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Review

Small molecules, big effects: the role of microRNAs in regulation of cardiomyocyte death

J Skommer[1,3], I Rana[1,3], FZ Marques[1], W Zhu[2], Z Du[2] and FJ Charchar*[,1]

MicroRNAs (miRNAs) are a class of small non-coding RNAs involved in posttranscriptional regulation of gene expression, and exerting regulatory roles in plethora of biological processes. In recent years, miRNAs have received increased attention for their crucial role in health and disease, including in cardiovascular disease. This review summarizes the role of miRNAs in regulation of cardiac cell death/cell survival pathways, including apoptosis, autophagy and necrosis. It is envisaged that these miRNAs may explain the mechanisms behind the pathogenesis of many cardiac diseases, and, most importantly, may provide new avenues for therapeutic intervention that will limit cardiomyocyte cell death before it irreversibly affects cardiac function. Through an in-depth literature analysis coupled with integrative bioinformatics (pathway and synergy analysis), we dissect here the landscape of complex relationships between the apoptosis-regulating miRNAs in the context of cardiomyocyte cell death (including regulation of autophagy–apoptosis cross talk), and examine the gaps in our current understanding that will guide future investigations.
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Facts

- There are altered cardiac microRNAs levels in various cardiovascular diseases states
- MicroRNAs can regulate cardiac cell proliferation, hypertrophy, death and cardiac fibrosis pathways.
- MicroRNAs have long biological half-lives, hence can be a good diagnostic marker of disease state

Open Questions

- What are the mechanisms leading to increase or decrease in cardiac microRNAs under pathological and physiological state?
- What are the mechanisms by which microRNAs contribute to cardiac pathological and physiological state?
- What are the cardiac microRNA changes associated with aging?
- How do microRNAs regulate the balance between pro-survival and death pathways in the heart?

Cell death processes are highly regulated in order to ensure proper tissue functioning and homeostasis. Loss of cardiomyocytes is an important factor in the pathogenesis of conditions such as myocardial infarction (MI),[1] and occurs through a variety of cell death pathways, induction of which depends not only on the type of stress stimuli, but also on the intracellular biochemical and genetic makeup.[2] Most importantly, the converging regulatory network of cell death pathways is potentially amenable for control and therapeutic intervention.[3]

Over the last several years, efforts have been directed toward elucidating the exact regulatory mechanisms that govern cell death processes, including protein oligomerisation, protein conformational changes, transient protein–protein interactions and protein relocations (for example, Vela *et al.*[4–8]), as well as non-genetic cell-to-cell variability.[9–12] Recently, microRNAs (miRNAs) have emerged as an important, perhaps critical, regulatory factor when it comes to cell death signaling. This is not surprising considering that miRNAs, a class of small non-coding RNAs, posttranscriptionally regulate messenger RNAs by inhibiting their translation or promoting their degradation, and thus represent a new paradigm that regulates the expression of up to 50% of human genes.[13,14] According to the latest version of miRNA database miRBase 20.0, there are over 2500 known mature miRNAs in the human genome.[15]

Increasing evidence indicates that miRNAs are linked with many pathological conditions, and play important roles in cardiovascular homeostasis and in initiation and progression of disease states, such as hypertrophy and heart failure.[16–18]

[1]Robert HT Smith Chair in Cardiovascular Science, School of Health Sciences, Federation University Australia, Ballarat, Victoria 3350, Australia and [2] Institute of Clinical Pharmacology, The Second Affiliated Hospital of Harbin Medical University, Harbin, China
*Corresponding author: F Charchar, Robert HT Smith Chair in Cardiovascular Science, School of Health Sciences, Federation University Australia, PO Box 663, Ballarat, Victoria 3353, Australia.Tel: +61 3 5327 6098; Fax: +61 3 5327 9242; E-mail: federation.edu.au/charchar-lab
[3]These two authors contributed equally to this work.
Abbreviations: STEMI, ST-elevation myocardial infarction; MI, myocardial infarction; DAXX, death domain-associated protein; HIF1A, hypoxia inducible factor 1, alpha subunit; MFF, mitochondrial fission factor; HSP, heat shock protein; PC, progenitor cells; PKC, protein kinase C; PDCD4, programmed cell death 4; SIRT1, sirtuin 1; SNP, single-nucleotide polymorphism; FasL, Fas ligand
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Considering the role of cell death processes in cardiovascular disease (CVD) as well as the emerging role of miRNAs in regulating cardiac physiological and pathological states, we will focus our review on the role of miRNAs in regulating myocardial cell death processes.

The World of miRNAs

Small non-coding RNA molecules of ~22–23 nucleotides in length, known as miRNAs, regulate the expression of protein-coding genes through sequence-specific recognition in the 3′ or the 5′ untranslated regions (UTRs) of mRNAs, thus regulating mRNA levels by posttranscriptional mechanisms.[14,19] Partial sequence complementarity between a miRNA and its target site in a specific mRNA is often sufficient for binding.[20] In animals, the binding to the 3′UTR lowers mRNA levels by preferentially decreasing the stability of the mRNA, thus increasing its degradation, or by repressing translation.[14] miRNAs were also described to bind to the promoter region in the 5′UTR of mRNAs.[21,22] The binding of miRNAs to 5′UTRs can either repress or, most likely, activate translation (reviewed by Da Sacco and Masotti[19] and Vasudevan[23]). Since no examples of this type of miRNA–mRNA interactions are known in CVD or cell death, this review will explore mechanisms involving miRNAs that bind to the 3′UTR of mRNAs only.

miRNA biogenesis has been extensively reviewed elsewhere.[24–26] Briefly, transcription of miRNA genes is usually performed by RNA polymerase (RNA Pol) II, or occasionally by RNA Pol III, resulting in a precursor miRNA, termed primary miRNA or pri-miRNA.[26] In the nucleus pri-miRNAs (~1000 bp) fold into hairpin structures, which act as a substrate for DGCR8 subunit of Drosha, a nuclear enzyme of the RNase III family, resulting in the cleavage of the pri-miRNA into a pre-miRNA (~60—70-bp long). The pre-miRNA is then exported to the cytoplasm, where Dicer, another enzyme of the RNAse III family, processes the pre-miRNA to a ~20-bp double-stranded miRNA duplexes.[24–26] The miRNA duplexes associate with specific members of the Argonaute (Ago) family of proteins, and together form the core of the RNA-silencing complex (RISC), which mediates the posttranscriptional regulation of gene expression by utilizing the guide strand (miR) of the miRNA duplex.[24–26] There is now evidence that the second miRNA strand, often called passenger or minor miRNA (miR*), before believed to be degraded, can also associate with Ago proteins and impact gene expression.[27]

Several algorithms are used for the discovery of miRNA–mRNA interactions (reviewed by Witkos *et al.*[28]), with computational predictions suggesting that a single miRNA can potentially regulate multiple mRNAs.[20,28] *In vitro* experimentation, however, is always necessary to validate the predicted targets as well as the role of miRNAs in regulation of cell signaling pathways and cellular phenotypes. Moreover, the general role of miRNAs in specific cellular or physiological processes can be examined by deleting or inhibiting miRNA-processing machinery. For instance, the cardiac-specific knockout of Dicer leads to rapidly progressive dilated cardiomyopathy, heart failure and postnatal lethality, emphasizing the importance of miRNAs for heart development and homeostasis.[29–31]

Types of Cell Death

Cell death is a group of fundamental processes in both normal physiology and pathological states. The mammalian cell death network comprises of many distinct functional modules,[32–34] including apoptosis (also referred to as programmed cell death), macroautophagy (commonly referred to as autophagy, and more appropriately as cell death dependent on autophagy genes), necrosis and an inflammatory form of cell death referred to as pyroptosis.[35] Of these, apoptosis has been studied most extensively and its molecular framework is relatively well defined.[36] Often more than one process of cell death is induced, and the particular extracellular milieu, as well as genetic and biochemical context of the intracellular environment, will determine which pathway is ultimately responsible for cell demise. Because of this, blocking one pathway of cell death may not prevent the final outcome of cell loss, as other simultaneously induced pathways may take over the executioner's role. At the same time, genetic and pharmacological inhibitor studies can identify the culprit pathway among all the signaling induced by a particular stress cue. Thus, detailed knowledge of multiple pathways, their regulators and cross talks between them, is of paramount importance when it comes to interfering with cell death process for therapeutic purposes.

Apoptotic Pathways in Cardiovascular Disease

The process of apoptosis is driven by a network of proteins connected to each other in an intricate manner, forming two main signaling pathways activated by a wide variety of stress signals that converge on mitochondria (the intrinsic pathway) or upon ligation of cell surface death receptors (the extrinsic pathway) (Figure 1). Caspase-9 activation by mitochondrial pathway and caspase-8 activation by extrinsic pathway lead to activation of the executioner caspases, such as caspase-3 or -7 which then directly degrade the proteome and commit cells to death (Figure 1).

Cytochrome *c* released from damaged mitochondria act as a trigger for the activation of the mitochondrial pathway. The mitochondrial pathway of apoptosis is heavily regulated by multiple proteins acting upstream and downstream of the mitochondria, determining the efficiency of apoptotic cell death (Figure 1). Control over the integrity of mitochondrial outer membrane is executed by the proteins of the B-cell lymphoma 2 (Bcl-2) family, divided into three subfamilies: anti-apoptotic (for example, Bcl-2, Bcl-Xl, Mcl-1, Bcl-w), pro-apoptotic effectors (Bax and Bak) and BH3-only proteins (for example, Bid, Puma, Bim, Bad and so on), which form a complex network of interactions (discussed in more detail in several reviews, including.[6,37–39] Downstream of mitochondria, activation of caspases is also heavily regulated, first by the low-probability event of apoptosome formation and second by inhibitor of apoptosis (IAP) proteins such as XIAP (Figure 1).

In the extrinsic pathway of apoptosis, the initial stress signal comes from the binding of a death domain-containing receptor, located at the plasma membrane, to its cognate ligand (for example, Fas ligand (FasL) or tumor necrosis factor-α (TNF-α)).[40] Upon binding, the receptor promotes the

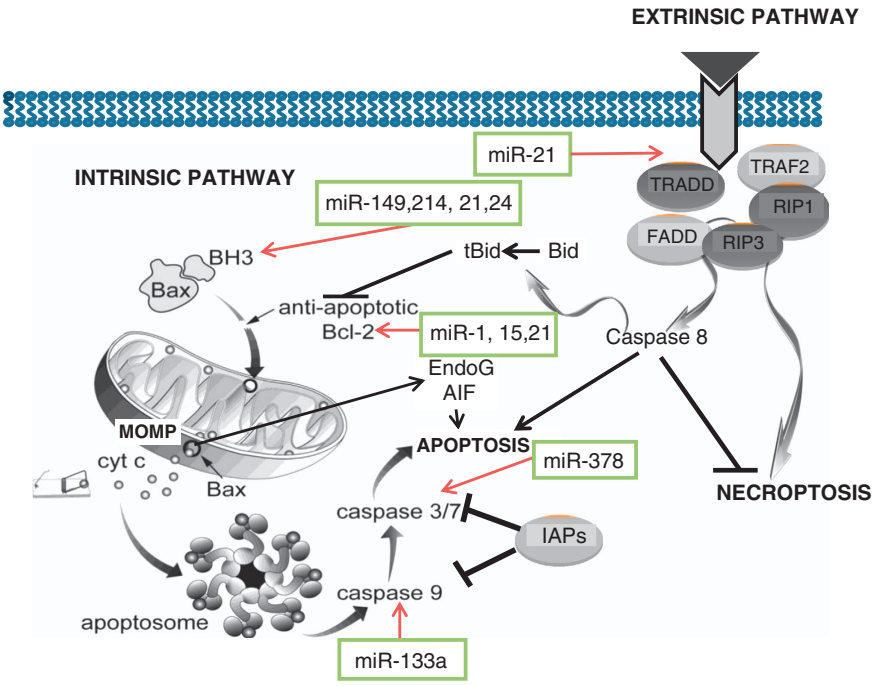

Figure 1 A simplified schematic representation of the extrinsic and the intrinsic (mitochondrial) pathways of apoptosis and necroptosis. The mitochondrial (intrinsic) pathway of cell death is regulated both upstream (Bcl-2 proteins) and downstream (e.g., IAP proteins) of mitochondria. In response to stress, mitochondria undergo permeabilisation of the outer mitochondrial membrane (MOMP), which leads to a release of a number of pro-apoptotic factors such as cytochrome *c*, AIF or EndoG. The extrinsic pathway of apoptosis is activated via a complex signal transduction from the plasma membrane, whereby the death receptors (e.g., Fas, TNFR) bind their cognate ligands (e.g., FasL, TNF), oligomerize, activate their intracellular death domains and recruit a number of receptor-associated proteins such as RIP1 or TRADD. The multiprotein complex then recruits the initiatior pro-caspase-8 leading to its activation. Active caspase-8 propagates the apoptotic signal either by direct activation of executioner caspases, or by cleaving a BH3 protein Bid which then leads to MOMP. In addition, components of the protein machinery that regulates the extrinsic pathway of apoptosis are also involved in regulation of necrosis. The figure also demonstrates apoptosis regulatory microRNA and their target genes in apoptotic pathways

formation of the death-inducing complex (DISC) that activates caspase-8 (Figure 1). In some cell types, caspase-8 cleaves the BH3 protein Bid, forming truncated Bid (tBid) and ultimately inducing the mitochondrial outer membrane permeabilisation. Thus, in some contexts, there is a cross talk between the extrinsic and the intrinsic pathway of apoptosis.

Cardiomyocytes undergo apoptosis in response to a plethora of stimuli including hypoxia (especially followed by reoxygenation), acidosis, oxidative stress, glucose deprivation and metabolic restriction, β_1-adrenergic agonists, stretch, angiotensin II (Ang II), TNF-α, Fas L and anthracyclines as reviewed in.[41] Even though it is often postulated that necrosis is the main pathway responsible for cardiomyocyte death following MI, several observations in human, rabbit, rat and mice hearts indicate that apoptotic cell death contributes to the overall cardiomyocyte loss following MI, particularly during subacute and chronic stages of infarction and after reperfusion,[42–47] as well as in end-stage cardiomyopathy and in terminally failing human myocardium.[48,49] Cardiomyocyte apoptosis has also been shown to contribute to left ventricle dysfunction following cardiac surgery,[50] particularly after retrograde cardioplegia.[51] Moreover, inhibition of post-ischemic cardiomyocyte apoptosis emerges as an effective therapeutic avenue to improve diastolic heart function after ischemia.[52]

Studies indicate the relevance of both the mitochondrial and the extrinsic pathway of apoptosis in cardiac pathologies. The mitochondrial pathway of apoptosis is activated following myocardial ischemia-reperfusion injury,[53] whereas TNF-α, a trigger of the death receptor pathway, is rapidly released from resident mast cells and macrophages in response to myocardial ischemia,[54] and can also be released from cardiomyocytes.[55] Overexpression of FasL was also shown to increase cardiomyocyte apoptosis *in vitro* and *in vivo*.[56] The fact that multiple cell death pathways are detectable and occur side-by-side is not surprising, and is a common phenomenon observed in contexts ranging from retrovirus-induced immunosuppression[57] to chemotherapy-induced cancer cell death.[58–60] Therefore, it is well recognized that activation of a particular cell death pathway does not constitute sufficient evidence to support its physiological importance in the pathological process, and instead needs to be supported by prevention of cell loss using pathway-specific pharmacological and/or genetic inhibition. In this regard, conflicting results have been published. For example, it was observed that Fas-deficient Ipr mice exhibit reduced infarcts and diminished apoptosis after myocardial ischemia-reperfusion (I/R) injury, both *ex vivo* and *in vivo*,[56,61] whereas in transgenic mouse lines with cardiomyocyte-specific overexpression of Bcl-2 or dominant-negative Fas-associated death domain (FADD)[53] only inhibition of the mitochondrial pathway provided a significant decrease in the size of infarction following coronary artery ligation. Of note, in the latter study, there was still a substantial decrease in the number of apoptotic (TUNEL positive) cardiomyocytes in FADD dominant-negative mouse line, suggesting that forms of cell death other than apoptosis

were contributing to the infarct size. In addition, in lpr mice Fas-deficiency is not limited to cardiomyocytes. Therefore, more studies in models where apoptosis contributes significantly to the infarct, and where it is blocked specifically in cardiomyocytes, are needed to provide more evidence as to the role of specific apoptotic pathways.

MiRNAs that Regulate Cardiovascular Sensitivity to Apoptosis

The role of miRNAs in regulating the expression of anti- and pro-apoptotic genes and regulation of cardiomyocyte and endothelial cell survival is well documented (Table 1). miRNAs appear to regulate cardiomyocyte apoptosis through multiple mechanisms. When it comes to the mitochondrial pathway of apoptosis, several miRNAs target the *BCL2* gene, including miR-1, miR-15 family and miR-21, whereas *PUMA* and *BIM*, which encode for the pro-apoptotic BH3 proteins, are targeted by miR-149 and miR-214 (as well as miR-24), respectively (Table 1). In addition, mitochondrial fission machinery is regulated by miR-30 family, which targets the *TP53* gene encoding for p53 protein which in turn regulates the expression of mitochondrial fission protein dynamin-related protein-1 (*Drp1*), and by miR-761, which targets a gene encoding for mitochondrial fission factor (*Mff*) (Table 1).[62] Of note, a particular recent study suggests that *Mff* is responsible for recruiting *Drp1* to the foci at the outer mitochondrial membrane.[63] Downstream of mitochondria, the expression of *CASP9*, which encodes for the initiator caspase-9, is regulated by miR-133a, whereas *CASP3*, which encodes for the executioner caspase-3, is regulated by miR-378 (Table 1). In addition, miR-21 regulates genes involved in the death receptor pathway of apoptosis, including death domain-associated protein gene *(DAXX)* and *FASLG*, which encode for death receptor-associated protein and Fas L, respectively (Table 1).

Genes encoding proteins that control ROS signaling are also enriched among the targets of apoptosis-regulating cardiac miRNAs. In particular, three miRNAs, that is, miR-34a, miR-195 and miR-199a, target *SIRT1* (Table 1), which encodes for a founding member of a family of NAD-dependent deacetylases called sirtuins. When expressed at low levels, *SIRT1* has been reported to protect cardiomyocytes from oxidative stress and apoptosis, and stimulation of Sirt1 protein mimics ischemic preconditioning and protects heart from I/R injury (reviewed in greater detail by Sundaresan *et al.*[64] and Yamamoto and Sadoshima[65]). Other ROS signaling genes targeted by cardiac miRNAs include *TXN* (encoding for thioredoxin) and *SOD* (encoding for superoxide dismutase). Interestingly, a study on mice with cardiac-specific overexpression of Sirt1 revealed that it upregulates the expression of both superoxide dismutase and thioredoxin 1.[66] Considering that multiple miRNAs associated with apoptotic regulation in cardiomyocytes impinge on the network of SIRT1-ROS signaling, while some other miRNAs affect ROS-induced signaling pathways, such as changes in free calcium (for example, miR-145[67]), more research is required to delineate the exact mechanism and causal role of miRNAs in maintaining cardiac redox homeostasis as well as the cell death regulatory role of redox-sensitive miRNAs.

Consistent with tissue-specific functions, many miRNAs have distinct biological effects depending on the cell type. For example, inhibition of miR-24 has been reported to promote cardiomyocyte apoptosis while decreasing the survival of endothelial cells.[68] Considering this, more work is needed to investigate the apoptosis-regulating effects of miR-17-92 cluster, which appears to be expressed at high level in the heart and has been extensively reported to exert anti-apoptotic effects in tumor cells.[69] Only few studies, however, have been reported so far with regards to the cardiac apoptosis regulation by this miRNA cluster (see Table 1). Of note, the variability in the role of miRNAs is observed not only between the cell types, but also between cellular processes, particularly as they relate to the effect on tissue functioning. For example, in cardiac myocytes, the signaling pathways that regulate hypertrophy impinge also on the balance between cell survival and cell death,[70] and many miRNAs may be involved in regulation of this delicate balance.

The striking abundance of miRNAs involved in the regulation of apoptosis in cardiomyocytes suggests the possibility of an extensive overlap of regulatory functions and effects on intracellular signaling. Such synergy between miRNAs has been recently investigated using receiver operating characteristic (ROC) analysis, which indicated that miR-21 scores high for its synergistic effects. Of particular interest, the synergistic effect of miR-21 and miR-1 was detected, and functionally validated in the context of regulation of myocardial apoptosis, cardiac hypertrophy and fibrosis.[71] Here, we have used the integrated parameter synergy score calculations (Figure 2), based upon the experimentally validated and confidently predicted miRNA-target interaction data obtained from miRSel[72] and TargetscanHuman 6.2, to calculate the synergy scores for the miRNAs linked to cardiomyocyte apoptosis (Figure 3). In total, we identified 35 synergistic (synergy score of more than 2.0) pair-wise combinations of miRNAs. This finding confirms that the synergistic action of these miRNAs on cardiomyocyte apoptosis should not be ignored. As observed before,[71] miR-21 was most commonly involved in synergistic interactions ($n=10$) (Figure 3a), with particularly strong synergy between miR-21 and miR-1 (Figure 3b), followed by two miR-15 family members miR-15a and miR-16 (each of them synergized with nine other miRNAs). In comparison, several miRNAs were not involved in synergistic interaction, as indicated by the synergy score <2.0. These miRNAs include miR-133a, miR-149, miR-19b, miR-210, miR-214, miR-30, miR-320, miR-378, miR-499 and miR-761 (example given in Figure 3c). Overall, this analysis indicates that there is a selective synergism among endogenous miRNAs in regulating complex biological processes such as apoptosis.

In addition, we used the Ingenuity Pathway Analysis (IPA) to determine network of connections between the identified apoptosis-regulating miRNAs of reported relevance to cardiovascular disease (Figure 4). We observed that many miRNAs are regulated through each other either directly or indirectly, and many are indirectly regulated by the same signaling molecules, for example, beta estradiol (Figure 4). In this context, it is interesting to note that to the best of our knowledge, no experimental studies have been published so far on estradiol-mediated regulation of cardiac miRNAs,

Table 1 Summary of miRNAs linked to apoptotic regulation in cardiomyocytes

miRNAs	Relevance to CVD	Targets relevant to apoptosis	Refs
miR-1	Serum expression upregulated in human AMI and in patients after open-heart surgery with cardiopulmonary bypass Overexpression enhances and inhibition attenuates apoptosis and infarct area after cardiac I/R injury in mice Overexpression inhibits apoptosis in a rat model of cardiac hypertrophy induced by pressure overload Ischemic post-conditioning upregulates miR-1 and inhibits cardiomyocyte apoptosis in rats Inhibition *in vitro* in cardiomyocytes reduces H_2O_2-induced and high-glucose-induced apoptosis Upregulation has pro-apoptotic effect in H9c2 cells exposed to oxidative stress miR-1 transfected ES cells protect host myocardium from MI-induced apoptosis overexpression enhances the angiogenic differentiation of human cardiomyocyte PC Upregulated in rat cardiomyocytes exposed to high glucose Downregulated in response to Tanshinone IIA	*PRKCE* (protein kinase C) *HSPD1* (HSP60) *BCL2*	147–159
miR-15 family	Upregulated in response to MI Silencing *in vitro* renders cardiomyocytes resistant to hypoxia-induced cell death Regulates angiogenic activity of endothelial cells	*BCL2*	160–163
miR-133a	Upregulation of miR-133a following ischemic post-conditioning miR-133a mimic attenuated IR-induced apoptosis in rats Anti-apoptotic effect in H9c2 cells exposed to oxidative stress Elevated levels of miR-133a in patients with ST-elevation myocardial infarction (STEMI) linked to more severe injury Increased expression in Tanshinone IIA-treated hypoxic neonatal cardiomyocytes	*CASP9*	155,156,164,165
miR-17-92 cluster	Overexpression of the cluster results in lethal cardiomyopathy Expression decreases in aging mice hearts miR-20a is upregulated in mechanically stretched neonatal rat cardiomyocytes and exerts anti-apoptotic effect Overexpression of miR-19b inhibits apoptosis in P19 cells		166–169
miR-21	Myocardial upregulation of miR-21 reduces MI size and apoptotic rate by increasing Bcl-2 levels Expression declines in cardiac myocytes upon exposure to hypoxia, and increases after ischemic preconditioning Overexpression diminishes murine coxsackievirus B3-induced myocardiatis Overexpression in transgenic mouse heart results in smaller infarct following ischemia Expression elevated in circulating endothelial progenitor cells from diabetic patients and protective from high-glucose-induced apoptosis Expressed in cardiac valve endothelium, where it regulates the development of AV valve	*BCL2 FASLG* (FASL) *PDCD4* *ANXA2* *SOD2 TXNDAXX*	170–176
miR-24	Expression lower in peri-infarct tissue in mouse model of MI Inhibition induces cardiomyocyte apoptosis *In vivo* overexpression inhibits cardiomyocyte apoptosis and attenuates infarct size Inhibition enhances EC survival	*BIM*	68,177
miR-30	Inhibits mitochondrial fission and apoptosis in cardiomyocytes	*TP53*	178
miR-150	Upregulated in cardiac myocytes treated with H_2O_2 Silencing protects from H_2O_2-induced apoptosis Dysregulated in human MI	*MYB* (c-myb)	147,179
miR-210	Upregulated in hypoxic cardiomyocytes Overexpression reduces cell death in response to oxidative stress Deregulated in human MI	?	147,180
miR-199a	Downregulated to undetectable levels during cardiac ischemia *in vitro* and *in vivo* Overexpression inhibits hypoxia-induced expression of several pro-apoptotic genes (e.g. *CASP3* and *CASP9*)	*HIF1A SIRT1*	181
miR-320	Downregulated in murine hearts following I/R Overexpression enhanced cardiomyocyte apoptosis	*HSPB6 (Hsp20)*	182
miR-149	Overexpression decreases apoptotic sensitivity G-allele of A > G SNP in pre-miR-149 decreases production of miR-149 and influences cardiac function in mouse model of MI	*BBC3* (*PUMA*)	183
miR-761	Inhibits mitochondrial fission Knockdown diminished H_2O_2-induced and I/R-induced cardiomyocyte apoptosis and infarct size in mice	*MFF*	62
miR-499	Inhibits cardiomyocyte apoptosis	*PPP3CA*, *PPP3CB*	184
miR-214	Protects cardiomyocytes from H_2O_2-induced apoptosis *in vitro* Genetic deletion in mice increases cardiac apoptosis	*PTEN BIM*	185,186
miR-145	Circulating levels reduced in patients with coronary artery disease Ameliorates ROS-induced apoptosis in cardiomyocytes	*CAMK2G*	67,187
miR-378	Downregulated in a rat model of myocardial ischemia Overexpression in H9c2 cardiomyocytes reduces apoptosis and necrosis Inhibition increases H_2O_2-induced apoptosis	*CASP3*	188
miR-195	Inhibition leads to decreased ROS production and apoptosis in palmitate-treated mouse cardiomyocytes	*SIRT1*	189
miR-34a	Expression increases with aging (in mice) Inhibition reduces cardiomyocyte apoptosis Levels higher in endothelial progenitor cells from coronary artery disease patients Regulates SIRT1 expression in endothelial progenitor cells and contributes to endothelial senescence	*SIRT1 PNUTS*	125,190,191

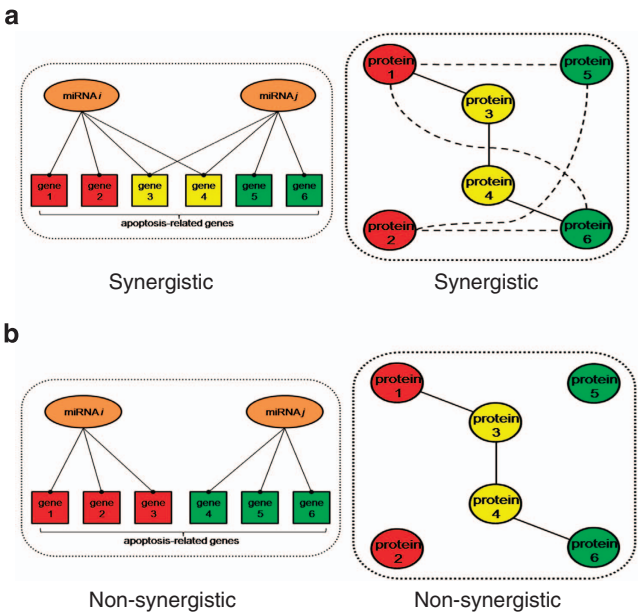

Figure 2 A graphic representation for quantitative assessment of miRNA synergy. (**a**) Targeting more common genes creates synergy between two miRNAs. (**b**) Denser functional association between proteins encoded by individual miRNA-target genes makes two miRNAs more likely to act synergistically

despite a well-recognized cardioprotective (via an anti-apoptotic mechanism) function of estrogen.[73–86]

Autophagy, a Double-Edged Sword in Cardiovascular Disease

Autophagy is an evolutionarily conserved mechanism of cellular self-digestion in which long-lived proteins and entire organelles are degraded through delivery to lysosomes (Figure 5). It is characterized by the formation of autophagosomes, cellular structures that encapsulate cytoplasmic cargoes. Autophagosomes ultimately fuse with lysosomes to form autolysosomes in which the contents are degraded into their constituent parts and delivered back into the cytosol for further biocatalysis or to be utilized in biosynthetic pathways.

It is well documented that basal levels of autophagy, operating in the majority, if not all, cells, serve to maintain homeostasis by removing misfolded or aggregated proteins, and by clearance of damaged organelles such as mitochondria and endoplasmic reticulum.[87,88] Both the rate of autophagy and the selection of cargoes destined for degradation can, however, change in response to specific external and internal cues. In this context, excessive autophagy, induced in response to stress signals, can have dual effects, acting either to maintain cell survival (for example, by providing essential energy for sustaining physiological function during the times of catabolic defects) or contributing to cell death (e.g., by diminishing cell volume or providing energy for the execution of apoptosis).

Defects in the process of autophagy have been implicated in numerous human diseases, including cardiovascular diseases such as hypertrophy and heart failure (reviewed in Marzetti *et al*.[89–92]). Moreover, induction of autophagy by perioperative ischemia/reperfusion has been observed.[93] Considering that autophagy can potentially exert both pro- and anti-survival function, it is not surprising that its role in cardiovascular disease remains relatively poorly understood, with some studies pointing toward its beneficial effects,[94–99] while others suggest its detrimental role.[100] Nevertheless, the preponderance of evidence supports the notion of beneficial role for autophagy in the heart.

MiRNAs and Cardiovascular Autophagy: The Causality Dilemma

The role of miRNAs in regulation of autophagy was first suggested in 2009, when *BECN1*, a gene encoding for Beclin 1 which is an important factor controlling vesicle nucleation (see Figure 5), was shown to be posttranscriptionally regulated by miR-30a.[101] Since then, multiple miRNAs have been linked to the autophagic pathway and have been associated with disease states, including cardiac pathologies (Table 2). In addition, there are several miRNAs that have been reported to modulate autophagy in other tissues, and have also known roles in cardiac function. For example, in hematopoietic cells, miR-17 has been shown to regulate the expression of SQSTM1 (p62), an ubiquitin-binding protein and regulator of autophagy-mediated protein degradation,[102] and the expression of *ATG7* in glioblastoma cells.[103] It also appears to modulate cardiac remodeling following MI.[104] Whether miR-17 regulates cardiac autophagy remains to be investigated.

We conducted pathway analysis of miRNAs linked to cardiac autophagy, using the Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Qiagen, Redwood City, CA, USA), and discovered two main nodes of interaction, that is p53 and STAT3 (signal transducer and activator of transcription 3) (Figure 6). Interestingly, both p53 and STAT3 are known as critical regulators of autophagy. Specifically, STAT3 inhibitors or genetic ablation stimulate autophagic flux,[105,106] and reduced levels of STAT3 are reported in patients with end-stage heart failure.[107,108] Moreover, the activation of STAT3 by angiotensin (Ang)II-induced Janus-activated kinase 2 (JAK-2) is impaired in failing human cardiomyocytes.[109] With regards to p53, its inhibition is cardioprotective against ischemic injury, and an increase in autophagic flux has been observed in p53 ($-/-$) heart under ischemic conditions.[110] This effect is suggested to occur through the p53-TIGAR axis,[110] with TIGAR (TP53-induced glycolysis and apoptosis regulator) being involved in regulation of the glycolysis and pentose phosphate pathway, ROS-levels, and inhibition of apoptosis and autophagy.[111,112] The p53-TIGAR axis has also been specifically shown to regulate cellular energy homeostasis and cell death in cardiomyocytes under ischemic stress.[113] There is a need for more detailed studies on the role of miRNAs in regulation of such complex signaling pathways, and their role in the context of myocardial cell death.

At the Crossroad of Apoptosis and Autophagy

It is a hackneyed expression that simultaneous induction of multiple cell signaling pathways occurs in biological systems, but it is nonetheless true, even in the context of concurrent induction of cell death and cell survival pathways. Concomitant

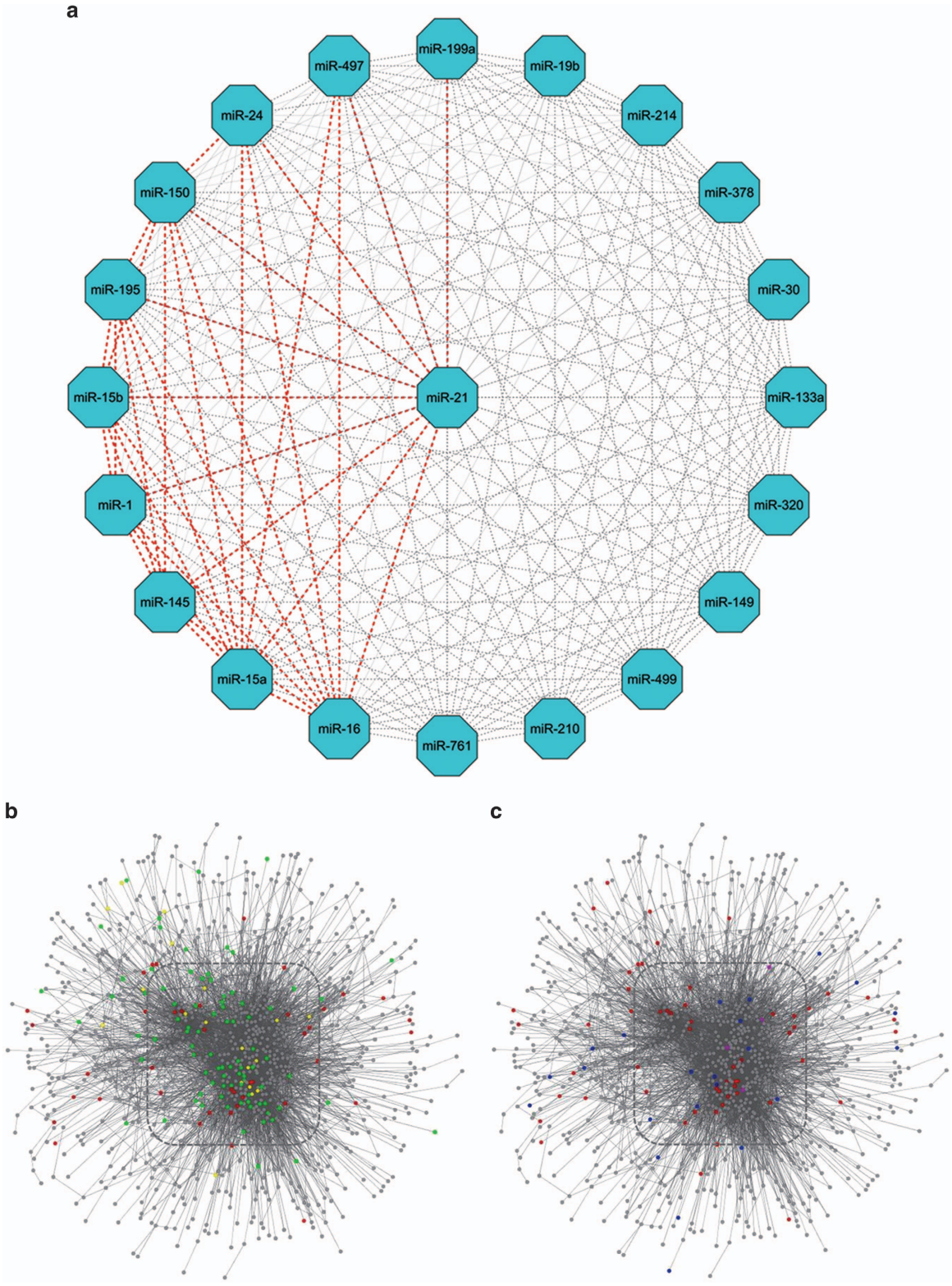

Figure 3. miRNA synergy in cardiac apoptosis. (**a**) Synergy score calculation for random combinations of validated miRNAs that are involved in cardiac apoptosis. Synergy scores of more than 2.0 are highlighted as thick red-dash lines. (**b**) Network location of apoptosis-related proteins encoded by target genes of miR-1 (red nodes), miR-21 (green nodes) and both (yellow nodes). (**c**) Network location of apoptosis-related proteins encoded by target genes of miR-1 (red nodes), miR-30 (blue nodes) and both (purple nodes). Synergistic apoptosis regulation should be expected for the pair of miR-1 and miR-21 instead of miR-1 and miR-30, due to more common target genes and denser functional association between gene-encoded products. Synergy score$_{\text{miR-1:miR-21}} = 3.23$; Synergy score$_{\text{miR-1:miR-30}} = 1.13$; Box shows the network core area

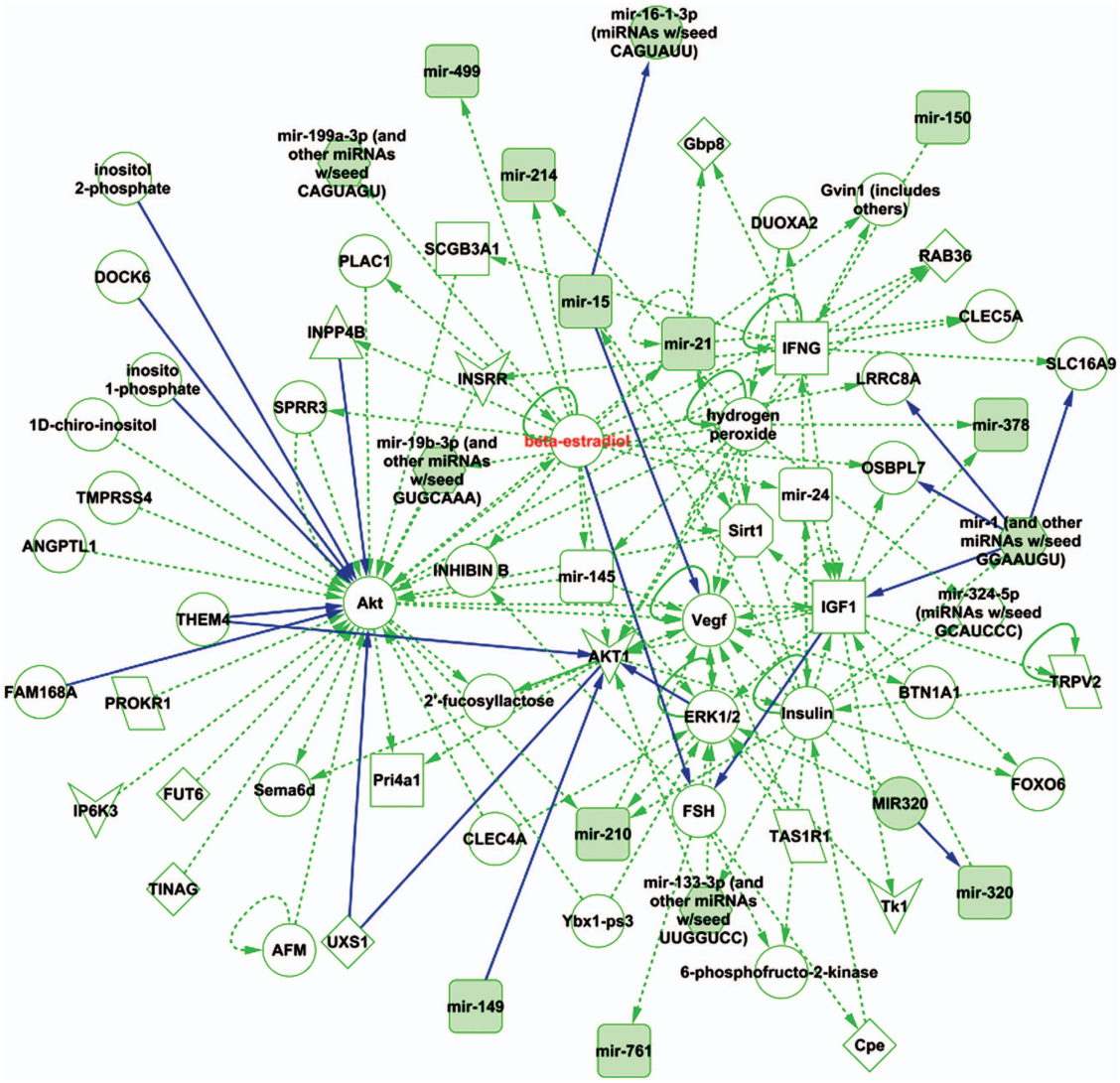

Figure 4 Ingenuity Pathway Analysis of the miRNAs identified as involved in regulation of cardiomyocyte apoptosis (see Table 1). The analysis revealed several regulatory nodes, with one particularly interesting being the oestradiol regulation (highlighted in red) of several of the miRNAs

induction of autophagic and apoptotic signaling, and the extensive cross talk between these two pathways have been reported extensively in cancer cells[114] and also in cardiac cells.[115] In general, the nodes of cross talk include Beclin-1–Bcl-2 interaction (reviewed in Marquez and Xu[116]), Beclin-1–Bim interaction,[117,118] caspase-mediated Beclin 1 cleavage,[119,120] caspase- and calpain-mediated Atg5 cleavage,[121] UVRAG–BAX interaction,[122] ATG12–Mcl-1 and ATG12–Bcl-2 interaction,[123] ATG5–FADD interaction,[124] p53-mediated cross regulation,[111] as well as regulation of Akt signaling that has differential effects on autophagy and apoptosis (Figure 7).

Several models can be put forward to suggest the possible role of miRNAs in regulation of autophagic–apoptotic cross talk. Firstly, different transcripts can share a common miRNA-binding site and compete for the same miRNA. It can be envisaged that overexpression of one of the transcripts, for example, one that regulates apoptosis, could leave less miRNAs free to bind the autophagy-related transcript (a buffering effect) and thus shift the balance between apoptosis and autophagy. Secondly, any miRNA regulating the expression of genes that encode the autophagy–apoptosis node proteins listed above can contribute to the switch between the two pathways. Finally, miRNAs that target histone deacetylases (such as sirtuins), which regulate the expression of both autophagic and apoptotic genes, are likely to impact the dynamic relationship between these two pathways.

As yet, the role of miRNAs that potentially affect the cross talk between apoptosis and autophagy on cardiac cell survival and tissue homeostasis remains largely unknown. Recent studies identified that genetic deletion of aging-associated miR-34a reduces cardiomyocyte cell death and improves functional recovery after MI, which is attributed to the function of a novel miR-34a target gene, *PNUTS*.[125] One of the cellular effects of Pnuts protein is nuclear sequestration of Pten,[126] a negative regulator of Akt, which in turn is a master regulator of both apoptosis and autophagy (Figure 7). Moreover, another miR-34a target, *SIRT1*, is known to regulate both apoptosis (via a p53 pathway and ROS signaling) and autophagy

(via p53 and the activation of FoxO transcription factor family members).[127,128] Thus, miR-34a-mediated regulation of *PNUTS* and *SIRT1* expression in cardiac cells could contribute to changes in apoptosis–autophagy switch in response to stress and during cardiac recovery and remodeling.

Interestingly, increased levels of the miRNA miR-34a appear to be also associated with telomere shortening, with overexpression of *PNUTS* in human cardiomyocytes leading to telomere attrition.[125] Telomere shortening is a well-known trigger of cellular senescence and has also been associated with several CVDs, including MI and the onset and progression of arterial hypertension.[129] Contrary to cell death, senescence is a process of irreversible cell cycle arrest which allows cells to remain viable and metabolically active for a long time (at least in *in vitro* cell culture). Cells undergoing senescence exhibit several phenotypic changes, including enhanced autophagy, the role of which, according to a recent study, is to process cytoplasmic chromatin fragments budded off nuclei.[130] Another miR-34a target gene, *SIRT1*, is also known to influence certain aspects of vascular ageing, including senescence.[131,132] This suggests another mechanism for miRNA-regulated cardiomyocyte autophagy involving senescence signaling pathways.

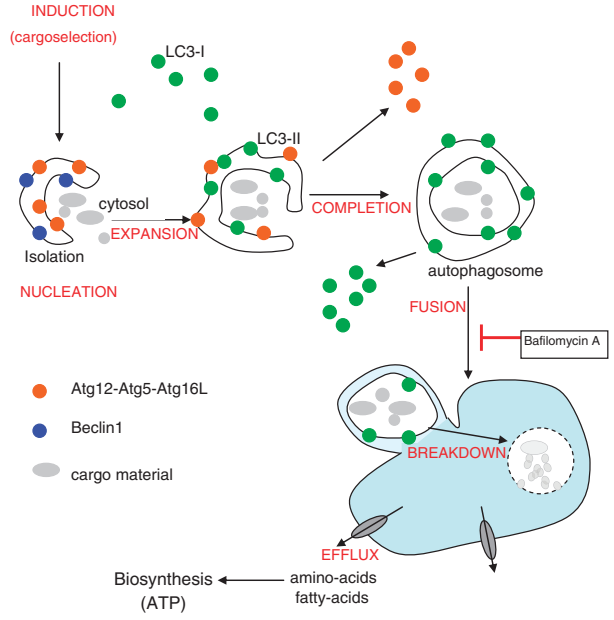

Figure 5 Key steps in the autophagic pathway. The process is initiated by the formation of autophagosomes, double-membrane vesicles that engulf fractions of the cytosol. Autophagosomes undergo a step-wise maturation associated with expansion and completion of the sequestering vesicle, which is regulated by many ATG proteins, particularly Beclin-1 and two ubiquitin-like conjugation systems Atg12-Atg5-Atg16L and Atg8(LC3)-PE. Autophagosomes then fuse with lysosomes to form single-membrane autolysosomes. In this process, autophagosomes acquire hydrolytic enzymes and are able to degrade the sequestered material. Recycling of the basic components, such as amino or fatty acids, helps the cell to maintain homeostasis

miRNAs in Cardiovascular Necrosis

Necrosis is a type of cell death which is morphologically characterized by increase of cell volume, dilation of organelles, rupture of the plasma membrane and subsequent loss of intracellular contents.[34] For a long time, necrosis was considered to be a passive, accidental and unregulated form of cell death, and as such was sharply contrasted against heavily regulated and tightly orchestrated apoptosis. This view has changed over the last few years, with increasing number of reports describing the regulatory network that governs necrotic cell death (reviewed in Henriquez *et al.*[133] and Wu *et al.*[134]). Many authors suggest that myocardial ischemia results predominantly in necrotic cell death,[135] due to depletion of ATP, increased calcium load, acidosis and severe oxidative stress, with apoptotic cell death playing a role following reperfusion. Accordingly, inhibitors of necrotic cell death appear to be effective at reducing myocardial cell death and infarct size in animal models.[136] In addition, myocardial necrosis is relatively common among infants who die as a result of congenital heart disease, perinatal asphyxia, sepsis or coronary artery abnormalities.[137]

So far, only limited evidence has been gathered on the role of miRNAs in regulation of cardiac necrosis. A recent study has reported that miR-874 inhibits cardiomyocyte necrosis, *in vitro* as well as in animal model of MI, via downregulation of

Table 2 Autophagy-modulating miRNAs with relevance to CVD

miRNAs	Relevance to CVD	Targets relevant to autophagy	Function	Refs
miR-30	Downregulated in a model of cardiac hypertrophy and by angiotensin II Circulating miR-30 elevated in patients with left-ventricular hypertrophy	*BECN1*	Vesicle nucleation	192
miR-204	Reduces cardiomyocytes autophagy in response to hypoxia-reoxygenation			193,194
	Concomitant downregulation of miR-204 and induction of autophagy following cardiac ischemia-reperfusion in rats Downregulated in pulmonary arterial smooth muscle cells from patients with PAH	*MAP1LC3A*	Vesicle maturation and fusion with the lysosome	195
miRNA-212/132	Impaired autophagy in starved cardiomyocytes Induced cardiac hypertrophy	*FOXO3 PTEN*	Pro-autophagic transcription factor	196
miR-21	Cell treatment with anti-miR-21 induced autophagy Not yet investigated with regards to cardiomyocte autophagy	*BECN1 MAP1LC3A PIK3C3* (*VPS34*)		197

Figure 6 Ingenuity network analysis of miRNAs identified as regulators of autophagy in cardiomyocytes (and miR-21)

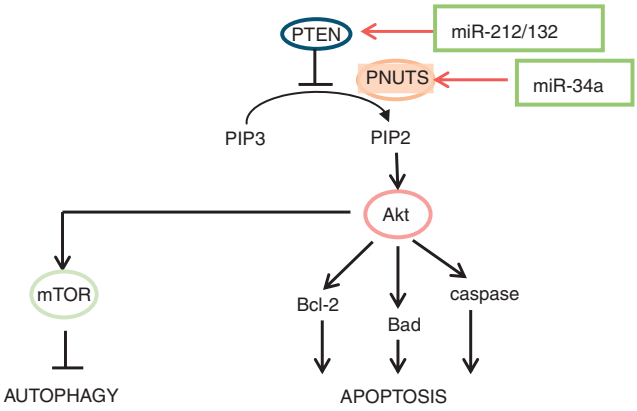

Figure 7 MiR-34a as potential regulator of the cross talk between autophagic and apoptotic signaling in cardiac cells. PNUTS is a PTEN-associated protein that sequesters PTEN to the nucleus[127]

the expression of caspase-8,[138] which normally serves not only as an element of the death receptor pathway, but also as a pro-survival regulator that supresses necrosis.[139] Apart from caspase-8, other components of death receptor pathway, for example, receptor interacting protein 1 (RIP1), are also involved in regulation of necrosis (see Figure 1). In this regard, overexpression of miR-155 was reported to attenuate necrotic cell death in cardiomyocyte progenitor cells exposed to hydrogen peroxide.[140] Considering the continuum and interdependence of cell death pathways, as well as the clinical importance of necrosis in conditions such as myocardial infarction, more work is needed to decipher the exact role of miRNAs in necrotic cell death.

Necroptosis is a specialized pathway of programmed necrosis (Figure 1), targeting the kinase RIP1 involved in extrinsic pathway of apoptosis.[141] Necroptosis shares characteristics of both necrosis and apoptosis.[142,143] A study on mice model has showing that a bolus dose of necroptosis inhibitor, necrostatin-1, in mice at the time of coronary reperfusion dramatically reduced infarct size after I/R njury.[144] These results indicates important role of necroptosis in regulation of cardiac cell death. Involvement of necroptosis in myocardia injury after Mi and in other cardiovascular conditions has not been investigated in detail. Further investigation on this pathway may hold promise for finding further clinical targets for treating heart diseases.

Can miRNAs Regulate Cell-to-Cell Variability in Cardiac Cell Death?

The performance of the apoptotic network determines the individual cell's probability to die, which, when assessed over a large population of cells, can be translated into the percent of cell death at a given time point. The apoptotic pathway consists of many processes that are stochastic in nature, that is, occur with certain probability rather than in a deterministic fashion. These processes include protein translocation (e.g., translocation of Bax from the cytosol to mitochondria) or formation of multiprotein complexes (e.g., the apoptosome). The architecture of the network includes also positive and negative feedback loops, which may buffer or enhance the inherent noise in the apoptotic signaling. Therefore, the net balance between the amount of pro- and anti-apoptotic proteins, the spatial and temporal availability of protein partners for interaction and functioning of the feedback loops are all critical for determining the performance of the apoptotic network and thus the threshold of stress signal required for successful execution of cell death at the level of cell population.

Gene expression is subject to stochastic variability even among the cells with the same genotype and environment.[145] The inherent stochastic nature of transcriptional regulation affects cell ability to control gene expression levels in response to extracellular stimuli, including stress cues. In the context of cell death, such cell-to-cell variability translates to dramatic differences in the time between exposure to stress and the execution of cell demise.[9,12] The differences in kinetics of cell death are particularly pronounced when critical events involve small numbers of molecules, for example, apoptosome formation. Importantly, for many genes, the noise in the expression pattern increases as their expression decreases. As miRNAs affect cell homeostasis by regulating gene expression, it is conceivable that they inherently contribute to the cell-to-cell variability in cell signaling as well as to the robustness of cell phenotypes and responses to stimuli, including pro-apoptotic stimuli. This field of research remains, as of now, largely unexplored, with only a few attempts to analyze the stochastic properties of regulatory clusters involving miRNAs.[146] It is, nevertheless, and exciting avenue with potentially far-reaching implications when it comes to regulation of cardiomyocyte cell death, particularly in the contexts of a delayed and slow-progressing response outcome of which can be substantially affected by changes in cell-to-cell variability. This line of research will require not only knowledge of the effects of specific miRNAs on oscillations in gene expression patters but also stochastic version of deterministic models employed traditionally to study cell signaling pathways.

Summary and Future Outlook

Through an in-depth analysis of the current evidence linking miRNAs with regulation of cardiomyocyte cell death pathways, and the understanding of regulatory nodes (including cross talks between the pathways and non-genetic regulation of cell-to-cell variability in cell death), we have identified several new research avenues, pursuit of which may be of pivotal importance to our understanding of regulatory circuits that govern survival and death of cardiomyocytes. These avenues include the role of redox-sensitive miRNAs, the effects of beta estradiol in regulation of miRNAs expression and action in the heart, the role of miRNAs in regulating the balance between pro-survival and death pathways as well as cell-to-cell variability in timing and extent of phenotypic response. The emerging systems-biology approach to studying the functional synergy between cardiac miRNAs in the context of cell death pathways should be extended to incorporate more extended network of signaling pathways, including ROS signaling. This should help to elucidate the full impact of miRNAs on gene expression networks and biological pathways. In the context of these observations, there is a need for investigation of aging-associated cardiac miRNAs that affect cell death pathways. Further studies are also required to understand mechanisms responsible for reported cardiac microRNA changes during myocardial infarction, chronic kidney disease, hypertension, diabetes and other cardiac pathological conditions of cardiac death. Analysis of the fine balance between the apoptotic and hypertrophic roles of cardiac miRNAs will contribute to the understanding of the complex interactions between multiple cardiac-specific miRNAs in aging. We envisage that all these studies will form a solid framework for development of future therapies for the treatment of cardiovascular diseases, based on anti-miR/miRNA overexpression approaches.

Conflict of Interest

The authors declare no conflict of interest.

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