LIN28/let-7 Axis as a Regulator of Myocardial Ischemic Injury

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UNIVERSITY OF MIAMI

LIN28/LET-7 AXIS AS A REGULATOR OF MYOCARDIAL ISCHEMIC INJURY

By

Shaurya Joshi

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida

May 2016
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

LIN28/LET-7 AXIS AS A REGULATOR OF MYOCARDIAL ISCHEMIC INJURY

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One of the prominent causes of heart failure is atherosclerosis of the coronary arteries. Obstruction of blood flow within the coronary arteries results in inadequate perfusion, or ischemia, of the heart muscle.

The let-7 family of microRNAs (miRs) regulates critical cell functions, including survival signaling, metabolic control and glucose utilization, which may be important during myocardial ischemia. MiR-let-7 expression is under tight temporal and spatial control through multiple redundant mechanisms. The mechanisms and functional consequences of miR-let-7 regulation in ischemic myocardium largely remain unknown.

Here we show that miR-let-7c, -7a and -7g are downregulated in the adult mouse heart early after coronary occlusion, and in neonatal rat ventricular myocytes subjected to hypoxia. This occurs post-transcriptionally and is independent of glucose depletion. Hypoxia also induced Lin28, a negative regulator of let-7. Both changes were abrogated by treatment with the histone deacetylase inhibitor trichostatin A. Let-7 repression and Lin28 induction were absent in cardiac fibroblasts. Restoration of let-7g to hypoxic myocytes and to ischemia-reperfused mouse hearts in vivo potentiated the activation of Akt and prevented cell death. Mechanistically, phosphotidyl inositol 3’kinase interacting
protein 1 (PIK3IP1), a negative regulator of PI3K, was identified as a novel target of miR-let-7 by a crosslinking technique the targeted PIK3IP1 to the myocyte RISC. Finally, in non-failing and failing human myocardium, we found specific inverse relationships between Lin28 and miR-let-7g, and between miR-let-7g and PIK3IP1.

This study reveals a conserved hypoxia-responsive Lin28-miR-let-7-PIK3IP1 regulatory axis that is specific to cardiac myocytes and promotes apoptosis during myocardial ischemic injury.
I would like to thank Dr. Salil Sharma and Dr. Sumit Jain, former graduate students in the laboratory. Their technical guidance was helpful in the initial stages of this project.

I would like to thank Dr. Jian Qin Wei for his assistance in the animal surgery and echocardiography.

I would like to thank Svetlana Speransky for her technical assistance and suggestions.

I would like to thank the American Heart Association for awarding me with a pre-doctoral fellowship (Award ID: 12PRE12080052 to Shaurya Joshi).
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Chapter 1

INTRODUCTION

1.1. Heart Failure

Heart failure (HF) is a condition in which the heart loses its ability to supply blood to the peripheral organs sufficient to maintain their physiological function. This decreased capacity results from impairment of myocardial contraction, relaxation or a combination of the two. HF is the leading cause of mortality and morbidity worldwide. HF affects almost 6 million people in the United States and 22 million worldwide [1]. Although advances have been made in understanding the basis of the disease, we are far from a cure. The mortality rate is a startling 50% within 5 years of diagnosis. In addition, with the rise in the aging population and prevalence of comorbid conditions such as diabetes mellitus and chronic hypertension, the associated financial and societal burden from HF is predicted to worsen. The cost associated with HF is estimated to rise to a whopping 70 billion dollars by 2030 [2].

The primary clinical manifestations of HF are dyspnea, fatigue, exercise intolerance and fluid retention in peripheral organs. Various disease processes contribute to HF, with ischemic heart disease (IHD) being one of the most important. In most cases of HF, regardless of the cause of the initial injury, there
is a severe impairment in the ability of the left ventricle to pump blood. This impairment activates compensatory physiological mechanisms such as activation of the sympathetic and renin-angiotensin systems, and production of excessive amounts of neurohormones [3]. These mechanisms are maladaptive in the failing heart as they grip it in a vicious cycle of inefficient pumping leading to structural remodeling. Current therapeutic strategies for HF target the hyperactivation of neurohormonal pathways and the abnormal hemodynamics of heart failure. These secondary therapeutic approaches have been shown to improve HF mortality [4]. Nevertheless, the progression of the disease still significantly shortens and compromises the quality of life; therefore, novel strategies to prevent or even reverse the structural and functional defects of the failing heart are imperative.

1.2. Acute myocardial infarction (AMI)

One of the major contributors to HF is atherosclerosis of the coronary arteries, which normally provide a steady supply of oxygen and nutrients to the myocardium. In coronary artery disease (CAD), high blood pressure and elevated cholesterol levels promote injury, inflammation and deposition of oxidized lipids within the coronary endothelium. The ensuing inflammatory response promotes the formation of platelet thrombi, which can cause sudden coronary vessel obstruction. In a parallel process, the expansion of smooth muscle cells and incursion of macrophages into the vessel wall gradually narrows
the lumen of the artery. Obstruction of blood flow within the coronary arteries results in inadequate perfusion, or ischemia, of the heart muscle. The resultant oxygen and nutrient deprivation and waste build-up jeopardize the viability of the downstream tissue. If the blockage is complete and the condition is sustained long enough (more than 20 minutes), a “wave front” of cardiac myocytes die beginning from the subendocardium and progressing transmurally towards the epicardium [5]. This culminates in a myocardial infarction, more commonly known as acute coronary event or heart attack. An acute coronary event is clinically identified as an acute myocardial infarction (AMI). Sudden cardiac death may occur. If not, the ensuing HF may be fatal.

1.2.1. Molecular consequence of ischemia

Ischemia deprives cardiac myocytes within the heart muscle of oxygen, a condition known as hypoxia. Oxygen is required for oxidative phosphorylation to generate ATP. Within the ischemic area, due to hypoxia, the ability of cardiac myocytes to maintain ATP levels is severely compromised. In order to generate ATP, cells switch from oxidative phosphorylation to anaerobic glycolysis[6]. Glucose becomes the preferred substrate. Its utilization is increased by 10-fold during ischemia/hypoxia and is cardioprotective[7]. Studies show that cardiac myocytes cultured in oxygen-deprived conditions have reduced survival when mechanisms to transport or metabolize glucose are perturbed[8]. Although this acute metabolic adaptation is beneficial for the cell, in the long run, it leads to
buildup of lactic acid. Due to reduced perfusion, this excess lactic acid accumulates and lowers the intracellular pH. These excess protons are extruded from the myoplasm to the interstitial space by the combined action of three ion-specific membrane transporters: the Na\(^+\)/H\(^+\) exchanger, the Na\(^+\)/HCO\(_3\)^- co-transporter and the vacuolar proton ATPase, by which the extrusion of H\(^+\) leads to increased intracellular Na\(^+\). When Na\(^+\) subsequently is extruded for Ca\(^{2+}\) through the Na\(^+\)/Ca\(^{2+}\) exchanger, Ca\(^{2+}\) overload results\[9\]. This imbalance in pH and Ca\(^{2+}\) overloading activates programmed cell death mechanisms in the cardiac myocyte, as discussed in later sections.

1.2.2. Reperfusion injury: molecular basis

The extent of the ischemic injury correlates with the length of the occlusion period\[5\]. Therefore, the most effective therapeutic intervention to limit the injury and the infarct size is effective and timely myocardial reperfusion using thrombolytic therapy or primary percutaneous coronary intervention (PPCI). Paradoxically, restoration of blood flow back to the ischemic area due to spontaneous thrombolysis or therapeutic intervention causes further damage. Reperfusion by oxygenated blood results in generation of reactive oxygen species (ROS) such as superoxides, hydrogen peroxides, hydroxyl ions and nitric oxides. Theses species are generated by components of the electron transport chain, cellular xanthine oxidase and NADPH oxidase\[10\]. ROS combined with the Ca\(^{2+}\) overloading that begins during the ischemia phase regulates the
opening and closing of two regulatory channels, the mitochondrial permeability transition pore (mPTP) and Bax, on the mitochondrial membrane. Opening of these pores releases factors, including cytochrome c, that initiate programmed cell death[11]. Thus, reperfusion-associated oxidative stress causes irreversible cardiac myocyte loss, partially offsetting the benefits of restoring blood flow. In fact, reperfusion injury accounts for 50% of the final infarct size[12]. The infarct size in the presence and absence of reperfusion is illustrated in Figure 1.1.

![Diagram](image)

**Figure 1.1.** Extent of ischemic injury determined by infarct size in presence and absence of reperfusion-mediated by PPCI therapy.
1.3. Cell death during ischemia-reperfusion

A principal feature of cardiomyopathies of both ischemic and non-ischemic origin is the progressive loss of cardiac myocytes. Cardiac myocyte death has been reported in various cardiac injury models such as ischemia-reperfusion, myocardial infarction, cardiac ageing etc. Cell loss may be further exacerbated by oxidative stress during reperfusion [13, 14]. As terminally differentiated cells, myocytes have limited intrinsic regenerative capacity. The extent of muscle damage determines the degree to which heart function is compromised as well as the eventual risk of congestive heart failure [13, 15]. Both apoptotic and necrotic pathways have been demonstrated to contribute to overall cardiac myocyte cell death.

1.3.1. Apoptosis and necrosis

Apoptosis is an energy-dependent cellular “suicide” mechanism characterized by nuclear condensation, DNA fragmentation, cell membrane preservation and absence of inflammatory response. The cardiac myocytes possesses cellular machinery to undergo apoptosis in response to a variety of pathological conditions including ischemia, ischemia-reperfusion, end- stage heart failure, post-infarction remodeling, diabetes, and hypertrophy[16]. Apoptosis has been histologically identified and biochemically verified in human heart failure samples, including ischemic heart disease. Typically, only marginal levels (0.1-0.5%) of
apoptosis are observed. However, considering factors such as duration of apoptosis, consistency of the implied death rate, and the contribution of cell replacement by proliferation, even marginal levels could lead to loss of 5-10% of the myocardium per year, which correlates with the progression and poor survivability of late-stage heart failure patients [17].

At the molecular level, apoptosis can be divided into two main types depending upon whether it is initiated by the activation of cell-surface receptors (the extrinsic pathway) or by permeability changes of mitochondria (the intrinsic pathway). Cardiac myocytes express members of the death receptor superfamily, of which Fas (APO-1/CD95) and TNFR1α (tumor necrosis factor receptor-1 α) are prominent members. The type 1 or extrinsic pathway is initiated when external factors bind to and activate these receptors. These receptors then recruit adaptor proteins and pro-caspases (caspase 8 and 10) leading to the formation of the death-inducing signaling complex (DIS). DIS then activates effector caspases, caspase 3 and 7[18].

The Bcl-2 family of proteins regulates the type 2 or intrinsic pathway. Bcl-2 proteins are classified as pro- or anti-apoptotic. In response to stress stimuli, pro-apoptotic Bcl-2 proteins (e.g. Bax and Bak) undergo a conformational change and relocate from cytosol to the mitochondrial membrane where they initiate the opening of Bax channels. This allows mitochondrial release of apoptogens such as cytochrome c, and SMAC/Diablo to activate caspase 9 and subsequently caspase 3 to activate nucleases and proteases. A majority of studies indicate
that the intrinsic pathway is predominant in determining cardiac myocyte fate decision during HF[19].

Necrosis is the result of loss in plasma membrane integrity, cell swelling and random degradation of DNA. It invokes a heightened inflammatory response[20]. The mPTP plays a major role in necrosis and has been verified as the source of Ca$^{2+}$ and ROS in experimental AMI [21, 22]. Opening of mPTPs releases H$^+$ ions from the mitochondria, which disrupts mitochondrial membrane potential. Reduction in intracellular pH due to metabolic switching to glycolysis exacerbates this process. A progressive reduction in ATP disrupts membrane integrity and allows for indiscriminate activation of nucleases and proteases that degrade cellular contents. Adjacent cells can also be affected by collateral damage[23].

It is well established that both apoptosis and necrosis contribute to overall cell death during ischemia-reperfusion injury in a time-dependent manner [24] Apoptosis is contingent upon the availability of an energy source and is a major cell death mechanism in the early hours following MI, whereas necrosis predominates later. Necrosis is limited to ischemic tissue, whereas apoptosis is observed in ischemic as well as non-ischemic tissue and is exacerbated by ROS[25].
1.4. Myocardial energetics and metabolic adaptation to ischemia

Metabolism governs cellular homeostasis, growth and survival. The myocardium is a highly metabolically active organ. It beats almost 100,000 times per day, pumping almost 10 tons of blood in 24 hours. The energy for myocardial pumping is derived from the conversion of chemical energy to mechanical work of the actin-myosin interaction in myofibrils. The chemical energy is provided by oxidation of substrates, mainly fatty acid and glucose, that the heart is able to utilize to generate ATP, the energy store of the cell. The myocardium has a voracious appetite for ATP, and consumes 6kg of ATP per day, which is 20-30 times its own weight of ATP. Under normal conditions, the major substrates for ATP generation by the myocardium are fatty acids. Generating a greater amount of ATP per gram, fatty acids are the preferred substrate for ATP generation over glucose; however, this process also demands greater oxygen consumption. Fatty acid β-oxidative metabolism contributes 60-90% towards ATP generation and the rest is derived from glucose oxidation[4]. Although both substrates feed into the Krebs cycle, they are differentially processed and regulated, especially under conditions where oxygen availability becomes limiting, as in ischemia.

Metabolism is significantly altered in the diseased heart, mainly at three levels: substrate utilization, oxidative phosphorylation and high-energy phosphate metabolism[4]. The myocardium adapts to ischemia by switching from β-oxidative metabolism to glycolysis [6, 26]. In HF, studies have found that fatty acid utilization is increased or unchanged in early stages [27, 28] but is substantially
decreased in advanced disease [29]. As such, due to reduced oxidative phosphorylation capacity, glucose oxidation is also hindered. This is compounded by insulin insensitivity that develops with HF, which results in a further decrease in ATP[30, 31]. Anaerobic glycolysis may permit short-term cell survival, but results in the production of acidic waste products and a fall in intracellular pH. Impairment in oxidative phosphorylation capacity reduces ATP availability for contraction. Insults such as ischemia alone or in combination with reperfusion result in disturbances of ion exchange and Ca^{2+} homeostasis. This impairs myocardial mitochondrial health and consequently, the electron-transport-chain (ETC) and ATP synthase capacity[32]. Uncoupling proteins that reduce oxidative phosphorylation efficiency are also elevated in heart failure[33]. Ischemia is accompanied by increased generation of reactive oxygen species even in the absence of reperfusion [34-36]. This acidic, oxidative environment leads to loss of cardiac myocytes and expansion of the area of myocardial damage [37]. At the level of energy transport, the creatine kinase system plays a key role. Creatine kinase uses ATP generated in the mitochondrion to transfer phosphate to creatine (Cr) to form phospho-creatine (p-Cr). P-Cr diffuses to myofibrils where the myofibrilar creatine kinase uses p-Cr to convert ADP to ATP. This ATP is utilized for actin-myosin cross-bridge cycling for contraction. Creatine kinase activity is severely comprised in HF and ADP levels are elevated. An imbalance in the critical levels of these metabolites leads to reduction in energy transfer; this metabolic deficit results in contractile dysfunction, the hallmark of heart failure[38].
1.5. *Transcriptional adaptation of myocardium to ischemia*

Oxygen deprivation or hypoxia is an obligate consequence of ischemia. Oxygen is required for the generation of ATP and consequently all aerobic organisms have an elaborate mechanism of oxygen sensing. Oxygen acts as the ultimate electron acceptor in the electron-transport-chain (ETC). As discussed earlier, as an energy-demanding organ, myocardial oxygen supply needs to be maintained at a constant level. Thus, several mechanisms are activated to cope with oxygen reduction in the myocardium and more specifically in the cardiac myocyte. These mechanisms allow the myocardium to modulate metabolism and function in order to increase cell survival, if need be at the expense of contractile function.

One key modulation occurs at the transcriptional level. An essential transcription factor required for activation of genes involved in adaptation to hypoxia is Hypoxia-Inducible Factor-1α (HIF1α). This bHLH (basic helix-loop-helix)-PAS (Per/ARNT/Sim) transcription factor is constitutively transcribed in human, rat, and mouse. However, in normoxia, HIF1α is continuously degraded, inhibiting its activation. However, under hypoxia, HIF-1α is stabilized. Lack of oxygen availability prevents hydroxylation of an oxygen-dependent death domain (ODDD) on HIF1α by an enzyme, prolyl hydroxylase (PHD) that requires molecular oxygen, iron and 2-oxoglutarate as co-factors. This hydroxylation is required for HIF-1α recognition by the E3 ligase VHL (von Hippel-Lindau) and subsequent ubiquitination and degradation by the proteosomal pathway. Stabilization enables HIF1α to binds to its co-activator HIF1β and enter the
nucleus. The HIF1 complex binds to hypoxia responsive elements (HREs) on promoters of a plethora of genes that encode growth and survival factors, extracellular matrix proteins, and modifying enzymes, cytoskeletal proteins, proapoptotic proteins, glucose transporters, glycolytic enzymes and other factors that participate in hypoxic adaptive mechanisms[39].

The energy requirements for the myocardium differ between normal physiological states and stress conditions such as ischemia, and are modulated by transcription of genes encoding regulators of metabolism. A key group of proteins regulating myocardial metabolism is the family of peroxisome proliferator-activated receptors (PPARs). These genes encode receptor proteins for fatty acid substrates on the plasma membrane and include PPAR-α, -β, -δ and -γ with PPAR-α and -γ being most important. Upon binding of ligands, predominantly long-chain fatty acids, these receptors dimerize with an obligate partner, retinoid-X-receptor (RXR) which translocates to the nucleus to bind to fatty acid response elements 1 (FARE 1) on gene promoter to encode for enzymes and transporters required for fatty acid uptake and β-oxidation. In cardiac myocytes, hypoxia reduces nuclear content of RXR, thereby reducing PPAR transcriptional activity[40]. Both PPARα and RXR are depressed in the failing hearts of mice and humans[29, 41, 42]. Another key protein involved in myocardial metabolism is the peroxisome proliferator-activated receptor gamma co-activator-1 (PGC-1α) that encodes genes required for mitochondrial biogenesis, uptake and oxidation of fatty acid and oxidative phosphorylation. PGC-1α is a master regulator of genes, including PPARα, that balance
mitochondrial biogenesis and function. PGC-1α inhibition leads to reduction in oxidative phosphorylation. Deficiency in this gene accelerates progression of HF\[42, 43\]. Altered activity of major metabolic regulators such as PPAR and PGC1 enhances glycolysis and reduces fatty acid oxidation (FAO) and is considered the main metabolic switch in substrate utilization in the failing heart.

1.6. Cardio-protective signaling

Ischemic stress initiates death of cardiac myocytes. As terminally differentiated cells, cardiac myocytes have limited regenerative capacity. Thus the loss of functional cardiac myocytes progressively leads to impairment in cardiac function. This subsequently leads to increases in mortality and morbidity in several clinical settings. At the same time, ischemia induces a wide variety of innate myocardial signaling pathways that can either mitigate or prevent cell death. Understanding these cardioprotective cellular signaling mechanisms that control cardiac myocyte survival has valuable clinical implications. One such cardio-protective signaling pathway that has received considerable attention is the PI3k-Akt pathway.

1.6.1. PI3k-Akt signaling

Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that have been shown to be involved in proliferation, differentiation, cell migration,
glucose metabolism, cell survival and growth, immune response and cell-to-cell communication. PI3Ks are divided into three classes based on their existence as combinations of different isoforms of catalytic and regulatory subunits. Class I and III PI3Ks exist as heterodimers with a catalytic subunit (p110) and a regulatory subunit (p85, p55, p50). Their role is to phosphorylate PtdIns(4,5)P2 (PIP2) to generate PtdIns(3,4,5)P3 (PIP3). Class I PI3Ks are further divided into Class IA and Class IB. Class IA are the major regulators of growth factor signaling and Class IB are activated by G-protein coupled receptors[44]. Class III PI3Ks only phosphorylate PtdIns and plays a role in agonist-independent membrane trafficking[45].

When insulin or insulin like growth factor (IGF) binds and activates tyrosine kinase receptors, a signaling cascade is initiated via adaptor molecule insulin receptor substrate-1 (IRS-1) to recruit PI3K regulatory subunits to the membrane. The association and activation of PI3K in the membrane leads to conversion of PIP2 to PIP3. PIP3 acts as a second messenger to recruit phosphoinositide dependent kinase-1 (PDK1) to the membrane via their peckstrin homology (PH) domain leading to their activation and downstream Akt [46].

One of the well-studied downstream effectors of PI3K is the AGC family of protein kinases, Akt or Protein Kinase B (PKB). Akt was first characterized as a proto-oncogene involved in the regulation of cell survival and proliferation. The three isoforms of AKT, Akt1, Akt2 and Akt3, have different expression patterns; Akt1 and Akt2 are expressed ubiquitously, with high levels in the
heart[47]. On recruitment to the membrane from the cytosol, Akt undergoes two sequential phosphorylations that lead to its activation. Phosphorylation by PDK1 at threonine (Thr) 305 primes Akt for activation and a subsequent phosphorylation at serine (Ser) 473 by mTOR2 complex lead to complete activation [48].

**Figure 1.2. PI3 kinase pathway and its regulation**

Akt is a serine/threonine kinase that regulates multiple cellular functions including metabolism, cell survival, cell growth and proliferation, through phosphorylation of a diverse set of targets. These include pro-apoptotic Bcl2 proteins such as Bad and Bax, caspase 3 and caspase 9; growth and survival regulators such as forkhead transcription factors (FOXO) and glucose
transporter GLUT4; inducers of protein synthesis mTOR1; and translation regulators S6K[49].

One of the best-studied targets of Akt is glycogen synthase kinase3 (GSK3). GSK3 is an Akt effector kinase in a variety of cellular functions that include glycogen synthesis, transcription, translation and apoptosis. PI3K-dependent phosphorylation of AKT leads to its activation and phosphorylation of glycogen synthase kinase3 (GSK3) and protection against myocardial ischemia and reperfusion damage in vitro and in vivo [50-52]. GSK3 comprises a family of two serine/threonine kinases, GSK3α and GSK3β, both of which are inactivated by phosphorylation at serine 21 and serine 9, respectively. In general, inactivation of GSK results in glycogen synthesis. In the case of myocardial ischemic injury, between the two isoforms, GSK3β is the most important participant. GSK3β inhibition is cardioprotective in cardiac myocytes challenged with hypoxia-reoxygenation[53, 54]. During ischemia and reperfusion, mice expressing a non-phosphorylatable mutant, GSKβ S9A, are more susceptible to injury and have greater extent of myocardial damage following coronary occlusion. Cyclosporine treatment reduces this injury[53], which suggests that phosphorylation of GSK3β regulates mPTP opening. Consistent with this view, phosphorylated GSK3β is known to increase the threshold for mPTP opening by directly interacting with mPTP components such as ANT thereby preventing intrinsic cell death[54].

PI3K/Akt regulates numerous cardiac functions, including cell growth, metabolism, survival, and remodeling. Various injury models such as ischemia-
reperfusion, pressure overload, and oxidative stress have been used to show that activation of PI3K/Akt confers cardioprotection[50, 51]. Activation of the PI3/Akt pathway by insulin administration protects cardiac myocytes and reduces infarct size post-ischemia-reperfusion[55]. Concurrent with its effects on survival, Akt activation also improves cardiac contractility by increasing Ca\(^{2+}\) influx through L-type calcium channels. In addition, Akt activation has also been shown to increase sarcoplastic reticulum Ca\(^{2+}\) ATPase2 (SERCA2) levels to regulate excitation-contraction coupling[56], and regulates substrate switching during nutrient deprived conditions, increasing glucose oxidation and decreasing fatty acid oxidation through transcriptional regulators PPAR\(\alpha\) and PGC-1\(\alpha\)[49].

1.7. MicroRNA as post-transcriptional regulators

Ischemic injury involves the coordinated regulation of genes that are presumed to be involved in adaptive responses. With the advent of whole genome sequencing and the realization that most genes do not encode proteins[57], intense research into the field of non-coding RNAs has commenced. Studies have shown the existence of a variety of non protein-coding RNA species that serve instead to regulate gene expression. These include short-interfering RNA (siRNA), piwi-interacting RNA (piRNA) and microRNA (miRNA or miR). Although they all regulate gene expression, there are innate differences in their biogenesis[58]. This study will focus on miRs.
MicroRNAs (MiRs) are 18-25nt, evolutionarily conserved non-coding RNAs that regulate gene expression by impeding translation from messenger RNA templates or by destabilizing mRNA in a sequence-specific manner[59]. The canonical pathway for microRNA biogenesis is now well understood and involves a series of processing stages leading to the formation of mature, functional miR[60]. MiR-encoding genes are present throughout the genome, and can exist within introns or exons of other genes, or stand alone. Most miR genes are intronic. MicroRNA genes are transcribed by a RNA polymerase II/III-dependent manner into the primary miR (pri-miR). These pri-miR transcripts are capped and poly-adenylated[61]. In the nucleus, pri-miR transcripts are processed by the Microprocessor complex, which consists of RNAses III, Drosha and a double stranded RNA-binding protein, DiGeorge critical region 8 (DGCR8), to yield a short hairpin ~70nt precursor microRNA (pre-miR)[62, 63]. As many as 42% miRs are polycistronic [64], i.e. multiple miR precursors are processed by Drosha from the same primary transcript. Exportin5, a protein responsible for nuclear export, exports the precursor miR transcripts to the cytoplasm where they are processed by a second RNAses III enzyme, Dicer, to generate the mature microRNA duplex[65]. One strand of the mature miR is then selected for loading on the miRNA induced silencing complex (miRISC). The key component of miRISC is Argonaute which is responsible for the translational repression or destabilization of target in a mRNA sequence-specific manner [58]. Argonaute protein possesses slicer and endonuclease activity. Among various isoform, Ago2 is essential for miR biogenesis as well as function. Biochemical assays
show that Dicer, Tar RNA binding protein (TRBP) and Ago2 exist as a complex; thus, dicer processing, duplex selection and miRISC loading occur as a concerted series of events[66, 67]. Figure 1.2 illustrates the biogenesis of miRs.

Since the discovery of the first microRNA, lin-41 back in 1993, thousands of miRs have been annotated across most species. The latest version (20.0) of miRBase, the largest web-accessible repository of miR nomenclature, sequence and annotation data, includes almost twenty-one thousand annotated miRs, and almost 2000 have been validated in humans [68]. Some have been observed to be tissue- or cell-type specific and others are ubiquitously expressed.

MiRs are predicted to regulate almost 30% of human protein coding genes. Nucleotides 2-8 at the 5’ end of each miR, called the “seed” sequence, bind to complementary bases on the 3'UTRs of its target mRNA. Since a short, incompletely complementary “seed” sequence is sufficient for target 3’UTR recognition, a single miR can target numerous genes. On the other hand, multiple miRs can target the same gene. In many cases, genes in a common biological pathway have been shown to have shared conserved 3’ UTR sequences that can be targeted by a single miR, indicating that miRs have evolved to control gene programs as well as individual genes [69, 70]. MiRs display coordinated spatial and temporal expression patterns and are also known to regulate acute, localized and transient cellular phenomena. Alterations in their expression patterns and the resultant co-dysregulation of their target genes are evident in derangement of vital cellular processes.
Figure 1.3. Biogenesis of microRNA.
MiRs have been implicated in development, differentiation, metabolism, and apoptosis, among other vital processes, and thus have pivotal roles in human physiology. Excess or depletion of miRs contributes to disorders of neurological, renal, metabolic, pulmonary and cardiac systems [71-74].

In the past decade, multiple studies have explored the role miRs play in cardiac biology. During murine heart development, deletion of Dicer causes lethality at different stages depending upon the timing of Cre transgene activation [75]. Postnatal deletion of dicer results in contractile protein disarray, dilated cardiomyopathy and heart failure. Similarly, deletion of trbp also led to a phenotype consistent with the Dicer knockout: progressive cardiomyopathy leading to heart failure. Trbp knockout upregulated Sox6, which resulted in increased expression of skeletal fast-twitch muscle fibers, atypical to the cardiac slow-twitch fibers. Cardiac myocyte-specific dgrc knockout mice have a fully penetrant phenotype with left ventricular malfunction that progresses to dilated cardiomyopathy and premature lethality[76]. This suggests the importance of miR processing for cardiac development and myofibril homeostasis. Moreover, the importance of miR regulation and function has been validated in stress-induced models such as ischemia, ischemia-reperfusion, pressure overload etc.[77-83].

1.7.1. MicroRNAs in ischemic heart disease

MiRs are now understood to exert regulatory control over many important aspects of cardiovascular biology, including myocyte growth, differentiation,
metabolism and the response to ischemic stress, [74, 82, 84]. Several miRs display a tissue-specific expression pattern. Certain miRs, notably miR-1, miR-133, miR-26a, and miR-208, are highly enriched in the heart[84]; miR-208a is expressed only in cardiac myocytes[82]. At the same time, the myocardium also expresses miRs with wide tissue distribution, e.g. let-7 family members, at high levels. High-throughput screens in experimental models of myocardial infarction, ischemia-reperfusion, and progressive end-stage heart failure have identified additional unique miRs that regulate cardiac myocyte survival, angiogenesis, and regeneration under such stress conditions, among other critical cell functions [79-82, 85-87]. A few of these miRs are briefly discussed below.

The miR-15 family includes miR-15a, -15b, -16, -197 and -497. These miRs were found to be upregulated in murine and porcine models of permanent coronary occlusion, and in human heart failure samples. Inhibition of this miR family using locked nucleic acid (LNA)–modified oligonucleotides that are perfectly complementary to the seed sequence of miR-15 family render cardiac myocyte resistant to hypoxia-reoxygenation-mediated cell death. In addition, systemic delivery of anti-miR-15 oligonucleotides reduced ischemia-reperfusion injury and improved cardiac function[80].

The miR-34 family also negatively regulates cardiac myocyte cell death. All members (miR-34a, -34b and -34c) are upregulated in human hearts during ageing, and in vivo inhibition or genetic deletion of miR-34a reduced age-related cardiac myocyte death and functional impairment. MiR-34 repression also
reduced cell death and fibrosis following both ischemia reperfusion and chronic permanent occlusion, thereby improved cardiac function [77, 85].

A microRNA array identified miR-320 to be downregulated in ischemia-reperfused murine hearts, and in an in vitro model of simulated ischemia. Overexpression of miR-320 increased cardiac myocyte cell death while knockdown promoted survival. Mice harboring a miR-320 transgene exhibited a heightened cell death phenotype following in vivo ischemia-reperfusion. In contrast, mice that were injected with antagomiR against miR-320 were protected from ischemia-reperfusion-induced injury as evidenced by reduced apoptosis and infarct size[81]. An extensive number of other miRs have been shown to regulate cardiac myocyte cell fate decision as well as other facets of ischemic injury including fibrosis, and angiogenesis. Table 1.1 displays miRs and their respective targets that have been validated in murine in vivo models. This is by no means an exhaustive list, as new miRs and their targets are constantly being discovered.
Table 1.1. MiRs involved in aspects of ischemic injury (reviewed in [84, 88])

<table>
<thead>
<tr>
<th>miR name/family</th>
<th>Direction</th>
<th>Target gene</th>
<th>Regulation</th>
</tr>
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<tr>
<td>miR-21</td>
<td>up</td>
<td>PDCD4</td>
<td></td>
</tr>
<tr>
<td>miR-199a</td>
<td>down</td>
<td>HIF1a</td>
<td>-</td>
</tr>
<tr>
<td>miR-24</td>
<td>down</td>
<td>Bim</td>
<td>-</td>
</tr>
<tr>
<td>miR-214</td>
<td>up</td>
<td>NCX1</td>
<td>-</td>
</tr>
<tr>
<td>miR-320</td>
<td>down</td>
<td>HSP20</td>
<td>+</td>
</tr>
<tr>
<td>miR-15</td>
<td>up</td>
<td>Bcl2, SIRT1</td>
<td>+</td>
</tr>
<tr>
<td>miR-1</td>
<td>up</td>
<td>Bcl2</td>
<td>+</td>
</tr>
<tr>
<td>miR-34</td>
<td>up</td>
<td>SIRT1</td>
<td>+</td>
</tr>
<tr>
<td>miR-140</td>
<td></td>
<td>MFN1</td>
<td>+</td>
</tr>
</tbody>
</table>

**Apoptosis**

- miR-21: up, PDCD4
- miR-199a: down, HIF1a
- miR-24: down, Bim
- miR-214: up, NCX1
- miR-320: down, HSP20
- miR-15: up, Bcl2, SIRT1
- miR-1: up, Bcl2
- miR-34: up, SIRT1
- miR-140: MFN1

**Fibrosis**

- miR-29: down, Collagens
- miR-21: up, Sprouty

**Angiogenesis**

- miR-92a: up, ITGA5
- miR-126: down, Spred-1, Pik3R2
- miR-210: up, Ephrin A3

1.8. MicroRNA Let-7

Among the first miRs to be identified and characterized were members of the miR-let-7 family, the founding member of which, let-7, was originally identified as a dose-dependent lethal mutation in *C. elegans*[89]. Later it was shown in *C. elegans* that let-7 controlled differentiation of a subset of skin cells, called seam cells, that are necessary for transitioning into the adult stage. A rise in let-7 levels was required for differentiation of these specialized cells. In a let-7 mutant, where let-7 could not be expressed, undifferentiated seam cells kept dividing[90]. Let-7
was later identified as a temporal regulator of gene expression, through its ability
to interact with the 3’ untranslated ends of target genes through a partially
homologous 7-8 nt “seed” sequence [90]. Several targets of let-7 have been
identified in C. elegans and depletion of these target genes, which include
transcription factors and signaling molecules, have confirmed the importance of
let-7 in multiple critical cell pathways[91-93].

Mature and functional let-7 is produced form polymerase II- dependent
transcription. Primary transcripts of let-7 are processed like other mRNA such
that they contain poly (A) tails and 5’ G-caps[94]. In addition, at least in the
worm, the primary transcript undergoes trans-splicing at its 3’ end generating a
splice-leader-1 (SL-1) sequence that is appended to the 5’ end of the let-7
primary transcript. This sequence acts as the substrate for Drosha in the nucleus.
Perturbation in this trans-splicing process results in accumulation of primary let-7
and reduction in mature levels[94, 95]. Following export of the Drosha-processed
transcript, pre-let-7, to the cytoplasm it is processed by Dicer to generate the
mature let-7 sequence[65]. A recent study has shown that let-7 is involved in an
auto-regulatory loop whereby it regulates its own expression. Mature let-7 loaded
on to RISC targets its own primary sequence. However, in this case, instead of
destabilizing primary let-7, this interaction leads to efficient processing and
subsequent increase in mature let-7 [96].

Orthologues of let-7 have been identified in many other higher organisms
including humans[90]. The mammalian miR-let-7 family includes at least 14
members with a common seed sequence, and all are probably functionally
redundant [97]. This redundancy exists in worms, as triple mutation of the let-7 family members mir-48, mir-84 and mir-241 has the highest penetrance for observed developmental defects compared to double or single mutants. Overexpression of one let-7 family member can rescue phenotypes caused by loss of other let-7 members [98]. Figure 3 displays the let-7 family members in the three species, Homo sapiens, Mus musculus and Rattus norvegicus, that were investigated in this study.

In mammals, by convention, a letter following let-7 such as let-7a, let-7b, designates let-7 family members originating from different genomic loci with slightly different sequences. Moreover, if the same sequence originates from different genomic loci then a number follows the letter such as let-7a-1, let-7a-2, let-7a-3. For example, let-7a is produced from 3 different precursor transcripts encoded from chromosome 9, 11 and 21. Although the characteristics of let-7 family members are conserved across species, there are noticeable differences. There are fewer let-7 members in C. elegans (nine members) and drosophila (one member) than in mammals. Another key difference is that in worms genomic clustering is absent, whereas flies and humans genomes have highly conserved let-7 clusters. Yet another difference lies at the level of regulation. Transcriptional regulation of let-7 is predominant in worms and flies. In worms, nuclear hormone receptor DAF-12 is known to regulate temporal regulatory elements (TRE) on the promoter of let-7 and thus regulate its transcription[99]. Primary and precursor let-7 are concurrently expressed with mature let-7 levels.
Figure 1.4. Sequences of all let-7 family members annotated thus far in three species investigated in this study. The “seed” sequence appears in red. Nucleotides that vary in a single member across species appear in different font color. The numbers of particular members that are generated from different precursors appear in parentheses. For example, the let-7a sequence is encoded at 3 different genomic loci (x3).

<table>
<thead>
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<th>3’</th>
<th>Accession</th>
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<td></td>
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<td>ugguuagguuagguuagguua</td>
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<td></td>
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</tbody>
</table>

Conserved: ******* * *** ***

Table 1.2 illustrates the various genomic loci for let-7 family members in mammals. Although uncommon, transcriptional regulation of mammalian let-7
does occur. In mammals, the let-7 cluster containing let-7a, -7f and 7d is known to be regulated by transcription factor MYC. MYC binds to the promoter of this cluster to negatively regulate it in B-cell lymphomas[100]. MYC also regulates the monocistronic let-7g[101]. Transcriptional control is also achieved by promoter methylation, as with let-7b[102].

Table 1.2. Genomic information of mammalian let-7 miR family

<table>
<thead>
<tr>
<th>miR name or cluster</th>
<th>Chromosome #</th>
<th>Strand</th>
<th>Location</th>
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<tr>
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<td>+</td>
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</tr>
<tr>
<td>hsa-miR-202</td>
<td>10</td>
<td>-</td>
<td>Intergenic</td>
</tr>
</tbody>
</table>

Nevertheless, expression of mammalian let-7 seems to be predominantly under post-transcriptional control. In support of this view, the primary sequences of let-7a-1-let-7d-let-7f cluster, let-7f-2-miR-98 cluster, let-7g and let-7i are all expressed throughout development; however, their mature sequences only rise later in embryonic development[103, 104]. The primary let-7 sequence contains extensively base-paired stem region harboring mature let-7. The complementary strands are connected through a terminal loop region termed the pre-element (preE). The preE region is variable among the various let-7 family members and
serves as a platform to interact with RNA-binding proteins involved in its post-transcriptional regulation (Figure 1.4) [105]. Post-transcriptional regulation of let-7 by a unique RNA-binding protein will be discussed later in section 1.8.4.

**Figure 1.5. Structure of the precursor let-7 sequence.** The different regions on the variable stem loop or the pre element (preE) region is labeled. The Dicer cleavage site and the region that harbors the mature let-7 are also indicated. Not drawn to scale.

### 1.8.1. Involvement of let-7 in mammalian development

The developmental role of let-7 has been extensively characterized beyond nematodes and across multiple species. Especially during nervous system development, when coordinated activation and repression of genes is imperative for neuronal diversity, let-7 expressional modulation is observed. During fly development, let-7 is activated at the time of metamorphosis, especially in the
nervous system. Deletion of let-7 results in defects in motor neuron remodeling, a process that is critical for peripheral nervous system development[106]. Following fertilization, let-7 is dramatically upregulated in the brain and spinal cord of zebrafish during development[107, 108]. Moreover, let-7 expression is also observed in mouse and human brain tissue[109]. In mice, let-7 is enriched in P19 cells that eventually differentiate into neurons[108]. Overexpression of let-7, among other miRs, was able to rescue phenotypic defects in retinal progenitors of conditional Dicer-KO mice and thereby, accelerate retinal development[110]. Developmental stages of an organism involve coordinated removal of cells through various death mechanisms. In line with its role in larval to adult transition (section 1.8), let-7 has also been implicated in cell death response involved in gonadal development in nematodes[111]. Several let-7 mediated pathways are conserved across many species that are involved in development-associated cell death. Reduction in let-7 levels caused increased cell death and reduced proliferation in chondrocytes impeding skeletal development in mice[112].

Let-7 has also been implicated in cardiac development. A study looking at expression pattern of miRs in 4 different stages of mouse heart development discovered that several members of the let-7a/7d/7e/7f cluster were upregulated in later cardiac development. A network analysis of the predicted targets of this cluster identified 5 genes known to be involved in cardiac development, including FOXP1, TBX5, HAND1, AKT2 and PPARGC1A[113]. In vitro model systems using embryonic stem cells (ESCs) have also been used to study let-7 role in cardiac development. In mouse ESCs, let-7 promotes mesoderm specification
and cardiac differentiation. Let-7 targets the mRNA of polycomb complex group, EZH2, which leads to modulation of histone marks on promoters of crucial cardiac transcription factors Nkx2.5, Mef2, and Tbx5 to promote cardiac differentiation[114]. In another study, overexpression of let-7g allowed for the maturation of human ESCs into adult-like cardiac myocytes. This observed phenotype was attributed to the ability of let-7g to downregulate insulin pathway; thereby, promote adult-like metabolic state evident by increased fatty acid oxidation[115].

1.8.2. Involvement of let-7 in metabolism

Transgenic animal models overexpressing let-7 have shed light on the involvement of let-7 in metabolic processes at the tissue or whole animal level. Two independent studies validated let-7 involvement in whole body glucose metabolism. Mice overexpressing let-7g and the let-7 cluster containing let-7a/-7f/-7d have retarded growth compared to wild-type littermates[97, 116]. These animals are also glucose intolerant and insulin insensitive. Let-7 transgenic animals have reduced insulin signaling in peripheral tissues, such as skeletal muscle, than wild type animals. The genes INSR, IRS1, and IGF1 in the insulin pathway were targeted by let-7 in these animals. This let-7 mediated insulin-resistant phenotype was attenuated by rapamycin, suggesting the involvement of PI3k-Akt-mTORc signaling [97, 116].
Knocking out let-7 has been a technical challenge because of the multiple genomic loci encoding let-7 family members. Therefore, various forms of sequences complementary to let-7 have been utilized to sequester mature let-7 and inhibit its function. Such sequences have been termed sponge, decoy, and antimiR[117]. AntimiRs were used to show that inhibition of let-7 function could both prevent and treat high fat diet-induced obesity and deficiency in glucose signaling. This was attributed to the derepression of let-7 targets in the Ins-PI3k-Akt pathway especially in skeletal muscle[97]. Endogenous “antimiR” sequences have also been discovered. The H19 long non-coding RNA sequesters endogenous let-7 to elevate insulin signaling in muscle. H19 was downregulated in T2D human muscle samples and muscle of mice fed a high fat diet[118]. This also explained the conundrum of why let-7 levels are not reduced in these animals. In addition to regulating glucose, let-7 is also implicated in the amino acid (AA) sensing pathway. By targeting RagD and Map4k3, components of the pathway, let-7 represses mTORc signaling and maintains metabolic homeostasis[119].

1.8.3. Let-7 as a tumor suppressor

In the nematodes, prominent phenotypes of lack of let-7 are failure of terminal differentiation and ongoing cell division, which are also hallmarks of tumorigenic cells. Initial evidence that let-7 could contribute to tumorigenicity appeared in expression profiling studies. Let-7 was found to be downregulated in a significant
number of lung cancer cell lines, and exogenous expression of let-7a and let-7f was able to suppress cell proliferation in these lines. The same study observed a significant correlation between low let-7 levels and poor prognosis following surgical treatment [120]. Let-7 also mapped to chromosomes that are frequently deleted in lung cancers [121].

The molecular basis of the role of let-7 as a tumor suppressor strengthened with studies showing that it directly targets well-established oncogenes. One such oncogene is RAS. Among many other proteins, RAS deregulation is implicated in lung cancer [122]. Let-7 targets all three isoforms of RAS, K-, H- and N-Ras. Let-7 and RAS also display reciprocal expression patterns in tumor and adjacent normal tissue[123]. Tail vein delivery of let-7b in the form of a lipid-encapsulated mimic reduced tumor burden and increased survivability in a murine model of lung cancer with Kras/p53 mutation[124]. Let-7 also regulates the MYC oncogene. MYC possesses a let-7 binding site on its 3’UTR and its mRNA is regulated by let-7c in Hepa-1 cells. Interestingly, MYC also prevents let-7 transcription by binding directly to the promoters of let-7a-1/-7f-1/-7d and let-7g in a model of MYC induced oncogenesis, implying a direct double-negative feedback loop[100, 101].

HMGA2 is yet another oncogene regulated by let-7. A reciprocal expression pattern is seen between let-7 and HMGA2, and the HMGA2 UTR is directly regulated by let-7 in a luciferase reporter assay. Several cancers harbor truncation mutations of the 3’UTR of HMGA2 associated with a heightened cancerous phenotype even in the presence of let-7[125]. Cell cycle regulators
are also direct targets of let-7. Functional effects of let-7 on cell proliferation have been observed in A549 lung cancer cells. CDC25 and CDK6, prominent cell cycle regulators, were identified as targets from a high throughput screen following modulation of let-7 levels[126]. In addition to directly regulating these genes, let-7 targets genes such as IMP1 that are required for stabilizing CDC25 and CDK6 expression suggesting a coordinated regulation of multiple targets to regulate a particular pathway [127]. Cyclin D1 is also a target of let-7[128].

1.8.4. LIN28-let-7 axis

Members of the mammalian let-7 family share a conserved mature sequence; however, the preE region that is removed by Dicer (section 1.8) exhibits sequence divergence. The non-conserved loop, bulge and stem structures in pre-miR transcripts serve as platforms for further enzymatic processing by RNA binding proteins (Figure 1.4). One such protein implicated in regulation of let-7 is Lin28. Like let-7, Lin28 was also discovered as a heterochronic gene that regulated developmental timing in C. elegans [129, 130]. These two genes were inversely expressed both spatially and temporally in various other systems[131, 132]. Lin28 has also been shown to possess conserved expression patterns and physiological roles in higher organisms including mammals. Such is its influence on gene expression that Lin28 is among the four factors that regulate pluripotency[133]. Variations in Lin28 have been linked to developmental traits such as height and timing of puberty onset in humans and mice[134].
Lin28 is encoded by two paralogous genes, Lin28A (Lin28 hereafter) and LIN28B, in mammals, compared to a single Lin28 gene in lower organisms. Lin28 (209 AA) and LIN28B (250 AA) are RNA binding proteins that consists of cold-shock domain (CSD) and two zinc knuckle domains (ZKD) comprising of the (Cys-Cys-His-Cys) motifs. Gain- and loss- of function studies of Lin28 have demonstrated that it promotes self-renewal and delays differentiation of hypodermal and vulvar cells in *C. elegans*[130]. Lin28 is highly expressed in early larval and embryonic stages, which gradually declines to undetectable levels in adult tissues[135]. Conversely, let-7 is minimally expressed during early developmental stages and is induced during differentiation into specialized adult tissue such as seam cells in *C. elegans* [98]. Consistently, let-7 loss-of function phenocopies lin28 gain-of function, which allows for uncontrolled self-renewal. Interestingly, let-7 also represses lin-28 in a 3'UTR sequence-dependent manner to promote differentiation[98, 130].

Post-transcriptional inhibition of let-7 by Lin28 is one important mechanism for the inverse association between the two proteins. Terminal loop or preE structures in the precursor forms of let-7 are specifically bound by LIN28, blocking their processing by Drosha and Dicer and/or inducing their degradation [104, 105, 136]. Lin28B is localized in the nucleus, and interacts with the primary transcript of let-7 to prevent its processing by Drosha/DGRC [103, 137, 138]. In the cytoplasm, Lin28 binds the pre- form of let-7 to prevent it from being loaded with Dicer and thereby reduces its processing into mature transcripts [103, 104, 139]. Crystallographic data from *Xenopus* and mouse species have shown that
the CSD domain of Lin28 binds to pyrimidine rich heptanucleotides on the preE loop region of pre-let-7. This interaction is sequence-independent. Binding of Lin28 initiates conformational changes in the preE bulge region to expose a conserved GAGG sequence. This sequence is then bound by the two Lin28 ZKDs, preventing the processing of let-7 by downstream Dicer (Figure 1.5)[140]. In addition, this interaction allows for the 3' polyuridylation of the precursor species by terminal uridyl transferase, TUT4/Zcchc11. The polyuridylated form of pre-let-7 is eventually degraded by an exonuclease identified as Dis3l2[105, 136, 141]. This reduces the mature levels of let-7 and antagonizes their functions. Figure 1.5 illustrates interaction of let-7 with Lin28. Regulation of let-7 by Lin28 is Zcchc11-dependent, while LIN28B is Zcchc11-independent. This difference in let-7 regulation is therapeutically significant as evident in cancer cells and xenograft models. Different cancer types have varying expression levels of these two isoforms and respond accordingly to Zcchc11 inhibition [142].
Figure 1.6. Molecular basis of Lin28 interaction with the precursor form of let-7. The CSD of Lin28 binds to pyrimidine-rich heptanucleotides on the let-7 preE loop and initiates a conformational change in the preE bulge to allow the two ZKDs of Lin28 to bind to conserved GAGG sequences. This interaction alters Dicer cleavage and prevents maturation of let-7.

Like let-7, Lin28 is strongly conserved through evolution from worms to humans, and its regulatory properties are similarly conserved. Through repression of let-7, LIN28 exerts control over multiple fundamental cell processes, including glucose metabolism, cell fate decisions, pluripotency, and tissue regeneration [131, 132].
Figure 1.7. Lin28 mediated processing of let-7.
1.8.5. Background and hypothesis for the study

Like other miRs, miR-let-7 is transcribed as a primary RNA molecule that is successively cleaved to a precursor hairpin form and ultimately to the mature 21 nt form that is incorporated into the RNA-induced silencing complex (RISC). Mammalian let-7 species are widely tissue-distributed and exhibit temporal regulation, increasing during development and differentiation. In keeping with the known role of let-7 in differentiation, in vitro models using mouse and human ESCs reveal that let-7 expression is increased as these cells attain a more cardiac myocyte phenotype[114, 115]. Let-7g induced fatty acid oxidation in the mature cardiac myocyte like cells. Moreover, upregulation of let-7c accompanies maturation of the developing heart, and repression of let-7 permits cardiomyocyte de-differentiation and regeneration[143]. Similarly, let-7 is highly expressed in cardiac tissue especially in myocytes. In addition, let-7 family members also demonstrate differential expression under stress in various tissues including the myocardium. MiR-let-7 is altered in the failing human heart including those of ischemic etiology[144]. Therefore, we postulated that ischemic stress in the heart would alter let-7 expression pattern. We were interested in determining how the components of ischemic stress, hypoxia, glucose deprivation, and acidosis, affect let-7 expression and function. In addition, we also wanted to determine whether the effects of let-7 were general or cell type-specific to myocyte or fibroblast compartments within the myocardium.
The timing of let-7 expression is determined both transcriptionally and post-transcriptionally, and the mechanisms regulating let-7 differ depending both on developmental stage and tissue type (reviewed in [145]). Other types of post-transcriptional modification, and even other microRNAs regulate let-7[146]. Hypoxia adaptation involves activation of HIF1α. HIF1 complex is a key transcriptional factor modulating the hypoxia adaptive processes. Therefore, it is likely that regulation of let-7 under hypoxia be under the control of HIF1. In addition, hypoxia also activates MAPKs and HDACs. Considering that these signaling mechanisms alter let-7 in other systems[147, 148], it could potentially regulate let-7 in the myocardium as well. The most well described negative regulator of let-7 is the RNA-binding protein, Lin28. This Lin28 mediated mechanisms helps to achieve specific local and time-dependent regulation of let-7. In Lin28 transgenic mice, Lin28 mediated repression in let-7 modulates glucose metabolism. This Lin28/let-7 axis is a downstream mediator of differentiation of neuronal cells under oxygen-deprived conditions and is activated by cancer cells to undergo aerobic glycolysis[149]. In light of these observations, we hypothesized that the Lin28/let-7 axis would be activated under ischemic conditions in the myocardium.

Let-7 has been implicated as a tumor suppressor. Let-7 loss is a major observation in cancer cells[120, 122]. Let-7 targets validated oncogenes such as Ras to modulate survival of cancer cells[123]. In addition, let-7 is also directly target Fas to modulate cell death. Let-7 family member, miR-98 represses hypertrophic stress induced apoptosis in the myocardium [87]. In other cell types,
miR-let-7 has been shown to regulate genes involved in glucose metabolism, autophagy, glycolysis and apoptosis [97, 116, 119, 149, 150] that are important for adaptation during stress conditions such as ischemia. **In these studies, we asked whether let-7 assists in the metabolic adaptation to hypoxia and if so, how it affects cardiac myocyte survival under ischemia.**

Here we show that myocardial ischemia *in vivo* activates LIN28, associated with repression of miR-let-7. We show that repression of miR-let-7c and -g occurs post-transcriptionally and is mediated by hypoxia in a cardiac myocyte-specific manner, concurrent with induction of Lin28 protein and mRNA. Repression of let-7 required ERK1/2 activity and was blocked by HDAC inhibitors. Lentiviral-mediated rescue of miR-let-7g levels during hypoxia protected myocyte from ischemia-induced cell death. Let-7 rescue using *in vivo* delivery of lenti-let-7g also prevented cell death in a murine model of ischemia-reperfusion. Here we show that let-7 elevation was associated with increased AKT activation both, in vitro and in vivo, resulting from positive regulation of the PI3 kinase pathway through inhibition of a novel target of miR-let-7, PIK3IP1.
Chapter 2

MATERIALS AND METHODS

2.1. Reagents

Antibodies against caspase 3, cleaved caspase 3, AKT, p-AKT (ser473), GSK, p-GSK (ser9), ERK, p-ERK and GAPDH were from Cell Signaling Technology; antibody against β-actin was from Sigma-Aldrich; antibody against HO1 was from Santa Cruz Biotechnology, Santa Cruz CA; Lin28 antibody from Abcam PLC, Cambridge, MA; antibody against AGO2 was from Wako USA. For chemiluminescence we used the West Pico ECL Western Detection System, Thermo Scientific. Turbo-RFP-tagged pLemir lentivirus constructs for microRNA-let-7g transduction and expression were from Open Biosystems Products Inc., Huntsville AL. Lentiviral expression constructs utilize the cytomegalovirus (CMV) promoter. The let-7 sponge sequence was obtained from a previously published study[151]. RNA and protein extractions used the MirVana miRNA Isolation Kit and MirVana Paris Kit from Applied Biosystems/Ambion, Austin TX. Reagents for Real-time Quantitative PCR were obtained from Applied Biosystems, Carlsbad, CA, including microRNA specific probes, transcript specific probes, Universal PCR Master Mix, and High Capacity cDNA Reverse Transcription kit. TUNEL assay for apoptosis was performed using the CardioTACs kit from Trevigen, Gaithersburg, MD.
2.2. Primary culture of rat neonatal cardiomyocytes

Methods for primary culture of neonatal rat cardiac myocytes have been previously described[152]. The final myocyte cultures contained >90% quiescent cardiac myocytes at partial confluence; 0.1 mmol/L bromodeoxyuridine (BrdU) was included in the medium for the first 5 days after plating to inhibit fibroblast growth. Fibroblasts from the same cultures were cultured in same media without BrdU and used for experiment within 2-3 passages.

2.3 Over-expression miR-let-7g in primary cardiac myocytes and hypoxia treatment

Transduction of NRVMs was carried out using lentiviral pLemir-let-7g (Open Biosystems) as described in[153]. Neonatal rat cardiac myocytes were cultured for 5 days in complete MEM media and 0.1mmol/L BrdU. On day 3, overexpression of miRs was done using lentivirus transduction at MOI 40. MicroRNA levels were assessed 48-hour post transfection by qPCR or were serum-starved 2 days with MEM supplemented with TIB (transferrin, vitamin B12, insulin) media before start of experiments. Neonatal cardiac myocytes and fibroblasts were exposed to hypoxia (0.5% O₂) in serum-free medium containing 5.5 mM glucose. For low glucose experiment, glucose free DMEM (Life Technologies) with 0.5mM D-glucose and TIB was used as described
previously[154]. Fresh deoxygenated media was used at the start of each hypoxia treatment. RNA and protein was collected within the hypoxia chamber.

*In vivo transduction and ischemia-reperfusion surgery*

All experiments were performed on wild type C57BL/6 mice under protocols approved by Institutional Review Board at University of Miami. Lentiviral transduction of neonatal pups was carried out described in[153]. Briefly, 2-day neonatal pups were placed on a transilluminator and 15x10^8 lentiviral vectors described earlier were injected using the external jugular vein. Mice were sacrificed 10 days and 9-10 weeks post injection. RNA and protein from whole heart was used to access the efficiency of injection. Ischemia-reperfusion was carried out as described in [155] with minor changes. Briefly, at 9-10 weeks of age, mice were anaesthetized with a cocktail of 40mg/kg ketamine and 5mg/kg xylocaine. Following thoracotomy, LAD artery was visualized and occluded using an 8-0 silk suture for 60 minute. Reperfusion was accomplished by releasing the suture and the chest wall was closed. After a reperfusion period of 24 hour, cardiac function was assessed using echocardiography. For echocardiography, mice were secured in a supine position. Mice were evaluated using 40-hertz transducer on a Visual Sonics 770 High Resolution Imaging System. B-mode in the short and long axis view of the ventricle was used to evaluate wall motion.
defects of ventricle and M-mode in long axis view used for the interventricular septal thickness, posterior wall thickness and the left ventricular dimensions in systole and diastole.

2.5. Real-time PCR and Western Blotting

NRVM and myocardial tissue RNA/protein was isolated using PARIS kit (Ambion). cDNA preparation and real time PCR and Western Blotting were performed as described in [153]. Primary and precursor let-7c were measured as described in [156] using primers from [157].

For primary let-7c sequence reverse primer for RT-PCR was used during cDNA synthesis. For gene expression, cycling parameters were: 2 minutes at 50°C, 20 seconds at 95°C, 40 cycles: 1 second at 95°C and 20 seconds at 60°C. Data was analyzed using the RQ Manager 1.2 from Applied Biosystems, CA. For microRNA quantitation, cycling parameters were: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles: 15 seconds at 95°C and 1 minute at 60°C.

The cell lysate fractions were subjected to SDS-PAGE and transferred to nitrocellulose. Membranes were blocked for 1 hour at room temperature in 5 % nonfat milk in a buffer containing (Tris 20 mM, sodium chloride 137 mM, 0.5% Tween-20 pH 7.5 (TBS-T)) and then incubated overnight at 4°C with primary antibodies followed by incubation for 1 hour with horseradish peroxidase-conjugated secondary antibody. Proteins were imaged by chemiluminescence.
2.6. *Measurement of cell death*

Cell death was determined by the measuring levels of LDH released into the media using a commercially available Cytotoxicity Detection KitPLUS (LDH) (Catalogue no. 04744926001) from Roche Applied Science, according to the manufacturer’s instructions.

2.7. *Apoptosis and morphometric analysis*

Mice were anesthetized and sacrificed by cervical dislocation at 24-hour reperfusion. Left ventricles were fixed in 10% NBF and embedded in paraffin. Paraffin embedded left ventricle sections were examined for apoptotic cardiac myocytes using a commercially available kit (Cardio- TACS, Trevigen) according to the manufacturer's instructions.

2.8. *Image acquisition and processing*

Myocardial tissue were collected and fixed as described in [153]. Apoptotic nuclei were measured using CardioTACs kit (Trevigen). Sections were imaged using a Retiga 4000R camera mounted on an Olympus IX73 and captured using Q-Capture Pro. Images were assembled using Adobe Photoshop CS5.
2.9. RNA immunoprecipitation: Modified Photo-Activable Ribonucleoside Crosslinking and Immunoprecipitation (PAR-CLIP)

RNA immunoprecipitation was carried out as described in [158] with minor changes. Briefly, cardiac myocytes were transduced by lenti-lent-7g and NT vector as described earlier. Following a 16-hour 100µM 4-SU treatment, NRVMs were washed in PBS and cross-linked at 365nm UV light in a Stratalinker 2400 (Stratagene). This was followed by lysis in lysis buffer ((50 mM HEPES, pH 7.5, 150 mM KCl, 2 mM EDTA, 1 mM NaF, 0.5% (v/v) NP40, 0.5 mM DTT, complete EDTA-free protease inhibitor cocktail (Roche)). 500ng of whole cell lysate was incubated overnight at 4°C with 2.5µG of Ago2 antibody and mouse IgG. 50uL Dynabeads (Applied Biosystems) were washed twice in citrate-phosphate buffer (4.7 g/l citric acid, 9.2 g/l Na₂HPO₄, pH 5.0) and resuspended in lysis buffer. Lysate was incubated with beads for additional 4-hour at 4°C. Beads were washed 3 times with 1 ml of IP wash buffer (50 mM HEPES-KOH, pH 7.5, 300 mM KCl, 0.05% (v/v) NP40, 0.5 mM DTT, complete EDTA-free protease inhibitor cocktail (Roche)) and washed 3 times with 1 ml of high-salt wash buffer (50 mM HEPES-KOH, pH 7.5, 500 mM KCl, 0.05% (v/v) NP40, 0.5 mM DTT, complete EDTA-free protease inhibitor cocktail (Roche)) and resuspended in 100 µl of SDS-PAGE Loading Buffer (10% glycerol (v/v), 50 mM Tris-HCl, pH 6.8, 2 mM EDTA, 2% SDS (w/v), 100 mM DTT, 0.1% bromophenol blue) in silicon coated tubes. Portion of the bead eluate not used for RNA isolation was used to access successful immunoprecipitation. The remaining bead eluate was treated to an equal volume of 2x Proteinase K Buffer (100 mM Tris-HCl, pH 7.5, 150 mM NaCl,
12.5 mM EDTA, 2% (w/v) SDS) followed by the addition of Proteinase K (Roche) to a final concentration of 1.2 mg/ml, and incubation for 30 min at 55°C. The RNA was recovered by acidic phenol/chloroform extraction followed by an ethanol precipitation. RNA was processed for real time PCR as described earlier.

2.10. Human tissue samples

Anonymized left ventricular myocardial samples and associated clinical information were harvested within 4 hours post-demise or explantation and obtained through the Cooperative Human Tissue Network under a protocol approved by the UM Institutional Review Board. Samples were maintained at -80°C until use.

2.11. Reporter assays

All luciferase assays were performed on 293T cells stably expressing miR-let-7g or control NT sequences. Forty-eight hours post-transfection of luciferase constructs, cell extracts were assayed for luciferase activity using the Dual-Glo luciferase assay system (Promega). Relative reporter activities were expressed as luminescence units normalized to Renilla luciferase activity. Luminescence was quantitated using a multimode microplate reader (EnSpire)
2.12. Statistical analysis

For all studies, analysis of variance (ANOVA) was used for comparison of multiple results within a single experiment, followed by Student's $t$-test using one- or two-tailed distributions as appropriate.
Chapter 3

RESULTS: Lin28/let-7 axis is a regulator of ischemia-reperfusion induced myocardial cell death

Ischemic stress initiates a plethora of molecular changes in the myocardium. Due to the reduction or complete obstruction of blood supply to the heart muscle, the cells within the ischemic zone become hypoxic, lose access to nutrients such as glucose and fatty acid, and build up lactic acid and other waste products. Subsequent reoxygenation due to spontaneous thrombolysis or therapeutic intervention results in additional oxidative stress. To a large extent, pathophysiology of the ischemic heart is governed by changes in the ischemic myocyte. These changes are part of signaling network that protect that myocardium from the ensuing stress long enough for the stress to subside or at least minimize damage. Although the initial changes are adaptive, unrestrained continuation of such signaling may be detrimental to the viability of the myocardial tissue due to obligate downstream consequences.

Under ischemia, due to limited oxygen availability cardiac myocytes switch from fatty acid oxidation to glycolysis to maximize ATP generation. This adaptation is governed by changes in gene expression that are modulated mainly by the master transcription factor, HIF1α (described in Section 1.5). For example, insulin dependent glucose transporter, GLUT4, is significantly upregulated to support the increased utilization of glucose. Although short-term glycolytic
adaptation is beneficial, it produces acidic waste products. At 48 hours, cardiac myocyte cultured under hypoxic conditions had significantly decreased media glucose and pH[9]. Due to the obligate activation of ion channels and pumps on the myocyte plasma membrane, this increase in intracellular pH disrupts physiological ionic homeostasis leading to Ca\(^{2+}\) overload. For example, the Na\(^{+}\)/H\(^{+}\) exchanger is quiescent under basal conditions but is activated under ischemia to extrude H\(^{+}\) in exchange for Na\(^{+}\), which is then exchanged for calcium to maintain normal intracellular Na\(^{+}\) levels. This produces cellular calcium overload, which can be reduced by inhibiting the activity of the Na\(^{+}\)/H\(^{+}\) exchanger [159]. Apoptotic and necrotic cell death, especially of the myocyte, both contribute to myocardial ischemic injury, and are initiated by alterations in energy status, disruption of ionic homeostasis, and improper Ca\(^{2+}\) handling, leading to the activation of caspases.

Variations in miR distribution may point toward their functional properties within tissues and cell types. Emerging data from our lab and others show that the miR expression profile is altered in the failing human heart[144, 160, 161]. In animal models, a striking feature is that miR expression is specifically altered within the areas of ischemic injury [79, 86]. Numerous reports have established associations between aberrant upregulation and downregulation of miRs in the heart and pathological ischemia-related conditions that include hypertrophy, arrhythmia, angiogenesis, and fibrosis [82, 88, 162, 163].

Let-7 has been implicated as a tumor suppressor [100, 120, 128, 146, 151], and in a multitude of cellular processes that may contribute to injury or to
adaptation, mainly cell death signaling, metabolic switching, and immune responses. Relevant let-7 targets in these scenarios have been validated in other cell types and organs[97, 116, 119, 149, 150]. The let-7 family member miR-98 reduced cell death in the murine myocardium following angiotensin-induced hypertrophy[87]. Consistent with the protective phenotype, miR-let-7b mimics protected cardiac myocytes subjected to simulated ischemia-reperfusion[164]. Previous studies looking at let-7 expression levels in rodent models have focused on time points relatively late after the ischemic insult[79]. Therefore, the role of let-7 in ischemic cardiac pathophysiology, especially in the early acute phase, has not previously been defined.

The RNA binding protein, LIN28, modulates let-7 at a post-transcriptional level. Lin28 binds to the terminal loop of the pre-let-7 transcript, which prevents it from being processed by Dicer. This mechanism reduces mature let-7 levels[104, 116, 165]. Studies looking at Lin28 in the myocardium have been very limited. Lin28 is among the four factors required to induce pluripotency[133], and some studies show that Lin28 modulates cardiac lineage selection and cardiac differentiation of mouse and human ESCs[166, 167]. However, the role of Lin28 in the adult myocardium has not been examined.

Briefly, in this chapter I will show that myocardial ischemia in vivo activates LIN28, associated with repression of miR-let-7. This repression of miR-let-7c and -g occurs post-transcriptionally and is mediated by hypoxia in a cardiac myocyte-specific manner, concurrent with induction of Lin28 protein and mRNA. Repression of let-7 required ERK1/2 activity and was blocked by HDAC
inhibitors. Lentiviral mediated rescue of let-7g levels during hypoxia protected myocytes from ischemia-induced cell death. We show that let-7 rescue using in vivo delivery of lenti-let-7g also prevents cell death in a murine model of ischemia-reperfusion.

3.1. Ischemic stress initiates acute changes in miR-let-7 expression in the myocardium.

In order to identify microRNAs regulated during acute myocardial ischemia, we compared ischemic and adjacent non-ischemic left ventricular myocardium in mice 2 hours after permanent coronary artery occlusion [168]. Induction of VEGF protein was used to confirm ischemia [169, 170]. Among the relatively small number of differentially regulated microRNAs in this model were multiple miR-let-7 family members, including let-7c, let-7g and let-7a (Figure 3.1), suggesting an important role for miR-let-7 in the early adaptive response to ischemia.
Figure 3.1. Acute myocardial ischemia *in vivo* represses multiple miR-let-7 species. MiR-let-7c, -7g and -7a were quantitated in mouse myocardium 2hr after LAD ligation (black bars) or a sham procedure (white bars) using a Taqman low-density microfluidic array (n=3).

3.2. Hypoxia time-dependently and myocyte-specifically downregulates let-7 family of miRs

Because miR-let-7 is ubiquitously expressed, we asked whether ischemic let-7 downregulation occurred equally in myocytes and non-myocytes (mostly fibroblasts) within the myocardium. Neonatal rat ventricular myocytes (NRVMs) and cardiac fibroblasts (CFs) derived from the pre-plating step in the same preparation were placed under normoxic or hypoxic (0.5% O2, 5% CO2, balance nitrogen) as previously described [152]. In myocytes, miR-let-7c levels declined in a time-dependent manner between 4 and 24 hours of hypoxia (Figure 3.2).
This decline was not seen in parallel cultures of fibroblasts (Figure 3.2). Reducing media glucose to 0.5mM from 5.5mM had no additional effect, and low glucose (0.5mM) alone was not sufficient to downregulate let-7 (Figure 3.2), indicating that miR-let-7 downregulation was dependent on hypoxia alone. Similar reductions were seen with miR-let-7g and miR-let-7a (Figure 3.3).

**Figure 3.2. Myocyte-specific repression of miR-let-7c is mediated by hypoxia.** Control neonatal rat ventricular myocytes were cultured under normoxia with 5 mM glucose. Experimental groups included normoxia without glucose (LG, diamonds), hypoxia with glucose (circles) and hypoxia without glucose (triangles). Cardiac fibroblasts (squares) were cultured under normoxia with glucose. MiR-let-7c was quantitated by RT-PCR at 4, 8 and 24 hours.
3.3. Hypoxia represses miR-let-7g and -7a. CMs were cultured as in (3.2) and miRNAs were quantitated at 24 hour.

Figure 3.3. Hypoxia represses miR-let-7g and -7a. CMs were cultured as in (3.2) and miRNAs were quantitated at 24 hour.

3.3. Hypoxia mediated let-7 downregulation is HIF1-independent and occurs at a post-transcriptional level

In looking for mechanisms of miR-let-7 downregulation, we found that precursor forms of let-7c were differentially affected. The processed precursor pre-miR-let-7c decreased, but levels of the primary transcript pri-miR-let-7c were not changed by hypoxia (Figure 3.5). As described in Section 1.5, HIF1 is a major regulator of hypoxic adaptation in the myocytes. To determine whether miR-let-7 was repressed by a hypoxia-inducible-factor-1α (HIF-1α)-dependent mechanism, we used cobalt chloride (CoCl₂) to achieve non-hypoxic induction of HIF-1α[171, 172]. HIF-1α activation was confirmed by upregulation of the known HIF-1α
target heme oxygenase 1 (HO-1) (Figure 3.4). However, mature miR-let-7c levels were not reduced by CoCl$_2$, (Figure 3.4, bottom), suggesting that HIF-1 is unlikely to be directly involved.

**Figure 3.4. Chemical induction of HIF1 is insufficient to downregulate let-7.** Cardiac myocytes were subjected to treatment with 50mM cobalt chloride (CoCl$_2$) and vehicle (NoCo), and expression of HO-1 protein and miR-let-7c were assayed by immunoblot and/or RT-PCR respectively. (Top) Representative Western blot. (Bottom) HO-1 protein/β-actin and miR-let-7c transcript levels (n=6). Hypoxia treated CM serves as positive control.
Figure 3.5. Hypoxia downregulates miR-let-7 at the post-transcriptional level. Primary and precursor miR-let-7c transcripts were quantitated by qPCR in CM cultured as in (3.2) and expressed relative to those in normoxic CMs (n=6). Norm=normoxia

3.4. Let-7 downregulation is prevented by Mek1/2 and class I and II HDAC inhibition

In other systems, miR-let-7 has been reported to be suppressed by MEK1/2 activation [147], and by histone deacetylase (HDAC) activity[148]. ERK activity has been shown to decrease let-7 levels, attributed to post transcriptional events that involve the phosphorylation of TRBP[147]. ERK/MAPK and HDAC have previously been reported to be activated by hypoxia in the myocyte[173, 174]. We confirmed the phosphorylation and activation of ERK1/2 in hypoxic cardiac
myocytes in our system (described later in Section 4.2). Congruent with these reports, we found that treatment with either the MEK1/2 inhibitor UO126 or the class I, II HDAC inhibitor trichostatin A (TSA) prevented hypoxic downregulation of miR-let-7c (Figure 3.6) suggesting that let-7 downregulation under hypoxia requires that activation of these pathways.

Figure 3.6. Hypoxic downregulation of let-7 requires MAPK/ERK and class I-II histone deacetylase activity. MiR-let-7c levels under hypoxic NRVMs in the presence and absence of the non-selective HDAC inhibitor trichostatin A (TSA) (10nM) and the MEK1/2 inhibitor U0126 (U0, (10µM)) (n=4). Norm= normoxic culture.
3.5. Ischemic stress induces Lin28 expression in the myocardium specifically in the myocytes

Mature microRNAs are generated through sequential transcriptional and enzymatic processing steps, each of which may be subject to regulation (discussed in Section 1.7 and reviewed in [175, 176]). The lack of impact on pri-miR-let-7 indicated a likely post-transcriptional regulatory mechanism. The RNA binding protein Lin28 regulates levels of mature miR-let-7 species in many tissues and organism [116, 177-179]. We observed that Lin28 protein was induced in ischemic myocardium (Figure 3.7), and in hypoxic NRVMs (Figure 3.8A) in a time-dependent manner, along with increased Lin28 mRNA (Figure 3.8B). Hypoxia failed to induce Lin28 in cardiac fibroblasts (Figure 3.8C). These findings suggest that hypoxia represses miR-let-7 through myocyte-specific induction of Lin28.
Figure 3.7. Acute myocardial ischemia in vivo induces Lin28. Lin28 was assayed by immunoblot in non-ischemic (white bar) and ischemic (black bar) zones of murine left ventricle following a 2-hour LAD occlusion. (Top) Representative Western blot. (Bottom) Graph summarizing 3 experiments.
Figure 3.8. A. Myocyte-specific induction of Lin28 is mediated by hypoxia. NRVMs were cultured under normoxic and hypoxic conditions for the indicated times and assayed for Lin28 by immunoblot. (Top) Representative Western blot. (Bottom) Graph summarizing 3 experiments. B. Hypoxia induces Lin28 mRNA in NRVMs. NRVMs were cultured in normoxic and hypoxic conditions and mRNA levels measured using RT-PCR (n=6). C. Hypoxia does not induce Lin28 in cardiac fibroblasts. Fibroblasts (CFs) were exposed to normoxic and hypoxic cultures for 24-hour and protein samples were assayed using immunoblot. (Top) Representative Western blot. (Bottom) Graph summarizing 4 experiments.
3.6. *Lin28 induction is prevented by class I and II HDAC inhibition*

It has been shown that Lin28 can be directly acetylated consequently leading to its destabilization[148]. HDAC inhibitor TSA prevented the hypoxic induction of Lin28 (Figure 3.9) indicating that hypoxia may regulate the myocyte Lin28/let7 axis through elevated HDAC activity. However, we were unable to see any difference in acetylated Lin28 protein between normoxic and hypoxic conditions.

![Figure 3.9. Lin28 induction in hypoxia requires class I-II histone deacetylase activity.](image)

Lin28 was assayed by immunoblot in normoxic and hypoxic myocytes cultured in the presence and absence of TSA (10nM). (Top) Representative Western blot. (Bottom) Graph summarizing 7 experiments. Lin28 protein was normalized to β-actin.
3.7. MiR-let-7 regulates cardiac myocyte cell death under hypoxic stress

Hypoxia and the associated acidic environment are detrimental to myocyte survival [9]. Certain miRs have been shown to modulate cell apoptosis in the heart [180], and let-7 can promote proliferation and survival of certain cells by directly targeting genes involved in regulation of cell death[150]. Our results suggest that hypoxia induces a gradual, time-dependent downregulation of let-7 in cardiac myocytes that continues over at least 24 hours. To assess the consequences of miR-let-7 loss in sustained hypoxia, we adopted a rescue strategy to restore miR-let-7g levels to normoxic levels in hypoxic myocytes, using lentiviral transduction. As expected, exogenous miR-let-7g was also subject to negative post-transcriptional control. However, we were able to achieve comparable levels of mature miR-let-7g in normoxic and hypoxic NRVM (Figure 3.10).
Figure 3.10. Lentiviral miR-let-7g transduction rescues miR-let-7g expression levels in hypoxic NRVMs. NRVMs were cultured as described above. 48 hours after transduction with lentiviral vectors expressing NT (non-targeting) and miR-let-7g, myocyte levels of miR-let-7g were measured using RT-PCR (n=5). Mature sequences for let-7g and let-7g are provided for comparison.

We initially hypothesized that loss of miR-let-7 might affect cell fate decisions in NRVMs during hypoxia. Overexpression of miR-let-7g in normoxic cells did not affect basal rates of cell death or apoptosis as determined by LDH release and caspase-3 cleavage, respectively (Figure 3.11). In contrast, both cell death and apoptosis increased in hypoxic cells transduced with a non-targeting (NT) vector,
but not in cells rescued with the miR-let-7g vector (Figure 3.11), implying that apoptotic signaling in hypoxic myocytes is due in part to loss of miR-let-7.

**Figure 3.11.** miR-let-7g rescue prevents myocyte apoptosis during hypoxia. Protein levels of caspase3 and its cleavage products were assayed using immunoblot. LDH release was measured by colorimetric assay as described in Methods, after 24 hours of hypoxia. (Top and bottom left) Representative Western blot and quantitation. (Bottom right) Quantitation of LDH release (n=6-9).
3.8. MiR-let-7 regulates cell fate decisions in the ischemic myocardium in vivo

The ability of intravenously injected miRs to target the vasculature in the myocardium and the myocardium itself has been reported by various groups, including our own [78, 83, 181]. Our lab has used viral vectors and chemical moieties to achieve overexpression and knockdown of miRs. Animal models for myocardial infarction and ischemia-reperfusion have been extensively used to elucidate pathophysiological mechanisms of cardiac remodeling following MI and evaluate potential therapeutic approaches [182, 183]. The effect of miR-let-7 on apoptosis was confirmed \textit{in vivo} using the same lentiviral vectors in a neonatal mouse transduction protocol [153, 184]. Transduction achieved a functionally significant increase in miR-let-7g, together with downregulation of the established let-7 target INSRβ (Figure 3.12, left and right). Elevated levels of miR-let-7g were sustained to at least 9-10 weeks of age (Figure 3.13). Although miR-let-7 transgenic mice have been reported to have smaller body weights [97, 116], we did not observe any difference in weight between NT- and miR-let-7g-overexpressing mice.
Figure 3.12. Lentiviral transduction of miR-let-7g in myocardium \textit{in vivo}. Transcripts levels of miR-let-7 family members and protein levels of INSR\(\beta\), a validated miR-let-7 target, were assayed in whole heart tissue using RT-PCR and immunoblot 10 days after lentiviral vector injection. (Left) Transcript levels relative to lenti-NT-transduced animals. (Above) Representative Western blot.

Figure 3.13. miR-let-7g expression is sustained in mouse myocardium till adulthood. MiR-let-7g transcript levels were measured in whole hearts 9-10 weeks after transduction (n=3-5).
Lentivirus-transduced mice were then subjected to a 60-min ischemia and 24-hour reperfusion injury protocol [168] at 9-10 weeks of age. NT-expressing myocardium contained large numbers of apoptotic cells in the ischemic zone, while miR-let-7g-expressing mice had low rates of apoptosis that were indistinguishable between ischemic and non-ischemic zones (Figure 3.14). While we did not specifically differentiate between apoptotic myocytes and fibroblasts, myocytes constitute the vast majority of apoptotic cells following ischemia-reperfusion injury [14, 185], whereas fibroblasts are relatively resistant to apoptosis under the same conditions [186]. Although let-7 “rescue” did not significantly affect calculated ejection fraction, both diastolic and systolic volumes were improved in these mice compared with mice receiving the non-targeting control vector (LViVd 71.9 ± 1.1 vs. 66.5 ± 2.4, p= 0.039; LViVs 22.8 ± 0.4 vs. 20.6 ± 0.91, p = 0.05, NT vs. Let7 n = 4,5) (Table 3.1), consistent with reduced post-infarct remodeling.
Figure 3.14. Increasing miR-let-7g reduces apoptosis in ischemia-reperfusion injury. Sections from sham-operated hearts, and from non-ischemic and ischemic zones of NT- and miR-let-7g-transduced mice were examined for apoptotic cells following 60 minutes of ischemia and 24 hours of reperfusion as described in Experimental Procedures. (Left) Representative sections and (top) enlargements. Sham = sham-operated. I/R NI = Ischemia-reperfusion, non-ischemic zone. I/R-NT, I/R-let-7g = I/R + non-targeting and let-7g vectors respectively. (Bottom right) Quantitation of apoptotic nuclei (n=2500-4000 nuclei) in n = 4 (NT) or 5 (let-7g) individual mice.
Table 3.1. Functional alterations induced by miR-let-7g restoration following ischemia-reperfusion. Nine to ten weeks after lentiviral transduction, mice were subjected to ischemia-reperfusion surgery as described in Methods. Echocardiographic parameters were assessed after 60 minutes of ischemia followed by a 24 hour reperfusion interval (I/R). HR, heart rate; LViDs, left ventricular internal dimension in systole; LViDd, left ventricular internal dimension in diastole; LVVs, left ventricular volume in systole; LVVd, left ventricular volume in diastole; SV, stroke volume; EF, ejection fraction; FS, fractional shortening; CO, cardiac output.

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<td><strong>FS (%)</strong></td>
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<td>38.29 ±0.31</td>
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<td><strong>CO (mL/min)</strong></td>
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<td>14.6 ±1.11</td>
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Chapter 4

RESULTS: Let-7 modulates myocardial cell death by regulating PI3k-Akt signaling by targeting its endogenous repressor

Ischemic stress in the myocardium initiates the activation of multiple signaling cascades. Some of these signaling cascades directly contribute to injury of the myocardium, as discussed in Section 1.3 and Chapter 3. Bioenergetic collapse, accumulation of intracellular calcium, oxidant free radicals and acidic waste all induce cell death in chronically ischemic myocytes and following myocardial ischemia-reperfusion. On the other hand, intrinsic signaling cascades can allow adaptation and protection of the myocardium during stress. A well-studied cardioprotective signaling cascade is the PI3k-Akt pathway.

Cardiac myocyte culture systems and animal models of myocardial ischemia and reperfusion have been used to validate the cardioprotective role of PI3k-Akt pathway. Akt activation is inhibited in the ischemic brain[187] and the ischemic and reperfused myocardium[188, 189]. Constitutive activation of PI3k and Akt conferred protection to hypoxic cardiac myocytes[50, 51, 190]. In keeping with these results, hearts of Akt transgenic mice are protected from acute ischemia-reperfusion injury and display increased systolic and diastolic function [51, 56, 190].

PI3k-Akt signaling is regulated at multiple levels (Figure 1.2). As discussed in Section 1.6.1, upstream tyrosine phosphorylation of the insulin
receptor (IR) initiates interaction with adaptor IRS-1, thereby recruiting PI3k to the plasma membrane. Subsequent conformational changes lead to PI3k activation. Phosphorylation of IRS-1 is a key regulatory event in the activation of PI3k. Serine phosphorylation of IRS-1 has been shown to inhibit its interaction with the upstream IR as well as downstream PI3k, resulting in dampened PI3k activation[191, 192]. Phosphorylation specifically blocks the interaction between IRS-1 and p85 subunit of PI3k [193]. In L6 myoblasts, conditions such as energy depletion, hypoxia and inhibition of oxidative phosphorylation induce this inhibitory serine phosphorylation of IRS. The catalytic subunit of PI3k, P110, encoded by Pik3ca, is critical for Akt activation. Cardiac myocyte-specific deletion of Pik3ca decreases PI3k levels and leads to contractile dysfunction[194]. Finally, the activation of Akt by PI3k is antagonized by the phosphatase PTEN. Pten is induced in murine models of myocardial ischemia-reperfusion, and pten knockout murine hearts are protected from ischemia-reperfusion injury[195]. Cardioprotective pre-conditioning protocols inactivate PTEN, allowing for increased Akt activation in the myocardium[196].

Determining the downstream targets of miRs is critical to understanding their physiological importance. Nucleotides 2-8 at the 5' end of each miR, called the "seed" sequence, bind to complementary bases on the 3'UTRs of its target mRNA. miR targets are predicted using computational algorithms based on three primary criteria. First is the requirement for Watson-Crick pairing between the 5' seed sequence and its complement in the 3'UTR of the target gene. MiR-mediated regulation is highly sensitive to disruption of the seed pairing. Second
is the extent of evolutionary sequence conservation of putative target binding sites across species. Most mammalian targets are predicted by scanning for a 7nt-conserved alignment. Third is the fact that highly conserved miRs have a high number of targets. Even after accounting for complementarity occurring due to chance, a single miR family has almost 300 target genes. More that half of human genes have conserved miR target sites on their 3'UTRs[58]. We used an algorithm available at Targetscan.org, to analyze possible targets of let-7 in this study.

Although computational target prediction is an excellent starting point, there are discrepancies among various computational prediction programs. This is due to inherent difference in algorithms, ranking systems, and usage of different databases. In addition, some features such as compensatory binding and UTR context may not be accounted for[58]. Therefore, approaches combining in silico bioinformatics with biochemical assays including co-immunoprecipitation, microarrays and RNA sequencing have also been used. Argonaute (Ago) is an integral component of the RISC, and co-immunoprecipitating mRNA associated with miR-programmed Ago is one such biochemical approach[197]. MiR programming of Ago is achieved by overexpressing a miR of interest[198]. This approach has been used successfully to identify miR targets in the myocardium in vivo[199] and is the approach utilized in this study.

Briefly, in this chapter I will show that let-7 elevation was associated with increased AKT activation in vitro and in vivo, resulting from positive regulation of
the PI3K pathway through inhibition of a novel target of let-7, PIK3IP1. I will also demonstrate that LIN28/let-7/PIK3IP1 axis exists in human myocardium. Although let-7 regulation of the PI3k-Akt pathway will be the focus of this chapter, I will also discuss some other cardioprotective signals regulated by let-7 in cardiac myocytes.

4.1. Restoring let-7 levels under hypoxia prevents hypoxia-induced reduction in Akt activation and downstream Gsk3

The serine/threonine kinase Akt plays a critical role in cardiomyocyte survival under stress [50-52]. AKT is initially activated under hypoxia and then progressively inhibited through a negative feedback mechanism involving the upstream adaptor IRS-1 [191, 192]. Consistent with this, we observed a decline in the activating phosphorylation of Akt at serine 473, as well as reduced phosphorylation on Ser9 of GSK3β, an Akt substrate during hypoxia (Figure 4.1). Transduction of miR-let-7g, but not the NT vector, prevented the hypoxic decline of both pSer473-Akt and pSer9-GSK3β. These findings suggest that restoring miR-let-7 during hypoxia protects against myocyte apoptosis by permitting AKT activation.
Figure 4.1. miR-let-7g rescue promotes Akt activation in hypoxia. NRVMs transduced with lenti-NT and lenti-let-7g were assayed after 24h for pSer473-AKT, pSer9-GSKβ, total GSKβ and total AKT by immunoblot. (Left) Representative Western blots. (Center and right) Quantitation of pAKT and pGSK (n=6-9 per treatment group). All phosphoprotein levels are normalized to their respective total proteins.

4.1.1. Let-7 mediated cardio-protection and Akt activation in vitro is sensitive to PI3k inhibition

We next asked whether miR-let-7g-mediated cytoprotection was dependent on PI3K, an important upstream activator of Akt. The miR-let-7g-dependent increases in pSer473-Akt and pSer9-GSK3β were abrogated by the selective PI3K inhibitor LY-294-002, but not by its vehicle (Figure 4.3). Similarly, the protective effect of miR-let-7g rescue against caspase3 cleavage was abrogated by LY-294-002 (Figure 4.2). These findings demonstrate that loss of miR-let-7g in hypoxia leads to loss of survival signaling through PI3K, upstream of AKT.
Figure 4.2. A. PI3K inhibitor LY-294-002 (LY) reverses cytoprotective effects of let-7 rescue. NRVMs were cultured and transduced as before and treated with 10µM PI3K inhibitor LY 294-002 (LY) or vehicle control. Protein samples were assayed for cleaved caspase3 and total caspase3 using immunoblot.
Figure 4.3. PI3K inhibition reverses effect of let-7 rescue on Akt Ser473 phosphorylation. NRVMs were cultured and transduced as before and treated with 10µM PI3K inhibitor LY 294-002 (LY) or vehicle control. Protein samples were assayed for phosphorylated Akt at serine 473 and GSK3β at serine9, total Akt and total Gsk3β using immunoblot.

4.1.2. Elevated let-7 levels sustain Akt activation in vivo following ischemia-reperfusion

Understanding mechanisms of cardioprotection is essential to counteracting the effects of detrimental signaling cascades and salvaging the myocardium under stress. The effect of miR-let-7 on Akt was confirmed in vivo using the same lentiviral vectors in a neonatal mouse transduction protocol [153, 184]. We observed that compared to the sham operated hearts, the pSer473 Akt was
significantly decreased NT transduced hearts. Transduction of miR-let-7g, but not the NT sequence, was associated with increased pSer473 AKT in the ischemic myocardium (Figure 4.4).

**Figure 4.4. AKT is activated in vivo with miR-let-7g overexpression.** Non-ischemic and ischemic zones of hearts from NT and miR-let-7g-transduced animals were assayed for pSer473-Akt, total Akt and β-actin. (Left) Representative Western blots. (Right) Graph summarizes data from n=4 (NT) and n=5 (let-7g) mice.

4.1.3. *Phosphoinositol-3-kinase interacting protein 1 (Pi3kip1) is induced by hypoxia*

Phosphatidylinositol 3' kinase (PI3K) transmits an activating signal to Akt (section 1.6.1). Phosphatidylinositol 3' kinase interacting protein 1 (PIK3IP1) is a transmembrane protein that contains a kringle motif allowing protein-protein interaction and shares domain homology with p85 regulatory subunit of PI3k. PIK3IP1 activation results in decreased PI3k as well as AKT activation [200-202]. A targetscan.org search reveals that the PIK3IP1 3'UTR contains a predicted
binding site for miR-let-7. The miR-let-7-induced degradation of PIK3IP1 would be predicted to increase AKT phosphorylation. Concomitant with loss of miR-let-7c/g, PIK3IP1 expression was increased in hypoxic NRVMs (Figure 4.5).

*Figure 4.5. PIK3IP1 is induced by hypoxia in NRVMs.* NRVMs were cultured in normoxia and hypoxia and mRNA levels of PIK3IP1 were measured using RT-PCR (n=4).

4.1.4. *Pik3ip1 is a target of let-7*

Because miRs repress the expression of their gene targets, another biochemical approach to determine the targets of let-7 is to perform transcriptomic analysis in cells overexpressing let-7. CMs expressing lenti-miR-let-7g had significantly decreased mRNA levels of Lin28, a previously validated target of miR-let-7 and
PIK3IP1 compared to CMs expressing the NT sequence (Figure 4.6). Although reduction in a specific mRNA can suggest that it is regulated by the overexpressed miR, there is a possibility that the repression is secondary and not direct. “Direct” miR targets may be identified by detecting mRNAs that are associated with Ago, a main functional constituent of the RISC [197, 198].

Figure 4.6. miR-let-7g modulates mRNA levels of PIK3IP1. NRVMs were cultured in normoxia and transduced with lenti-let-7g and NT vector and mRNA levels of Lin28 and PIK3IP1 were measured using RT-PCR (n=4).

To validate PIK3IP1 as a “direct” miR-let-7g target in cardiac myocytes, we used a direct biochemical approach based on Photoactivatable Ribonucleoside
Crosslinking and Immunoprecipitation (PAR-CLIP) [158]. CLIP a robust method for validating miR and target interaction in the cardiac RISC[199]. Myocyte RISC complexes were programmed with miR-let-7g or a non-targeting sequence using lentiviral vectors, and Argonaute 2 (Ago2), a major component of the RISC, was immunoprecipitated from NRVM lysates 48 hours later. Co-precipitated RNA was extracted and analyzed by RT-PCR for the presence of potential miR-let-7 targets. In myocytes programmed with the miR-let-7g vector, miR-let-7 was enriched in Ago2-associated immunoprecipitates relative to NT-transduced cells; unrelated miR-142-5p and snoRNA species were not (Figure 4.7, top). PIK3IP1 mRNA was also significantly enriched in the miR-let-7g-programmed RISC, as was Lin28, while control β-actin and 18S RNAs were not (Figure 4.7, bottom). The wild type 3’UTR of PIK3IP1 was also functionally targeted by miR-let-7g but not NT sequence (Figure 4.8). Thus, in cardiac myocytes, miR-let-7g promotes Akt activation and cell survival by directly targeting PIK3IP1 to the RISC.
Figure 4.7. miR-let-7g targets PIK3IP1 to cardiac myocyte RISC. NRVMs were cultured in normoxia and transduced with lenti-let-7g and NT vector so as to program the myocyte RISC. Following immunoprecipitation with Ago2 antibody as described in Methods levels of various miRs and genes were quantified in the immunoprecipitated RNA fraction using RT-PCR. (Top) Transcript levels of miR-let-7g, miR-142 and snoRNA. (Bottom) mRNA levels of PIK3IP1, Lin28, β-actin and 18S. Transcript levels were normalized to immunoprecipitated AGO2 protein (n=3).
Figure 4.8. miR-let-7g functionally targets WT PIK3IP1 3’UTR. 293Ts expressing miR-let-7g and NT were transfected with pGLO dual luciferase vector. Renilla luciferase units were normalized to firefly luciferase units (n=5).

4.1.5. MiR-let-7 modulates its target genes in human heart failure

Previous studies have separately shown that that miR-let-7 family members are dysregulated in human heart failure [144] and that Lin28 expression is
upregulated in the ischemic human heart [203]. To obtain evidence for a functional LIN28-let-7 regulatory axis in human heart failure, we determined the protein levels of Lin28 and mature transcript levels of miR-let-7g and miR-let-7a in a series of failing and non-failing human hearts. Clinical characteristics of samples are provided in Table 4.1.

A significant inverse relationship was found between Lin28 expression and levels of both let-7 family members (Figure 4.9A, left and right, respectively). Likewise, PIK3IP1 protein content varied inversely with miR-let-7 family members, miR-let-7g and miR-let-7a in the same human heart failure samples (Figure 4.8B, left, and right, respectively). The inverse relationship between let-7g and both Lin28 and PIK3IP1 was observed across all samples and was particularly evident in the failing heart group. In contrast, there was no relationship between Lin28 or PIK3IP1 and levels of the unrelated miR-141 (Figure 4.8C left and right, respectively).
Table 4.1. Characteristics of human subjects analyzed for this study.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Condition</th>
<th>Heart Weight (g)</th>
<th>Age</th>
<th>Ethnicity</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>39306A1D</td>
<td>Male</td>
<td>non-failing</td>
<td>414</td>
<td>55</td>
<td>White</td>
<td>Normal anatomy</td>
</tr>
<tr>
<td>322A20D</td>
<td>Male</td>
<td>non-failing</td>
<td>N/A</td>
<td>46</td>
<td>Black</td>
<td>Stent in LAD, apical scarring</td>
</tr>
<tr>
<td>51565T_004</td>
<td>Male</td>
<td>non-failing</td>
<td>472</td>
<td>40</td>
<td>White</td>
<td>Triple vessel CAD, septal infarct</td>
</tr>
<tr>
<td>1030528A2</td>
<td>Male</td>
<td>ICM</td>
<td>518</td>
<td>42</td>
<td>Black</td>
<td>Biventricular dilatation and hypertrophy, interstitial fibrosis</td>
</tr>
<tr>
<td>1050968B3</td>
<td>Male</td>
<td>Hypertrophic, failing</td>
<td>840</td>
<td>51</td>
<td>Unknown</td>
<td>Stent in LAD, aortic valve prosthesis</td>
</tr>
<tr>
<td>1040221A2</td>
<td>Female</td>
<td>Hypertrophic, failing</td>
<td>280</td>
<td>33</td>
<td>White</td>
<td>Posterior infarction, fibrosis</td>
</tr>
<tr>
<td>1050508A3</td>
<td>Female</td>
<td>CHF</td>
<td>unknown</td>
<td>59</td>
<td>White</td>
<td>Concentric hypertrophy, patchy fibrosis, ICD in place</td>
</tr>
<tr>
<td>1040319A3</td>
<td>Male</td>
<td>ICM, aortic valve disease</td>
<td>548</td>
<td>67</td>
<td>White</td>
<td>Triple vessel CAD, septal infarct</td>
</tr>
<tr>
<td>1030952A3</td>
<td>Male</td>
<td>ICM</td>
<td>547</td>
<td>62</td>
<td>White</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.9. miR-let-7 microRNA family members modulate levels of Lin28 and PIK3IP1 in human myocardium. A. miR-let-7 family members inversely correlate with Lin28. Scatter plot showing the protein levels of Lin28 and transcript levels of miR-let-7g (left) and miR-let-7a (right) in corresponding human myocardial samples. B. miR-let-7 family members inversely correlate with PIK3IP1. Scatter plot showing the protein levels of PIK3IP1 and transcript levels of miR-let-7g (left) and miR-let-7a (right) in corresponding human myocardial samples. C. No inverse correlation between Lin28 or PIK3IP1 and miR-141. Scatter plot showing the protein levels of Lin28 (left) and PIK3IP1 (right) and transcript levels of miR-141 in corresponding human myocardial samples. (n=3 non-failing, n=6 failing, p≤0.05 α at 5%).
4.2. Let-7 represses hypoxia induced ERK signaling in vitro

Let-7 directly targets and regulates Ras [123], which is implicated in survival signaling through extracellular signal-regulated kinase (ERK) in the heart. Activation of ERK by ischemia attenuates apoptosis in cardiac myocytes and in intact hearts, and its inhibition sensitizes cardiac myocytes to apoptosis [173]. Hypoxia activates MAPK ERK1/2 in cardiac myocytes and this activation is associated with cardioprotection; however, the protection mediated by ERK activation appears to be context-dependent. For example, inhibition of ERK did not affect the cardioprotective effects mediated by β2 adrenergic receptor activation in hypoxia[204].

Figure 4.10. miR-let-7g rescue inhibits ERK1/2 activation in hypoxia. NRVMs transduced with lenti-NT and lenti-let-7g were assayed after 24h for p-ERK1/2, total ERK1/2 by immunoblot. (Left) Representative Western blots. (Right) Quantitation of pERK1/2 (n=6-9 per treatment group). All phosphoprotein levels are normalized to their respective total proteins.
Under normoxia, there was minimal difference in ERK activation in NRVMs treated with control lenti-NT and lenti-let-7g vectors. As expected, ERK was significantly activated in the NRVMs that were made hypoxic. Compared to control lenti-NT treated NRVMs, this activation was significantly attenuated in NRVMs treated with lenti-let-7g (Figure 4.10). This reduction is likely due to direct targeting of Ras, an upstream activator of ERK.

4.3. *Let-7 potentiates induction in heme oxygenase-1 (HO-1) in vitro*

Heme oxygenase-1 is an inducible enzyme that converts prooxidant heme to carbon monoxide and biliverdin/bilirubin[205]. These catalytic products, respectively, possess anti-inflammatory and anti-oxidant properties. Thus, induction of HO-1 is considered to be cardio-protective under stress conditions, such as ischemia, where ROS and chronic inflammation are detrimental to cell viability. HO-1 transcription is negatively regulated by the transcription factor, BACH1. *Bach1* null mice have increased expression of HO-1 and are protected during pressure overload and ischemia-mediated cell injury[206]. Let-7 directly targets and regulates BACH1[207].
Figure 4.11. miR-let-7g rescue potentiates HO-1 activation in hypoxia. NRVMs transduced with lenti-NT and lenti-let-7g were assayed after 24h for HO-1 and β-actin by immunoblot. (Left) Representative Western blots. (Right) Quantitation of HO-1 normalized to β-actin (n=6-9 per treatment group).

Under normoxia, HO-1 protein expression was not affected by lenti-let-7g. As expected, HO-1 was induced by hypoxia in both the lenti-NT and lenti-let-7g transduced NRVMs. However, HO-1 induction was significantly potentiated in lenti-let-7g treated NRVMs (Figure 4.11).

4.4. Potentiating the downregulation of let-7 during hypoxia promotes insulin signaling in vitro

We observed a trend toward downregulation of let-7 beginning at 8 hours of hypoxia. This downregulation reached statistical significance at 24 hours, the maximal time point under investigation. To assess the consequences of further potentiating this downregulation, we used a let-7 sponge sequence to sequester functional let-7 from the system at or before 8 hours [117, 151]. As expected, compared to normoxia, hypoxic NRVMs had heightened insulin signaling
indicated by increased levels of insulin receptor and phosphorylated Akt. NRVMs that were treated with the let-7 sponge vector had a further increase in these signals (Figure 4.12). This suggests that potentiating the repression of let-7 likely contributes to the elevation of insulin signaling and glucose oxidation in the initial phase of hypoxia consistent with other systems[97, 116].

**Figure 4.12. Potentiating the initial downregulation of let-7 promotes Akt activation in hypoxia.** NRVMs treated with PBS control and transduced with lenti-empty and lenti-let-7 sponge were assayed after 8h for insulin receptor β, β-actin, pSer473-AKT, and total AKT by immunoblot. (Top) Representative Western blots. (Center and bottom) Quantitation of pAKT and insulin receptor B (n=4 per treatment group). All phosphoprotein levels are normalized to their respective total proteins.
4.4. Restoration of let-7 levels under hypoxia switches metabolic preference in cardiac myocyte

Because of limited availability of oxygen under ischemic conditions, the cardiac myocyte switches from fatty acid oxidation to glycolysis to generate ATP. This switch is modulated by transcriptional factors that coordinately activate or repress genes involved in both pathways. We observed that rescuing the levels of let-7 was protective in cardiac myocyte under simulated ischemia. We wanted to assess if let-7 modulates genes involved in metabolic pathways. The PPARα direct transcriptional target, medium chain acyl co-A dehydrogenase (MCAD), is responsible for oxidizing medium-chain fatty acids. The medium-chain fatty acid oxidation is not reliant on carnitine palmitoyltransferase 1 (CPT-1), a transporter of fatty acids[208]. Under normoxic conditions, let-7g has no effect on expression of this gene. As expected, under hypoxia, MCAD was repressed suggesting that fatty acid oxidation is suppressed in these conditions. However, when we rescued let-7g, MCAD expression was significantly elevated (Figure 4.13). In tandem, pyruvate dehydrogenase kinase 1 (PDK1), which is a HIF-1α direct target, is induced under hypoxia. PDK1 inhibits PDH to prevent conversion of pyruvate to acetyl co-A thereby increasing glycolysis[209, 210]. This induction was significantly potentiated when we rescued let-7g levels (Figure 4.13). These data suggest that let-7g elevates both glycolysis and fatty acid oxidation.
Figure 4.13. Alteration in metabolic preference in hypoxic cardiac myocytes with let-7g restoration. mRNA levels of MCAD (medium chain acyl dehydrogenase) and PDK1 (pyruvate dehydrogenase kinase 1) were measured in cardiac myocytes under normoxia and hypoxia following let-7g transduction (N=5).
Chapter 5

DISCUSSION

MiR-let-7 expression is dysregulated in human heart failure [144], and several miR-let-7 species decline after myocardial injury in zebrafish and rat [143, 211, 212]. The biological significance of these changes is unknown, although loss of let-7 has been proposed to initiate a regenerative process [143]. The regulatory mechanisms and key targets of miR-let-7 in the diseased heart are also unknown. Here we show that miR-let-7 is controlled in the postnatal heart through myocyte-restricted, hypoxia-dependent induction of the pluripotency factor Lin28. Activation of this pathway results in suppression of Akt through a direct let-7 target, PIK3IP1, increasing myocyte vulnerability to apoptosis that can be reversed by restoring let-7g levels. Finally, we show that Lin28, let-7, and PIK3IP1 are specifically co-regulated across a spectrum of normal and diseased human hearts. Whether hypoxic activation of this pathway potentiates metabolic adaptation or regenerative mechanisms, it clearly does so at the expense of PI3K/Akt-mediated survival signaling.

5.1. Loss of miR-let-7 is potentially adaptive to hypoxia

The fall in miR-let-7 and rise in Lin28 coincide with a period of rapid adaptation to ischemic stress in which the myocyte is required to generate ATP through
glycolysis. MiR-let-7 is well-established as a repressor of glucose utilization by targeting multiple genes in the PI3K-mTOR pathway regulating insulin signaling and glucose metabolism, including INSRβ, IGF1R and IRS2 [97, 116]. Global let-7 transgenic animals are glucose intolerant and insulin-resistant, and let-7 inhibitors prevent and treat high-fat induced glucose intolerance in vivo [97, 116].

In cancer cells, loss of miR-let-7 promotes high rates of glycolysis even when oxygen is abundant, a phenomenon known as the Warburg effect [213] through de-repression of pyruvate dehydrogenase kinase (PDK), a key glycolytic switch [149]. The observed loss of let-7 in hypoxic cardiac myocytes would thus act to facilitate the adaptive switch from β oxidation to anaerobic glycolysis [8, 214], and to increase glucose uptake through insulin signaling (Figure 4.12).

5.2. A myocyte-specific mechanism for let-7 downregulation in ischemia

Multiple pre- and post-transcriptional mechanisms exist to permit close control of the timing and quantity of miR-let-7. Our results show that hypoxia acts post-transcriptionally to repress let-7, but only in cardiac myocytes and not in fibroblasts from the same tissue. Processing of mature miR-let-7 from its precursor forms is potentially sensitive to extracellular signals and cellular context. One potential control point for this process is binding of the KH-type splicing regulatory protein (KSRP) to the terminal loop of precursor species, which is required for accumulation of mature let-7 in multiple cell types, and for promotion of myogenic differentiation of C2C12 cells[215, 216]. This interaction
is regulated in part by PI3K/Akt signaling and thus potentially by other extracellular signals [216]. The same terminal loop is targeted by Lin28a and heteronuclear ribonucleoprotein A1 (hnRNP A1) to inhibit production of miR-let-7 and maintain cell stemness [217]. Functioning of mature let-7 is directly inhibited by the IncRNA H19, which contains multiple let-7 binding sites and thus acts as an endogenous sponge [219]. Our findings show that the Lin28/let-7 regulatory axis can be invoked in a cell type-specific and stimulus-specific manner to regulate the adaptation to myocardial hypoxia, and may be important in regulating miR-let-7 levels in the failing human heart. This axis is evolutionarily ancient, being conserved from worms to mammals, and appears to be preserved in adult mammalian heart as a means of responding to extracellular conditions.

Lin28 itself is subject to repression by miR-let-7, setting up a mutual negative feedback loop that can facilitate rapid adaptations. In our system, upregulation of Lin28 protein and downregulation of miR-let-7 were immediate-early responses to myocardial ischemia, occurring within 2 hours of coronary occlusion in vivo and beginning at 8 hours in hypoxic myocytes in vitro. Although we did not see any effect of hypoxia on Lin28 acetylation, hypoxic modulation of both Lin28 and miR-let-7 was sensitive to broad-spectrum HDAC inhibition, suggesting that other post-transcriptional modifications such as acetylation could regulate let-7 directly or indirectly [148]. MEK1/2 and/or its upstream activators, which also respond rapidly to ischemia, further facilitate this fast response [220].

MEK/ERK activity is known to repress let-7, despite generally increasing
microRNA expression through effects on Dicer [147]. Using the MEK1/2 inhibitor U0126, Paroo et al. [147] showed that active ERK, through a DICER-dependent mechanism, downregulates multiple let-7 family members. Our experiments provide evidence that a similar pathway may be activated in myocytes by hypoxia. ERK activation has been observed in myocytes under hypoxic and oxidative stress [220, 221]. However, ERK activation is likely cardioprotective, thus the enhanced vulnerability of myocytes following downregulation of let-7 must result from a mechanism downstream of let-7 itself. We propose that myocyte-selective inhibition of survival signaling through PI3 kinase is a major contributor to the selective apoptosis of myocytes when let-7 levels are reduced by hypoxia (Figure 5.1).

5.3. Preventing let-7 downregulation reduces myocyte apoptosis

As noted above, the immediate loss of let-7 in hypoxic cardiac myocytes could have short-term value in the ischemic myocardium by facilitating glucose uptake and glycolysis. However, at later time points, preventing the fall in miR-let-7g reduced, rather than increased, cardiac myocyte death both in hypoxia in vitro, and following ischemia-reperfusion injury in vivo. This observation is consistent with a recent study in which overexpression of let-7b protected against cell death in an in vitro model of simulated ischemia-reperfusion [164]. Similarly, overexpression of the let-7 family member miR-98 conferred survival benefit in a
model of angiotensin II-induced myocardial hypertrophy [87]. Efforts to
downregulate let-7 have produced discordant results. Aguirre et al described

Figure 5.1. Summary diagram. Hypoxia induces expression of the RNA binding
protein Lin28 by a mechanism that is sensitive to ERK signaling and class 1/2
HDAC activity. Lin28 destabilizes members of the miR-let-7 family, potent
repressors of genes (Insrβ, Irs2, Igf1r) involved in glucose utilization, promoting
an adaptive shift to glycolysis. However, let-7 also represses PIK3IP1, an
inhibitor of Akt, thus cell survival signaling is impaired by let-7 loss. Restoration
of let-7g during hypoxia boosts Akt signaling and improves cell survival.
improved systolic function with combined intracardiac injection of anti-miR-let-7a/c and anti-miR-99/100 late in the course of experimental myocardial infarction, associated with evidence of increased cardiomyocyte mitosis [143]; Tolonen reported that intravenous infusion of a miR-let-7c antagomiR 4 weeks after coronary occlusion prevented post-MI remodeling, associated with reduced apoptosis and fibrosis[222]. These conflicting results could be due to the significant difference in timing of intervention in these latter studies, and possibly also in model systems and microRNA manipulation strategies. The balance of competing signals and outcomes following myocardial infarction in vivo depends greatly on the intensity and duration of hypoxia. Moreover, the targets and functions of let-7 are strongly influenced by concentration and by other factors in the cellular milieu [145], and potentially involve other cell types present at lower abundance in the myocardium, such as endothelial cells[223, 224].

Our data show that transduction of miR-let-7 markedly reduces apoptosis rates. Although we did not identify significant improvement in ejection fraction, both systolic and diastolic dimensions were significantly reduced, indicative of an anti-remodeling effect after infarction. It is important to note that end-systolic and end-diastolic volumes are also excellent predictors of adverse cardiovascular events after infarction[225]. As a technical point, it has been reported that treatments targeting myocardial infarctions directly (e.g. stem cell injections) result in local improvement in contractility without affecting measurements of global ejection fraction [226]. Hence it is possible that beneficial effects of let-7 were exerted locally within the infarcted area, a measurement technically
inaccessible to our probe. It is also possible that the effect size on global contractility was below the threshold of measurement and would have been increased by greater let-7 transduction, or by co-transduction of let-7 and other ischemia-regulated microRNAs, including miR-99 and miR-100, as suggested by the work of Aguirre et al. [143]. Understanding the targets and regulation of the Lin28-let-7 axis may shed light on how the complex myocardial injury response is regulated in vivo.

5.4. MiR-let-7 reduces apoptosis by promoting Akt signaling via targeting PIK3IP1

One novel finding of this study is that miR-let-7 can act to promote Akt signaling in the heart. Although miR-let-7 negatively regulates the INSRβ-PI3K pathway in many cell types, in cortical neurons, let-7 “mimics” failed to decrease AKT phosphorylation [119], suggesting additional cell-type specific or context-dependent factors. A protein, PIK3IP1 that shares significant homology with the PI3K p85 regulatory subunit, negatively regulates PI3K. PIK3IP1 binds to the catalytic p110 subunit to prevent PI3K activation and phosphorylation of Akt in multiple cell types, including cardiac myocytes [200-202, 227]. Our study provides substantial evidence for PIK3IP1 as a novel target for let-7 in the myocardium. The PIK3IP1 3’ UTR contains a functional miR-let-7 binding site; overexpression of let-7g represses expression of PIK3IP1 in cardiac myocytes; PIK3IP1 and miR-let-7g are reciprocally expressed in human myocardium; gain
of miR-let-7g induces AKT phosphorylation both in cultured myocytes and *in vivo*. Finally, PIK3IP1 is specifically enriched in the RISC of cardiac myocytes programmed with miR-let-7g. We propose that among other mechanisms targeting of PIK3IP1 by miR-let-7g enhances Akt activation and reduces loss of myocytes through apoptosis.
Chapter 6

SUMMARY AND CONCLUSION

6.1. Summary

- Hypoxia induces Lin28 expression in a cardiac myocyte-specific manner to downregulate members of the miR-let-7 family.
- Downregulation of let-7 promotes cardiac myocyte cell death during hypoxia/ischemia.
- MiR-let-7g targets a negative regulator of PI3K, PIK3IP1 to the RISC.
- Hypoxia represses PI3K-Akt signaling in cardiac myocytes through a fall in let-7, and exogenous restoration of miR-let-7g restores PI3K signaling.
- A similar LIN28-let-7 axis appears to control PIK3IP1 in human myocardium.

6.2. Conclusion

Important work to date has focused on the role of let-7 in differentiation, de-differentiation and maturation of the cardiac myocyte[115, 143]; however, it is not clear that these processes contribute meaningfully to repair of the adult mammalian heart, so that the physiologic significance of altered miR-let-7 levels in conditions such as ischemic damage and heart failure remains obscure. My
data demonstrates that ischemic stress downregulates let-7 in the myocardium and that let-7 regulates myocardial adaptation to ischemic injury by modulating an established cardioprotective signaling in a novel manner. This may have significant implications towards development of heart failure.

As demonstrated in other systems, let-7 negatively regulates glucose metabolism and insulin pathway signaling. As demonstrated by the sponge data, it seems that myocytes also downregulate let-7 to heighten insulin signaling thereby promoting glycolytic adaptation to ischemia. However, chronic continuation of this initial downregulation becomes maladaptive and leads to cardiac myocyte cell death. This likely is due to the reciprocal induction of PI3k-Akt signaling suppressor, PIK3IP1. Ischemia is known to reduce Akt activation downstream of IRS1. Our let-7 “rescue” data demonstrating sustained Akt activation and cardioprotection under ischemia suggests an additional let-7 mediated regulation of Akt signaling. In addition we also demonstrated induction in genes including MCAD and PDK1 suggestive of a metabolic switch in cardiac myocytes with let-7 rescue. The direct targets of let-7 in mediating such metabolic switch can be explored in future studies.

My study displays that having an elevated level of let-7, especially, let-7g reduced apoptotic nuclei in the myocardium flowing ischemia-reperfusion insult. Reducing infarct size by salvaging myocardial tissue still remains a prioritized approach to preventing the onset of HF. Advancements in chemical and structural biology have enabled the use of special chemical entities such as mimics and antimiR to, respectively, increase or repress let-7. Tail-vein delivery
of let-7b in form of lipid encapsulated mimic reduced tumor burden and increased survivability in a murine model of lung cancer with Kras/p53 mutation[124]. Future studies exploring the ability of such modified mimics administered during myocardial reperfusion would be interesting to assess the therapeutic benefits of let-7. Our demonstration of a LIN28/let-7/PIK3IP1 axis in human hearts makes it a tempting area to explore for potential clinical application.

My data demonstrates that Lin28 protein is induced in the ischemic myocardium and hypoxic myocytes. As a negative regulator of let-7, it is likely that Lin28 induction is the sole reason for let-7 downregulation. However, other factors also regulate let-7, which include KSRP. KSRP is known to mediate the maturation of let-7[215]. Whether KSRP is downregulated by ischemia to affect let-7 was not explored in this study. In addition, Lin28 is known to stabilize mRNAs including HMGA1, IGFBP1 and function independent to let-7[228]. It would be interesting to explore if novel Lin28-bound and -regulated genes contribute to myocardial adaptation to ischemia.

As in the developing organism, the timing and amplitude of miR-let-7 changes may be critical to its functions in the adult heart. Our study shows that Lin28/miR-let-7 are involved in the short-term response of the cardiac myocyte to ischemia, which may permit acute metabolic adaptation to hypoxia independent of its role in cardiac regeneration.
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