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MicroRNA-34 induces cardiomyocyte apoptosis and accounts for the anti-apoptotic effect of Tanshinone IIA in myocardial infarction

par
GUORONG CHEN

Département de médecine
Faculté de médecine

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Présenté par :
GUORONG CHEN

évalué par un jury composé des personnes suivantes :

Bruce G. Allen, président-rapporteur
Zhiguo Wang, directeur de recherche
Edwin Wang, membre du jury

Résumé

MicroARN (miARN) ont récemment émergé comme un acteur central du gène réseau de régulation impliqués dans la prise du destin cellulaire. L'apoptose, un actif processus, par lequel des cellules déclenchent leur auto-destruction en réponse à un signal, peut être contrôlé par les miARN. Il a également été impliqué dans une variété de maladies humaines, comme les maladies du cœur, et a été pensé comme une cible pour le traitement de la maladie. Tanshinone IIA (TIIA), un monomère de phenanthrenequinones utilisé pour traiter maladies cardiovasculaires, est connu pour exercer des effets cardioprotecteurs de l'infarctus du myocarde en ciblant l'apoptose par le renforcement de Bcl-2 expression. Pour explorer les liens potentiels entre le miARN et l'action anti-apoptotique de TIIA, nous étudié l'implication possible des miARN. Nous avons constaté que l'expression de tous les trois membres de la famille *miR-34*, *miR-34a*, *miR-34b* et *miR-34c* ont été fortement régulée à la hausse après l'exposition soit à la doxorubicine, un agent endommageant l'ADN ou de pro-oxydant H_2O_2 pendant 24 heures. Cette régulation à la hausse causé significativement la mort cellulaire par apoptose, comme déterminé par fragmentation de l'ADN, et les effets ont été renversés par les ARNs antisens de ces miARN. Le prétraitement des cellules avec TIIA avant l'incubation avec la doxorubicine ou H_2O_2 a empêché surexpression de *miR-34* et a réduit des apoptose. Nous avons ensuite établi BCL2L2, API5 et TCL1, en plus de BCL2, comme les gènes nouveaux cibles pour *miR-34*. Nous avons également élucidé que la répression des ces gènes par *MiR-34* explique l'effet proapoptotique dans les cardiomyocytes. Ce que la régulation positive de ces gènes par TIIA realisée par la répression de l'expression de *miR-34* est probable le mécanisme moléculaire de son effet bénéfique contre ischémique lésions cardiaques.

Mots-clés : microARN; *miR-34*; tanshinone IIA; Apoptose; Bcl-2; Bcl-w; Api.

Abstract

MiRNAs (miRNAs) have recently emerged as a central player of gene regulatory network involved in decision of cell fate. Apoptosis, an active process that leads to cell death, has been shown to be controlled by miRNAs. It has also been implicated in a variety of human disease, such as heart disease, and established as a target process for disease therapy. Tanshinone IIA (TIIA), a monomer of phenanthrenequinones used to treat cardiovascular diseases, is known to exert cardioprotective effects in myocardial infarction by targeting apoptosis through enhancing Bcl-2 expression. To explore the potential link between miRNAs and the anti-apoptotic action of TIIA, we studied the possible involvement of miRNAs. We found that expression of all three members of the *miR-34* family, *miR-34a*, *miR-34b* and *miR-34c* that have been known to mediate the apoptotic effect of p53 in cancer cells, were robustly upregulated after exposure to either the DNA-damaging agent doxorubicin or pro-oxidant H_2O_2 for 24 hr in cultured neonatal rat ventricular myocytes. This upregulation caused significant apoptotic cell death, as determined by DNA fragmentation, and the effects were reversed by the antisense to these miRNAs. Pretreatment of cells with TIIA prior to incubation with doxorubicin or H_2O_2 prevented upregulation of *miR-34* and reduced apoptosis. We then established BCL2L2, API5 and TCL1, in addition to BCL2, as the novel target genes for *miR-34*. We further unraveled that repression of these genes by *miR-34* accounts for its proapoptotic effect in cardiomyocytes whereas upregulation of these genes by TIIA through downregulating *miR-34* is likely the molecular mechanism for its beneficial effect against ischemic myocardial injuries.

Keywords : miRNA; *miR-34*; tanshinone IIA; apoptosis; Bcl-2; Bcl-w; Api.

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LIST OF ABBREVIATIONS

ACE: angiotensin I converting enzyme

Akt: v-akt murine thymoma viral oncogene homolog

AMO: anti-miRNA oligonucleotide

Ang: angiotensin

ANOVA: analysis of variance

AP-1: activator protein 1

Apaf-1: apoptotic protease activating factor-1

Api5/Aac11: Apoptosis inhibitor-5/antiapoptosis clone-11

API5: Protein Apoptosis inhibitor 5

ARC: Apoptosis Repressor with Caspase Recruitment Domain

Bak: Bcl-2 homologous antagonist/killer

Bax: Bcl-2-associated X protein

Bcl-2: B-cell lymphoma/leukemia-2

Bcl-w: protein product of gene BCL2L2

BH3-only protein: BH stands for BCL2 homology domains.

Bid: BH3 interacting domain death agonist

CA: caffeic acid

c-fos: FBJ osteosarcoma oncogene

CT: cryptotanshinone

CTAR1: C-terminus activation region 1

CTAR2: C-terminus activation region 2

DED: death effector domains

DGCR8: DiGeorge syndrome critical region gene 8

DISC: death-inducing signaling complex

DLA: 3,4-dihydroxyphenyl lactic acid

DMEM: Dulbecco's Modified Eagle Medium

Dnd1: Dead End 1

EAT : MCL1

ECG: electrocardiogram

eIF4E: eukaryotic translation initiation factor 4E

eIF6: eukaryotic translation initiation factor 6

ELISA : Enzyme-Linked Immunosorbent Assay

ERK: extracellular-signal-regulated kinases

FADD: Fas-associated via death domain

FDA: U S Food and Drug Administration

FGF2: fibroblast growth factor 2

FLICE: FADDLike IL-1β-converting enzyme

FLIP: FLICE-inhibitory protein

GJA1: gap junction protein, alpha 1

HCC: hepatocellular carcinoma

HDL: High-density lipoprotein

HIF-1alpha: hypoxia-inducible factor 1alpha

Hsp20: Heat shock protein 20

HuR: Human antigen R

HUVEC: Human Umbilical Vein Endothelial Cell

ICAM: Inter-Cellular Adhesion Molecule

IL: interleukin

InsP3R: inositol trisphophate receptor

ISDN: Isosorbide Dinitrate

ITI: isotanshinone I

KCNJ2: Kir2.1, potassium inwardly-rectifying channel, subfamily J, member 2

LA: lithospermic acid

LDL: low density lipoproteins

LMP1: latent membrane protein 1

LNA: locked nucleic acid

MAP: Mitogen-activated protein

mcl-1: myeloid cell factor-1

MDA: malondialdehyde

MI: myocardial infarction

miRNA: microRNA

MMP-1: matrix metalloproteinase-1

MTCP1 : mature T-cell proliferation 1

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-κB: Nuclear factor-kappa B

NO: Nitric oxide

NRVC: neonatal rats ventricular myocyte

ODN: oligodeoxynucleotides

Omi/HtrA2: Omi/High temperature requirement protein A2

PA: protocatechuic acid

PAl: protocatechuic aldehyde

PDCD4: Programmed cell death 4

PDGF: platelet-derived growth factor

PIO : Pioglitazone

PKA: Protein Kinase A

PPAR: peroxisome proliferator-activated receptor

PTEN: phosphatase and tensin homolog

RA: rosmarinic acid

RISC: the RNA-Induced Silencing Complex

SalA: salvianolic acid A

SalB: salvianolic acid B

siRNAs: short interfering RNAs

Smac/DIABLO: second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI

SOD: serum superoxide dismutase

Sprt1: Sprouty homologue 1

TC: tanshindiol C

TCL1: T-cell leukemia/lymphoma protein 1

TCL1b: T-cell leukemia/lymphoma protein 1b

TGF: Transforming growth factor

TI: tanshinone I

TIIA: tanshinone IIA

TIIB: tanshinone IIB

TNF: tumor necrosis factor

TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

TRBP: TARRNA-binding protein

VCAM: vascular cell adhesion molecule

VEGF: vascular endothelial growth factor

XIAP: x-linked inhibitor of apoptosis

XPO: eXPOrtin (nuclear export receptor)

15,16-DTsI: 15,16-dihydrotanshinone I

2'-MOE: 2'-O-methoxyethyl

2'-OMe: 2'-O-methyl

3'UTR: 3'-untranslated region

5'UTR: 5'-untranslated region

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DEDICATION

This thesis is dedicated to:

My mother, my father, my sisters, my brother-in-law, and my cousins, for their lifetime of love, patience, understanding, encouragement, and support. The strength from them turned this work into reality.

1. INTRODUCTION

1.1 Cell death

Cell death was commonly regarded as an unregulated process until a challenging point of view brought by Horvitz HR's group, saying that at least a subset of cell death is controlled by the cell itself. Such mind exploding idea has brought intense studies on the underlying molecular and biochemical mechanisms of a process called apoptosis--the programmed cell death. The parameters defining cell death are controversy. From observation perspective, cell dying presents characteristics including disruption of membrane integrity, fragmentation of cell and phagocytosis by neighboring cells. But not all of these characteristics will be present at the same time. One thing about cell death that is not controversial is its irreversibility. Active or passive, the ways of cells' dying can be divided into apoptosis and necrosis.

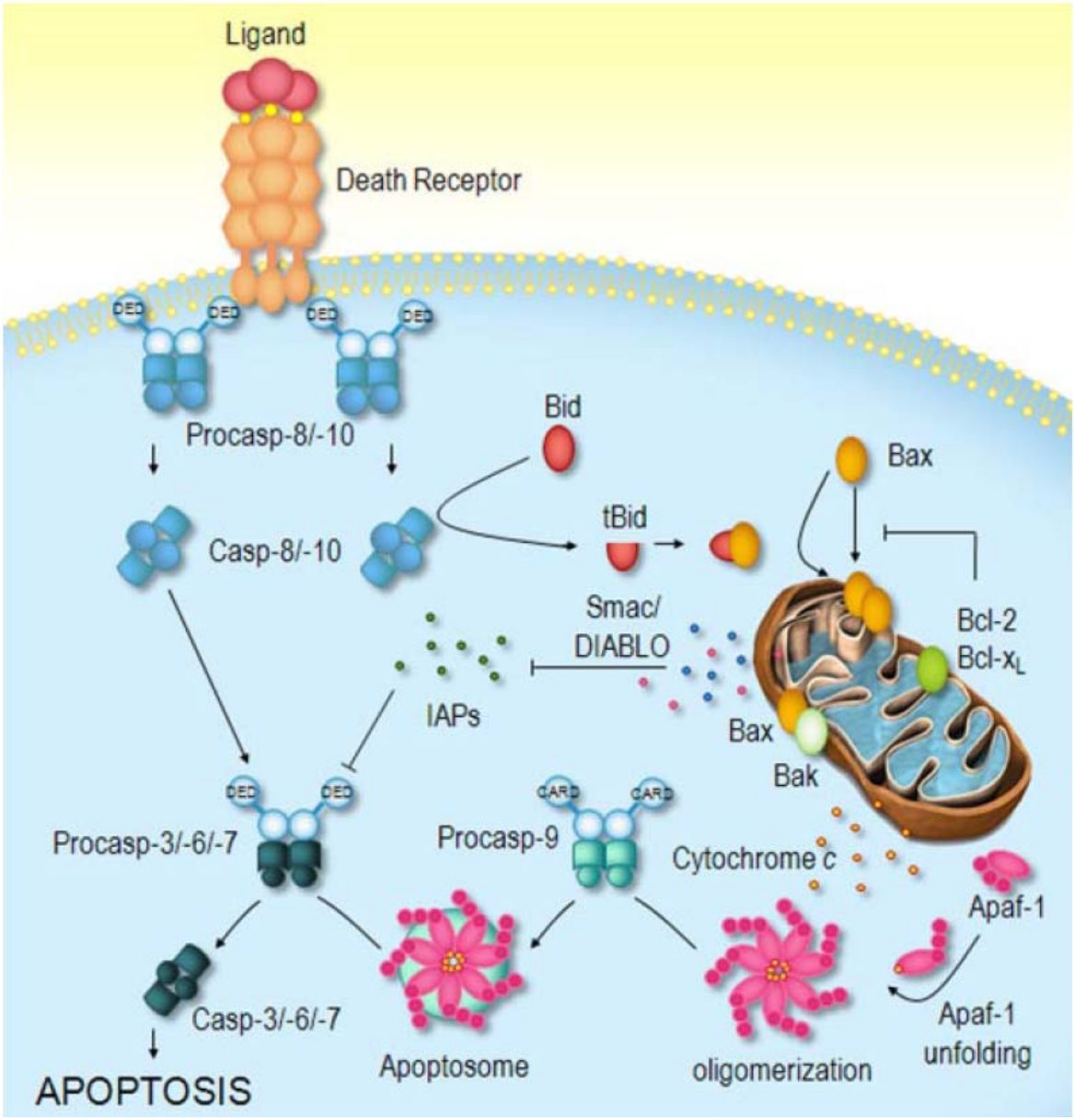


Figure 1. Apoptosis Pathways (173).

1.1.1 Apoptotic pathways

Two major pathways are involved in apoptosis: extrinsic and intrinsic pathways. The extrinsic pathway requires the binding of extracellular stimulating factors such as tumor necrosis factor (TNF) to its corresponding receptors. On the contrary, the intrinsic pathway responds to the changes of cell status including inadequate nutrition or energy, hypoxia, DNA damage and oxidative stress. Thus, the intrinsic pathway accounts for most of the

apoptotic events. A model of the extrinsic pathway is the Fas-Fas ligand model. In this model, upon binding of Fas ligand, Fas undergoes conformational changes and associates with the Fas-associated death domain (FADD) with its intracellular death domain. Afterwards, FADD utilizes its death effector domains (DED) to recruit caspases to initiate the final steps in apoptosis. Unlike the extrinsic pathway, the intrinsic pathway requires participation of mitochondria and endoplasmic reticulum (ER) by translocating two types of protein Bax (Bcl-2-associated X protein) and BH3-only proteins into them (16). Constitutively inactive, Bax is activated via conformational changes upon apoptotic signals in the cell and translocated into mitochondrial and ER (17, 18). Precise details on Bax activation are unknown but many Bax proteins regulating Bax conformation state have been reported. The translocation of Bax and BH-3-only proteins changes mitochondrial outer membrane permeability, allowing release of apoptogens. Other than Bax, mitochondria outer membrane permeability relies on another protein on the membrane itself called Bcl-2 homologous antagonist/killer (Bak) (19). How Bax and Bak permeabilize the mitochondrial outer membrane is not known. But what is released from permeabilized mitochondria is well known as apoptogens. One well-recognized apoptogen is cytochrome *c*. Cytochrome *c* functions as a component of the electron transport chain in oxidative phosphorylation in mitochondria. Once released into the cytosol, cytochrome *c* binds to apoptotic protease activating factor-1 (Apaf-1), brings about polymerization of paf-1 and further recruits procaspase-9 to form apoptosomes. The apoptosome activates downstream caspase to execute apoptosis.

1.1.2 Anti-apoptotic molecules

There are endogenous molecules counteracting apoptosis. FLICE (FADDLike IL-1β-converting enzyme)-inhibitory protein (c-FLIP) prohibits the extrinsic pathways with its two isoforms. One consists of two DED and the other is homologous to procaspase-8 with defects in enzymatic activity. The former binds to the DEDs on FADD and procaspase-8 and prevents formation of death-inducing signaling complex (DISC) (20). With regard to the intrinsic pathway, antiapoptotic Bcl-2 proteins play an important role by inhibiting release of apoptogens. Bcl-2 protein was also reported to interact with inositol trisphophate receptor (InsP3R) Ca^{2+} release channels on the ER. This interaction significantly enhances

the Ca^{2+} release rate through InsP3R and lowered ER Ca^{2+} content. The anti-apoptotic effect of Bcl-2 thereby is partially ascribed to maintaining Ca^{2+} homeostasis. Anti-apoptotic protein mcl-1 mediated apoptosis protection also via associating with InsP3R. Some other proteins such as x-linked inhibitor of apoptosis (XIAP) have multiple actions against apoptosis. IAP can bind to activated caspase-3 and caspase-7 to separate them from their substrates (21-23) and to procaspase-9 for prevention of activation (24). It can also use its E3 ubiquitin ligase activity to tag downstream caspases (25, 26). But IAP function can be undermined by its inhibitors Smac/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) (27, 28) and Omi/HtrA2 (Omi/High temperature requirement protein A2) (29, 30). Xq28 region on the chromosome encodes a family of protooncogenes (anti-apoptotic) including TCL1, MTCP1 and TCL1b. In Laine *et al*'s report, TCL1 increased cell proliferation by augmenting the activity of Akt both *in vivo* and *in vitro* and stabilizing the mitochondrial transmembrane potential (114). TCL1 associates with Akt kinase to aid transphosphorylation and is an anti-apoptotic regulator acting as Akt kinase coactivator. Protein apoptosis inhibitor 5 (API5) has activity against apoptosis. Api5/antiapoptosis clone-11 (Api5/Aac11) was upregulated in tumors. Genetic depletion of Api5 is lethal to cancer cells and API5 inhibition counteracts tumorigenesis (115).

1.1.3 Apoptosis regulators

Many endogenous factors do not directly lead to apoptosis but they play a role in apoptosis by regulating the activity or abundance of apoptotic factors. One typical example is miRNA. More detailed discussion about miRNA is presented the section <1.3 MiRNAs>. One study reported that in chronic lymphocytic leukemia downregulated miRNAs *miR-29* and *miR-181* are expressed with inverse relation with the oncogene TCL1 and in fact TCL1 is the target of both *miR-29* and *miR-181* (116). LMP1 is the crucial protein expressed by Epstein-Barr virus (EBV), responsible for transforming the normal cell into cancer cells. One of the growth inhibitory mechanisms of LMP1 was proposed to be LMP1-mediated upregulation of *miR-29b* and subsequent miRNA-mediated downregulation of TCL1. Data presented for this mechanism include upregulation of *miR-29b* in miRNA profilings of LMP1 transfectants and mutation of CTAR1 and CTAR2 domain of LMP1 abolishing both

miR-29b upregulation and TCL1 downregulation (120). The apoptotic role of *miR-29* through repressively targeting anti-apoptotic protein Bcl-2 and Mcl-1 was further illustrated in hepatocellular carcinoma (HCC). Reinforcing expression of *miR-29* promoted apoptosis through the disruption of mitochondrial potential and the release of cytochrome *c* to the cytoplasm (117). *MiR-29* apoptotic function via Mcl-1 repression was also examined in other cell types including human immortalized non-malignant H69 cholangiocyte and malignant KMCH cholangiocarcinoma cell lines. *MiR-29* also sensitized the KMCH cell line to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) cytotoxicity (119).

1.1.4 Specific apoptotic pathways in myocardial infarction

Both extrinsic and intrinsic pathways play pivotal roles in cardiomyocyte apoptosis during myocardial infarction. Infarct size in mice without functional Fas is significantly reduced after I/R compared to control (31). However, the TNF pathway, expected to have the same effect as the Fas pathway, seems to limit infarct size because loss of both TNF receptor 1 and 2 leads to infarct size augmentation (32). In terms of intrinsic pathways, cardiac overexpression of bcl-2 reduces infarction, cardiomyocyte apoptosis and cardiac dysfunction (33, 34). Depletion of a p53-responsive BH-3 protein in isolated heart attenuates cardiac infarction (35). Calpain was reported to have increased activity during ischemia/reperfusion injury and over-activated calpain elevated cleaved fragments of BH3 interacting domain death agonist (bid), which can lead to release of cytochrome *c* from mitochondria (36). Transgenic mice with overexpression of IAP-2 showed less infarction compared to control (37). Moreover, cIAP-1 has been shown to protect cells from TNFalpha induced cell death via influencing NF-kappaB activation (38). Omi/HtrA2, an inhibitor of IAP-1, was demonstrated to translocate from mitochondria to cytosol during myocardial infarction, promote apoptosis by cleaving IAP-1 and remove the inhibition of downstream caspase activities (39). Similarly, antagonizing Omi/HtrA2 with pharmacological compound UCF-101 reduces infarct size, recovers blood pressure and restores contractile function in rat model with I/R injury (40). There are several reports of infarction attenuation by targeting caspases. But inhibiting some of the caspases failed to stop cell death in some experimental settings and this is probably because mitochondrial damage was irreversible. Another effective inhibitor of apoptosis, ARC (Apoptosis

Repressor with Caspase Recruitment Domain) was studied since it differs from common apoptosis inhibitor targeting intrinsic or extrinsic pathways. ARC is abundantly expressed in cardiac muscle cells (41). It restricts apoptosis via blocking DISC assembly, Bax activation/mitochondria translocation (42), translocation of p53 into cytoplasm (43) and cytochrome *c* release (44). Transgenic overexpression of ARC renders protection to transgenic mice suffering ischemia/hypoxia damages (45). Akt is critical in apoptosis since phosphorylation of apoptotic molecules such as Bax and Bad inactivates them. Therefore, overexpressing ARC *in vivo* significantly improves cell survival ability and ameliorates cardiac function after I/R injury (46, 47). The anti-apoptotic protein EAT/mcl-1, a bcl-2 related early gene, was found expressed more abundantly in the non-ischemic region in the early stage of rat myocardial infarction (174).

1.1.5 Cell death therapy for myocardial infarction

Inhibiting cell death as therapy for myocardial infarction includes targeting apoptosis and necrosis. Although therapeutic suppression of cell death may increase the risk of cancer, it is still worthy of practising considering the high rate of mortality of patients with infarcted hearts. A number of compounds were shown to have beneficial effects on myocardial infarction via attenuation of apoptosis. UCF-101 is a small molecule that possesses cardiac function improving properties by inhibiting apoptosis. Another compound vanadium elicits cardioprotection via upregulation of Akt expression (48-50).

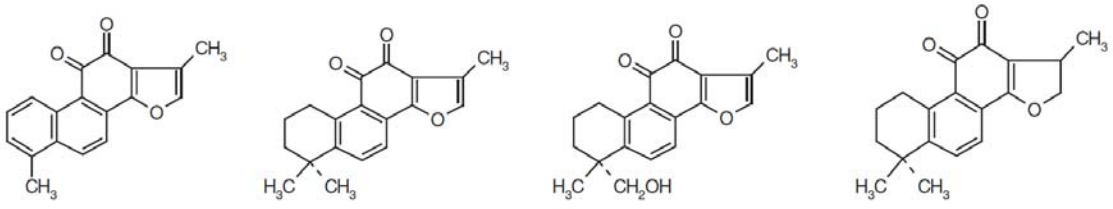
1.2 Role of Danshen in alleviating ischemic injuries

Salvia miltiorrhiza is an annual sage plant that is mainly distributed in China, Mongolia, Korea and Japan. Growing in the west, southwest and southeast of China, it is used as a medicinal herb. Its dried root is called Danshen in Chinese. Since the first case of using Dansen in treating stroke in 1970 (1), Danshen is used to treat angina and heart attack (2). Danshen is one of the components of a popular herbal medicine fufang danshen tablet (Fufang means a composition of multiple herbs). Salvia miltiorrhiza's relative, Salvia columbariae is seeded by Californian Indians in California, USA. It contains tanshinones and can be used to treat the strokes as well.

Commercial markets of Danshen are located in China, Vietnam, Russia, Cuba, the Korean Republic, and Saudi Arabia while China consumes the most. Danshen is packed into many different dosage forms in China, such as tablets, dripping pills, liquid drinks, capsules and injections. Among these, fufang danshen dripping pills and danshen tablets are most popular and they are the first Chinese traditional medicine that pass FDA phase II and phase III clinical trials (15).

1.2.1 Components of Danshen

According to the properties of solvent, components separated from Salvia miltiorrhiza can be categorized into two parts: water soluble and lipophilic diterpenoid quinines. Identified compounds are listed below. Aqueous compounds include protocatechuic aldehyde (PAl), protocatechuic acid (PA), caffeic acid (CA), and 3,4-dihydroxyphenyl lactic acid (DLA, or danshensu in Chinese pinyin), rosmarinic acid (RA), lithospermic acid (LA), salvianolic acid A (SalA), salvianolic acid B (SalB), and other salvianolic acids. Lipophilic diterpenoid quinines soluble compounds are composed of tanshinone I (TI), tanshinone IIA (TIIA), tanshinone IIB (TIIB), cryptotanshinone (CT), tanshindiol C (TC), 15,16-dihydrotanshinone I (15,16-DTI), isotanshinone I (ITI), isotanshinone II and other tanshinones. Danshensu and Tanshinone IIA, two major components of Danshen, can be quickly uptaken by the gastro-intestinal system whereas the remaining major component, Salvianolic acid B, has poor absorption via oral administration (146).



Tanshinone I Tanshinone IIA Tanshinone IIB Cryptotanshinone

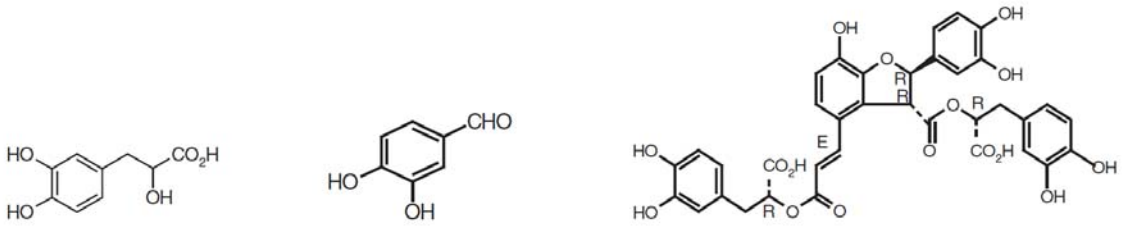


Figure 2. Chemical Structure of both hydrophilic and lipophilic components from Danshen (172).

1.2.2 Effects of Danshen on microcirculation

Amelioration of microcirculation by Danshen has been reported in numerous studies. Most of the pharmacological studies on the components of Dansen are focused on danshensu, salvianolic acid B, and tanshinone IIA (102, 103, 104). Danshen decreases platelet aggregration by affecting the expression of adhesion molecules on the surface of platelets (8). 15,16-dihydrotanshinone I (15,16-DTI) inhibits mast cell degranulation, which is detrimental to vascular endothelial function (9,10). Danshensu was shown to dilate coronary arteries, protect the heart from ischemia/reperfusion injury, suppress platelet aggregation (131) and improve microcirculation in animals (132). These effects are achieved via preventing incidence of calcium overload (130), eliminating free radicals in vessel (133, 134) and protecting endothelial cells from homocysteinemia (135). It can induce production of NO in vessels (137), abrogate angiotensin II-mediated hyperplasmia, prevent LDL from oxidation and consequent uptake by microphages (138, 139). It can also inhibit DNA synthesis in noncardiocytes and relieve I/R injury by inhibiting the stress-activated protein kinase (140). Zhang's group examined the ACEI inhibitor in Salvia miltiorrhiza and found that salvianolic acids in the aqueous extract inhibited ACEI activity (4). Extract of Danshen root helps to lower cell adhesion to the vascular wall. Salvianolic acid B (Sal B), can protect the vascular endothelial cells from attack of oxidative species and suppress endothelial expression of ICAM (Inter-Cellular Adhesion Molecule) and VCAM (vascular cell adhesion molecule), which are atherosclerotic risk factors (6). Protocatechuic aldehyde (PAl) prevents U937 human monocytic cells from adhering to

HUVECs (Human Umbilical Vein Endothelial Cells) (6). Similarly, Sal B prevents adhesion of U937 to TNF treated human aortic endothelial cells (7). Inflammatory factors such as IL-1β and TNF-α are inhibited by TIIA in RAW264.7 cells (11). Tanshinone IIA significantly reduces infarct size in *in vivo* studies via scavenging the free radical in the mitochondrial membranes (141). It is also able to keep LDL from oxidation (142), inhibit angiotensin system and abrogate incidence of hypertrophy (143).

1.2.3 Effects of Danshen on the other systems

In addition to its effects on the cardiovascular system, Danshen also has beneficial effects on other organs such as brain, lung, kidney and liver. It was reported by Lam's group that the tanshinone IIA and IIb can penetrate the brain barrier and reduce the size of an infarct (12). Salvia miltiorrhiza root extract protects the lung by prohibiting the production of oxidant, accumulation of peroxides and production of inflammatory factors (13). Livers from the rats pretreated with Salvia miltiorrhiza root extract showed decreased bile duct ligation, reduced scission-induced rat hepatic injury and significantly decreased apoptosis due to decrease in Bax protein level and augmentation of Bcl-2 protein (14). Salvianolic acid B has cerebroprotection effect (136).

1.2.4 Tanshinone IIA—the most studied active component of Danshen

The major active component of Danshen is the tanshinone IIA (TIIA). Tanshinone IIA has cardioprotective effects, anticancer effects and neuroprotective effects. It has been shown that TIIA enhances hypoxia-inducible factor 1alpha (HIF-1alpha) mRNA abundance, promotes upregulation of vascular endothelial growth factor (VEGF) expression and increases the survival rate of MI rats (108). TIIA protects from cardiac injury induced by antineoplastic agent doxorubicin, which causes a toxic effect in the heart by inducing production of reactive oxygen species. TIIA increases H9C2 cell line viability and ameliorates electrocardiogram parameters such as ST interval and QRS interval in an animal model of chronic cardiomyopathy created by doxorubicin (109). Yang *et al* (110) tested the antioxidant property of TIIA sodium sulfonate on prevention of cardiac fibrosis. The study demonstrated that Ang II induced enhancement of collagen type I expression, collagen synthesis and reduction of matrix metalloproteinase-1 (MMP-1) expression and

activity. These changes were blocked by TIIA sodium sulfonate and the interruption was achieved via suppressing intracellular generation of reactive oxygen species, NADPH oxidase and subunit p47 expression (110). Analogous to the Ang II receptor antagonist Valsartan, TIIA sodium sulfonate abrogated cardiac hypertrophy induced by Ang II of neonatal rat cadiomyocytes and enhanced protein synthesis rate and proto-oncogene c-fos mRNA level (111). In another study, adriamycin significantly induced hypertrophy in primary cultured cells. TIIA sodium sulfonate was able to attenuate the hypertrophy via reduction of reactive oxygen species production. The elevated protein ratio of Bcl-2/Bax by TIIA sodium sulfonate was confirmed with western blot and apoptosis was reduced in a dosage dependent manner (112). Similarly, tanshinone IIA has an anti-hypertrophic effect on H_2O_2-induced hypertrophic myocytes and I/R rat model via elevating the ratio of Bcl-2 to Bax protein and enhancing antioxidation of lipid, likely due to increased serum superoxide dismutase (SOD) activity and a downregulation of serum malondialdehyde (MDA) level (113). Another study also used adriamycin to induce damage in cellular and animal models. But their purpose was to investigate how TIIA protects against lipid peroxidation and their data showed that adriamycin-induced mitochondrial lipid peroxidation as well as swelling was abrogated by TIIA.

1.3 miRNAs

1.3.1 General information about miRNAs

MiRNAs are 21-23nt long noncoding functional small RNAs that are ubiquitously expressed in cells. They mainly impose negative control on expression of protein by recognizing targets according to base pairing, repressing target gene translation efficiency and influencing mRNA stability. The first miRNAs lin-4 and let-7 are found in studies of Caenorhabditis elegans development in which these miRNAs can control C. elegans development steps. Since then, thousands of miRNAs have been discovered in all kinds of cells with cloning and sequencing approaches. Till today, more than 700 human miRNAs are sequenced and sequences are stored in public databases facilitating the sharing of information. Bioinformatics are extremely important tools in the miRNA research, helping idenfication of potential miRNAs, predicting miRNAs biogenesis pathways and predicting

miRNAs targets. Some informative analysis even brings about the idea that more than 60% of human protein encoding genes are under miRNA regulation (51).

1.3.2 MiRNA biogenesis

MiRNAs can be generated from their own coding sequences or from the intron of protein coding gene. Transcripts containing mature miRNAs with a stem loop structure, as long as thousands of nucleotides, termed pri-miRNAs, are primarily generated by RNA polymerase II (52) while some of them are reported to be transcribed by RNA polymerase III (53). Such transcripts are subject to cleavage from Drosha-DGCR8/Pasha complex to generate approximate 100nt hairpin structured pre-miRNAs. In this Drosha-DGCR8/Pasha complex, DGCR8/Pasha functions as RNA binding protein while Drosha cleaves the RNA. Intriguingly, some pre-miRNAs dodge Drosha-mediated cleavage and mature in unconventional pathways (54). After formation of pre-miRNAs, pre-miRNAs are transported into the cytosol by nuclear export factor XPO-5 (Exportin 5) (55). In the cytoplasm, pre-miRNAs go through the final steps in maturation: it is cleaved by ribonuclease III Dicer associated with TRBP (TARRNA-binding protein) and turned into a 22nt miRNA:miRNA* duplex (56). The duplex then fulfills its duty by incorporating into RISC (the RNA-Induced Silencing Complex) and guiding RISC (57) towards its target with the miRNA strand while the miRNA* strand is degraded (58).

1.3.3 Mechanisms of actions of miRNAs

Mechanisms of actions of miRNAs in different species are different. They silence target genes by two actions: translation repression or mRNA cleavage. Most metazoan miRNAs bind to their target in an imperfect complementarity and repress translation, leaving the mRNA intact. But in plant, miRNAs base pair with the 3'UTR almost in a perfect manner and lead to mRNA cleavage. The translation repression mechanism is complicated. In the initiation step of translation, it is essential for mRNA cap to be recognized by cap-binding protein eIF4E (eukaryotic translation initiation factor 4E). RISC may competitively bind to this critical cap and block translation initiation (59). Besides, RISC recruits eIF6, a notable ribosome assembly inhibitory protein, to prevent ribosome formation (60). Some also reported that RISC can move the mRNAs to P bodies where many mRNA catabolising

enzymes are concentrated and give mRNAs special treatment, resulting in translational repression (61). Since many exonucleases are contained in the P body, miRNA-targeted mRNAs are likely subject to degradation and quantitatively reduced. With regard to cleavage mechanism, the miRNA strand guides the Argonaute component of RISC to 'scissor' a phosphodiester bond within complementary RNA molecules. The cleavage fragments are then released, unleashing the RISC to attack another transcript.

1.3.4 Determination of miRNA targets

MiRNAs are involved in a wide range of biological functions including development, apoptosis, differentiation, metabolism, cell growth, and cell proliferation. Understanding the function of miRNAs relies on identification of their targets. But identification of their targets is proven to be difficult. Although we know that miRNAs regulate mRNAs containing their binding sites, we also know that the base pair formed by miRNA and targeted mRNA can be imperfect. To what degree the complementary should be and what crucial nucleotides in the binding sites are for miRNAs to take actual silencing actions on this target mRNA are not precisely understood. What we know is that the 2-7 nucleotides starting from the 5' of miRNA strand are pivotal to their target selection, termed seed sites. Numerous computational algorithms are developed based on different standards, such as base pairing stability, duplex formation free energy level and the degree of conservation of seed sites. However, prediction results generated by these algorithms are not consistent and due to the existence of false positive or false negative results, experimental verification of these prediction results is inevitable (62). Most current miRNA targets studied contain miRNA binding sequence in their 3'UTR. Exceptional binding sequences are reported embedding in the mRNA encoding regions and even in the 5'UTR (63, 64, 65 66).

1.3.5 Additional factors affecting miRNA actions

Not only the binding site itself, but also the sequence in the vicinity of the binding site, could be involved in influencing miRNA-mRNA interaction. Such sequence can attract corresponding functional proteins and prevent the approach of RISC or, by contrast, facilitate the binding of RISC. Kedde *et al* (168) demonstrated in human germline cells Dead End 1 (Dnd1) relieved repression of several mRNA from related miRNAs. Their

immunoprecipitation result showed that Dnd1 binds to the U-rich regions and these regions are in the vicinity of miRNA binding sites. Conversely, Kim *et al* (105) presented another protein HuR (Human antigen R, also a RNA binding protein), through interaction with putative binding sites near let-7 binding sites, recruit RISC complexes to repress gene expression of c-myc. In this report, they also used immunoprecipitation and found that c-myc mRNA is significantly higher in pull-downs with HuR protein compared with the relevant control. Knocking down HuR with siRNA led to significant increases in c-myc mRNA and protein abundance. Artificial elevation of let-7 made no change in HuR binding to c-myc mRNA and overexpression of HuR did not affect the abundance of let-7. These results suggest that let-7 and HuR do not interact with each other. Ago2 coimmunoprecipitated with HuR. Moreover, degradation of RNA disrupts such association and low abundance of let-7 weakens this interaction. Taken together, HuR does affect action of let-7 on its target mRNA (105).

1.3.6 Complexity about miRNAs

One miRNA can affect multiple mRNAs and one mRNA can be regulated by multiple miRNAs. Such properties dramatically increase the complexity of the miRNA regulation network. One can imagine that in a single pathway, many different miRNAs can act in a synergetic manner to regulate different components in the pathway. However, similarly, one miRNA can counteract the effect of another miRNA. By targeting proteins that are not abundantly expressed, miRNAs can completely switch off gene expression. Meanwhile, miRNAs targeting abundant proteins serve as a fine-tuning mechanism. Like mRNAs, miRNAs have unique expression profiles in different cell types, in different stages of cell and in different cellular status. Thus, changes in the expression pattern of miRNAs in a certain cell type may indicate a dramatic alteration in physiological conditions. On the other hand, alterations in the pattern of miRNA expression suggests that miRNAs can respond to changes in environmental condition such as hypoxia and ischemia.

1.3.7 Ischemic miRNAs

In ischemic diseases of the heart, miRNAs participate in all aspects such as arrhythmia, fibrosis, neo-angiogenesis, and cell death. *MiR-1* upregulation was reported in acute

myocardial infarction (83, 84). Connexin 43 (GJA1), potassium channel subunit Kir2.1 (KCNJ2) and the cardiac transcription factor Irx5 have been identified as direct targets of miR-1 (83). Given their important functions in maintaining normal membrane resting potential, impulse conduction and expression of ion channels, upregulation of *miR-1* during myocardial infarction could increase the incidence of arrhythmia. Results from Yang B's group (83) revealed that *miR-1* upregulation during MI is stimulated by the beta-adrenoceptor-cAMP-protein kinase A (PKA) signaling pathway. Blockade of this pathway with beta blocker propranolol prevent *miR-1* upregulation, restored membrane depolarization and conduction slowing. This further confirmed *miR-1* role in arrhythmogenesis during MI. Another miRNA *miR-21* is responsible for fibrosis during MI. Using *In situ hybridization*, *miR-21* was found highly expressed in the infarct area (85). Upregulated *miR-21* represses PTEN (86) and sequentially leads to increases in matrix metalloprotease-2, which promotes fibrosis. *MiR-21* is also upregulated in fibroblast in the failing heart. *MiR-21* targets Sprouty homologue 1 (Sprt1) and thus release ERK–MAP kinase pathway from inhibition of Sprouty homologue 1. As a result, fibroblast survival increases significantly and induces fibroblast growth factor 2 (FGF2) secretion. The *miR-29* family mainly regulates the expression of collagens, fibrillins, and elastin and manipulation of *miR-29 in vivo* and *in vitro* could influence collagens' expression (86). MiRNAs correlated with angiogenesis are potential therapeutic targets for MI. Artificial overexpression of miR-92a in endothelial cells blocks angiogenesis *in vivo* and *in vitro*. Meanwhile, antagonizing *miR-92a* in an MI mouse model significantly increases vessel growth and reduces damage brought by infarction. Proangiogenic protein integrin subunit alpha5 was demonstrated as the target of *miR-91a* in angiogennesis (87).

1.3.8 MiRNAs regulating cell death in myocardial infarction

As previously stated, cell death is one of the crucial pathological aspects of MI. MiRNAs are critical regulators of cell death during MI via interacting with survival factors. MiR-1 is involved in pathogenesis after MI as a pro-apoptotic mediator (89-91). MiR-1 was found significantly higher in rat model after ischemia/reperfusion injury (89) and regulates anti-apoptotic factor Bcl-2 (90) and IGF-1 (91). Meanwhile, *miR-320* downregulated significantly after I/R injury *in vivo* and *in vitro* and it seems to be the cardiac self-

protection mechanism by downregulating *miR-320* and releasing *miR-320* suppression target Hsp20 (92). Consistent with these data, enforced expression of *miR-320* in mice worsens myocardial infarction. Interestingly, *miR-21* was downregulated in the infarct area but upregulated in the boarder zone of the infarct area. The cardioprotective effect of *miR-21* is mediated through inhibition of AP-1 and programmed cell death 4 (PDCD4) (93). As mentioned in the <apoptosis> section, *miR-21* is known to mediate apoptosis. Consistent with this, downregulation of *miR-21* in I/R was shown to be beneficial. In Ye's study (118), antisense downregulation of *miR-29* has the same protective effect in H9C2 cell line as a PPAR-gamma agonist pioglitazone (PIO) does. They also found that *miR-29* expression is significantly lower in the rat heart after administration of PIO and dramatic downregulation of *miR-29* in H9C2 cell line by PIO is abrogated with a selective PPAR-gamma inhibitor GW9662. All these data suggest that the beneficial effect of PIO to I/R is acting through changes in expression of apoptotic *miR-29*. Van Rooij *et al* (121) showed that *miR-29* is significantly reduced in acute myocardial infarction and downregulation of *miR-29* causes increased collagen expression. Thus *miR-29* is established as a potential target of fibrogenesis during myocardial infarction (121).

1.3.9 MiRNAs as potential therapeutic targets or medicine

MiRNAs are implicated in various kinds of diseases and hence they are considered as therapeutic targets. Suppressing aberrantly expressed or pathogenic miRNAs and enforced expressing beneficial miRNAs are feasible by employing previously developed RNAi technologies for siRNAs. SiRNAs and miRNAs are similar in gene silencing mechanisms. However, siRNAs are exogenous whereas miRNAs are endogenous. Endogenous miRNAs have properties superior to siRNAs in the following aspects: 1. siRNAs often trigger the interferon response. 2. siRNAs can have significant off-target effects. 3. Transfection of large amount of siRNAs can saturate endogenous small RNA processing machinery, disrupt global miRNAs expression and lead to eventual cytotoxicity (94). All of these drawbacks hamper siRNAs from becoming therapeutic agents. Conversely, miRNAs are naturally accepted by cells. Single molecule-multiple targets properties of miRNAs render the possibility of manipulation of the whole network simply via moving one single point.

However, one should be cautious with such property since undesirable effects could also be produced by miRNAs.

1.3.9.1 Enhancement of miRNAs

To restore or overexpress miRNAs inside the cell, transfection of miRNA duplex (termed mimics) directly or vector carrying precursor miRNA is applicable. In terms of miRNA mimics, because of the weak stability of RNAs, proper chemical modification of RNA to enhance half life within the cell is necessary. Chemical modifications should not hinder the incorporation of miRNAs in to RISC or the binding of miRNA to its target. LNA (locked nucleic acid) technology significantly increases miRNA intracellular half life and augments miRNAs binding affinity as well as cell uptake. Other chemical modification methods applicable include modification of the –2OH residue on the ribose with 2′-*O*-methyl (2′-OMe), 2′-*O*-methoxyethyl (2′-MOE).

1.3.9.2 Suppression of miRNAs

Antisense inhibition of miRNAs with modified anti-miRNA oligonucleotides (AMOs) has been successfully demonstrated *in vitro* and *in vivo*. Since AMOs` action is analogous to pharmacological molecules to antagonize targets, AMOs are also called antagomirs. Modification methods for AMOs are the same as those for miRNA mimics. The exact mechanism for AMOs to inhibit miRNA remains unclear. Some reports suggest that AMOs bind to the guide strand of miRNA in the RISC complex and intervene miRNA binding to targets (95, 96, 97). There also exists evidence that AMOs cause miRNA degradation whereas the mechanism needs further clarification (98).

1.3.9.3 Delivery of miRNAs into cells

Even though effective inhibition methods and overexpression methods of miRNAs are well developed, the delivery of miRNA into cells or even into specific cell types is a main barrier in front of us. One strategy is to link miRNAs with delivery lipid and proteins and they are actively absorbed by target cells (101). Using viruses packing therapeutic miRNAs, receptor binding RNA aptamers and antibody-protamine are all also applicable

(99, 100). Alternatively, instead of direct application of double stranded miRNA mimics, miRNA carrying vectors are delivered into cells and miRNAs of interest are produced constantly and stably. This approach is achieved by placing polymerase II or polymerase III promoters upstream of a short hairpin RNA coding sequence. Transcribed short RNAs (shRNAs) are processed by Dicer into miRNAs. The miRNA expression vector renders persistent suppression of the target gene and easily expresses several miRNAs at the same time. However, it was reported that overexpressing shRNAs in mouse liver results in liver injury and consequential death, concomitant with a global downregulation of miRNAs. The author suggested that the shRNAs can occupy the small RNA processing machinery inside the cell and disrupt normal production of other miRNAs. To what level the shRNAs are expressed that can avoid toxicity is unclear. Seemingly associated with the compromised miRNA expression, toxic effect truly becomes a hurdle for this expression vector to become an applicable therapeutic approach.

1.3.9.4 Avoidance of miRNAs side effects

As stated above, one miRNA-multiple target feature of miRNAs is a double edged sword. It may also bring about undesirable side effects. Therefore, understanding the whole picture of targets of an miRNA becomes extremely crucial. Even after this target map is drawn, it is necessary to develop technologies to exempt undesirable targets from regulations. Recently, after development of direct RNA sequencing technology, Chi et al (106) sequenced RNA isolated by crosslinking the RNA with the Ago protein in mouse brain. Then they analyzed the result with bioinformatic tools and generate the map of miRNA-mRNA target sites. Their data indicate that on average there are 2-3 miRNA binding sites on a single mRNA 3'UTR. The miRNA-masking antisense approach developed by Xiao *et al* (107) is an elegant way of controlling miRNA actions on particular targets. They employed antisense oligodeoxynucleotides (ODN) with locked 5' and 3' ends, covering miRNA target sites on 3`UTR of HCN2 and HCN4 and successfully restored expression of these two genes. The principle of this technology is that the ODN masks the miRNA target site and stops duplex formation between miRNA and mRNA.

1.4 Hypothesis

We proposed that the miR-34 family induces cardiomyocyte apoptosis by post-transcriptionally repressing BCL2L2, API5 and TCL1 and the TIIA can block oxidative stress-induced miR-34 upregulation, thereby increasing the cell survival. This work was aimed to test this hypothesis.

2. EXPERIMENTAL PROCEDURES

2.1 Myocyte Isolation and Primary Cell Culture

The enzymatic dispersion techniques used to isolate single ventricular myocytes from neonatal rats (NRVCs) have been previously described in detail (83). Briefly, 1-3 days old rats were decapitated and their hearts were aseptically removed. The atria were dissected, minced and trypsinized at 37°C for 10 min. Dissociated cells were plated in 24-well plates in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) containing 10% FBS and 0.1 mM bromodeoxyuridine (Sigma) to inhibit the growth of fibroblasts and the non-adherent cardiomyocytes were removed. The cells ($1x10^5$/well) were seeded in a 24-well plate for further experiments. This procedure yielded cultures with 90±5% myocytes, as assessed by microscopic observation of cell beating. The cardiomyocytes were also verified by positive staining with an anti--actin monoclonal antibody through immunocytochemistry. All procedures are in accordance with the guidelines set by the Animal Ethics Committee of the Montreal Heart Institute and of Harbin Medical University.

2.2 Cell Culture

Isolated NRVCs were stored in KB solution (in mM: glutamic acid 70, taurine 15, KCl 30, KH_2PO_4 10, HEPES 10, $MgCl_2 \cdot 6H_2O$ 0.5, glucose 10, and EGTA 0.5; pH 7.4 with KOH) at 4°C until use. Rat ventricular cell line H9c2 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM).

2.3 Induction of Apoptosis in NRVCs

NRVCs in culture were incubated with doxorubicin (0.5 μM) or H_2O_2 (200 μM) for 48 h to induce apoptotic cell death.

2.4 Treatment of Tanshinone IIA in NRVCs

NRVCs were incubated with Tanshinone IIA (TIIA; 1 μM; Leawell International Limited) for 5 h before exposure to Dox or H_2O_2 to induce apoptosis.

2.5 Synthesis of MiRNAs and Anti-miRNA Antisense Inhibitors and Their Mutant Constructs

miR-34a, *miR-34b* and *miR-34c*, and the mutant *miR-34b* construct were synthesized by Integrated DNA Technologies, Inc. (IDT) (Fig. 8A). Because the mature miRNAs sequences are highly conserved (nearly identical) in sequence among members of the *miR-34* family and across human, rat and mouse, the miRNAs synthesized were only based on human sequences. And the mutation to *miR-34* was made only to *miR-34b* to have seven nucleotide mismatches at the 5'-end, which disrupts its binding to the target sites (Fig. 8B) and thus turns the miRNA into a negative control. The sequences of anti-miRNA antisense inhibitor oligonucleotides (AMOs) are the exact antisense copies of the two strands of the mature *hsa-miR-34b* sequences (Fig. 8B). Five nucleotides at both ends were locked (the ribose ring is constrained by a methylene bridge between the 2'-*O*- and the 4'-*C* atoms) (128).

2.6 Construction of Chimeric miRNA-Target Site–Luciferase Reporter Vectors

To construct reporter vectors bearing miRNA-target sites, we synthesized (by Invitrogen) fragments containing the exact target sites for *miR-34* or the mutated target sites and inserted these fragments into the multiple cloning sites downstream the luciferase gene (HindIII and SpeI sites) in the pMIR-REPORTTM luciferase miRNA expression reporter vector (Ambion, Inc.).

2.7 Transfection and Luciferase Assay

We plated the cell lines and rabbit cardiac myocytes in primary culture with density of 0.5-2 x 10^5 cells in 500 μl of growth medium in 24 well or 12 well flat bottom plates. Before transfection, cells were starved to synchronize growth by incubating in serum- and antibiotics-free medium for 12 h. By the time of transfection, the cells had reached

approximately 90-95% confluency. Commercial transfection reagents lipofectamine 2000 and the accompanying optimal medium Opti-MEM I Reduced Serum Medium were purchased from Invitrogen. Two complexes were prepared during transfection steps. A. A mixture of DNA with 50 µl of Opti-MEM I Reduced Serum Medium without serum. B. A mixture of Lipofectamine 2000 and Opti-MEM I Medium in 50 µl for 5 minutes incubation. After 5 min of incubation, these two complexes were combined and left at room temperature for 20 min. This final mixture (100 µl) was added to the appropriate cell culture well and mixed gentlely with cell culture medium. The volume of transfection reagent as well as the transfection medium can vary to maximize transfection efficiency. After 18-48 hours incubation at 37 °C in a CO_2 incubator, cells were harvested with 100 µl passive lysis buffer from dual luciferase kit purchased from Promega. Generally, 50 µl Luciferase Assay Reagent II and 30 µl lysate were added to the detection tube. Luminescence was read with luminometer and then 50 µl Stop & GloTM Reagent was added to the luminometer tube to simultaneously quench the firefly luciferase reaction and activate the Renilla luciferase reaction. The second luminescence was read immediately. The second luminescence was used as an internal DNA loading control.

2.8 siRNA Transfection

Cells were plated at $2x10^5$ cells/well and BCL2L2 siRNA, API5 siRNA (Dharmacon, Lafayette, CO; ON-TARGETplus siRNA reagents), and control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) were each diluted to 100 nM. Transfection was performed according to Dharmacon's protocol using Lipofectamine 2000 (Invitrogen), whose details have been provided in section 2.7. Twenty-four hours after transfection, cells were treated with H_2O_2 for 48 hr.

2.9 Quantification of mRNA and miRNA Levels

The procedures for quantification of BCL2, BCL2L2 (Bcl-2-like protein 2), API5 (apoptosis inhibitor 5), and TCL1 (T-cell leukemia/lymphoma 1) transcripts by conventional Taqman real-time RT-PCR were the same as previously described (83,169).

The mirVana™ qRT-PCR miRNA Detection Kit (Ambion), a quantitative reverse transcription-PCR (qRT-PCR) kit was used in conjunction with Taqman real-time PCR for quantification of *miR-34* and *miR-133* (for control) transcripts. The total RNA samples were isolated with Ambion's mirVana miRNA Isolation Kit from cultured neonatal rat ventricular cells (NRVCs) and H9c2 cells. RNA concentrations are determinate with Nanodrop 2000. RNA quality is controlled by agarose gel electrophoresis. Fold variations in expression of *miR-34a*, *miR-34b* and *miR-34c* between RNA samples were calculated after normalization to U6.

Details with the miRNA extraction procedure are as the following:

(1) Aspirate the culture medium from the well and rinse the cell culture with PBS twice carefully. Remove the PBS solution and the culture plate is put on ice for next steps.
(2) Add 300-600 ul Lysis/Binding Solution directly to the well according to the number of the cells or roughly to the size of the well. The cells are expected to lyse upon exposure to the Lysis/Binding Solution. Cell lysates in the culture plate are collected with a rubber spatula and move to a tube.
(3) Vortex the tube to lyse the cells completely.
(4) 1/10 volume of miRNA Homogenate Additive is added to the cell lysate and mix completely and incubate on ice for 10 min.
(5) A volume of Acid-Phenol:Chloroform that is equal to the the lysate volume before addition of the miRNA Homogenate Additive is added and mix well by vortexing.
(6) 5 min centrifuge (10,000g) at room temperature. Centrifuge for a longer time to obtain a compact interphase between the aqueous and organic phases.
(7) Carefully move the aqueous phase to a new tube and mark down the volume. The organic phase is discarded. Make sure the organic phase is not disturbed.
(8) 1.25 volumes of room temperature 100% ethanol is added to the aqueous phase and mix by inverting the tube.
(9) Place the Filter Cartridge into the Collection Tubes provided.
(10) Pipette the mixture from step 8 into the Filter Cartridge.
(11) Centrifuge for 15s at 6000 rpm to allow the mixture pass through the filter.

(12) Discard the flow-through and repeat the steps from 10 until all the mixture goes through the filter.
(13) Wash with 700 ul miRNA Wash Solution 1 (prepared with 100% ethanol) by centrifuging for 10s at 6000 rpm and the flow-through is discarded.
(14) Wash with 500 ul Wash Solution 2/3 with similar methods to the previous step. Wash twice with Wash Solution 2/3.
(15) Centrifuge the Filter Cartridge without any solutions for 1 min under 10,000 rpm to remove residual fluid from the filter.
(16) Pre-heat the Elution Solution to 95°C.
(17) Place the Filter Cartridge in a new tube and apply 100 ul pre-heated Elution Solution to the center of the filter. Close the cap and leave the tube to stand for 1 min.
(18) Centrifuge 1 min at 10,000 rpm to obtain the final eluate and place the tube on ice for further usage.

2.10 Western Blot

The procedures for semi-quantification of Bcl-2, Bcl-w Api5, and Tcl-1 protein levels were the same as described in detail elsewhere (83,169,170). Membrane protein samples were extracted from the left ventricular wall of rabbits and SKBr3 cells. The goat polyclonal antibodies against Bcl-2 and Bcl-w were both purchased from Cell Signaling, Tcl-1 from Abcam Inc (Cambridge, MA, USA) and Api5 from Santa Cruz Biotechnology, Inc.

The protein extraction procedure:

(1) Aspirate the culture medium from the cell culture plate and add proper volume of cold PBS to the cell culture. Leave the plate on ice for next steps.
(2) Scrap the cell off the bottom with rubber spatula and transfert the suspension to a fresh tube.
(3) Centrifuge at 500g for 5 min and remove the PBS.
(4) Add 200 ul lysis buffer, mix well and break the cell by sonication.
(5) Centrifuge at 1000g 4 °C to for 10 min.

(6) Take the supernatant, and centrifuge at 45 000 rpm for 1 h at 4 °C (Beckman TL-100 Ultracentrifuge with rotor TLA-100.3). Save supernatant (cytosolic fraction), and the pellets (membrane fraction).
(7) Re-suspend pellets in TE with 2% Triton X-100 1 h at RT.
(8) Centrifuge 13,000g for 10 min at 4ºC. Keep the supernatant which is the membrane fraction.
(9) Measure the protein concentration by Bradford with a Bio-Rad protein assay kit, make aliquots and store at –80 °C.

Electrophonetic transfer is performed on Thermo Scientific Owl HEP-1 Semidry Electroblotting System. After transfer, the membrane with samples is incubated with primary antibodies over night and secondary antibodies for one hour. Final signal is detected with Western Lightning® Plus–ECL, Enhanced Chemiluminescence Substrate (680 ul) from PerkinElmer Inc.

2.11 MTT Assay for Cell Viability

Cell Proliferation Kit I (3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT); Roche Molecular Biochemicals, Laval, PQ, Canada) was used to quantify the number of cells surviving from oxidative stress (170,128). The steps from the protocol is as the following:

(1) Grow the cells in microplates (96 wells, flat bottom) with a final volume 100 ul of culture medium per well. The incubation period of the cell cultures is modified according to our experiment needs and cell line.
(2) Add 10 ul of the MTT labeling reagent to each well to achieve final concentration 0.5 mg/ml after incubation.
(3) 4 h incubation in the cell culture incubator.
(4) Add 100 ul solubilization solution to each well.
(5) Incubate the plate overnight in cell culture incubator.
(6) ELISA microplate reader is used to measure the absorbance of the samples. The wavelength is between 550 and 600 nm to measure formazan product and wavelength longer than 650 nm is employed as reference.

2.12 Enzyme-Linked Immunosorbent Assay (ELISA)

The Cell Death Detection ELISA kit (Roche Molecular Biochemicals) was employed to quantify DNA fragmentation on the basis of antibody detection of free histone and fragmented DNA.

The detailed procedure is as the following (21,128):

(1) Treat cells with apoptosis-inducing agent in the well of a microplate (1–24 h, 37°C).

(2) Centrifuge microplate (200g) and remove supernatant (10 min, Room Temperature).

(3) Incubate treated cells with lysis buffer (30 min, Room Temperature).

(4) Repeat microplate centrifugation (200g) (10 min, Room Temperature).

(5) Transfer aliquot of supernatant (lysate) to streptavidin-coated microplate.

(6) Incubate supernatant with immunoreagent (containing anti-histone and anti-DNA) (2h, Room Temperature).

(7) Wash microplate wells three times with incubation buffer at Room Temperature.

(8) Add substrate solution to wells and incubate (approx. 15 min, Room Temperature).

(9) Measure absorbance at 405 nm.

2.13 Computational Prediction of MiRNA Target

We used seven miRNA databases and target-prediction websites for our initial analysis, as described in detail elsewhere (171). These algorithms include DIANA-microTv3.0, miRanda, MirTarget2, MicroCosm, PicTar, PITA, and TargetScan. The criterion for a gene to be considered a candidate target for a miRNA was set to have positive hits from at least four out of seven algorithms.

2.14 Data Analysis

Group data are expressed as mean $\pm$S.E. Statistical comparisons (performed using ANOVA followed by Dunnett's method) were carried out using Microsoft Excel. A two-tailed $p<0.05$ was taken to indicate a statistically significant difference.

3. RESULTS

3.1 Initial Analysis of Candidate miRNAs

To pinpoint miRNAs that could possibly link TIIA to Bcl-2 for the anti-apoptotic efficacy, we performed an initial analysis on the miRNAs known to regulate apoptosis in cardiac cells. Among these miRNAs, *miR-1*, *miR-29* and *miR-320* are proapoptotic whereas *miR-133*, *miR-21* and *miR-199a* are antiapoptotic (178-181). There is a possibility that TIIA acts to downregulate the proapoptotic miRNAs so as to relieve the repression of Bcl-2. However, our analysis excluded major involvement of these miRNAs since none of these miRNAs have been shown by experimental studies, or are predicted by our computational analysis, to target Bcl-2. Instead, we identified the *miR-34* family as a possible candidate based on the following points. First *miR-34* has been shown to repress Bcl-2 in tumor cell lines so as to mediate the proapoptotic effect of p53 (144-146). The *miR-34* family contains three members including *miR-34a*, *miR-34b* and *miR-34c*, and these miRNAs share the same seed motif and therefore have virtually the same, or nearly the same, set of target genes. Second, transcription of these miRNAs is directly activated by p53 in response to DNA damage and other cellular stress such as oxidative stress. Doxorubicin is known to activate p53 by inducing reactive oxygen species and DNA damage. Third, numerous studies have documented enhanced expression and activity of p53 and its deleterious effect in several cardiac pathological conditions associated with cellular stress (147-158). And coincidently, *miR-34* has also been found to be upregulated in failing heart (159). Finally, our target prediction revealed that *miR-34* has the potential to regulate other genes encoding anti-apoptotic proteins (BCL2L2 for Bcl-w, API5 for apoptosis inhibitor–Api, and TCL1 for T-cell leukemia/lymphoma 1–Tcl-1) in addition to BCL2 encoding Bcl-2 protein (Fig. 9). We therefore focused our subsequent study on the *miR-34* family.

3.2 Upregulation of miR-34 in Response to Cellular Stress in Cardiomyocytes

All three members of the *miR-34* family were found expressed at low levels, relative to the muscle-specific miRNA *miR-133*, under normal conditions in NRVCs (Fig. 3). However, their expression was robustly upregulated after exposure to either doxorubicin (0.5 μM) or H_2O_2 (200 μM) for 24 hr. In contrast, *miR-133* level was reduced. Strikingly, pretreatment

of cells with TIIA (1 μM) prior to incubation with doxorubicin or H_2O_2 prevented upregulation of *miR-34*, but failed to alter the downregulation of *miR-133*.

3.3 Role of *miR-34* in Cardiomyocyte Apoptosis

Cells treated with doxorubicin or H_2O_2 demonstrated significant apoptotic death, as indicated by the shrunken cell body, decreased cell survival, and DNA fragmentation (Fig. 4A & 4B). The apoptosis, however, was largely mitigated in cells pretreated with TIIA for 5 hr, consistent with previous finding by other groups (160-163). Noticeably, apoptosis was consistently observed in cells transfected with the *miR-34* family miRNAs (Fig. 4C & 4D), which is in agreement with the proapoptotic action of these miRNAs in cancer cells (164-167). This proapoptotic effect in cardiomyocytes was abrogated by co-transfection of the LNA antisense to *miR-34b* (AMO-34b). As expected, AMO-34b was able to antagonize the effects of all three members of the *miR-34* family as they share high degrees of sequence homology and have an identical seed motif (Fig. 8). More importantly, transfection of AMO-34b alone was able to diminish the apoptotic cell death induced by doxorubicin or H_2O_2 (Fig. 4E & 4F).

We reasoned that if downregulation of *miR-34* accounts for the anti-apoptotic effect of TIIA, then supplying exogenous *miR-34* should render TIIA a loss-of-effect. This was indeed supported by the following experiments. First, TIIA treatment failed to antagonize the ability of *miR-34a* to induce apoptosis in cells transfected with this miRNA (Fig. 5A & 5B). Second, co-application of AMO-34b and TIIA produced the same magnitude of antiapoptotic effects as either of these two agents (Fig. 5C & 5D).

As negative controls, the mutant *miR-34b* failed to cause apoptosis. And the mutant AMO-34b failed to affect the apoptosis induced by any of the *miR-34* family members either (Fig. 4C & 4D). The efficacy of AMO-34b to knockdown all three members of the family *miR-34a*, *miR-34b* and *miR-34c-5p* were confirmed (Fig. 10).

3.4 *miR-34* Targets Multiple Anti-apoptotic Protein-Coding Genes

BCL2 has been validated as a target gene for the *miR-34* family miRNAs in cancer cells (144-146). Here we confirmed this result in cardiomyocytes (Fig. 6A). To further establish the other candidate genes BCL2L2, API5 and TCL1 as cognate targets for *miR-34*, we first performed luciferase reporter activity assays in H9c2 cells with the vectors engineered to carry the 3'UTR of these genes downstream of the luciferase gene. Transfection of *miR-34a*, *miR-34b*, or *miR-34c* reduced luciferase reporter activity by >50% and co-transfection with AMO-34b reversed the inhibitory effects (Fig. 6A).

The targeting was confirmed at the protein level by Western blot analysis in NRVCs (Fig. 6B), in particular, transfection of AMO-34b alone increased the protein levels of Bcl-2, Bcl-2l2 and Api by 15~25% in the absence of H_2O_2 and by 50~65% in the presence of H_2O_2 (Fig. 6C). The mRNA levels of these genes were not significantly altered by *miR-34* and AMO-34b in NRVCs. Tcl-1 was not detectable at either protein or mRNA levels in NRVCs. In all these experiments, the negative control miRNA and AMO failed to affect significantly the expression of these genes.

3.5 Role of Bcl-w and Api in Cardiomyocytes Survival

While Bcl-2 has long been known to transduce survival signals in cardiac cells, the potential role of and Bcl-w and Api has not yet been studied. To clarify whether these two proteins actually produce antiapoptotic effect and could mediate cytoprotective effect of TIIA through *miR-34* downregulation, we used the siRNAs with proven efficacy in silencing BCL2L2 and API5, respectively to knockdown these genes (201) (Fig. 11). Treatment of cells with either of these two siRNAs did not induced any significant cell death in the absence of H_2O_2; but these siRNAs exaggerated the apoptosis induced by H_2O_2 (Fig. 7A & 7B). Moreover, these siRNAs mitigated, though did not abolish, the ability of AMO-34b to rescue cell death induced by H_2O_2 (Fig. 7C & 7D).

4. DISCUSSION

miRNA-mediated gene regulation is now considered a fundamental layer of genetic programs that operates at the post-transcriptional level. Our current understanding of the functions of miRNAs primarily relies on their tissue-specific or developmental stage-specific expression patterns as well as their evolutionary conservation, and is thus largely limited to biogenesis and oncogenesis. Target finding and function discovery are two major challenges to researchers in miRNA research. This study led us to several novel findings.

4.1 Discovery of the Role of *miR-34* in Cardiomyocyte Apoptosis

The members of the *miR-34* family are well known as apoptosis regulators in cancer. But their contribution to cardiomyocyte apoptosis is first reported by this work. These miRNAs can be activated by stress including DNA damage as well as reactive oxygen species. P53 binding sites were found upstream of *miR-34,* and *miR-34* expression is under the activation of p53 (123), a well-known tumor repressor gene inducing apoptosis in many types of cancer. The *miR-34* family contains three members sharing nearly completely identical sequence. Similarity in their sequences suggests that these three miRNAs may share a similar set of targets. This view indeed is experimentally supported. Three miRNAs were transfected into cell line separately and the transciptome of these cell lines were determined. Data showed that the changes in the mRNA profiles were nearly identical (124). Not surprisingly, most of the changed mRNAs are cell cycle related. Expression profile of these three members is tissue specific. *MiR-34a* is ubiquitously expressed all over the brain while miR-34b/c are largely expressed in the lung. Intriguingly, all three members of the *miR-34* family are constitutively expressed in low level in cardiac cells. Only upon exposure to extreme stress are all members of *miR-34* significantly upregulated and able to exert their functions. Under pathological conditions such as ischemia/reperfusion injury and heart failure, reactive oxygen species are produced at levels far more than in the normal heart. In such a scenario, p53 protein content as well as its phosphorylation increase, inducing *miR-34* family expression. Due to this specific *miR-34* expression pattern, the *miR-34* family can be detected for prognostic purpose. There exists evidence that during the

transition from hypertrophy to heart failure, p53 protein level is significantly increased (127). Although no data suggest that *miR-34* upregulation is coincidence with this transition, from our data, we can predict that *miR-34* is significantly upregulated in this transition and can be employed as prognostic index. There are also signs that the *miR-34* family may participate in the pathogenesis of heart failure because upregulation of both p53 and *miR-34* have been observed in separate studies of heart failure (125, 126).

4.2 **Identification of Novel Target Genes of *miR-34* in Cardiomyocytes**

To reveal specific miRNA function, miRNA targets should be validated with experimental methods. But these experiments require large amounts of time and labor. Therefore, only a limited number of mRNAs have been experimentally confirmed as miRNAs targets. This work added a small piece to this complex puzzle but is valuable in understanding of miRNA function and their therapeutic potential in heart disease. We identified BCL2L2, API5 and TCL1 as new target genes, in addition to BCL2. Unfortunately, we were unable to detect TCL1 mRNA or protein in our cardiomyocytes. Whether TCL1 is constitutively expressed in cardiac tissue requires further investigation. The functions of BCL2L2 and API5 in the heart have never been mentioned before. For the first time, our data established antiapoptotic roles for BCL2L2 and API5 in the heart during oxidative stress. There are far more targets than BCL2, BCL2L2, API5 for the *miR-34* family. A long list of determined targets is described in Hermeking's review (129). The author also mentioned that many target mRNA's 3'UTRs carry multiple seed sequences for *miR-34*. In our case, all of these target mRNAs only contain one binding site for members in *miR-34* family. Thus it seems that all three *miR-34* members target the mRNAs at the identical binding site. We used one single miRNA inhibitor, AMO-34b, to knock down all members from *miR-34* family. Tests were conducted to examine the efficacy of AMO-34b in knocking down all three members of *miR-34* family (Fig. 10). It seems that *miR-34a* and *miR-34c* are more abundant than *miR-34b* under normal condition and *miR-34a/c* responded to oxidative stress more robustly than *miR-34b*. Difference in constitutive and induced expression might be ascribed to their different transcriptional control elements. Although they are all activated by p53, their different genome location and different upstream sequences indicate that they are possibly controlled by different sets of transcriptional regulators. We were not able to

dissect the relative contribution of these three miRNAs to apoptosis due to lack of member specific AMO. Whether these three members made unequal contribution to cardiac apoptosis needs further examination.

4.3 Elucidation of miRNAs as A Molecular Mechanism for TIIA's Anti-apoptotic Efficacy

We demonstrated that TIIA was able to prevent the upregulation of apoptotic *miR-34* and apoptosis induced by oxidative stress. The underlying mechanism is likely to be TIIA-mediated blockade of oxidative stress-induced *miR-34* upregulation, preservation of antiapoptotic protein such as Bcl-2, Bcl-w and Api and eventual increased cell survival. Numerous reports have proven TIIA is effective in ameliorating cardiac function after ischemic/infarction injuries in human subjects and in animal models. This work has provided a mechanistical model that TIIA, by altering miRNA expression inhibits apoptosis, supports more cardiac cells to survive the harsh environment imposed by ischemia and infarction, preserves the building material and functional material of the heart and achieves cardioprotection. This finding also provided evidence for the ability of a therapeutic drug to regulate a signaling pathway via influencing miRNA expression. But we did not go further to explore the details about TIIA influence on *miR-34* expression. Some reports point out that TIIA has anti-oxidant properties and we can postulate that TIIA acts as an anti-oxidant and neutralizes the reactive oxidative species that will trigger upregulation of p53 and subsequent upregulation of *miR-34*. We knew that TIIA can directly affect p53 protein level within cancer cell line but whether it is also the case in cardiac cells needs further examination. TIIA kills cancer cells via upregulation of p53 and subsequent promotion of apoptosis. If somehow TIIA affects p53 expression in cardiac cells, the regulating direction is expected to be opposite to that in cancer cells, since TIIA is antiapoptotic to cardiac cells.

4.4 Possible Limitations of the Study

It should be noted that our study was conducted in *in vitro* conditions and whether the same results can be extrapolated to *in vivo* is yet to be determined; nonetheless, it lays the

groundwork for future investigations into the matter. The fact that the role of *miR-34* in apoptosis is manifested in the presence of cellular stress suggests that *miR-34* may participate in the pathological process of the heart. However, the present study failed to establish this link; further studies using animal models of cardiac disease are warranted for the purpose.

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6. FIGURES AND FIGURE LEGENDS

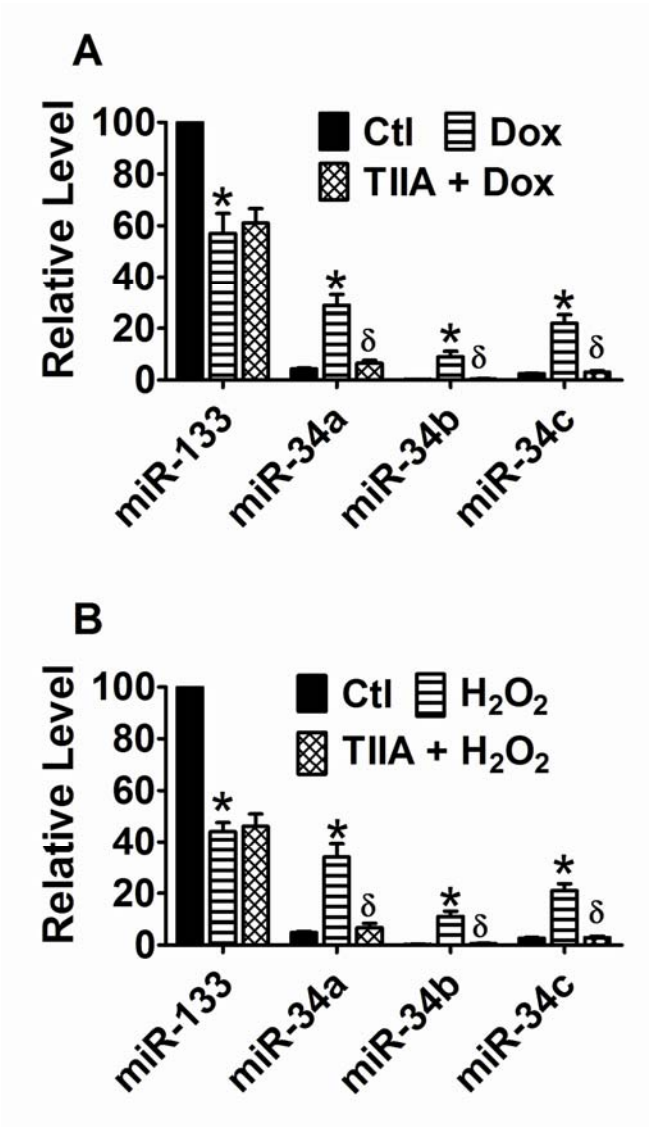

Figure 3. Upregulation of the *miR-34* family members in response to cellular stress in cardiomyocytes. (A) Expression level of the *miR-34* family miRNA in the mature form (*miR-34a*, *miR-34b* and *miR-34c*) after 24 h incubation with doxorubicin (Dox, 0.5 μM, an activator of p53 inducing DNA damage) in neonatal rat ventricular cells (NRVCs), as measured by quantitative real-time RT-PCR. *miR-133* was used as a control *p<0.05 *vs.* Ctl; δp<0.05 *vs.* Dox alone; n=4 batches of cells for each group. (B) Expression level of mature *miR-34a*, *miR-34b* and *miR-34c* after 24 h incubation with H_2O_2 (200 μM) in NRVCs. *p<0.05 vs. Ctl; δp<0.05 *vs*. H_2O_2 alone; n=5 batches of cells for each group.

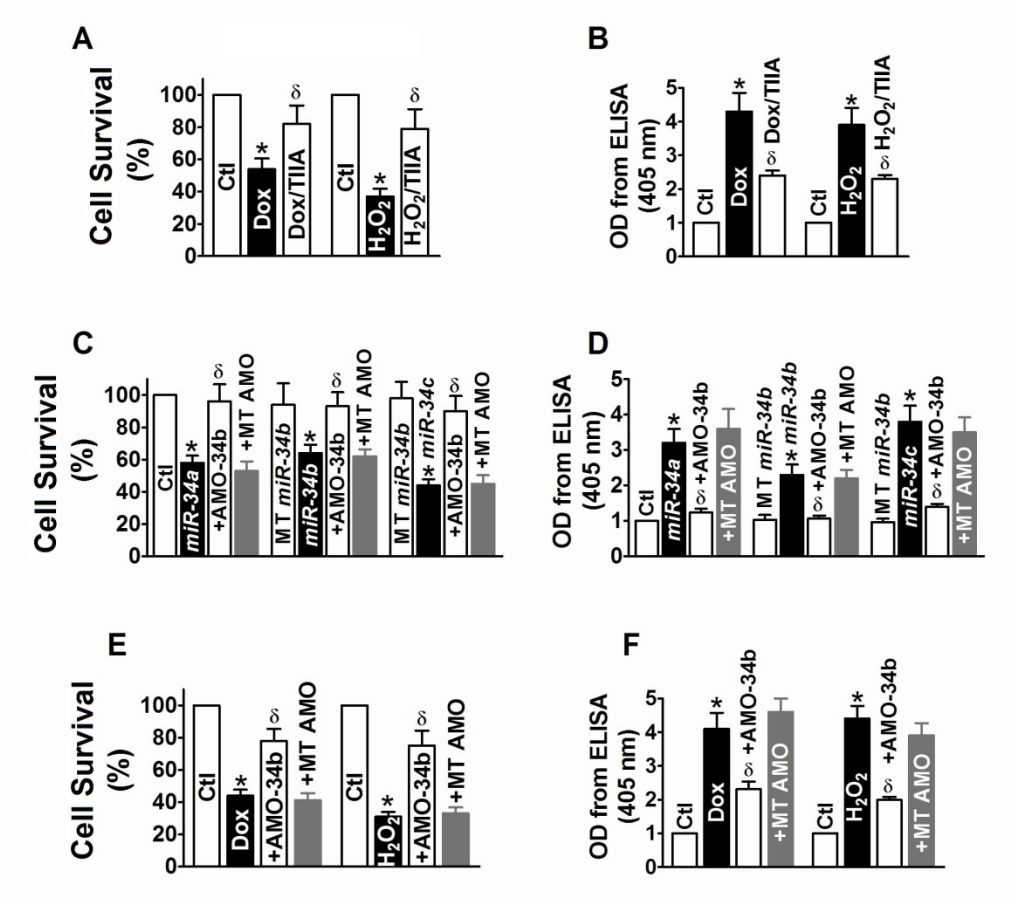

Figure 4. Role of *miR-34* in cardiomyocyte apoptosis. (A) Protective effect of tanshinone IIA (TIIA, 1 μM) on cell survival in the presence of doxorubicin (0.5 μM) or H_2O_2 (200 μM) in NRVCs, as determined by MTT methods. *p<0.05 *vs.* Ctl; δp<0.05 *vs.* Dox or H_2O_2; n=5 batches of cells for each group. TIIA/Dox or TIIA/ H_2O_2: cells were pretreated with TIIA for 5 h followed by incubation with Dox or H_2O_2. (B) Rescuing effect of tanshinone IIA (TIIA) on apoptosis induced by doxorubicin (0.5 μM) or H_2O_2 (200 μM) in NRVCs, as determined by ELISA quantification of DNA fragmentation indicated by the OD (optical density) values. *p<0.05 *vs.* Ctl; δp<0.05 *vs.* Dox or H_2O_2; n=6 batches of cells for each group. (C) Damaging effects of the *miR-34* family miRNAs (100 nM) on cell survival in the presence of doxorubicin (0.5 μM) or H_2O_2 (200 μM) in NRVCs. +AMO-34b: co-transfection of *miR-34* (100 nM) and AMO-34b (30 nM), anti-miRNA antisense oligonucleotide to *miR-34*; + MT AMO: co-transfection of *miR-34* and mutant AMO-34b; MT *miR-34b*: mutant *miR-34b* (Fig. 8). The constructs were transfected using lipofectamine 2000; Control cells

were mock-treated with lipofectamine 2000 (same below). *p<0.05 *vs.* Ctl; δp<0.05 *vs. miR-34* alone; n=5 batches of cells for each group. (D) Proapoptotic effects of the *miR-34* family miRNAs (100 nM) in the presence of doxorubicin (0.5 μM) or H_2O_2 (200 μM) in NRVCs. *p<0.05 *vs.* Ctl; δp<0.05 *vs. miR-34* alone; n=5 batches of cells for each group. (E) Protective effect of the anti-miRNA antisense oligonucleotide to *miR-34* (AMO-34b, 30 nM) cell survival in the presence of Dox or H_2O_2 in NRVCs. *p<0.05 *vs.* Ctl; δp<0.05 *vs.* Dox or H_2O_2; n=5 batches of cells for each group. (F) Rescuing effect of AMO-34b on apoptosis induced by Dox or H_2O_2 in NRVCs. *p<0.05 *vs.* Ctl; δp<0.05 *vs.* Dox or H_2O_2; n=5 batches of cells for each group.

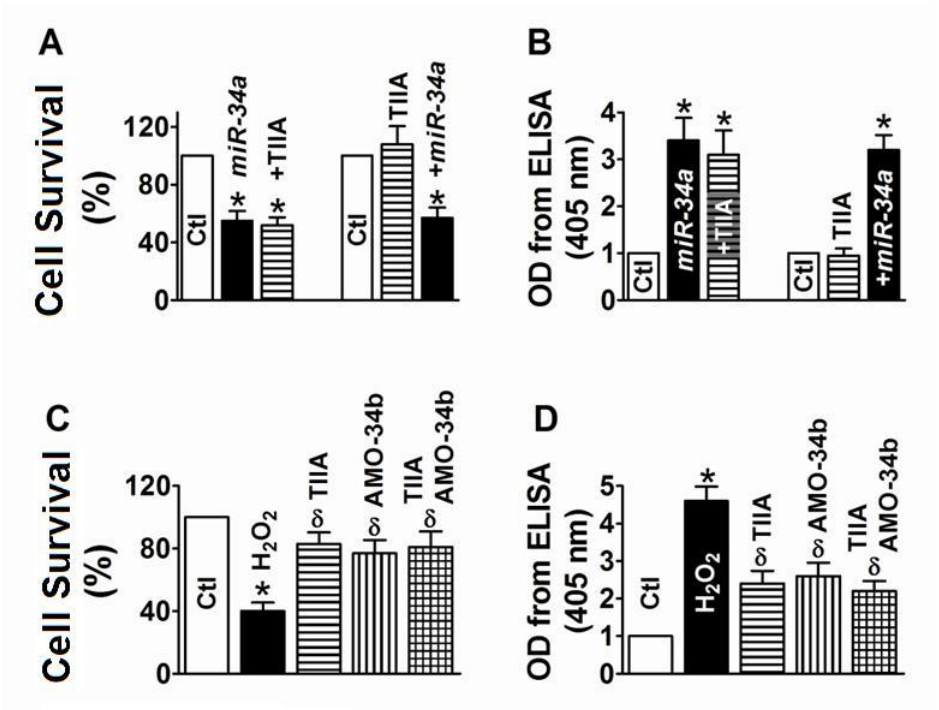

Figure 5. Relationship between tanshinone IIA (TIIA) and *miR-34* in terms of their effects on cell death. (A) Loss of cardioprotective effect of TIIA (1 μM) on reduced cell survival caused by exogenous *miR-34a* (100 nM) in NRVCs. Cells were transfected with *miR-34a* for 12 h, followed by TIIA treatment for 5 h. *p<0.05 vs. Ctl; n=6 batches of cells for each group. (B) Loss of rescuing effect of TIIA on apoptotic cell death induced by exogenous *miR-34a* in NRVCs. *p<0.05 vs. Ctl; n=5 batches of cells for each group. (C) Comparison of effects of TIIA (1 μM) and AMO-34b (30 nM) on cell survival in the presence of H_2O_2 (200 μM). *p<0.05 *vs.* Ctl, δp<0.05 *vs.* H_2O_2; n=5 batches of cells for each group. (D) Comparison of effects of TIIA (1 μM) and AMO-34b (30 nM) on apoptosis induced by H_2O_2 (200 μM). *p<0.05 *vs.* Ctl, δp<0.05 *vs.* H_2O_2; n=5 batches of cells for each group. Note that application of TIIA and AMO-34b together produced the same magnitude of effects as application of TIIA or AMO-34b alone.

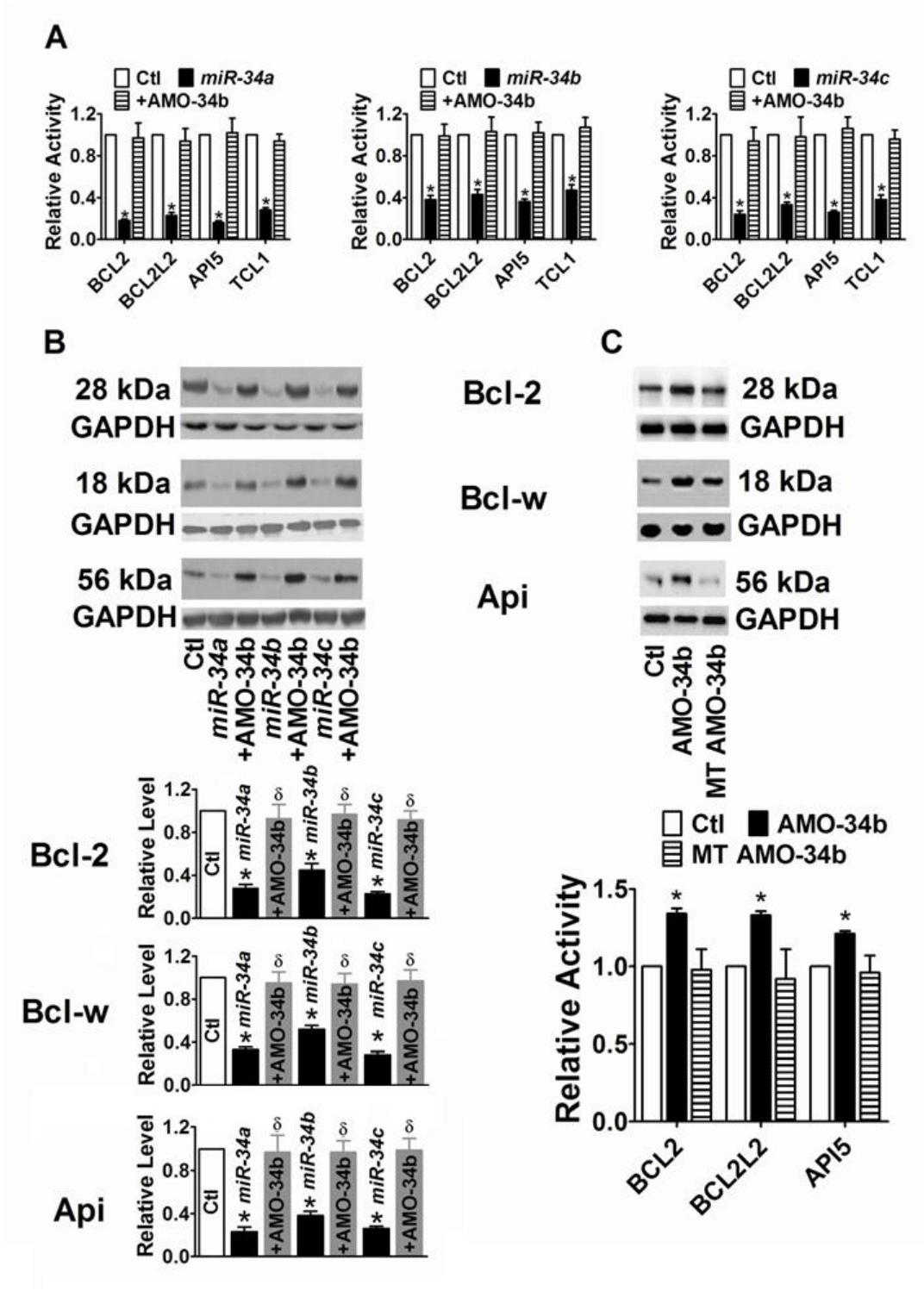

Figure 6. Establishment of multiple anti-apoptotic protein-coding genes as targets for the *miR-34* family miRNAs. (A) Role of *miR-34a* (left), *miR-34b* (middle), and *miR-34c* (right) in repressing the candidate target genes BCL2, BCL2L2 (Bcl-2-like protein 2), API5 (apoptosis inhibitor 5), and TCL1 (T-cell

leukemia/lymphoma 1) (Fig. 9), determined by luciferase activity assays with the pMIR-REPORTTM luciferase miRNA expression reporter vector carrying the 3'UTR of the candidate target genes in neonatal rat ventricular cells (NRVCs). +AMO-34b: co-transfection of *miR-34* (100 nM) and AMO-34b (30 nM), anti-miRNA antisense oligonucleotide to *miR-34*. **p*<0.05 *vs.* Ctl; n=6 batches of cells for each group. (B) Role of *miR-34a*, *miR-34b*, and *miR-34c* in repressing BCL2, BCL2L2 (Bcl-2-like protein 2), and API5 (apoptosis inhibitor 5), determined by Western blot analysis. Upper panel: representative Western blot bands for Bcl-2, Bcl-w and Api proteins, respectively; lower panels: averaged data showing relative levels of protein levels. +AMO-34b: co-transfection of *miR-34* (100 nM) and AMO-34b (30 nM), anti-miRNA antisense oligonucleotide to *miR-34*. **p*<0.05 *vs.* Ctl, δ*p*<0.05 *vs. miR-34* alone; n=3 batches of cells for each group. (C) Role of AMO-34b to knockdown endogenous *miR-34* to relieve the tonic repression of BCL2, BCL2L2 (Bcl-2-like protein 2), and API5 (apoptosis inhibitor 5), determined by Western blot analysis. Upper panel: representative Western blot bands; lower panels: averaged data showing relative levels of protein levels. MT AMO-34b: mutant AMO-34b. **p*<0.05 *vs.* Ctl; n=3 batches of cells for each group.

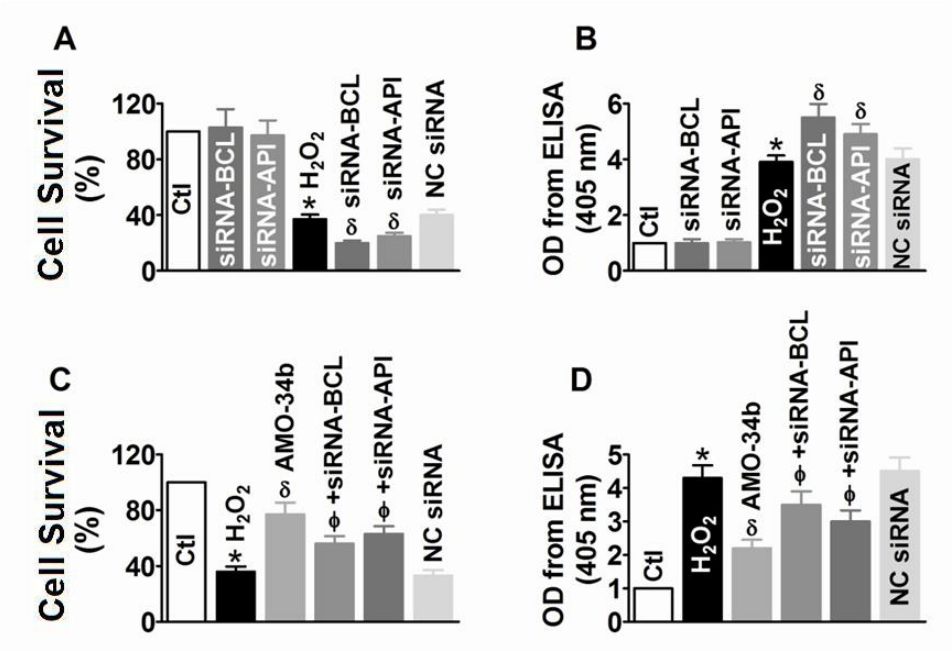

Figure 7. Role of Bcl-w and Api in cardiomyocytes survival and in mediating the effects of *miR-34*. (A) Effects of siRNAs to BCL2L2 and API5, respectively, on cell survival in the absence or presence of H_2O_2 (200 μM). siRNA-BCL: siRNA to BCL2L2; siRNA-API: siRNA to API5; NC siRNA: negative control scrambled siRNA. Note that the siRNAs exaggerate the reduction of cell survival in the presence of oxidative stress. *p<0.05 *vs.* Ctl, δp<0.05 *vs.* H_2O_2; n=5 batches of cells for each group. (B) Effects of siRNAs to BCL2L2 and API5, respectively, on apoptosis in the absence or presence of H_2O_2 (200 μM). Note that the siRNAs exaggerate apoptosis induced by oxidative stress. *p<0.05 *vs.* Ctl, δp<0.05 *vs.* H_2O_2; n=5 batches of cells for each group. (C) Effects of AMO-34b and siRNAs to BCL2L2 and API5, respectively, on cell survival in the presence of H_2O_2 (200 μM). +siRNA-BCL: co-transfection of AMO-34b and siRNA-BCL2L2; +siRNA-API: co-transfection of AMO-34b and siRNA-API5. Note that the siRNAs abrogate the protective effect of AMO-34b on cell survival. *p<0.05 *vs.* Ctl, δp<0.05 *vs.* H_2O_2, ϕp<0.05 *vs.* AMO-34b; n=5 batches of cells for each group. (D) Effects of AMO-34b and siRNAs to BCL2L2 and API5, respectively, on apoptosis induced by H_2O_2 (200 μM). Note that the siRNAs abrogate the antiapoptotic effect of AMO-34b. *p<0.05 *vs.* Ctl, δp<0.05 *vs.* H_2O_2, ϕp<0.05 *vs.* AMO-34b; n=5 batches of cells for each group.

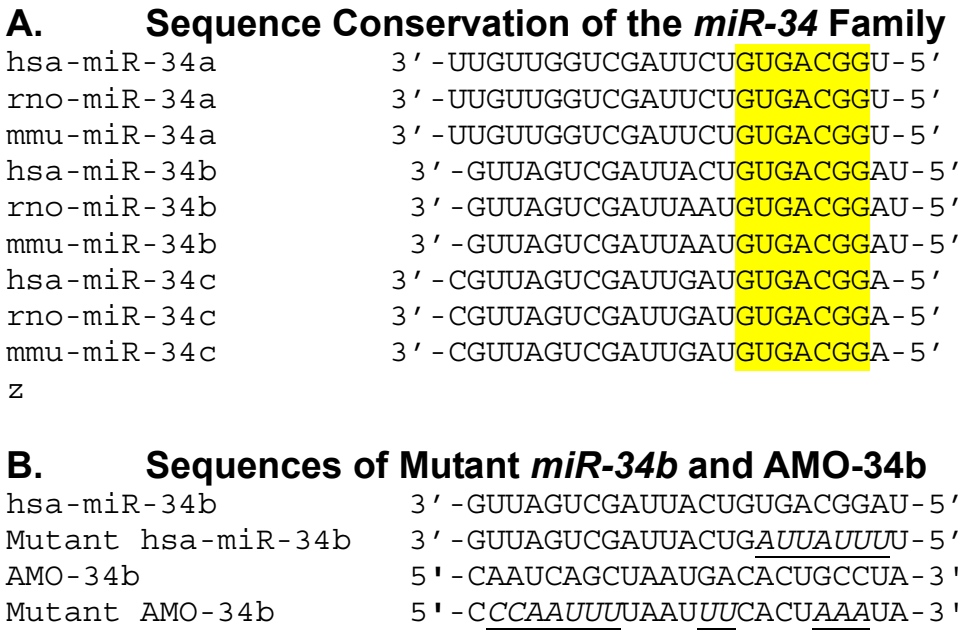

Figure 8. Sequences of the *miR-34* family miRNAs and the antisense oligonucleotide. (A) Alignment of the sequences of *miR-34a*, *miR-34b* and *miR-34c* from human, rat and mouse. The seed sites were highlighted in grey. Note that the seed motifs are identical among the three different members of the *miR-34* family and among the three different species. (B) Sequences of the mutated *miR-34b*, the antisense oligonucleotide to *miR-34b* (AMO-34b), and the mutated AMO-34b. The mutated nucleotides are indicated by the underlined letters in italics.

BCL2 (3'UTR)

```
hsa-miR-34a      3'-UUGUUGGUCGAUUCUGUGACGGU-5'
Human             190-GAAUCAGCU-AUUUACUGCCAA-211
Rat               182-AAAUCAGCU-AUUUACUGCCAA-203
Human            1098-AGCUCAGAAUUCCACUGUCA-1112
Rat             5255-AGCAGCUAUGAAUUCCAUUGCCU-5281

hsa-miR-34b       3'-GUUAGUCGAUUACUGUGACGGAU-5'
Human           190-GAAUCAGCUAUU--UACUGCCAA-211
Rat             182-AAAUCAGCUAUU--UACUGCCAA-203
Human          1098-AGCUCAGAAUU---CCACUGUCA-1112
Rat            5255-AGCAGCUAUGAAUUCCAUUGCCU-5281

hsa-miR-34c       3'-CGUUAGUCGAUUGAUGUGACGGA-5'
Human           190-GAAUCAGCUA--UUUACUGCCAA-211
Rat             182-AAAUCAGCUA--UUUACUGCCAA-203
Human          1098-AGCUCAG---AAUUCCACUGUCA-1112
Rat            5255-AGCAGCUAUGAAUUCCAUUGCCU-5281
```

BCL2L2 (3'UTR)

```
hsa-miR-34a      3'-UUGUUGGUCGAUUCUGUGACGGU-5'
Human           585-AAC-AAGGGCCAGUUCACUGCCC-607
Rat             669-AGACUAGGGCUCUGGCACUGCUG-691

hsa-miR-34b       3'-GUUAGUCGAUUACUGUGACGGAU-5'
Human            586-ACAAGGGCCAGUU-CACUGCCCU-608
Rat             669-AGACUAGGGCUCUGGCACUGCUG-691

hsa-miR-34c       3'-CGUUAGUCGAUUGAUGUGACGGA-5'
Human            585-AACAAGGGC-CAGUUCACUGCCC-607
Rat              670-GACUAGGGCU-CUGGCACUGCUG-691
```

TCL1 (3'UTR)

```
hsa-miR-34a      3'-UUGUUGGUCGAUUCUGUGACGGU-5'
Human            463-AACACGCCUGCAAACGCUGCCUG-486
Rat               462-CACCCAGCAACUCCACUGCCUG-483

hsa-miR-34b       3'-GUUAGUCGAUUACUGUGACGGAU-5'
Human           463-AACACGCCUGCAAACGCUGCCUG-486
Rat             462-CACCCAGC-AACUCCACUGCCUG-483

hsa-miR-34c       3'-CGUUAGUCGAUUGAUGUGACGGA-5'
Human           463-AACACGCCUGCAAACGCUGCCUG-486
Rat             462-CACCCAGC-AACUCCACUGCCUG-483
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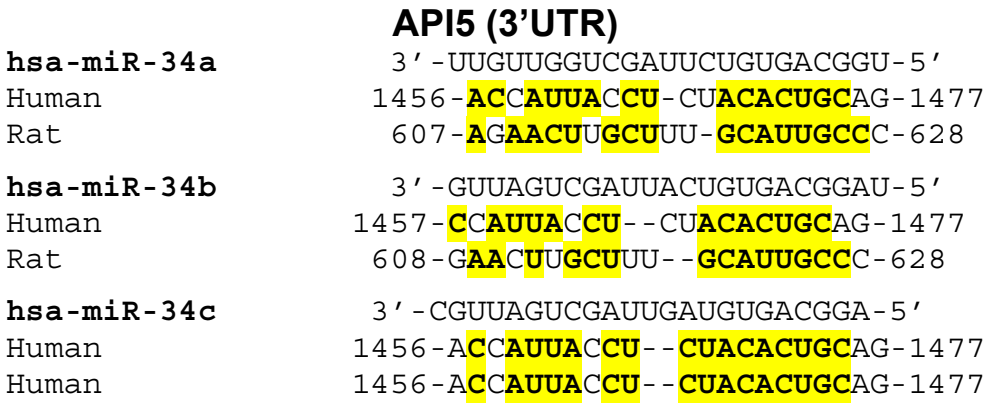

Figure 9. Complementary motifs between the three members of the *miR-34* family and the 3'UTR of the predicted target genes BCL2, BCL2L2 (Bcl-2-like protein 2), API5 (apoptosis inhibitor 5), and TCL1 (T-cell leukemia/lymphoma 1). Matched nucleotides and wobble matches are highlighted in grey.

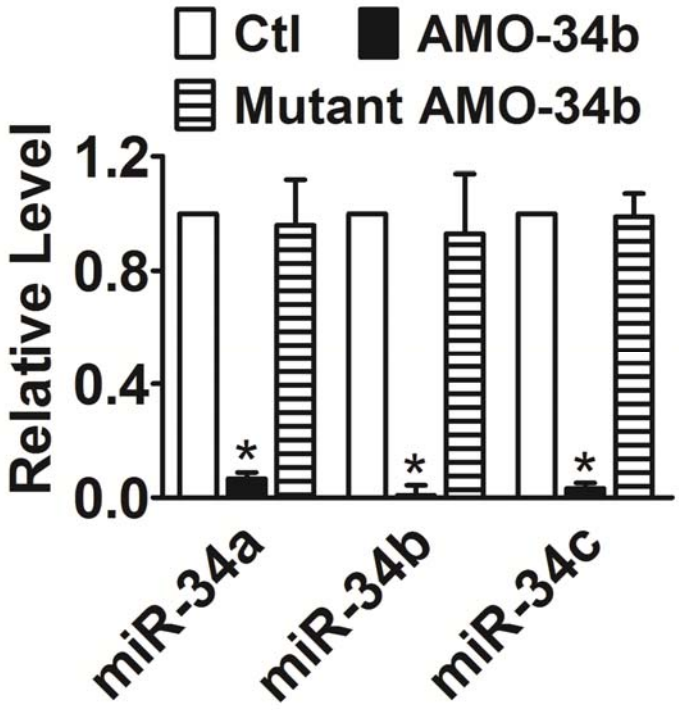

Figure 10. Verification of the efficacy of AMO-34b, and the inability of the mutant AMO-34b, to knockdown all three members of the *miR-34* family, as determined by quantitative real-time RT-PCR methods.

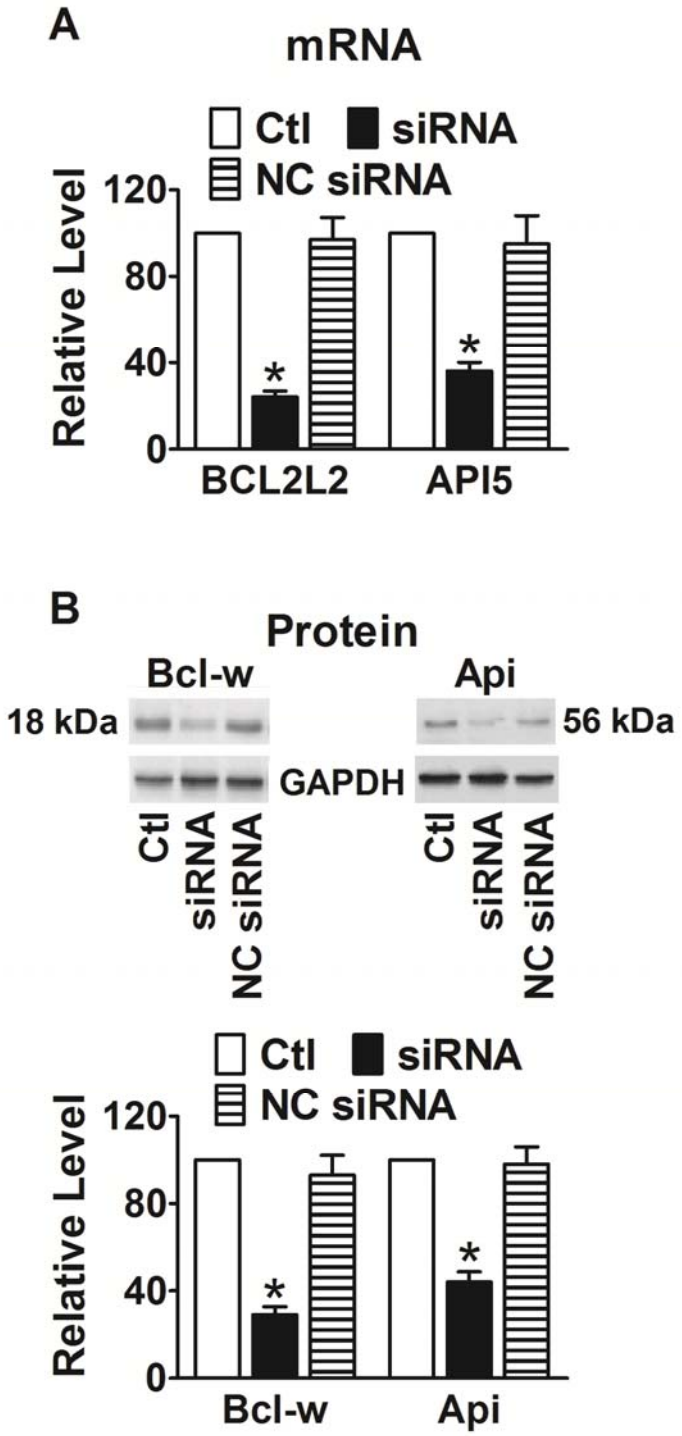

Figure 11. Verification of the efficacy of the siRNAs to BCL2L2 and API5, respectively, to silencing the genes at both mRNA and protein levels, measured by qPCR and Western blot analysis, respectively. The scrambled siRNA was used as a negative control. *p<0.05 *vs.* Ctl.