliable, automated, and quantifiable[22]. Vascular endothelial growth factor (VEGF) is a well-known angiogenic growth factor[23,24] and endothelial tube formation promoter. In this study, it was identified that the combinatorial treatment of miR-143 and miR-506 abrogates VEGF-induced angiogenesis. Indicative images of tube formation and the effects of treatment are presented in **Figure 6**.

[Place **Figure 6** here]

FIGURE AND TABLE LEGENDS:

Figure 1: Relative expression of CDK1, CDK4, and CDK6 genes as detected by qPCR, and gel electrophoresis analysis of the DNA amplification products. miR-143 and miR-506 transfection of A549 cells induced downregulation of CDK1 (A), CDK4 (B), and CDK6 (C) downregulation at 24 h and 48 h post-transfection. DNA amplification products were evaluated by gel electrophoresis (D) to confirm single gene amplification. GAPDH was used as reference gene. Average ± SEM, *p < 0.05; **p < 0.01, two-tailed *t*-test. This figure has been modified from Hossian et al.[11]

Figure 2: Cell cycle analysis of A549 cells transfected with miR-143 and miR-506 at 24 h and 48 h post-transfection. Cell populations percentages for each cell cycle were determined by flow cytometry and DNA-binding propidium iodide. Average ± SEM. This figure is reprinted with modifications from Hossian et al.[11]

Figure 3: Illustrative analysis of apoptotic cells. Transection with miR-143 and miR-506 increased the percent of Annexin V positive A549 cells. Average ± SEM. *p < 0.05, **p < 0.01, two-tailed *t*-test. This figure has been modified from Hossian et al.[11]

Figure 4: Gene dysregulation as detected by microarray and RNA sequencing analysis. (A) Heatmap of cell cycle pathway gene expressions as detected by microarray analysis in protein extracts from A549 cells transfected with miR-143 and miR-506, at 24 h and 48 h post-transfection. (B) Fold change of cell cycle pathway gene expressions from A549 cells transfected with miR-143 and miR-506 at 24 h post-transfection as detected by RNA sequencing. (C) Pathway activity as analyzed by pathway analysis software from data obtained from RNA sequencing. This figure has been modified from Hossian et al.[11]

Figure 5: Illustrative pathway and mechanistic analysis as presented by pathway analysis aoftware. RNA sequencing data was analyzed from A549 cells transfected with miR-143 and miR-506, 24 h post-transfection, using pathway analysis software and identified canonical pathways with the lowest (A) or highest (B) activation score. The software also provided predicted functions (C) and potential upstream regulators/targets (D). This figure is reprinted from Hossian et al.[11]

Figure 6: Representative images of endothelial sprouts of VEGF-treated vs. non-treated HUVECs transfected with scramble miRNA, miRNA-143, miRNA-506, or a combination thereof. Pictures were obtained under a brightfield microscope equipped with a digital camera under 4x magnification.

Table 1: Materials for cDNA synthesis from RNA samples. Required quantities of respective ingredients to prepare a master mix for one sample for cDNA synthesis.

Table 2: Materials for quantitative real-time PCR from cDNA samples. Required quantity of ingredients to prepare a master mix for one sample for qPCR.

DISCUSSION:

miRNAs can operate as targeted therapies for cancer treatment, recognizing the dysregulation of expression levels in diseased vs. normal tissues. This study aimed to determine miRNAs that potentially halt cell cycle progression during multiple stages. It was identified that miR-143 and miR-506 halt the cell cycle of cancer cells, and the presented protocols aimed to comprehend the activity of this combinatorial miRNA treatment.

The described methodologies provide an overarching understanding on the function of miRNAs. The challenges of studying miRNAs are associated with their capacity to target multiple genes and thus affect multiple pathways. The described qPCR analysis allows identification of the expression of specific genes of interest, if specific targets are identified prior to treatment. For example, the main focus here was the expression of CDK1 and CDK4/6 and the cell cycle.

Thus, cell cycle analysis using propidium iodide and flow cytometry protocol is a reliable approach to detect alterations in the cell populations according to their stage in the cell cycle. The method relies on the proportionate increase of fluorescence signal by the PI, which binds to DNA, and the stage of the cell cycle. Briefly, cells in the S phase synthesize DNA, inducing higher signals than cells in the G0/G1 phase, and cells in the G2 phase have duplicated their DNA, producing the most intense signal.

Accumulating evidence indicates the connection of damage to the cell cycle and triggering of apoptosis[25]. The flow cytometry method using Annexin VI/PI has consistently been used for the identification of induced apoptosis in cells from chemotherapy treatment. Indicatively, a strong apoptotic response was identified due to the combinatorial miRNA therapy, which was more potent compared to the individual miRNAs.

The semi-quantitative protein antibody microarray is a sensitive and reliable method to identify protein expression alterations related to specific biological responses[26,27]. This protocol used a cell cycle pathway-specific antibody microarray, which detected expression changes in ~60 genes between treated and untreated cells. Caution is required during the washing steps to ensure that the process has been thoroughly performed and to minimize the background signal. Additionally, the slides should not become dry until completion of the experiment.

In contrast, RNA sequencing provides quantitative gene expressions analysis of multiple genes, at the post-transcription level. The significantly large number of analyzed genes (>18,000 for RNA-seq vs. 60 for microarray) allows for the simultaneous analysis of multiple pathways and molecular targets, and with increased accuracy. Such broad analysis is important, as a single miRNA can bind to and target different mRNAs. In contrast, the pathway analysis of large numbers of gene dysregulations is inherently challenging. For example, although the RNA sequencing confirmed our qPCR data regarding CDK1 and CDK4/6 downregulation due to the miRNA treatment, the analysis also provided data on thousands of genes that were also down- or up-regulated. To provide context to such numerous gene dysregulations, pathway analysis software was used to determine overall effects of the treatment on different pathway and cellular functions. Indicatively, the software provided scores representative to activation

(positive z score) or inactivation (negative z score) of specific functions or pathways, as well as statistical significance of the analysis (**Figure 5**)[28].

In conclusion, the study of miRNA activity is a challenging procedure. The inherent capacity of miRNAs to affect multiple genes requires the utilization of multiple elaborate and complicated analytical methods to identify potential activity. Not surprisingly, further work is required to fully comprehend the activities of miR-143 and miR-506 in lung cancer.

DISCLOSURES:

No conflicts of interest are declared.

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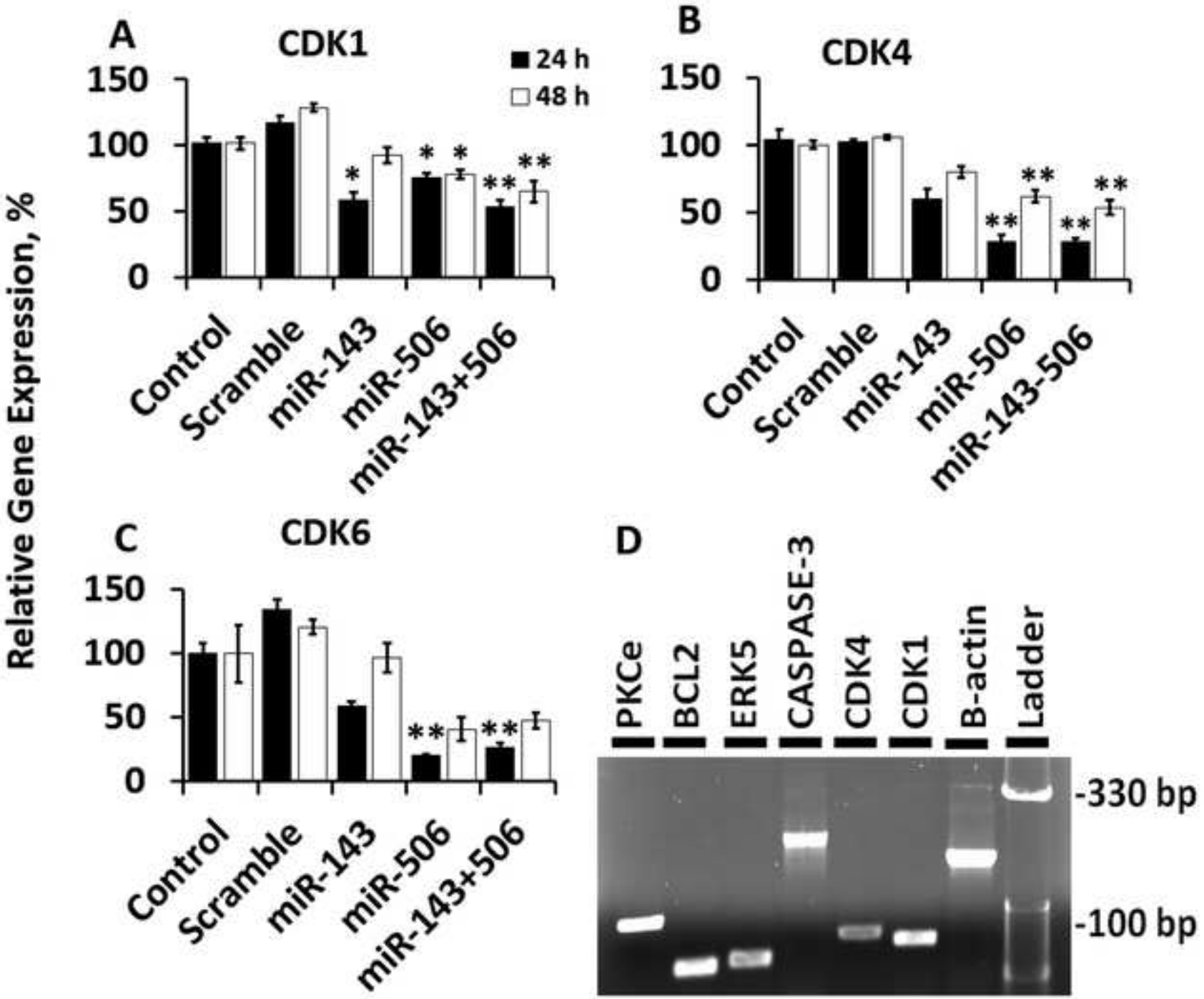
Figure 1



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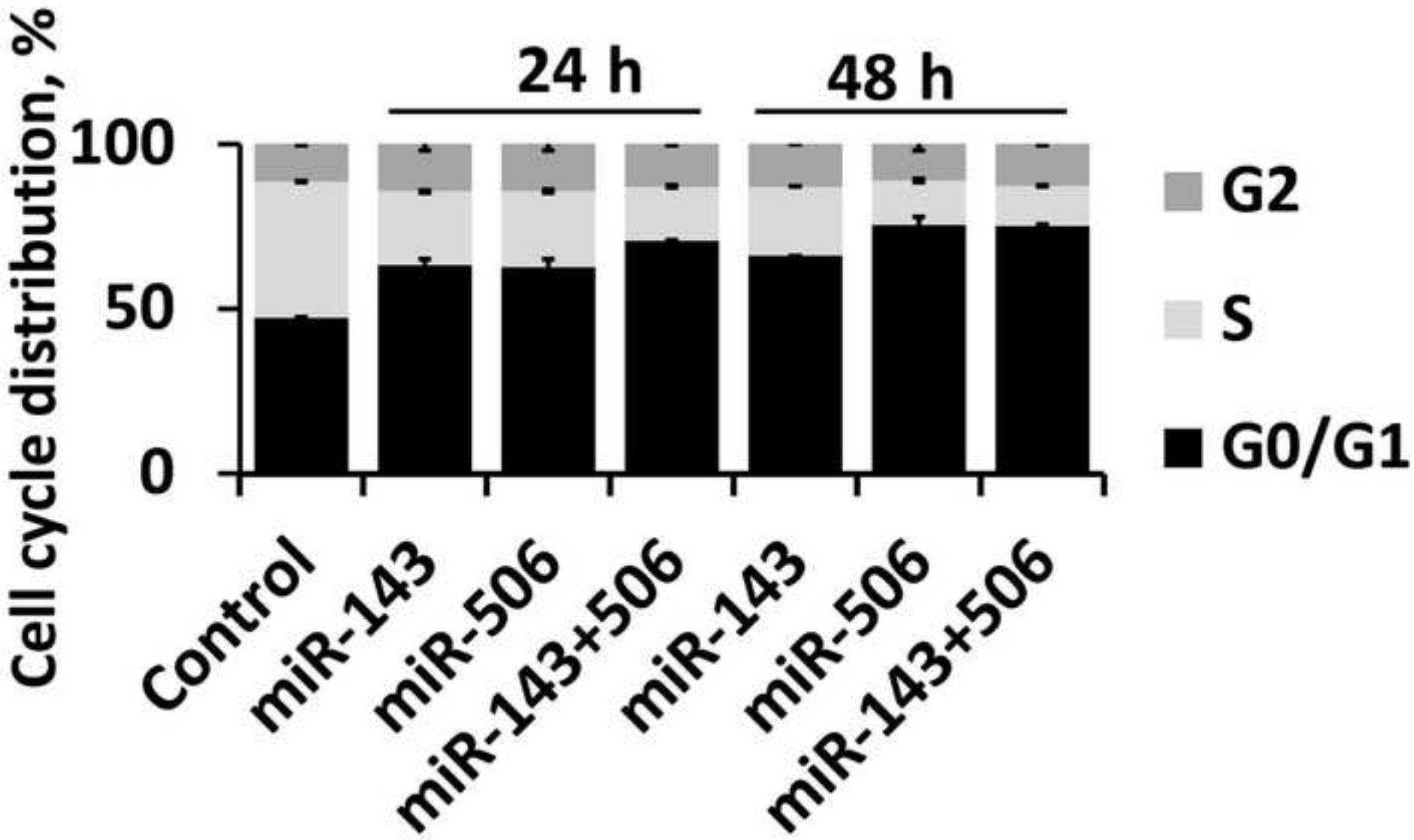

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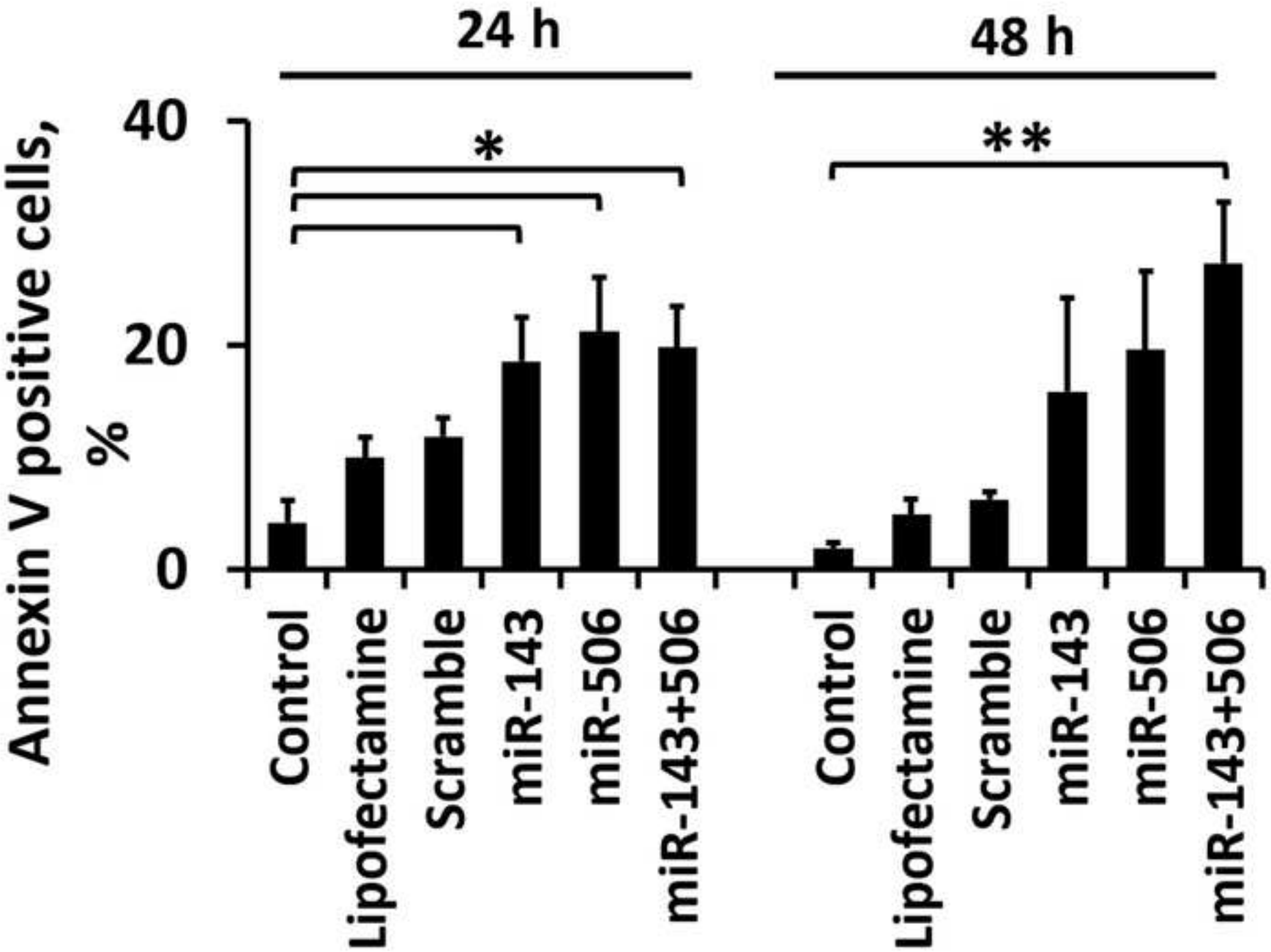

Figure 4

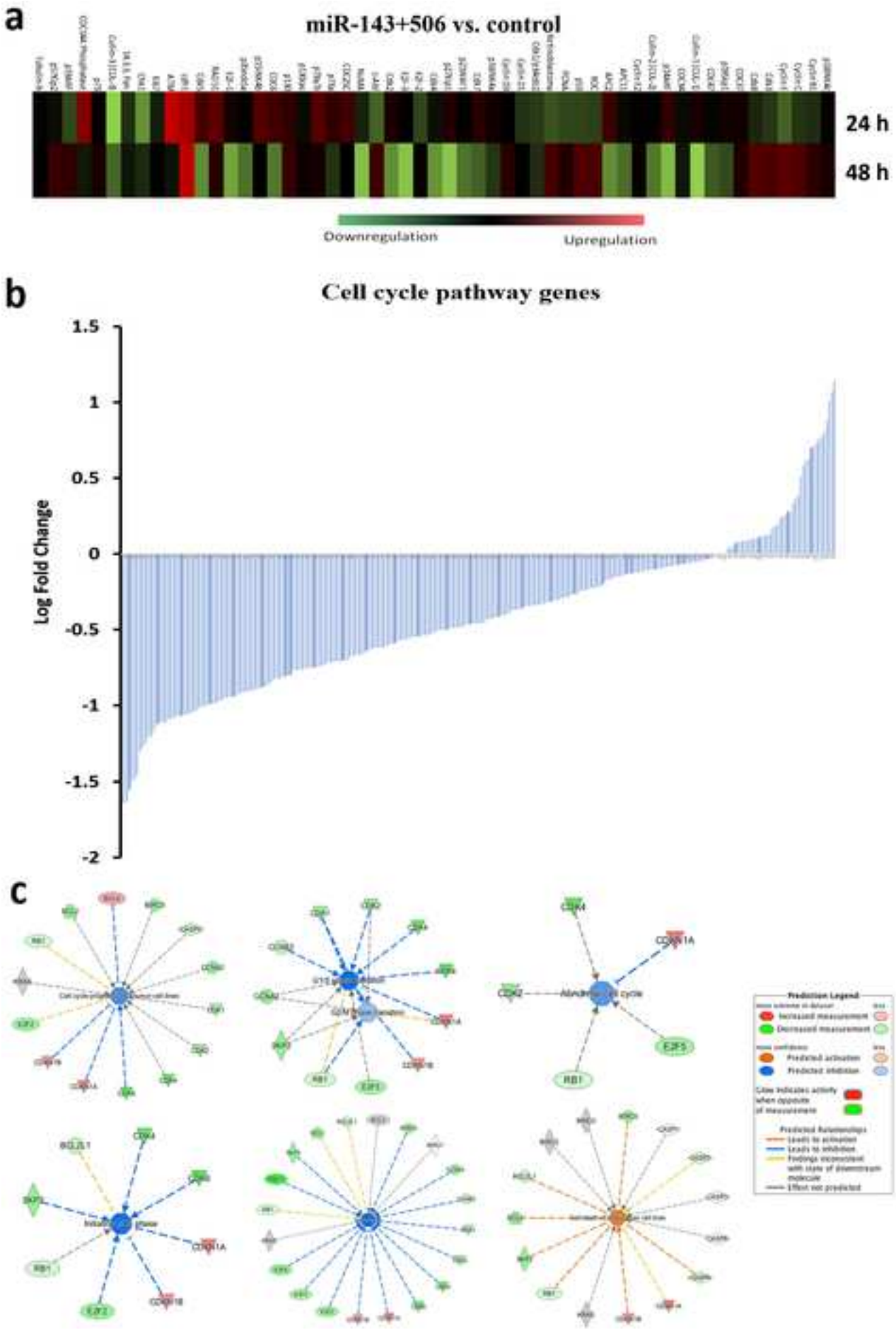

Figure 5

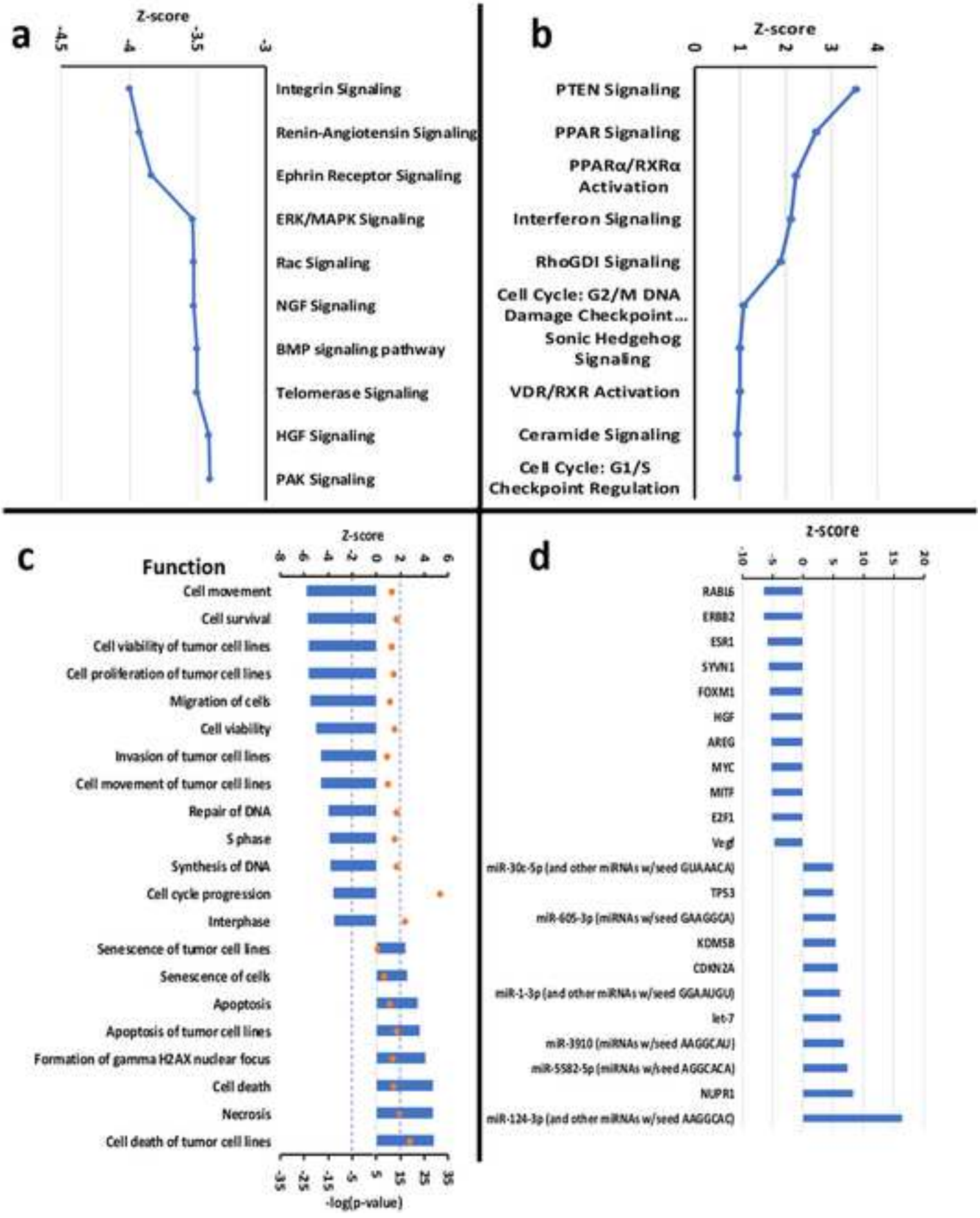

Figure 6

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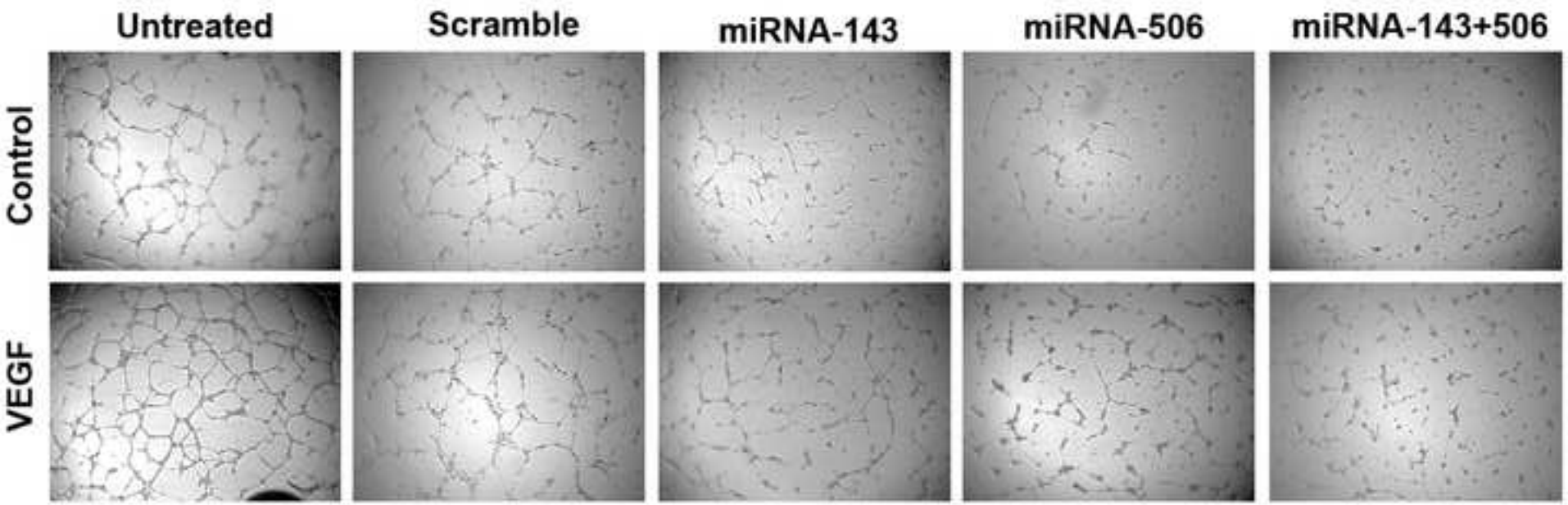

Table 1

Ingredients	Quantity (µL)/sample (20 µL)
5X cDNA Master mix	4
dNTPs	2
Random hexamers	1
RT enhancer	1
Verso enzyme mix	1
DNase and RNase free water	Required qty after adding RNA to make 20 µL

Table 2

Ingredients	Quantity (µL)/sample (20 µL)
SYBR master mix	10
Forward Primer - 10 µM	2
Reverse Primer - 10 µM	2
DNase and RNase free water	3
cDNA sample	3

Table of Materials

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Name of Material/ Equipment	Company
-80 °C Freezer	VWR
96 well plate	CELLTREAT Scientific
96-well Microwell Plates	Thermo Scientific
A549 Non Small Cell Lung Cancer Cells	ATCC
Agarose	VWR
Agilent 2100 Bioanalyzer	Agilent Technologies
Ambion Silencer Negative Control No. 1 siRNA	Ambion
Antibiotic-Antimycotic Solution (100x)	Gibco
Antibody Array Assay Kit, 2 Reactions	Full Moon Bio
Bright field microscope	Microscoptics
Bright field microscope	New Star Environment LLC
Cell Cycle Antibody Array, 2 Slides	Full Moon Bio
Cell Logic+ Biosafety Cabinate	Labconco
Cellquest Pro	BD bioscience
CFX96 Real Time System	BioRad
Chemidoc Touch Imaging System	BioRad
CO2 Incubator	Thermo Scientific
Cultrex Reduced Growth Factor Basement Membrane Matrix	Trevigen
Digital Camera	AmScope
DMEM 4.5 g/L Glucose, w/out Sodium Pyruvate, w/ L-Glutamine	VWR
DNAse I	Zymo Research
Endothelial Cell Growth Supplement (ECGS)	BD Biosciences
Eppendorf Pipette Pick-A-Pack Sets	Eppendrof
Ethanol, Absolute (200 Proof), Molecular Biology Grade,	Fisher BioReagents
Ethidium bromide	Alfa acar
F-12K Nutrient Mixture (Kaighn's Mod.) with L-glutamine, Corning	Corning
FACS Calibur Flowcytometer	Becton Dickinson
Fetal Bovine Serum - Premium	Antlanta Biologicals
Fetal Bovine Serum (FBS)	Fisher Scientific
Fisherbrand Basix Microcentrifuge Tubes with Standard Snap Caps	Fisherbrand Basix
Forma Series II water Jacket CO2 incubator	Thermo Scientific
Heparin Solution (5000 U/mL)	Hospira
Horixontal Electrophoresis system	Benchtop lab system

hsa-miR-143-3p miRNA Mimic	ABM
hsa-miR-506-3p miRNA Mimic	ABM
Human Recombinant Vascular Endothelial Growth Factor (VEGF)	Thermo Scientific
Human Umbilical Vein Endothelial Cells (HUVEC)	Individual donors
HyClone Phosphate Buffered Saline (PBS)	Fisher Scientific
Ingenuity Pathway Analysis	Qiagen
Invitrogen UltraPure DNase/RNase-Free Distilled Water	Invitrogen
Lipofectamine 2000	Invitrogen
Loading dye 10X	ward's science+
Medium M199 (with Earle's salts, L-glutamine and sodium bicarbonate)	Sigma Aldrich
Microscope Digital Camera	AmScope
Modfit LT	Verity Software
Nanodrop	Thermo Scientific
Opti-MEM	Gibco by life technologies
Penicillin-streptomycin 10/10	Antlanta Biologicals
Power UP sybr green master mix	Applied Biosystems
Propidium Iodide	MP Biochemicals LLC
Proscanarray HT Microarray scanner	Perkin elmer
q PCR optical adhesive cover	Applied Biosystems
Quick-RNA Kits	Zymo Research
Ribonuclease A from Bovine pancreas	Sigma
ScanArray Express	PerkinElmer
Shaker	Thermo Scientific
SimpliAmp Thermal Cycler	Applied Biosystems
SpectraTube Centrifuge Tubes 15ml	VWR
SpectraTube Centrifuge Tubes 50ml	VWR
TBS Buffer, 20x liquid	VWR
Temperature controlled centrifuge matchine	Thermo Scientific
Temperature controlled micro centrifuge matchine	Eppendrof
Thermo Scientific BioLite Cell Culture Treated Flasks	Thermo Scientific
Thermo Scientific Pierce BCA Protein Assay	Thermo Scientific
Thermo Scientific Pierce RIPA Buffer	Thermo Scientific
Thermo Scientific Thermo-Fast 96-Well Full-Skirted Plates	Thermo Scientific
Thermo Scientific Verso cDNA synthesis Kit (100 runs)	Thermo Scientific

Ultra Low Range DNA Ladder	Invitrogen
VWR standard solid door laboratory refrigerator	VWR

Catalog Number

VWR40086A
50-607-511
12-556-008
ATCC CCL-185
0710-25G
G2938c
AM4611
15240-062
KAS02
IV-900

ACC058
342391100
Steps 5.14; 6.13: Used for calculating the population distrubution according to the cell cycle phase and for calculating the population
CFX96 Optics Module
Chemidoc Touch Imaging System
HERAcell 150i
3433-010-01
FMA050
VWRL0100-0500
E1010
356006
05-403-152
BP2818500
L07462
45000-354

S11150
10438026
02-682-002

NDC#63739-920-11
BT102

MCH01315
MCH02824
PHC9394
IRB# A15-3891
SH30256FS
Results: Used for bioinformatics pathway analysis
10-977-015
11-668-027
470024-814
M4530
MU130
Step 5.15: Alternative software for analysis of cell cycle population distributions
NanoDrop one C
31985-070
B21210
A25780
IC19545825
ASCNPHRG. We used excitation laser wavelength at 543 nm.
4360954
R1055
R6513-50MG
Step 7.33: Microarray analysis software
2314

470224-998
470225-004
10791-796
ST16R
5415R
12-556-009
PI23225
PI89900
AB0800WL
AB1453B

10597012

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Analysis of combinatorial miRNA treatment to regulate cell cycle and angiogenesis

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RESPONSE TO REVIEWERS' COMMENTS

We thank the reviewers for their time, effort and for their insightful comments. We have revised our manuscript according to their suggestions. Following each of the reviewers' comments (in italics), we present our specific responses.

Editorial and production comments:

Note the change in numbering (only the protocol should be numbered).

1. Please include comments in the Table of Materials so its clear which software is used for which analysis step (6.13, 7.33, 9.13).

Corrected

2. 1.5: 4 mL culture media, as appears to be the case in the video?

Corrected. Information added to the text protocol

3. 1.9: Steps 2 and 3 are mentioned here, but don't appear to be correct (even in the original numbering).

Corrected

4. 2: The bench is being sprayed down in the video; is this important for the protocol (e.g., if an RNAse inhibitor is being used)? Please mention in the written protocol if so.

Added to the protocol

5. 2.5: How fast and how long do you centrifuge?

Information added

6. 2.12: Is this referring to section 8? This appears to be the case in the 7. Section 3: How is analysis done here?

This line was removed from the protocol, as it is explained in section 8.

8. 7: The order in the video is confusing-rinsing with deionized video is shown after what appears to be incubation in the coupling chamber but is before this step in the written protocol. Please clarify.

We added this information at step 7.26, which should better align with the video. There are multiple washing steps in this protocol. We included a clarification that ddH20 is the washing media, when not mentioned otherwise.

9. 7.32: Which wavelengths?

Information added

10. 8.5: The bioanalyzer does not appear to be in the Table of Materials.

Information added

11. 8.7-8.8: Please provide more details or references here.

References added

12. 8: Pathway analysis is mentioned in the heading, but is not detailed in the protocol itself.

The heading was corrected and the protocol section removed. The pathway analysis was part of a service. Indicative figure 5 demonstrates the potential analysis of the RNA -seq relative expression data.

13. Results, RNA sequencing…: Do you have any idea of the statistical tests done here?

Appropriate references were added.

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Multipronged activity of combinatorial miR-143 and miR-506 inhibits Lung Cancer cell cycle progression and angiogenesis in vitro, A. K. M. Nawshad Hossian, Md. Sanaullah Sajib, Paul E. Tullar, Constantinos M. Mikelis & George Mattheolabakis, Scientific Reports volume 8, Article number: 10495 (2018)

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