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# Bioengineered miR-34a modulates mitochondrial inner membrane protein 17 like 2 (MPV17L2) expression toward the control of cancer cell mitochondrial functions

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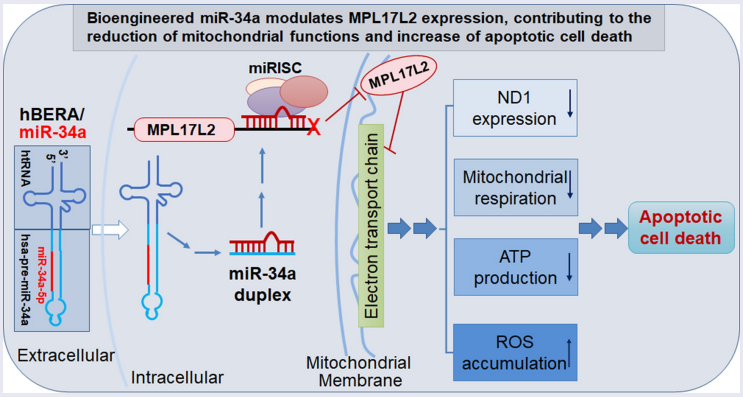
**ABSTRACT**

Genome-derived microRNAs (miRNAs or miRs) control post-transcriptional gene expression critical for various cellular processes. Recently, we have invented a novel platform technology to achieve high-yield production of fully humanized, bioengineered miRNA agents (hBERAs) for research and development. This study is aimed to produce and utilize a new biologic miR-34a-5p (or miR-34a) molecule, namely, hBERA/miR-34a, to delineate the role of miR-34a-5p in the regulation of mitochondrial functions in human carcinoma cells. Bioengineered hBERA/miR-34a was produced through in vivo fermentation production and purified by anion exchange fast protein liquid chromatography. hEBRA/miR-34a was processed to target miR-34a-5p in human osteosarcoma and lung cancer cells, as determined by selective stem-loop reverse transcription quantitative polymerase chain reaction analysis. The mitochondrial inner membrane protein MPV17 like 2 (*MPV17L2*) was validated as a direct target for miR-34a-5p by dual luciferase reporter assay. Western blot analysis revealed that bioengineered miR-34a-5p effectively reduced MPV17L2 protein outcomes, leading to much lower levels of respiratory chain Complex I activities and intracellular ATP that were determined with specific assay kits. Moreover, Seahorse Mito Stress Test assay was conducted, and the results showed that biologic miR-34a-5p sharply reduced cancer cell mitochondrial respiration capacity, accompanied by a remarkable increase of oxidative stress and elevated apoptotic cell death, which are manifested by greater levels of reactive oxygen species and selective apoptosis biomarkers, respectively. These results demonstrate the presence and involvement of the miR-34a-5p-MPV17L2 pathway in the control of mitochondrial functions in human carcinoma cells and support the utility of novel bioengineered miRNA molecules for functional studies.

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**Research highlights the following features:**

- A new fully humanized miR-34a was bioengineered and purified for functional studies.
- MPV17L2, a mitochondrial protein, was verified as a new direct target for miR-34a-5p.
- Bioengineered miR-34a altered MPV17L2 protein outcomes, Complex I assembly and activity.
- MiR-34a reduced mitochondrial respiration and increased oxidative stress and apoptosis.

## Introduction

MicroRNAs (miRs or miRNAs) are a superfamily of genome-derived noncoding RNAs (ncRNAs) around 18–25 nt in length [1,2]. Through imperfect complementary base pairings with the miRNA response elements (MREs) that are usually present within the 3'untranslated regions (3'UTR) of target transcripts, functional miRNAs modulate post-transcriptional gene expression toward the control of protein outcomes. Therefore, regulatory miRNAs play important roles in essentially all cellular processes, such as cell cycle, differentiation, proliferation, migration, senescence, apoptosis, autophagy, and metabolism [3,4]. Furthermore, some miRNAs are dysregulated in various types of human diseases, including cancer in which upregulated miRNAs are prone to promote tumor initiation, progression, and metastasis, whereas tumor-suppressive miRNAs are usually downregulated in carcinoma cells [3,4]. With an improved understanding of miRNA actions in tumor development and progression as well as establishment of viable technologies for the manipulation of target miRNA expression or function, efforts are underway to develop novel miRNA-based therapies for lethal cancer diseases [4–7].

Among those cancer-related miRNAs, miR-34a-5p or miR-34a is a direct downstream target of p53 protein [8] and miR-34a is commonly downregulated in many types of cancers, including lung, breast, prostate, colon, hepatocellular carcinoma, and sarcomas (see reviews in Refs. 9,10 and). Oncolytic miR-34a has been shown to control cancer cell stemness, cell cycle, proliferation, invasion, apoptosis, and senescence through the regulation of respective targets, such as cyclin-dependent kinase 6 (CDK6) [11,12], silent information regulator 1 (SIRT1) [13,14], hepatocyte growth factor receptor or tyrosine-protein kinase Met (c-MET) [15], B-cell lymphoma-2 (BCL-2) [16–18], and cell surface glycoprotein (CD44) [19]. Recent studies have also demonstrated that miR-34a is involved in the modulation of cancer metabolism and tumor immune responses through the regulation of several metabolic enzymes [20,21] and programmed death-ligand 1 (PD-L1) [22,23], respectively. As a result, restoration of miR-34a function has been shown to be effective in controlling many types of malignancies including lung cancer [24–26], osteosarcoma (OS) [14,27,28], prostate cancer [19,29], liver cancer [15], and colorectal cancer [30] in animal models.

At present, miRNA research and experimental therapy depend predominantly upon the use of synthetic miRNA "mimics." Noticing the fundamental differences between miRNA "mimics" synthesized chemically or biochemically *in vitro* and natural RNAs produced and folded *in vivo* [31–33], we have established a novel technology, based on the stable tRNA-fused precursor miRNA (pre-miRNA) carriers identified in our laboratory, to achieve *in vivo* fermentation production of bioengineered miRNA agents (BERAs) [26,34,35] that should better recapitulate the physicochemical and biological properties of natural RNAs because both are produced and folded in live cells. Further identification of proper human tRNAs and optimal hsa-pre-miRNA coupled carriers leads to high-yield and large-scale production of "humanized" BERAs (hBERAs) that are more compatible to human cells [28]. Omics-based studies and targeted analyses have demonstrated that oncolytic miRNAs or small interfering RNAs (siRNAs) are precisely released from the BERAs or hBERAs in human cells to selectively control target gene expression and regulate various cancer cellular processes *in vitro* and subsequently suppress tumor growth and metastasis and improve chemotherapy in animal models *in vivo* [25,26,28,34,36–44].

While confirming the functions and selectivity of bioengineered miR-34a-5p in the regulation of target gene expression, our RNA sequencing study has revealed that mitochondrial inner membrane protein MPV17 like 2 (MPV17L2) transcript is reduced by miR-34a to the greatest degree in human cells [26]. Although the function of mitochondrial protein MPV17L2 remains elusive, one study has demonstrated that MPV17L2 is critical for the biogenesis of mitochondrial ribosome, playing an important role in the assembly of oxidative phosphorylation (OXPHOS) complexes [45]. It is hypothesized that miR-34a-5p may disturb mitochondrial functions via directly targeting MPV17L2. Therefore, this study is aimed at establishing the interaction between miR-34a-5p and *MPV17L2* and defining the impact of miR-34a-MPV17L2 signaling on mitochondrial functions in human cancer cells. Herein, we report the bioengineering and application of a new fully humanized miR-34a agent (hBERA/miR-34a) to functional studies, in which MPV17L2 is verified as a direct target of miR-34a-5p. Downregulation of MPV17L2 by bioengineered miR-34a-5p disrupts the assembly of the mitochondrial inner membrane in human cancer cells and leads to the reduction of mitochondrial respiration function and induction of oxidative stress that is associated with enhanced apoptotic cell death. These findings shall improve the understanding of miR-34a functions in cancer metabolism and support the utility of novel bioengineered miRNA molecules for research and development.

## Materials and methods

### Chemicals and materials

RPMI 1640 medium, fetal bovine serum, Lipofectamine 3000, 0.05% trypsin-EDTA phenol red, RIPA lysis buffer, Trizol reagent, and BCA Protein Assay Kit were bought from the Thermo Fisher Scientific (Waltham, MA). Protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Bovine serum albumin and dimethyl sulfoxide were purchased from VWR (Radnor, PA). The polyvinylidene fluoride (PVDF) membrane, ECL Substrate Kit, and blotting-grade blocker for Western blots were purchased from Bio-Rad (Hercules, CA). Direct-zol RNA MiniPrep kit was purchased from Zymo Research (Irvine, CA). The Luminescent ATP Detection Assay Kit (ab113849) and colorimetric Complex I Enzyme Activity Microplate Assay Kit (ab109721) were purchased from Abcam (Boston, MA). The Fluorometric Intracellular ROS Kit was purchased from Sigma-Aldrich (St. Louis, MO). The Seahorse XF Cell Mito Stress Test Kit was purchased from Agilent (Santa Clara, CA). All other organic solvents and chemicals were of analytical grade and purchased from Thermo Fisher Scientific or Sigma-Aldrich.

### Cell culture

Human embryonic kidney HEK293, osteosarcoma 143B, and lung carcinoma A549 cells were purchased from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM or RPMI 1640 medium containing 10% fetal bovine serum in a humidified atmosphere with 5% $CO_2$ and 95% air, at 37°C. Lipofectamine 3000 reagent was used to carry plasmids or bioengineered RNAs for transfection, according to the instructions from the manufacturer.

### Bioengineering of ncRNA agents

Expression and purification of hBERAs bearing the target miR-34a-5p (hBERA/miR-34a) and corresponding Sephadex aptamer-tagged human leucyl-tRNA ($htRNA^{Leu}$; control RNA) were conducted as described recently [28,35,42]. Briefly, insert that encodes the pre-miR-34a-fused human $tRNA^{Leu}$ was cloned into the pBSTNAV vector linearized with EcoRI-HF® and PstI-HF® (New England Biolabs, Ipswich, MA) by using an In-Fusion® HD Cloning Kit (Takara, Mountain View, CA). After plasmids were confirmed by DNA sequencing (GenScript, Piscataway, NJ), recombinant hBERA/miR-34a and control RNA were overexpressed in *E. coli* HST08 (0.5 L). Target RNAs were separated from total RNAs on an Enrich-Q 10 × 100 anion exchange column by using a NGC QUEST 10PLUS fast protein liquid

chromatography (FPLC) system (Bio-Rad), as described [26,35,42]. The purity of recombinant RNA was estimated by denaturing urea (8 M) polyacrylamide (8%) gel electrophoresis (PAGE) and quantitatively determined by the high-performance liquid chromatography (HPLC) method reported previously [25]. A Limulus Amebocyte Lysate Pyrogent-5000 kinetic assay kit (Lonza, Walkersville, MD) was employed to determine endotoxin levels. Bioengineered RNAs showing high homogeneity (≥ 98%) and low endotoxin activity (≤ 5 EU/μg RNA) were used in the following studies.

### RNA isolation and reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR)

HEK293 and 143B cells were seeded in 24-well plates at a density of $4 \times 10^4$ cells/well. After incubating overnight, the cells were treated with 15 nM of hBERA/miR-34a, control RNA, or the lipofectamine 3000 (vehicle). Following 48-h transfection, total RNAs were isolated by using a Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA), and RNA concentrations were determined by using a NanoDrop spectrophotometer (Thermo Scientific, Rockford, IL). Reverse transcription was carried out with NxGen M-MuLV reverse transcriptase (Lucigen, Middleton, WI) and random hexamers (for U6) or stem-loop primer 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACA CAA CC-3' (for miR-34a-5p). The qPCR analyses were conducted on a CFX96 Touch real-time PCR system (Bio-Rad, Hercules, CA) by using iTaq™ Universal SYBR® Green Supermix (Bio-Rad) as well as gene-specific primers: forward 5'-CGC GCT GGC AGT GTC TTA GCT-3' and reverse 5'-GTG CAG GGT CCG AGG T-3' for miR-34a-5p and forward 5'-CTC GCT TCG GCA GCA CA-3' and reverse 5'-AAC GCT TCA CGA ATT TGC GT-3' for U6. The formula $2^{-\Delta\Delta Ct}$ [25] was used to determine the relative level of miR-34a-5p over U6 (internal standard), in which ΔΔCt equals the treatment group $\Delta C_T$ (analyte minus internal standard) diminished by corresponding control group ΔCt.

### Identification of the microRNA response element (MRE) and luciferase reporter assay

Putative MRE for miR-34a-5p within the 3'UTR of *MPV17L2* was identified by using TargetScan (http://www.targetscan.org/). The full length of human MPV17L2 3'UTR was inserted downstream of the firefly luciferase gene within a firefly/*Renilla* dual-luciferase reporter vector (pEZX-MT06) (GeneCopoeia, Rockville, MD), and the correct plasmid was named pEZX-MT06-MPV17L2 -3'UTR. Luciferase reporter assays were performed in HEK293 cells and 143B cells as previously reported [36,42,44], with minor modifications. Briefly, after seeding in 96-well plates ($1.5 \times 10^4$ cells/well) and maintaining overnight, cells were transfected with pEZX-MT06-MPV17L2-3'UTR alone or along with bioengineered miR-34a or control RNA. After 48-h treatment, the firefly and *Renilla* luciferase activities were quantitated with a dual-luciferase reporter assay kit (Promega, Madison, WI) on a SpectraMax M3 Microplate Reader (Molecular Devices, LLC., San Jose, CA). The firefly luciferase activity was normalized to respective *Renilla* luciferase activity and further to control treatment (3'UTR-expressing plasmid alone) for comparison. Individual treatments were carried out in five replicates.

### Protein isolation and western blot analyses

A549 ($3 \times 10^5$ cells/well) and 143B ($2.5 \times 10^5$ cells/well) cells were seeded in 6-well plates and treated with 15 nM of hBERA/miR-34a, control RNA, or vehicle for 48 h. Cells were harvested and lysed with RIPA lysis buffer supplemented with complete protease inhibitors, and whole cell protein concentrations were measured with a BCA Protein Assay Kit. Total proteins (30 μg/lane) were separated on a 10% SDS-PAGE gel and transferred onto PVDF membranes. The membranes were first incubated in 5% nonfat milk for 2 h and then blotted with primary antibodies against MPV17L2 (1:500 dilution, Thermo Fisher Scientific), mitochondrial NADH dehydrogenase 1 (MT-ND1) (1:500, Thermo Fisher Scientific), B-cell lymphoma-extra large (Bcl-xl) (1:1,000, Santa Cruz Biotech Inc., Texas, TX), cleaved poly-ADP-ribose polymerase (c-PARP) (1:1,000, Santa Cruz Biotech Inc.), cleaved-Caspase-7 (c-Caspase-7) (1:1,000, Cell

Signaling Technology, Inc.), or β-actin (1:5,000, A5441, Sigma-Aldrich) overnight at 4°C. After incubating with horseradish peroxidase-labeled antibodies (anti-mouse, 1:3,000, Cell Signaling Technology; anti-rabbit, 1:10,000, Jackson ImmunoResearch, West Grove, PA) at room temperature for 2 h and Clarity Western ECL substrates, the images were acquired immediately with a ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA). Protein band intensity was determined using Image Laboratory software (Bio-Rad, Hercules, CA) and further normalized to the β-actin level in the respective sample and then to vehicle control for comparison [42].

### Measurement of Complex I activity

The mitochondrial respiration chain Complex I enzyme activity was determined with a Complex I Enzyme Activity Microplate Assay Kit (ab109721; Abcam, Boston, MA), according to the manufacturer's instructions. Briefly, A549 ($3 \times 10^5$ cells/well) and 143B ($2.5 \times 10^5$ cells/well) cells were seeded in 6-well plates and transfected with 15 nM of hBERA/miR-34a, control RNA, or vehicle. Cells were harvested at 48 h post-transfection to extract proteins, and protein concentrations were measured using the BCA Protein Assay Kit. Equal amounts of proteins were loaded on the assay plates and incubated at room temperature for 3 h. After washing three times, 200 µL of Assay Solution was added into each well, and the optical density (OD450 nm) was recorded using a SpectraMax microplate reader in a kinetic mode for 30 min at room temperature. Complex I activity was calculated and expressed as the changes in absorbance per minute per 80 µg of proteins loaded into the well (mOD/min) for the A549 cells or 100 µg of protein for 143B cells [46]. Each treatment was conducted in five replicates.

### Quantification of intracellular ATP levels

Intracellular ATP levels were measured with a Luminescent ATP Detection Assay Kit (ab113849; Abcam), according to the manufacturer's instructions [47]. Briefly, cells ($1.5 \times 10^4$ cells/well) were seeded in a 96-well plate and treated with 15 nM of hBERA/miR-34a, control RNA, or vehicle for 48 h. Cells were harvested, lysed, and exposed to the ATP substrate solution. Luminescence was determined by using a SpectraMax microplate reader and normalized to the protein concentration of the respective sample. ATP levels were calculated as the percentages of vehicle control that were set as 100%. Each treatment was performed in five replicates.

### Real-time live cell mitochondrial functions

A549 and 143B cells were seeded in Seahorse XFe24 cell culture microplates at a density of $1.3 \times 10^4$ cells/well and $1.4 \times 10^4$ cells/well, respectively, and then transfected with 15 nM of hBERA/miR-34a, control RNA, or vehicle. After 48 h, the oxygen consumption rate (OCR) was monitored in real time before and after the sequential injection of oligomycin, carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone (FCCP), and a mixture of rotenone and antimycin A (ROT/AA) through separate ports in the XFe24 Assay cartridge for the evaluation of mitochondrial functions by using an Agilent Seahorse XFe24 Analyzer and a Cell Mito Stress Test Kit (103,015–100, Agilent Technologies), following the manufacturer's protocol [48]. Basal respiration, maximal respiration, and ATP production values were calculated for individual samples. Each treatment was performed in triplicates.

### Quantification of intracellular ROS levels

The intracellular ROS levels were measured with a Fluorometric Intracellular ROS kit (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, A549 and 143B cells were seeded in 96-well plates at a density of $1.5 \times 10^4$ cells/well. After treating with 15 nM of hBERA/miR-34a, control RNA, or vehicle for 48 h, the fluorescence intensities were recorded with a SpectraMax microplate reader (Molecular Devices, Sunnyvale, CA) at the excitation of 490 nm and emission of 525 nm. The ROS level was normalized to the vehicle control treatment group (set as 100%) [43]. Individual treatments were carried out in five replicates.

### Immunofluorescence assay

Cells ($2.5 \times 10^4$ cells/well) were seeded in 8-well chamber slides with removable wells (Thermo Fisher Scientific) and then treated with 15 nM of hBERA/

miR-34a, control RNA, or vehicle for 48 h. After fixing with 4% paraformaldehyde, permeabilizing with 0.5% tween-20, and blocking with 1% BSA, cells were incubated overnight with the primary anti-cleaved-Caspase-3 (c-Caspase-3 or c-Cas-3) antibody (Cell signaling Technology, Beverly, MA) and subsequently with a secondary antibody (anti-rabbit IgG Alexa Fluor® 488 Conjugate, Cell signaling Technology). After washing three times, cells were stained with DAPI (Cell signaling Technology), and images were obtained with a Zeiss Axio Observer.z1 Microscope coupled to a Zeiss LSM 710 Scanning Device (Zeiss, Oberkochen, Germany) [36]. C-Caspase-3-positive cells were counted directly in five random views under the confocal microscope.

### Statistical analyses

All values are presented as means ± SD. Different treatment groups were compared by one- or two-way ANOVA with Bonferroni *post hoc* tests (Prism, GraphPad Software Inc., San Diego, CA). The statistically significant difference was noted when the *P* value was less than 0.05 ($P < 0.05$).

## Results

The aim of this study is to test the hypothesis that miR-34a-5p directly modulates the expression of mitochondrial protein MPV17L2 and thus alters mitochondrial functions of human carcinoma cells. A novel humanized miR-34a-5p agent, namely, hBERA/miR-34a, has been bioengineered, purified, and used in this study. The results demonstrate that MPV17L2 is a direct target of miR-34a-5p and miR-34a-5p-caused downregulation of MPV17L2 protein expression leads to the disruption of assembly of mitochondrial respiratory chain complexes and consequently, reduction of mitochondrial respiration capacity, an increase of oxidative stress, and an enhanced apoptotic cell death. These findings on the miR-34a-MPV17L2 pathway not only offer new insight into understanding the mechanistic functions of miR-34a in the control of cancer metabolism and apoptotic cell death but also support the broad applications of novel bioengineered RNA agents to research and development.

### Mitochondrial MPV17L2 is a direct target of miR-34a-5p

Our recent RNA sequencing study [26] has revealed that mitochondrial *MPV7L2* was the transcript downregulated to the greatest level by miR-34a in human 293 T cells. Computational analysis was thus conducted, and it identified one putative MRE for miR-34a-5p within the 3'UTR of *MPV17L2* (Figure 1(a)), suggesting *MPV17L2* as a direct target for miR-34a-5p. Using the *in vivo* fermentation-based RNA bioengineering platform we established very recently [28], high-quality (> 98% pure, determined by HPLC) hBERA/miR-34a and the control RNA were produced at high yield and on a large scale (> 10 mg per liter of microbial fermentation). Selective stem-loop RT-qPCR was further performed to determine if hBERA/miR-34a could be processed to target miR-34a-5p in 143B and HEK293 cells. The results showed that mature miR-34a-5p levels were remarkably higher in both HEK293 and 143B cells treated with hBERA/miR-34a than control RNA or vehicle treatments at 48 h post-transfection (Figure 1(b)), indicating a selective release of miR-34a-5p from bioengineered hBERA/miR-34a in human cells. Dual-luciferase reporter assays were then carried out to assess potential interactions between miR-34a-5p and *MPV17L2* 3'UTR. As shown in Figure 1(c), hBERA/miR-34a treatment (5 and 15 nM) remarkably suppressed the *MPV17L2* 3'UTR-luciferase reporter activities in a dose-dependent manner in both HEK293 and143B cells, supporting the direct action of miR-34a-5p on the *MPV17L2* 3'UTR.

### Bioengineered miR-34a-5p controls MPV17L2 protein outcomes and modulates mitochondrial ND1 levels

Western blot analyses were then performed to delineate the impact of miR-34a-5p on MPV17L2 protein levels in human cancer 143B and A549 cells. The MPV17L2 protein levels were revealed to be around 50% lower in hBERA/miR-34a-treated 143B and A549 cells than control RNA-treated cells, whereas no differences were observed between the control RNA and vehicle treatment groups (Figure 2). Since MPV17L2 modulates mitochondrial protein synthesis and

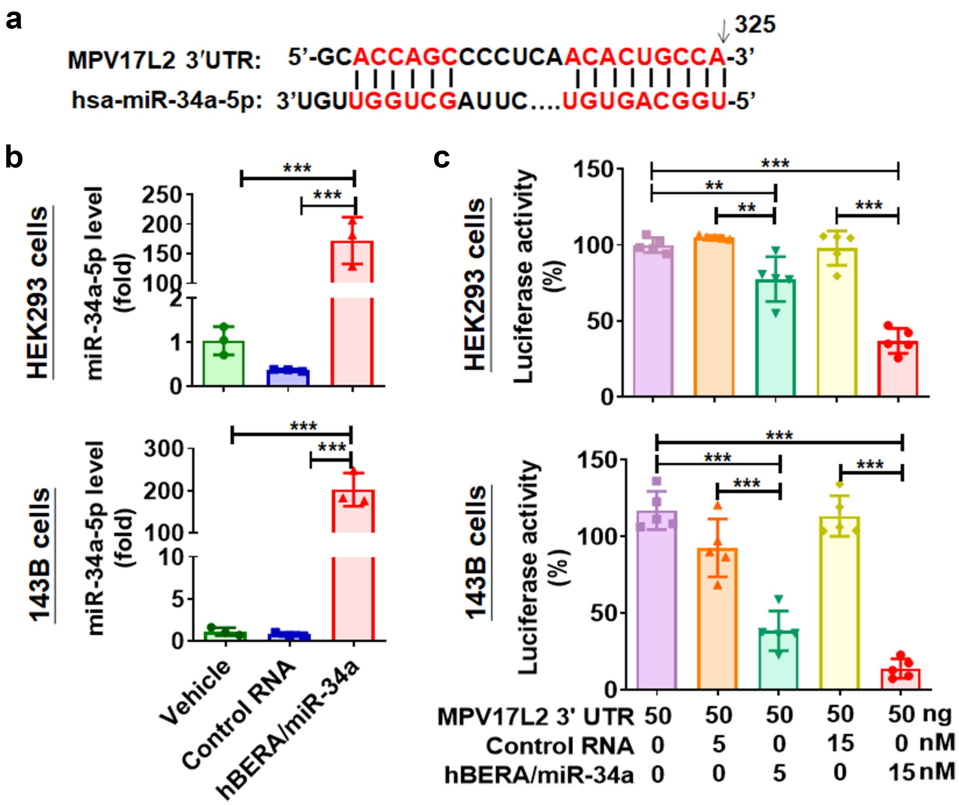


**Figure 1.** Mitochondrial MPV17L2 is verified as a direct target for miR-34a. (a) Computational analyses identified one putative miRNA response element (MRE) for miR-34a-5p within the 3'UTR of MPV17L2 transcript. (b) Levels of miR-34a-5p were remarkably higher in human HEK293 and 143B cells treated with recombinant hBERA/miR-34a than the controls, as determined by specific stem-loop RT-qPCR assay. Cells were transfected with 15 nM of hBERA/miR-34a, control RNA, or vehicle for 48 h. Values are mean ± SD (N = 3/group). (c) MiR-34a-5p significantly inhibited the MPV17L2 3'UTR-luciferase reporter activities in HEK293 and 143B cells, as compared to controls (N = 5/group). ***P < 0.01* and ****P < 0.001*, one-way ANOVA with Bonferroni *post hoc* tests.

assembly of OXPHOS [45], we further investigated the consequent effects on the protein levels of ND1, a subunit of respiratory chain Complex I that is synthesized in mitochondria [49,50]. The results showed that ND1 protein levels were suppressed by approximately 55% and 40% in hBERA/miR-34a-treated A549 and 143B cells, respectively, as compared with respective control RNA treatments (Figure 2). Taken together, these findings demonstrate that the bioengineered hBERA/miR-34a agent is effective in downregulating the MPV17L2 protein level and subsequently inhibiting the assembly of respiratory chain Complex I.

### *Biologic miR-34a-5p suppresses respiratory chain complex I activity and intracellular ATP level in human cancer cells*

To determine to what degree the Complex I activity would be changed following the suppression of ND1 by miR-34a-5p, the mitochondrial respiration chain Complex I enzyme activities were measured. The data showed that, as compared with control RNA treatments, Complex I activities were decreased by hBERA/miR-34a-5p to approximately 70% in both 143B and A549 cells (Figure 3(a)). Meanwhile, the control RNA treatment group was not different from the vehicle control group. Since the mitochondrial OXPHOS complexes offer energetic ATP [51], we further examined the consequent effects on intracellular ATP levels. Compared with the control RNA, bioengineered miR-34a-5p treatments reduced the ATP levels by 30–40% in 143B and A549 cells (Figure 3(b)). These results indicate that miR-34a-5p-mediated downregulation of MPV17L2 leads to a significant reduction of respiratory chain Complex I activities and intracellular ATP levels in human cancer cells.

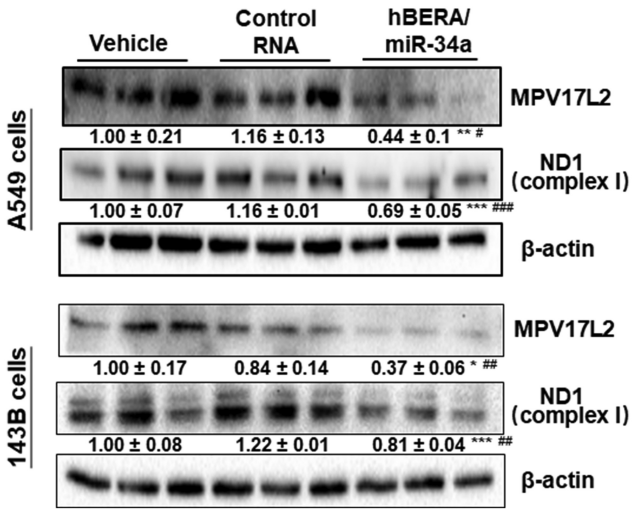


**Figure 2.** Bioengineered miR-34a-5p suppresses mitochondrial MPV17L2 protein levels in human lung carcinoma and osteosarcoma cells and subsequently decreases mitochondrial ND1 protein levels. Cells were treated with 15 nM of hBERA/miR-34a, control RNA, or vehicle for 48 h, and Western blots were conducted with selective antibodies. β-actin was used as a loading control. Protein levels were normalized to respective β-actin levels and then vehicle groups for comparison. Values are mean ± SD (N = 3/group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared with control RNA and vehicle treatment, respectively (one-way ANOVA with Bonferroni *post hoc* tests).

### *MiR-34a-5p controls cancer cell mitochondrial respiration*

To delineate the impact of miR-34a-MPV17L2 signaling on the mitochondrial function of cancer cells, we employed an Agilent Seahorse XF24 Analyzer and the Mito Stress Test Kit to monitor live cell mitochondrial respiration after cells were treated with miR-34a for 48 h. The real-time oxygen consumption rate (OCR) data demonstrated that mitochondrial respiration capacity was remarkably inhibited by miR-34a-5p in both 143B and A549 cells, when compared with control RNA treatments (Figure 4(a)). In particular, the basal respiration (Figure 4(b)) and maximal respiration (Figure 4(c)) rates were around 50% lower in cells treated with miR-34a-5p than control RNA. In addition, the calculated mitochondrial ATP production values were revealed to be approximately 50% lower in both 143B and A549 cells treated with miR-34a-5p than the control RNA (Figure 4(d)). These results indicate the importance of the miR-34a-MPV17L2 pathway in the control of mitochondrial respiration of human osteosarcoma cells and lung carcinoma cells.

### *Bioengineered miR-34a-5p induces oxidative stress and apoptotic cell death*

Since mitochondria are also the major generators of ROS and oxidative stress may cause apoptotic cell death, experiments were carried out to determine whether biologic miR-34a-3p alters ROS levels and subsequently induces apoptosis. The fluorometric intracellular ROS assay revealed that ROS levels were increased by 100% and 30% in A549 and 143B cells, respectively, at 48 h post-treatment with miR-34a-5p, as compared with control RNA treatments (Figure 5(a)), indicating the induction of oxidative stress by miR-34a-5p. Therefore, Western blots were performed to examine the influence of bioengineered miR-34a-5p on the protein levels of apoptosis markers, Bcl-xl, c-PARP, and c-Caspase-7 in A549 and 143B cells. The data showed that levels of antiapoptotic protein Bcl-xl were significantly ($P < 0.05$) lower in biologic miR-34a-treated A549 and 143B cells, compared with either vehicle or control RNA treatments (Figure 5(b)). In contrast, the protein levels of apoptotic markers c-PARP and c-Caspase-7 were remarkably higher ($P < 0.05$) in both A549 and 143B cells treated with biologic miR-34a-5p than control RNA or vehicle (Figure 5(b)). In addition, we employed immunofluorescence assay and confocal microscope imaging to examine another apoptosis marker, c-Caspase-3 (Figure 6(a)). The numbers of c-Caspase-3-positive cells in miR-34a-5p-treated A549 and 143B cells were revealed to be around 3-fold higher than those treated with either control RNA or vehicle (Figure 6(b)). Taken together, these results demonstrate that bioengineered miR-34a-5p elevates the intracellular ROS levels and enhances apoptosis of cancer cells.

## Discussion

Mitochondrial MPV17L2, an essential element for the assembly of mitochondrial ribosome and protein synthesis [45], was validated as a new direct target for miR-34a-5p by using a novel bioengineered miR-34a agent in the present study. It is

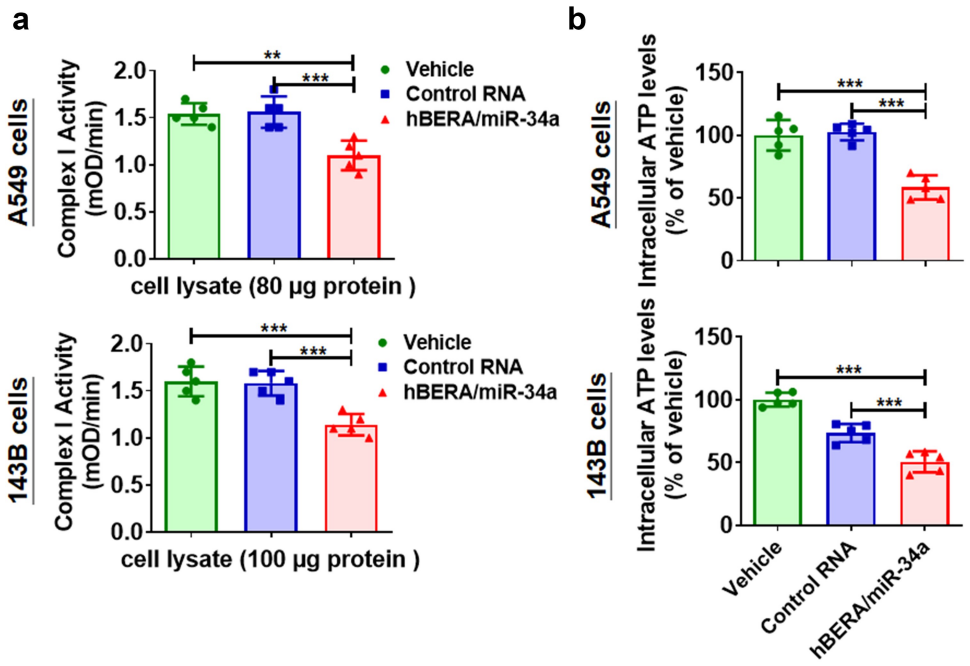


**Figure 3.** Respiratory chain Complex I activities and intracellular ATP levels are prominently inhibited by hBERA/miR-34a-5p in human cancer cells. (a) Complex I activities were reduced by 30% in miR-34a-5p-treated A549 and 143B cells, as compared with control RNA or vehicle treatments, which were determined by using a Complex I Enzyme Activity Microplate Assay Kit at 48 h post-treatment. (b) Bioengineered miR-34a reduced the intracellular ATP levels by 30–40% in A549 and 143B cells, as compared with control RNA or vehicle treatments, which were determined by using a Luminescent ATP Detection Assay Kit at 48 h post-treatment. Values are mean ± SD (N = 5/group). $^{**}P < 0.01$ and $^{***}P < 0.001$, one-way ANOVA with Bonferroni *post hoc* tests.

notable that bioengineered miR-34a was produced and folded in live cells, and it is distinguished from chemically synthesized miR-34a mimics made *in vitro*. The results showed that suppression of MPV17L2 expression by hBERA/miR-34a led to a significant decrease of ND1 protein levels, a subunit of the largest multimeric enzyme complex of the mitochondrial respiratory chain [50,52]. Further studies demonstrated that, following the miR-34a-controlled downregulation of MPV17L2, the respiratory chain Complex I activity, mitochondrial respiration capacity, and ATP production were sharply reduced in human cancer cells. In addition, biologic miR-34a-treated cancer cells exhibited a much greater degree of oxidative stress and apoptosis, as manifested by higher levels of intracellular ROS and apoptotic biomarkers (e.g. cleaved Caspases 7 and 3), respectively. These results indicate an important role of miR-34a-MPV17L2 signaling in the control of mitochondrial respiration, energetics, and redox balance, providing new insights into miR-34a functions in cancer metabolism and the development of miRNA-based therapeutics [4,6].

Mitochondria are multifunctional organelles that are responsible for the production of bioenergetic molecules (e.g., ATP), biosynthesis of building blocks, and maintenance of redox balance and cell signaling to retain regular cell functions and drive cell proliferation [53]. While many cancer cells are revealed to attain excess glucose uptake and undergo glycolysis even in aerobic conditions rather than complete oxidation through mitochondrial OXPHOS, namely, "Warburg effect"' or aerobic glycolysis [54], mitochondrial respiration of most cancer types may not be necessarily impaired, and the implication of mitochondrial respiration in tumor progression and drug resistance has been verified in many studies [55]. In addition, mutations of mitochondrial proteins could lead to the production of oncogenic metabolites [56]. Indeed, mitochondria are essential mediators during tumorigenesis, and thus, targeting mitochondria represents a new viable strategy

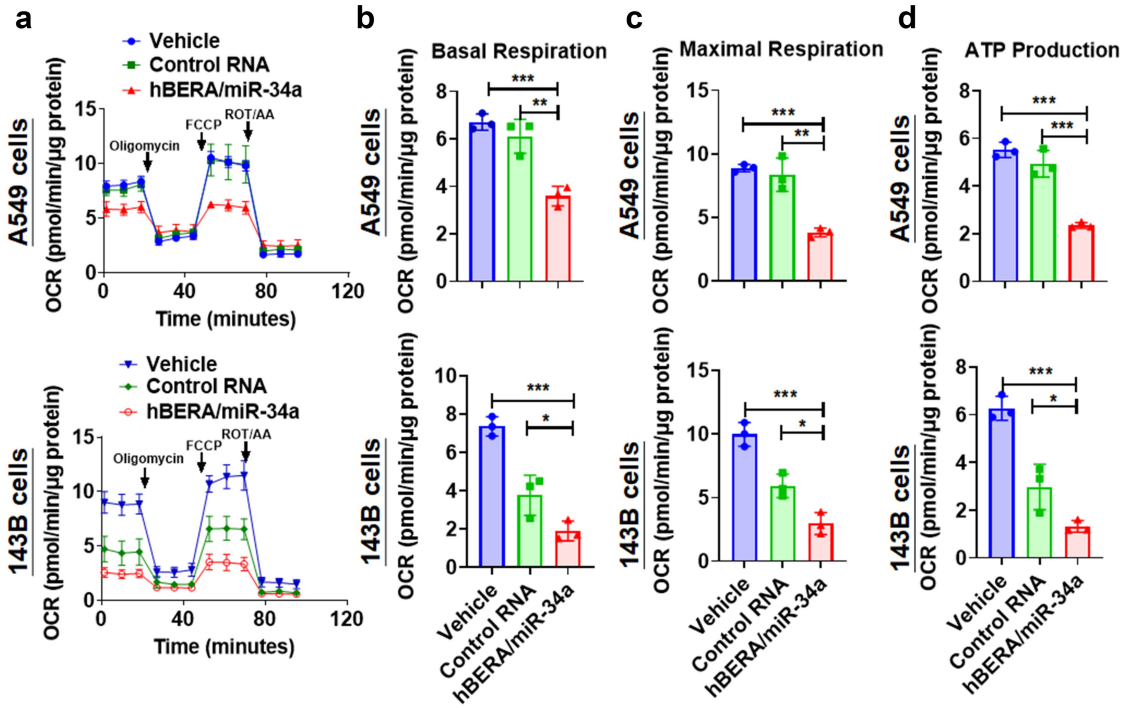


**Figure 4.** Mitochondrial respiration is significantly inhibited by miR-34a-5p in human lung carcinoma and osteosarcoma cells. (a) Real-time oxygen consumption rate (OCR) levels of live A549 or 143B cells at the basal level and after sequential addition of oligomycin, FCCP, and a mixture of rotenone and antimycin (ROT/AA). The basal (b) and maximal (c) respiration capacities were remarkably lower in cells treated with miR-34a-5p, as compared with control RNA treatments. (d) ATP production was inhibited by miR-34a-5p in A549 and 143B cells, as compared with control RNA treatments. Cells were treated with 15 nM of hBERA/miR-34a, control RNA, or vehicle. A Seahorse XFe24 Analyzer and a Cell Mito Stress Test Kit were employed to determine mitochondrial respiration at 48 h post-treatment. Values are mean ± SD (N = 3/group). *$P < 0.05$, **$P < 0.01$, *and* ***$P < 0.001$, compared to control RNA or vehicle (one-way ANOVA with Bonferroni *post hoc* tests).

for the treatment of cancer [57,58]. The current study established the involvement of miR-34a-5p in the control of cancer cell mitochondrial respiration through the regulation of MPV17L2.

MPV17L2, an inner mitochondrial membrane protein, belongs to the integral membrane protein family that consists of four members, MPV17, MPV17L, MPV17L2/FKSG24, and peroxisomal membrane protein 2 (PXMP2) [59]. Although its complete functions remain elusive, it has been revealed that MPV17L2 participates in the assembly and stability of mitochondrial ribosome and plays an essential role in mitochondrial protein translation [45]. Our recent RNA sequencing study identified MPV17L2 as the most downregulated transcript by miR-34a-5p in human cells [26], and in the present study, we successfully validated MPV17L2 as a direct target for miR-34a-5p and defined the consequent effects on mitochondrial functions. Being transcriptionally regulated by the protein p53, tumor-suppressive miR-34a-5p has been reported to control multiple cancer cellular processes, such as the alteration of the cell cycle, induction of apoptosis, and inhibition of proliferation, migration, and invasion through the regulation of related genes [9]. The miR-34a-5p-MPV17L2 signaling established in the current study is not only an indication of miR-34 action in the control of mitochondrial functions in cancer cells but also an addition to previously disclosed pathways behind tumor-suppressive actions of miR-34a, supporting the development of miR-34a therapeutics.

Complex I, the largest enzyme complex of the mitochondrial respiratory chain, functions as one of the entry points for electrons into the OXPHOS system [52,60]. Mammalian mitochondrial Complex I is composed of at least 45 subunits, among which seven subunits including ND1 are encoded by mitochondrial genome [61,62]. Given the role of MPV17L2 in mitochondrial protein synthesis [45], the suppression of MPV17L2

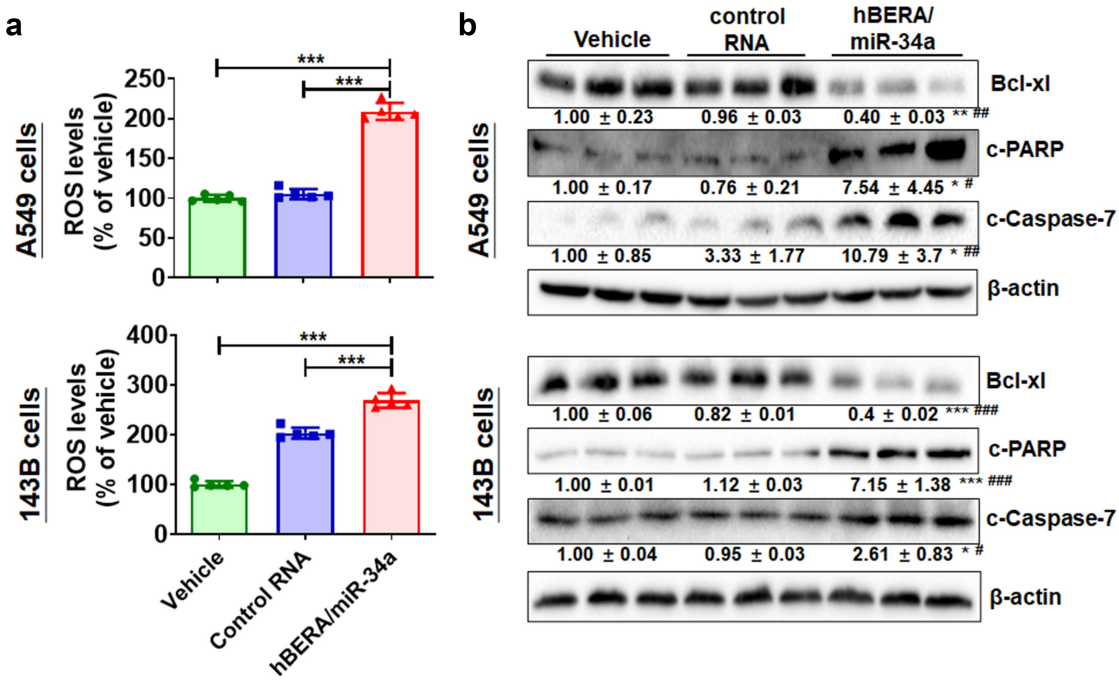


**Figure 5.** Bioengineered miR-34a-5p increases the intracellular ROS levels in human cancer cells and subsequently induces apoptosis. (a) ROS levels were increased approximately 100% and 30% by miR-34a-5p in A549 and 143B cells, respectively, as compared to respective control RNA treatments. ROS levels were measured at 48 h post-transfection. $^{***}P < 0.001$, compared with control groups, N = 5/group. (b) Antiapoptotic biomarker (Bcl-xl) levels were significantly reduced in miR-34a-5p-treated cells, whereas apoptosis biomarkers (c-PARP and c-Caspase-7) were elevated. Western blot analyses were carried out with selective antibodies, and β-actin was used as a loading control. Protein levels were normalized to corresponding β-actin levels and then vehicle groups for comparison. Values are mean ± SD (N = 3/group). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$, compared to control RNA and vehicle, respectively (one-way ANOVA with Bonferroni *post hoc* tests).

protein outcomes by miR-34a was shown to cause a lower level of ND1 protein expression as well as Complex I activity in the present study. Because Complex I provides the motive force for Complex V-mediated ATP synthesis through pumping four protons per NADH oxidation into the inter membrane space [62], the impaired Complex I activity is consistent with the reduced ATP synthesis and mitochondrial respiration, as demonstrated in the present study. Therefore, MPV17L2 deficiency caused by bioengineered miR-34a-5p is effective to alter the production of essential proteins involved in the assembly of OXPHOS complexes, and the inhibition of Complex I activity is at least one of the possible mechanisms by which miR-34a controls mitochondrial respiration. Nevertheless, the dependence of miR-34a-5p-caused changes in mitochondrial functions on MPV17L2 warrants further evaluation.

While mitochondrion is the major source of ROS, excessive ROS is also associated with mitochondrial dysfunction that might be accompanied by the damage of the ROS removal system [63,64]. Furthermore, disruption of mitochondrial function including ROS-induced oxidative stress may play a crucial role in apoptotic cell death [65]. Antiapoptotic Bcl family member proteins (e.g., Bcl-2, Bcl-xL, Bcl-w, and Mcl-1) are known to block the release of cytochrome c from mitochondria to cytosol [56,66]. Release of cytochrome c triggers the activation of Caspases (e.g. Caspase-3 and Caspase-7) to initiate apoptotic signaling pathways [65] and subsequently, leads to the cleavage/activation of PARP at the intermediate stage of apoptosis [67]. Results obtained from this study illustrate the actions of miR-34a in the control of ROS balance and oxidative stress that are associated with enhanced apoptosis. The latter was manifested by the lower levels of Bcl-xl and higher levels of activated/cleaved Caspase-3 and Caspase-7 as well as cleaved PARP, similar to recent findings on the effects of miR-193b-3p on oxidative stress and mitochondrial damage [68] as well as miR-506 on mitochondrial apoptosis [69].

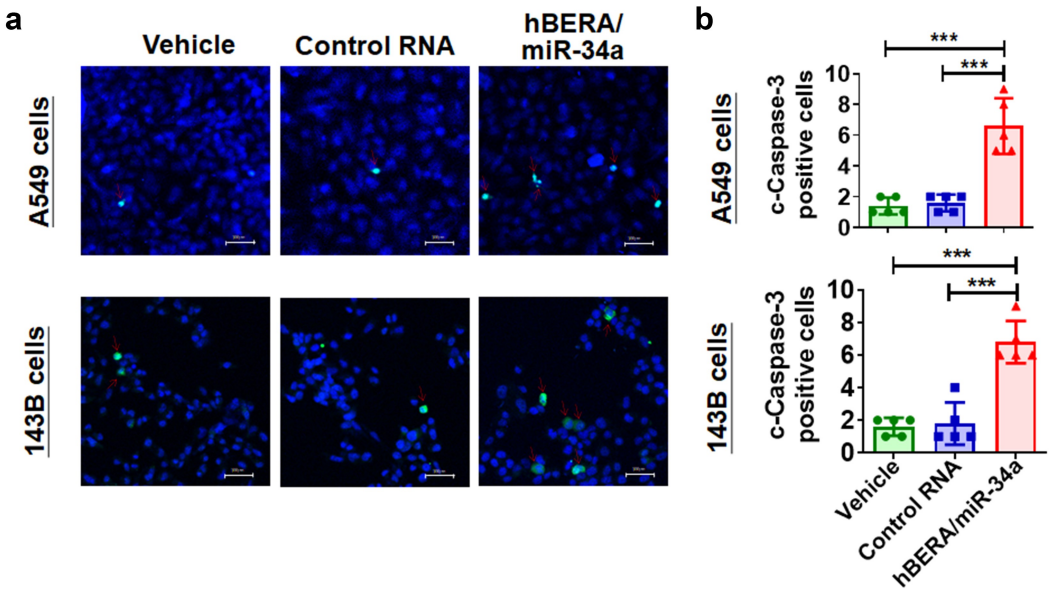


**Figure 6.** Biologic miR-34a promotes the activation of Caspase-3 in human carcinoma cells. (a) Representative images of c-Caspase-3 foci (green) and DAPI-stained nucleus (blue) in A549 and 143B cells with different treatments. (b) Numbers of c-Caspase-3 positive cells were increased by approximately 3-fold in both A549 and 143B cells following miR-34a-5p treatment, as compared with control RNA or vehicle treatments. Immunofluorescence analyses of c-Caspase-3 were conducted with selective antibody. Images were acquired with a Zeiss confocal microscope. Values are mean ± SD (N = 5/group). ***$P < 0.001$ (one-way ANOVA with Bonferroni *post hoc* tests). Scale bar, 100 µm.

In addition, miR-34a-5p has been shown to directly induce apoptosis through the regulation of some apoptosis genes, such as BCL-2 [16–18] and SIRT1 [13]. Rather, our findings are consistent with previous findings on the promotion of apoptosis by miR-34a-5p through the enhancement of ROS production in cancer cells [70], which further highlight the role of miR-34a in the regulation of mitochondrial functions.

miRNA functional studies and translational research have been dominated by using chemo-engineered mimics or analogs synthesized *in vitro* that are literally different from natural miRNA molecules produced and folded in living cells *in vivo* [7,31,32,71,72]. The new hBERA/miR-34a molecule utilized in the current study was made through *in vivo* fermentation production, which is a novel RNA bioengineering platform technology permitting large-scale, high-yield, and cost-effective manufacture of miRNA agents [28]. These bioengineered miRNA agents are unique and an addition to traditional tools for basic and experimental therapeutic studies, as exemplified in the present and recent studies [28,42–44]. As a broad-spectrum tumor suppressor and showing direct actions on cancer energetics, miR-34a could also be combined with other anticancer drugs, such as metabolic modulators, to improve therapeutic outcomes. The biologic miR-34a produced *in vivo* on a large scale is an unparalleled molecular entity for the exploration of potential experimental therapeutics for the treatment of lethal cancer diseases.

## Conclusions

The present study has established an important role for miR-34a in the regulation of mitochondrial functions by using a new, fully humanized, bioengineered miR-34a agent produced through *in vivo* fermentation production. Mitochondrial protein MPV17L2 is validated as a direct target for miR-34a, and the downregulation of MPV17L2 protein outcomes by miR-34a leads to the disruption of Complex I activity, mitochondria respiration, ATP production, and ROS balance, which are linked to an enhanced apoptotic cell death. Our finding on the involvement of the miR-34a-MPV17L2 pathway in the control of mitochondrial functions using the unique bioengineered miR-34a molecule shall not only improve the mechanistic understanding of miR-34a functions in cancer metabolism but also

offer clue to bioengineering of new miRNA entities for anticancer therapies.

## Abbreviations

BCL2, B-cell lymphoma-2; Bcl-xl, B-cell lymphoma-extra large; BERA, bioengineered miRNA agent; CD44, cell surface glycoprotein 44; CDK6, cyclin-dependent kinase 6; c-Met, hepatocyte growth factor receptor or tyrosine-protein kinase Met; c-Caspase-3, cleaved-Caspase-3; c-Caspase-7, cleaved-Caspase-7; c-PARP, cleaved poly-ADP-ribose polymerase; FCCP, carbonyl cyanide-4(trifluoromethoxy) phenylhydrazone; FPLC, fast protein liquid chromatography; hBERA, humanized bioengineered miRNA agent; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; miR or miRNA, microRNA; MPV17L2, mitochondrial inner membrane protein MPV17-like 2 protein; MRE, miRNA response element; MT-ND1, mitochondrial NADH dehydrogenase 1; ncRNA, noncoding RNAs; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PAGE, polyacrylamide gel electrophoresis; pre-miRNA, precursor microRNA; ROS, reactive oxygen species; RT-qPCR, reverse transcription quantitative real-time polymerase chain reaction; SIRT1, silent information regulator 1; 3'UTR, 3'-untranslated region.

## Author contributions

W-R Yi and M-J Tu conducted the experiments, acquired data, analyzed data, and wrote the manuscript, and they contributed equally to this work. J Lin conceived the idea, analyzed data, and edited the manuscript. A-X Yu and A-M Yu conceived the idea, designed the studies, edited the manuscript, and provided research funding.

## Disclosure statement

No potential conflict of interest was reported by the author(s).

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