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An Investigation into the role of microRNAs in Myocardial Ischemia-Reperfusion Injury in Late Pregnancy

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An Investigation into the role of microRNAs in Myocardial Ischemia-Reperfusion Injury

in Late Pregnancy

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Molecular, Cellular, & Integrative Physiology

by

Laila Aryan

2024

ABSTRACT OF THE DISSERTATION

An Investigation into the role of microRNAs in Myocardial

Ischemia-Reperfusion Injury in Late Pregnancy

by

Laila Aryan

Doctor of Philosophy in Molecular, Cellular, & Integrative physiology

University of California, Los Angeles, 2024

Professor Mansoureh Eghbali, Chair

Maternal mortality remains high in the US and pregnancy-associated myocardial infarction accounts for over 20% of maternal cardiac deaths. The relative risk of myocardial infarction during pregnancy is approximately 3 to 4-fold higher than the rates of age-matched non-pregnant individuals in the reproductive age group. During myocardial infarction, ischemia restricts blood flow to the myocardium, necessitating timely reperfusion therapy to restore blood flow. However, reperfusion therapy can exacerbate tissue damage. Our lab has demonstrated that cardiac vulnerability to ischemia reperfusion injury drastically increases in late pregnancy, and one bolus of

intralipid at reperfusion reduces the infarct size in late pregnant rats. However, the mechanisms underlying the higher vulnerability of late pregnancy to ischemia reperfusion injury and the cardioprotective role of intralipid are unknown.

In **Chapter 2**, we employ a rat model to investigate the reasons behind the heightened susceptibility of the heart in late pregnancy to ischemia-reperfusion injury. We identify microRNA-98-5p, whose expression increases during late pregnancy upon ischemia reperfusion injury, and promotes cardiomyocyte apoptosis, mitochondrial oxidative stress, and inflammation via its targets Stat3 and Pgc-1α. In an *in vivo* late pregnant ischemia-reperfusion injury rat model, we show the therapeutic potential of microRNA-98-5p inhibition at the onset of reperfusion. In late pregnant patients with acute myocardial infarction, plasma microRNA-98-5p was significantly higher than healthy late pregnancy and it was correlated with troponin levels. In **Chapter 3**, we study the therapeutic role of intralipid in attenuating ischemia-reperfusion injury in a rat model of late pregnancy. We demonstrate that the protective effects of intralipid are mediated by microRNA-122-5p. MicroRNA-122-5p exhibits its protective effects in late pregnancy by mitigating cardiomyocyte apoptosis and oxidative stress through its target Pkm2. In humans, plasma microRNA-122-5p levels were lower in healthy late pregnant compared to healthy non-pregnant individuals and even lower in late pregnant patients with acute MI and negatively correlated with troponin levels. In an *in vivo* late pregnant ischemia-reperfusion injury rat model, we show the therapeutic potential of microRNA-122-5p overexpression at the onset of reperfusion.

Taken together, this dissertation offers insights into the heightened susceptibility of late pregnancy to ischemia-reperfusion injury and the cardioprotective role of intralipid.

The dissertation of Laila Aryan is approved.

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University of California, Los Angeles

2024

This dissertation is devoted to my family, whose love and support remain steadfast and unconditional.

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CHAPTER 1: Introduction

This chapter includes a portion of text adapted from Aryan L, Medzikovic L, Umar S, Eghbali M. *Pregnancy-associated cardiac dysfunction and the regulatory role of microRNAs. Biol Sex Differ.* 2020 Apr 6;11(1):14. doi: 10.1186/s13293-020-00292-w. PMID: 32252821; PMCID: PMC7137306.

1. Cardiovascular System

The heart, a vital organ in the human circulatory system, comprises four chambers that work synergistically to maintain a continuous and efficient blood flow. The upper chambers, known as the atria, play a crucial role in the heart's function. The right atrium receives deoxygenated blood returning from the body through the superior and inferior vena cavae, while the left atrium receives oxygenated blood from the lungs via the pulmonary veins. This differentiation ensures that oxygen-rich and oxygen-poor blood are appropriately routed within the heart, facilitating the efficient pumping of blood throughout the circulatory system.

These atria then contract, propelling the blood into the lower chambers, the ventricles. The left ventricle (LV), being the most robust, pumps oxygenated blood into the systemic circulation, ensuring that every cell and tissue in the body receives the necessary nutrients and oxygen. Simultaneously, the right ventricle pumps deoxygenated blood into the pulmonary circulation for oxygenation (1).

The coronary circulation plays a pivotal role in sustaining the heart's function. Comprised of a complex network of coronary arteries and veins, this intricate system serves the critical function of ensuring a continuous and adequate supply of oxygen and nutrients to the myocardium. Among the coronary arteries, the left anterior descending artery (LAD) holds particular significance. Originating from the left coronary artery, the LAD courses down the anterior surface of the heart, supplying oxygenated blood to the myocardium, especially the LV. The LV, being a major pumping chamber responsible for systemic circulation, relies heavily on the nourishment provided by the LAD. As blood flows through the LAD, it carries essential resources vital for the metabolic demands of the LV

myocardium, thereby sustaining its contractile function and overall cardiac performance (1). This intricate interplay within the coronary circulation underscores its pivotal role in maintaining myocardial viability and preserving cardiac health.

2. Physiological hypertrophy

Physiological hypertrophy may occur in response to maturation, exercise, or pregnancy and is considered a normal adaptation of the heart to increased workload. It is not associated with fibrosis, impaired function, or heightened morbidity and mortality risks, and enables the heart to adapt to increased demands without long-term adverse effects on cardiac function (2) **(Figure 1)**. Importantly, despite the increased workload, ejection fraction is not reduced in physiological hypertrophy. Both pregnancy-induced and exercise-induced hypertrophies share similarities in their physiological responses. However, pregnancy is further characterized by significant hormonal fluctuations, with estrogen and progesterone levels peaking towards the end of gestation. Unlike exercise, pregnancy imposes continuous demands on the heart throughout its duration.

Hypertrophy can be further classified based on the type of overload the heart experiences. Pressure overload results in concentric hypertrophy, characterized by an increase in wall thickness to compensate for increased pressure within the heart. On the other hand, volume overload leads to eccentric hypertrophy, characterized by chamber dilation to accommodate the increased volume of blood. Physiological hypertrophy can manifest in both volume and pressure overload (3).
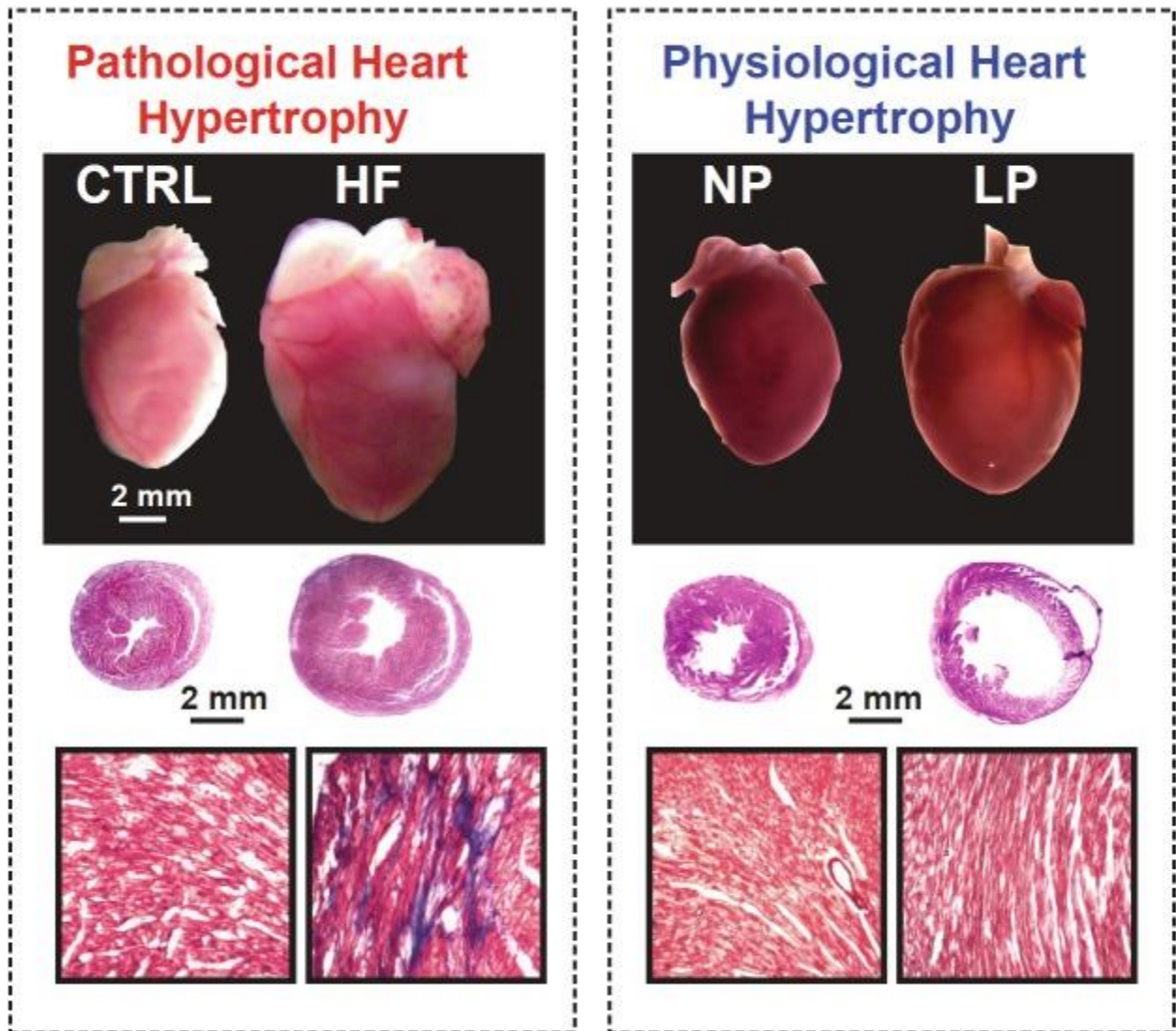
Figure 1. Pathological hypertrophy *vs.* pregnancy induced physiological hypertrophy. Top panels: Images of the whole heart in pathological hypertrophy and physiological hypertrophy in late pregnancy in mice; Middle panels: Hematoxylin-eosin staining of heart cross-sections; Bottom panels: Masson trichrome staining of heart cross sections, blue color indicates fibrosis. CTRL: control; HF: heart failure; NP: non-pregnant; LP: late pregnant. Note that the heart develops concentric hypertrophy in pressure overload and eccentric hypertrophy in physiological hypertrophy, in which no fibrosis is present. *Adapted from Li et al., Am J Cardiovasc Dis. 2012.*

3. Physiological Cardiovascular Changes during Pregnancy

<u>Hemodynamics of the maternal cardiovascular system during pregnancy</u>

The maternal cardiovascular system undergoes several changes during pregnancy, adapting to the evolving needs of both mother and fetus. Blood flow increases to meet the metabolic needs of the maternal organs and fetus, leading to volume overload hypertrophy, which is eccentric in nature due to the expansion of cardiac chambers in response to increased blood volume (4,5). Blood volume increases approximately 45% above pre-pregnancy levels (6). Stroke volume, heart rate, and end-diastolic volume all increase, resulting in enhanced cardiac output (6). Indeed, cardiac output rises up to 50% above pre-pregnancy levels at about 16–20 weeks of gestation (7). Both systolic and diastolic arterial blood pressure decrease in the first and second trimesters (7,8). However, arterial blood pressure rises in the third trimester, returning to baseline by the end of pregnancy (8). To meet these hemodynamic changes during pregnancy, the heart undergoes structural and functional changes.

<u>Structural changes in the heart during pregnancy</u>

Natural volume overload, mechanical stretch, and hormonal changes during pregnancy induce physiological cardiac hypertrophy (9,10). Importantly, myocardial capillary density remains normal. Furthermore, pregnancy-induced physiological hypertrophy is not associated with fibrosis, cardiomyocyte sarcomere disarray, or enhanced re-expression of the cardiac fetal gene program (11). Notably, the changes in cardiac structure and function during normal healthy pregnancy are rapidly reversed post-partum (2).

Role of pregnancy hormones on the cardiovascular system

Hormones play a crucial role in maintaining the physiological cardiovascular function throughout pregnancy (12,13). When properly regulated, hormones facilitate the physiological adaptation of the cardiovascular system (6). There is a relationship between increased levels of estrogen and progesterone and vasodilation, and certainly levels of both rise substantially during pregnancy, reaching their peak in the final trimester (12,14). Fluctuations in estrogen and progesterone levels are connected to adaptations in the cardiovascular system to sustain the developing fetus and mitigate the stresses of pregnancy (12). Nonetheless, these pregnancy hormones are also associated with various pathophysiological effects on the cardiovascular system (12).

Oestrogens, a class of steroid hormones, are primarily produced by the ovaries and the placenta during pregnancy (15). However, oestrogens can also be synthesized by various non-reproductive tissues such as the liver, heart, muscle, bone, and brain (16). Estradiol (E2) is the primary form of estrogen and plays a role in various physiological processes (12). Estrone (E1) is predominantly secreted after menopause, while estriol (E3) is produced by the placenta during pregnancy (12). E2 exerts its effects through both genomic and non-genomic actions (12). E2 binds to the classical estrogen receptors (ERs), estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) in the cytosol. The E2-bound ER complexes undergo conformational changes to dimerize, translocate to the nucleus, and either directly bind to DNA sequences known as estrogen response elements (EREs) or indirectly bind to DNA through other transcription factors to differentially regulate gene transcription (17,18). Traditionally, E2 has been known to

exert its cardioprotective effects by binding to the nuclear receptors ERα and ERβ. However, G protein–coupled receptor GPR30 (G protein–coupled estrogen receptor 1 or GPER) has also gained increased research attention over the past decade. GPR30 is localized in the endoplasmic reticulum and plasma membrane, and is known to be expressed in cardiomyocytes (19–21). E2 binds to GPR30 to exert rapid non-genomic events, triggering intracellular signaling cascades that alter gene expression downstream (22,23). A wide array of studies have highlighted several beneficial effects of E2 treatment on the cardiovascular system (24,25). Such effects are associated with reduced fibrosis, reduced oxidative stress, improved mitochondrial function, attenuation of cardiac hypertrophy, and stimulation of angiogenesis and vasodilation (25,26). Although numerous animal studies have demonstrated the beneficial cardioprotective effects of estrogen, two extensive clinical trials, Heart and Estrogen/progestin Replacement Study (HERS) and Women's Health Initiative (WHI), have both demonstrated a lack of evidence supporting the efficacy of hormone replacement therapy in postmenopausal women for reducing the risk of cardiovascular disease (25,27,28). Recent reviews examine the reasons why hormone replacement therapy was found to lack protective effects, considering factors such as a wide age range (50-80 years old), varying times of administration after menopause, dosage, hormonal composition, method of administration, and lifestyle factors of women, which were not adequately addressed. However, a recently popularized hypothesis known as the "Critical Window of Hormone Therapy" has garnered significant attention. This hypothesis suggests that hormone replacement therapy could potentially be effective if initiated early at the onset of

menopause (25,29). Further studies are needed to investigate the protective role of estrogen in cardioprotection in postmenopausal women.

The corpus luteum secretes high levels of progesterone, while smaller amounts are produced by the adrenal glands (12). Progesterone induces various physiological and protective responses within the cardiovascular system, these include increasing blood volume, promoting vasodilation, and protecting against apoptosis (12,30). However, many studies have shown increased levels of progesterone being associated with higher pathological effects in both men and women (31,32). For example in female mice, but not male mice, progesterone was shown to attenuate and slow cardiomyocyte contraction and reduce myofilament calcium sensitivity in female hearts. This could negatively impact heart function, particularly during periods of elevated serum progesterone levels observed in pregnancy (31). Furthermore, increased physiologic concentrations of progesterone were found to be associated with an increased prevalence of congestive heart failure in men and women, independent of inflammatory factors, markers of renal function and insulin metabolism (32). Progesterone exerts its effects through genomic (nuclear) and non-genomic (extranuclear) receptor pathways (33). In the genomic pathway, progesterone, a lipophilic molecule, diffuses into the cytoplasm and interacts with two specific nuclear progesterone receptors (PRs), PR-A, and PR-B. When progesterone binds, both isoforms of nuclear PR undergo a conformational change. Subsequently, the receptors form homo- or hetero-dimers and attach to hormone response elements within the promoter regions of target genes (34). Progesterone also exerts rapid non-classical effects on various signaling pathways, which operate independently of transcriptional or

genomic regulation (35). This non-genomic mechanism is facilitated by membrane-bound progesterone receptors (36).

4. Pathological Hypertrophy

Pathological hypertrophy represents an aberrant response of the heart to various stressors, leading to structural and functional alterations that are maladaptive and detrimental to cardiac health (37). This type of hypertrophy is characterized by an increase in cardiomyocyte size, fibrosis, and impaired contractile function, ultimately contributing to the progression of heart failure. In pathological hypertrophy, the heart becomes stiff and less efficient at pumping blood, which can further exacerbate cardiovascular complications. Pathological hypertrophy can manifest as either eccentric or concentric hypertrophy, depending on the underlying etiology and the specific hemodynamic stressors involved **(Figure 1).**

5. Ischemic Cardiac Pathologies

In the realm of cardiac pathologies, a myriad of conditions exists, encompassing both ischemic and non-ischemic categories. In this dissertation, our primary focus centers on ischemic cardiac pathologies. Ischemic heart disease results from an inadequate blood supply to the myocardium due to coronary artery obstruction, often caused by atherosclerosis (38). This process gradually narrows the arteries, impairing blood flow to the myocardium and predisposing individuals to myocardial ischemia, particularly during periods of increased demand such as physical exertion or emotional stress. Furthermore, ischemic heart disease can also manifest acutely as a result of a blood clot blocking suddenly the coronary artery.

Myocardial Infarction:

Myocardial infarction (MI), commonly known as a heart attack, is a severe manifestation of ischemic heart disease (39). MI occurs when a coronary artery becomes occluded, leading to ischemia and subsequent necrosis of myocardial tissue. The LAD artery, a branch of the left coronary artery, is particularly vulnerable to occlusion, and can lead to extensive myocardial damage and potentially fatal outcomes.

The clinical presentation of MI is typically characterized by a sudden onset of severe chest pain or pressure, which may radiate to the left arm, neck, jaw, or back. Other symptoms such as shortness of breath, nausea, vomiting, and diaphoresis commonly accompany chest pain. Prompt recognition and timely intervention are paramount in the management of MI to salvage viable myocardium, minimize myocardial damage, and prevent complications such as heart failure, arrhythmias, and death. Reperfusion therapy, aimed at restoring blood flow to the ischemic myocardium, is the cornerstone of treatment and may involve thrombolytic therapy or primary percutaneous coronary intervention (40).

Experimental animal models play a pivotal role in elucidating the pathophysiological mechanisms of ischemic cardiac pathology, particularly focusing on MI related to the occlusion of the LAD (41–46). These models allow researchers to mimic human disease conditions, investigate underlying mechanisms, and evaluate potential therapeutic interventions in a controlled environment. Rodent models, such as the mouse and rat LAD artery ligation model, have been extensively utilized to develop ischemic cardiac models by inducing coronary artery occlusion due to their cost-effectiveness, ease of handling, and genetic manipulability, enabling researchers to study the molecular

pathways involved in ischemic injury and cardiac remodeling (41–46). These studies underscore the importance of experimental animal models in translational research aimed at developing novel therapies for ischemic cardiac pathologies, ultimately benefiting patients with acute MI and other ischemic heart diseases, by providing insights into disease mechanisms, evaluating the efficacy of potential interventions, and facilitating the translation of preclinical findings into clinical practice.

6. Hallmarks of Myocardial Infarction

Central to the understanding of MI pathology are the hallmark processes that underscore the complex cascade of events involved in its pathogenesis. By dissecting and comprehensively analyzing these hallmark processes, clinicians and researchers can gain invaluable insights into the underlying mechanisms of MI, facilitating accurate diagnosis, risk stratification, and targeted therapeutic interventions. Among the myriad of processes implicated in MI pathophysiology, three major hallmarks emerge as pivotal players: apoptosis, inflammation, and the generation of reactive oxygen species (ROS) (47–50). These hallmarks not only serve as key markers of MI but also provide valuable targets for therapeutic intervention aimed at mitigating the adverse effects of MI and improving patient outcomes.

<u>Apoptosis</u>

Apoptosis, or programmed cell death, contributes to cardiomyocyte loss and myocardial damage, exacerbating the progression of MI. Studies have elucidated the intricate molecular mechanisms underlying apoptosis in the context of MI, highlighting its role as a hallmark of this condition. It has been shown that increased apoptosis occurs in

myocardial tissue in patients with acute MI, suggesting a direct association between apoptotic cell death and infarct development (51). Furthermore, experimental studies using animal models of MI have provided valuable insights into the signaling pathways involved in apoptosis-mediated cardiomyocyte death (52,53). These findings underscore the significance of apoptosis as a critical mechanism driving cardiomyocyte loss and myocardial injury in the setting of MI, highlighting its potential as a therapeutic target for mitigating adverse cardiac remodeling and improving outcomes in affected individuals.

Inflammation

Inflammation plays a pivotal role as a hallmark of MI, contributing to the progression of cardiac injury and remodeling (49). Studies have demonstrated the involvement of inflammatory processes in various stages of MI, from the initial ischemic insult to the subsequent tissue repair and remodeling phases (54). Furthermore, experimental studies using animal models have provided mechanistic insights into the role of specific inflammatory mediators in MI pathophysiology. For example, studies have demonstrated the importance of monocyte-derived macrophages in the resolution of inflammation and the promotion of infarct healing in a mouse model of MI, highlighting the therapeutic potential of targeting inflammatory pathways for improving cardiac outcomes post-MI (55,56). These findings underscore the significance of inflammation as a critical component of MI pathogenesis and suggest that modulating inflammatory responses may offer novel therapeutic strategies for attenuating myocardial injury and promoting cardiac repair in affected individuals.

Reactive Oxygen Species

Free oxygen radicals are highly toxic and their metabolites, including hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), singlet oxygen, and hydroxyl radical (OH^-), are involved in the pathogenesis of myocardial I/R injury (57). ROS play a pivotal role as a hallmark of MI, contributing to the pathophysiological processes underlying myocardial injury and remodeling. Studies have consistently demonstrated the involvement of ROS in the pathophysiology of MI, where excessive ROS production during ischemia-reperfusion contributes to myocardial damage and impaired cardiac function (48). For instance, research has shown that ROS generation is markedly increased in the ischemic myocardium during reperfusion, leading to oxidative stress and cellular injury (58). Additionally, studies in animal models of IRI have provided mechanistic insights into the detrimental effects of ROS on cardiac function. For example, studies have shown that antioxidant therapies targeting ROS can attenuate myocardial injury and improve cardiac function in rodents subjected to IRI (59). These findings underscore the importance of ROS as key mediators of myocardial injury in MI and highlight the potential therapeutic value of ROS-targeted interventions in mitigating IRI and improving outcomes in affected individuals.

By unraveling the intricate interplay between these hallmark processes, researchers aim to elucidate the underlying mechanisms of MI and identify novel therapeutic targets for intervention.

7. Cardiovascular Complications during Pregnancy

The drastic changes in the cardiovascular system during pregnancy are usually well-tolerated in healthy individuals. However, some healthy individuals can develop severe

adverse cardiac events in late pregnancy. Cardiovascular complications reflect an inability to adapt to the various changes in systemic physiology that are associated with pregnancy (60). Maladaptation of the cardiovascular system during pregnancy in previously healthy women can lead to complications that may cause maternal and fetal mortality (60,61). Cardiovascular complications during pregnancy may put the mother at risk to develop cardiac dysfunction and subsequent heart failure (HF) (61,62). These complications include preeclampsia, gestational diabetes mellitus, gestational hypertension, peripartum cardiomyopathy, and MI (63–66). Cardiac complications in pregnancy are becoming increasingly common (67). In the USA, about 12% of pregnancy-related deaths have been attributed to cardiac dysfunction, and cardiac dysfunction during pregnancy has been associated with a 7.7-fold increase in the risk of death (67,68). Furthermore, the adverse effects of cardiovascular complications on the heart can be long-lasting, pre-disposing the mother to heart failure (HF) later in life (69,70).

Myocardial Infarction during Pregnancy

While MI during pregnancy remains relatively rare, its incidence has been on the rise, particularly among individuals of advanced maternal age and those with comorbidities such as smoking, obesity, diabetes, and chronic hypertension (71–74). Notably, pregnancy-associated MI now contributes to over 20% of maternal cardiac-related deaths (75), highlighting its significant impact on maternal health outcomes. Furthermore, late-pregnant (LP) individuals face a 3 to 4-fold higher relative risk of MI compared to age-matched non-pregnant (NP) individuals (73,74,76). Alarmingly, MI occurring during the third trimester carries a substantially higher maternal mortality rate of 45% compared to

earlier trimesters, which have mortality rates of around 23% (74,77–79). These statistics underscore the urgent need for innovative approaches to better understand and manage this potentially lethal condition, emphasizing the critical importance of advancing our knowledge and therapeutic strategies to safeguard the health and well-being of both mothers and infants during pregnancy.

Our lab has successfully modeled myocardial ischemia reperfusion injury (IRI) in nonpregnant (NP) and late pregnant (LP) rats and showed that the myocardial infarct size was ~4 fold larger in LP rodents compared to NP rodents (80) **(Figure 2).** However, the mechanisms responsible for the high vulnerability of LP animals to IRI remain unclear.

8. The Role of microRNAs in Cardiovascular Complications in Pregnancy

While up to 75% of the genome is transcribed into RNA, only 2% of the genome consists of protein-coding genes (81). Consequently, non-coding RNAs, and in particular small non-coding microRNAs (miRs), have emerged as critical regulators of cellular processes in both health and disease (81). MiRs are small endogenous noncoding RNAs that regulate gene expression through translational repression or through degradation of target mRNAs (81). Various cell types actively secrete miR into the circulation, and thus can both mediate crosstalk between different cell-types or organs, and simultaneously represent disease biomarkers (82). MiRs have emerged as a powerful regulator of gene expression as each miR can regulate the expression of multiple genes (83).

Throughout the past two decades, circulating and tissue-specific miRs have become of growing interest as modulators of pathophysiology, diagnosis, and prognosis in a variety of cardiovascular disorders including heart failure (84–86). In recent years, research has
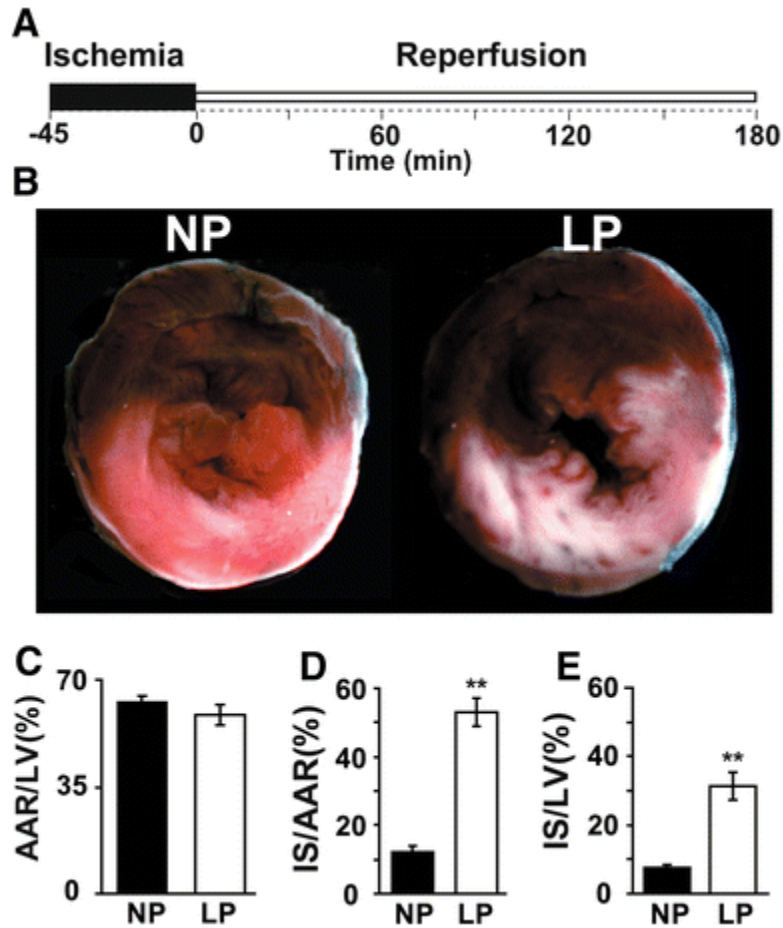
Figure 2. Cardiac vulnerability to ischemia/reperfusion injury drastically increases in late pregnancy. The infarct size is larger in LP rats subjected to IRI than NP. **A.** Experimental protocol: the LAD was occluded in NP and LP rats for 45 min followed by 3 h of reperfusion. **B.** Representative heart cross sections of NP and LP rats. Percentage of area at risk (AAR) divided by LV (**C**), infarct size (IS) divided by AAR, (**D**) and infarct size (IS) divided by LV (**E**). **$P < 0.01$ versus LP ($n = 4–6$). *Adapted from Li et al. Basic Res Cardiol, 2012.*

increasingly illuminated the role of miRs in IRI within the cardiac milieu. The identification of specific miRs implicated in the intricate cascade of events underlying IRI underscores their potential as promising targets for innovative cardioprotective interventions (65,87). These miRs act as key mediators in the intricate interplay between cellular signaling cascades, dictating the balance between adaptive responses and maladaptive remodeling following ischemic insult. Through their ability to fine-tune the expression of target genes involved in diverse cellular processes, miRs exert profound influence over the myocardial response to ischemia and subsequent reperfusion injury (43). Furthermore, dysregulation of miR expression profiles has been implicated in the pathogenesis of various cardiovascular disorders, underscoring their clinical relevance as potential diagnostic and therapeutic targets (88). However, the intricate network of miR-mediated regulatory mechanisms presents challenges in deciphering their precise roles and therapeutic implications in myocardial IRI. This understanding not only highlights the multifaceted regulatory roles of miRs within the intricate network of cellular responses to ischemic insults but also underscores their feasibility as druggable targets for the development of novel therapeutic strategies aimed at mitigating myocardial damage in conditions such as MI.

A wide array of research has underscored the intricate association between miRs and the hallmarks of MI, including apoptosis, ROS, and inflammation. MiRs, as key regulators of gene expression, play pivotal roles in modulating these pathological processes, thereby influencing the pathogenesis and progression of MI. Studies have demonstrated that specific miRs are dysregulated in response to myocardial IRI, leading to aberrant expression profiles associated with apoptotic cell death (89–91). Furthermore, miRs have

been implicated in the regulation of ROS production and oxidative stress in the ischemic myocardium, with dysregulated miR expression contributing to ROS-mediated myocardial injury (92,93). Additionally, miRs have emerged as critical regulators of inflammatory responses in the context of MI, modulating the expression of pro-inflammatory cytokines and chemokines involved in leukocyte recruitment and activation (90,94). Collectively, these findings highlight the multifaceted roles of miRs in orchestrating the molecular mechanisms underlying the hallmarks of MI and suggest their potential as therapeutic targets for mitigating myocardial injury and improving outcomes in affected individuals.

Indeed, an extensive body of research has consistently demonstrated alterations in miR profiles during pregnancy (95–97). For example, circulating miR-122 levels have been shown to be decreased in women with premature acute coronary syndrome with a history of preeclampsia during pregnancy (98). Furthermore, preeclamptic patients exhibit increased circulating levels of miR-21 compared to healthy patients (99). These studies have shed light on the dynamic changes occurring in miR expression patterns, reflecting the intricate interplay between maternal physiology and fetal development.

Despite their profound significance in regulating gene expression, the role of miRs in pregnancy-related cardiovascular pathologies remain relatively understudied (10,100). Further elucidation of the complex interplay between miRs and their target genes is imperative for advancing our understanding of cardiovascular pathophysiology and developing novel strategies for myocardial protection and repair in pregnancy. This gap in knowledge underscores the pressing need for comprehensive studies elucidating the specific contributions of miRs to the development and progression of cardiovascular

complications in pregnancy, thereby paving the way for targeted interventions aimed at safeguarding maternal and fetal health.

MiRs offer a versatile and promising approach for therapy and biomarker discovery across a wide range of diseases in pregnancy, offering unique advantages in the context of maternal cardiovascular health (101). Notably, investigations have revealed that many of the differentially expressed miRs detected in maternal serum or plasma originate from the placenta (97,102), suggesting a crucial role for placental miRs in modulating maternal physiological responses during gestation. Due to their stability and specific expression patterns, circulating miRs hold potential as sensitive and specific biomarkers. Additionally, it is important to note that while miRs are utilized as biomarkers, they also play dual roles in disease processes; while some may serve as therapeutic targets, others may promote the progression of certain conditions (103,104). It is noteworthy that several miRs are already being utilized as biomarkers in pregnancy for various conditions including peripartum cardiomyopathy, gestational hypertension, gestational diabetes mellitus, and preeclampsia, underscoring the potential versatility and clinical relevance of miRs in maternal health monitoring (95,101,105–108).

To date, there are no studies demonstrating miRs as biomarkers in pregnancy in the context of acute MI. The aim of this dissertation is to investigate the potential utility of miRs as both biomarkers and therapy for acute MI. By profiling miRs signatures associated with acute MI, clinicians can develop non-invasive diagnostic assays tailored to the unique physiological and pathological changes occurring during pregnancy. Tools to predict who can develop an acute MI during pregnancy is crucial for timely intervention and management, as delays in diagnosis can exacerbate maternal and fetal risks.

Leveraging the diagnostic capabilities of miRs may enable clinicians to implement targeted interventions, such as reperfusion therapy or pharmacological treatments, to mitigate the adverse consequences of AMI on both maternal and fetal health. Continued research efforts aimed at validating the diagnostic utility of miRs in pregnant individuals hold promise for enhancing clinical care and improving outcomes in patients with acute MI.

9. Cardioprotective role of Intralipid in Pregnancy

Intralipid (ITLD), recognized as the first safe fat emulsion designed for human use, constitutes an emulsion composed of soybean oil, egg phospholipids, and glycerin. ITLD has been integral in meeting the nutritional needs of individuals unable to consume food orally or digest fats effectively. Its safety profile and nutritional efficacy have solidified its status as a cornerstone in clinical nutrition, providing essential lipids and calories for more than 5 decades to patients requiring parenteral support for various medical conditions (109).

Notably, ITLD's versatility extends to pregnancy, as it is capable of traversing the placental barrier without eliciting harmful effects on either the mother or the developing fetus (109). ITLD's safety profile renders it an invaluable resource in providing essential nutrients to premature and underweight infants, contributing significantly to their growth and development. As a trusted component of medical nutrition, ITLD plays a pivotal role in supporting the health and well-being of both patients and newborns, underscoring its enduring importance in clinical care.

Over a decade ago, our laboratory pioneered groundbreaking research by demonstrating that post-ischemic administration of ITLD significantly reduces myocardial infarct size by approximately 60% in both male mice and rats, as well as in LP rats subjected to IRI *in vivo* (41,44) **(Figure 3).** However, a thorough understanding of the mechanism by which ITLD confers its protective effect remains elusive. These findings garnered significant attention within the scientific community, prompting numerous investigators, including our own research team, to further explore the protective effects of ITLD on the heart in rodent models and even in porcine models (41,44,110–119).

Our group has also shown ITLD therapy reduces inflammation in the experimental model of pulmonary hypertension by reducing IL-6 transcript expression and accumulation of macrophages/monocytes in the lungs, therefore improving RV function. Furthermore, ITLD prevented the upregulation of cleaved caspase 3 protein (111). We have also shown that postischemic treatment of ITLD decreases cardiac ROS generation as well as mitochondrial superoxide production in the context of IRI (110). The overwhelming body of evidence from these studies solidifies the notion of ITLD as a potent therapeutic intervention for mitigating cardiac damage in various experimental settings, thus laying the foundation for its potential translation into clinical practice.

More recently, a few small-scale studies in humans have shown that ITLD attenuates myocardial injury in patients undergoing valve replacement surgery and off-pump coronary revascularization (120,121). These studies, albeit preliminary in scale, have offered promising insights into the therapeutic efficacy of ITLD as an adjunctive treatment in cardiac surgical settings. By demonstrating a reduction in myocardial injury markers and improved cardiac outcomes following ITLD administration, these investigations
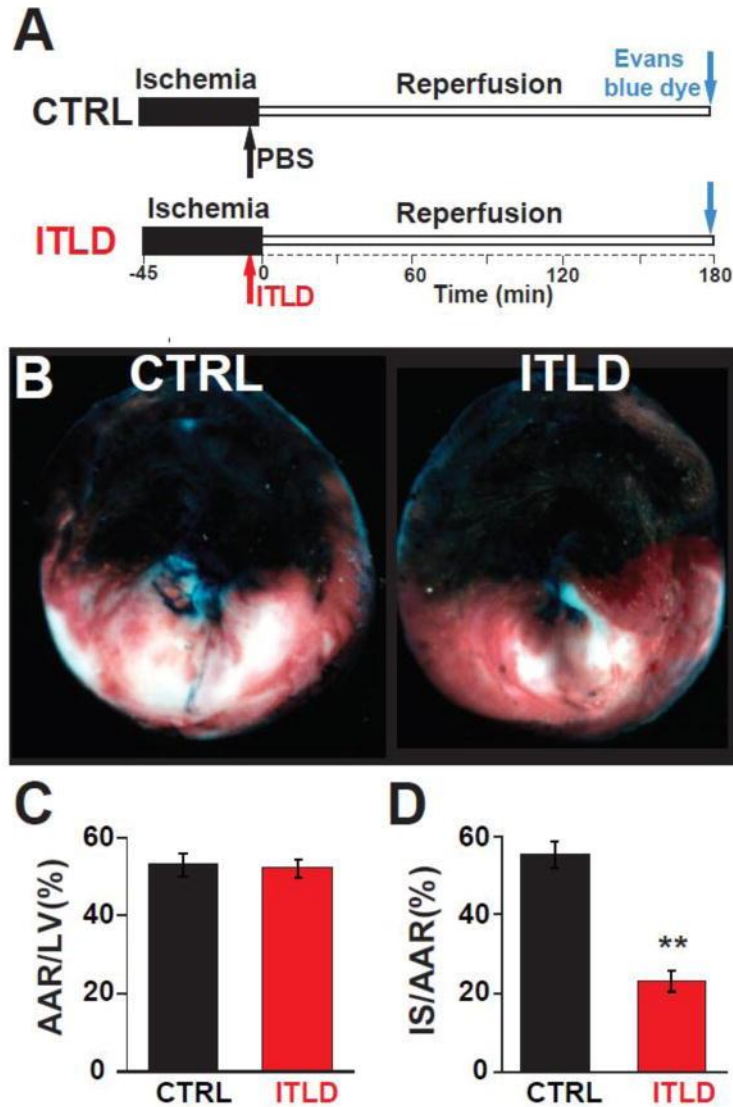
Figure 3. ITLD reduces the myocardial infarct size in LP rats subjected to *in vivo* IRI. A. Experimental protocol, the left coronary artery was occluded for 45 minutes followed by 3 hours of reperfusion. One single bolus of PBS (control group, CTRL) or 20% ITLD (5ml/kg body weight, ITLD) was administered 5 min before reperfusion. **B.** Representative cross sections of LP hearts in CTRL and ITLD groups. **C.** Percentage of area at risk (AAR) divided by LV and **D.** infarct size (IS) divided by AAR in CTRL and ITLD groups. [**]$p < 0.01$ *vs.* CTRL (n=6–7). Adapted from *Li et al. J Mol Cell Cardiol, 2017.*

underscore the potential utility of ITLD in enhancing perioperative cardiac care strategies. However, further extensive research is needed to confirm these findings and understand the exact molecular mechanisms through which ITLD offers cardioprotection. Nonetheless, these preliminary studies represent a significant step toward harnessing the therapeutic potential of ITLD in optimizing cardiovascular surgical outcomes.

10. Summary

This chapter delves into the intricacies of heart structure and function, exploring physiological changes during pregnancy and distinguishing them from pathological hypertrophy. It also delves into ischemic cardiac pathologies, with a particular focus on myocardial infarction, emphasizing the involvement of reactive oxygen species (ROS), apoptosis, and inflammation. Furthermore, it discusses cardiovascular complications during pregnancy and explores the role of miRs as both therapeutic tools and biomarkers. Additionally, the chapter examines the potential cardioprotective effects of ITLD, highlighting its safety and efficacy in reducing myocardial infarct size in animal models.

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Chapter 2: Myocardial Ischemia-Reperfusion Injury in Late Pregnancy is regulated

by microRNA-98-5p Targeting Stat3 and Pgc-1α

Abstract

Background

Maternal mortality remains high in the US and pregnancy-associated myocardial infarction (MI) accounts for over 20% of maternal cardiac deaths. The relative risk of MI in late pregnancy (LP) is 3-4 fold higher than non-pregnant (NP). We have previously modeled this clinical observation in LP rodents and found the cardiac infarct size in LP was ~4 fold larger compared to NP rats. However, the underlying mechanisms are unknown.

Methods

We performed microRNA (miR) microarray analysis on the left ventricle (LV) of NP and LP rats in sham and upon IRI. Female adult rat ventricular myocytes (ARVMs) and H9c2 rat cardiomyoblasts were incubated with serum from NP and LP rats, and subjected to hypoxia/reoxygenation. We administered miR inhibitor intravenously at the onset of reperfusion. Plasma was collected from healthy NP and LP humans, and LP patients with acute MI.

Results

We identified a novel microRNA-98-5p (miR-98) using our microRNA-microarray analysis and experimentally validated that upregulation of miR98 in IRI is only specific to LP, but not NP. In humans, plasma miR-98 levels were significantly higher in healthy LP compared to healthy NP individuals. Interestingly, in LP patients with acute MI, plasma miR98 expression was significantly higher than healthy LP and it was correlated with troponin levels. Stat3 and Pgc-1α were identified as the targets of miR-98 both *in vivo*

and *in vitro*. We found that miR-98 promotes cardiomyocyte apoptosis, mitochondrial oxidative stress, and inflammation via its targets Stat3 and Pgc-1α. A single dose of miR-98 inhibitor in LP rats at the onset of reperfusion significantly reduced infarct size and was associated with upregulation of Stat3 and Pgc-1α.

Conclusions

We show miR-98 regulates myocardial infarct size via Stat3 and Pgc-1α and could serve as a novel cardio-protective strategy or biomarker in LP.

Introduction

The drastic changes in the cardiovascular system during pregnancy are usually well-tolerated in healthy individuals. However, healthy individuals can develop severe adverse cardiac events in late pregnancy. In fact, cardiovascular disease has emerged as the leading cause of maternal mortality in the US, accounting for 26.5% of US pregnancy-related deaths (1). Pregnancy-related myocardial infarction (MI) is a devastating event that can claim the life of both the mother and fetus. While MI is uncommon in pregnancy, its prevalence is increased recently with advanced maternal age, smoking, obesity, diabetes, and chronic hypertension (2–4). Pregnancy-associated MI accounts for over 20% of maternal cardiac deaths (5). The relative risk of MI during pregnancy is approximately 3 to 4 fold higher than the rates of age-matched non pregnant (NP) individuals in the reproductive age group (4,6,7). MI has been reported to occur more frequently with a worse prognosis and a higher maternal mortality during the third trimester of pregnancy (45%) as compared to the first and second trimesters (23%) for unclear reasons (6,8–10). These unsettling facts call for innovative approaches to understand and cure this lethal disease.

During MI, an insufficient amount of blood to the myocardium due to obstruction of arterial inflow results in ischemia (11). Timely restoration of coronary blood flow to ischemic tissue, i.e. reperfusion, remains the foundation of clinical therapy. Although reperfusion is necessary to reestablish oxygen and nutrient delivery and salvage ischemic tissues, it paradoxically exacerbates the injury caused by ischemia alone (12). One of the most significant pathological mechanisms in reperfusion injury is oxidative stress, which

damages cardiomyocytes by inducing cell death and inflammation, leading to irreversible cardiac dysfunction (13,14).

Our lab has successfully modeled myocardial ischemia-reperfusion injury (IRI) in NP and late-pregnant (LP) rats and showed that the myocardial infarct size was ~4 fold larger in LP rodents compared to NP rodents (15). We also found that reactive oxygen species (ROS) production in the heart was significantly higher in LP mice subjected to IRI (15). However, the mechanisms responsible for the high vulnerability of LP animals to IRI remain unclear.

MicroRNAs (miRs) are small endogenous noncoding RNAs that regulate gene expression through translational repression or through degradation of target mRNAs. miRs have emerged as a powerful regulator of gene expression as each miR can regulate the expression of multiple genes (16). In the heart, several miRs have been reported to be involved in IRI (16–18), suggesting that miRs could be targeted as a novel cardioprotective strategy. However, the role of miRs in pregnancy-related cardiovascular pathologies remain understudied (18).

By combining bioinformatics, experimental, and pre-clinical approach in rat, we identified microRNA-98-5p (miR-98) that promotes cardiomyocyte apoptosis, mitochondrial oxidative stress, and inflammation via its targets signal transducer and activator of transcription 3 (Stat3) and peroxisome proliferator-activated receptor-gamma coactivator (Pgc-1α). Moreover, in a pre-clinical *in vivo* LP IRI rat model, we show the therapeutic potential of miR-98 inhibition at the onset of reperfusion. Our data also demonstrates that miR98 could serve as a novel biomarker in LP patients with acute MI.

Materials and Methods

<u>Animals</u>

NP and timed LP female rats (Sprague-Dawley, 2-3 months old, day 20-21 of pregnancy) were obtained from The Jackson Laboratory. The institutional Animal Research Committee approved all animal procedures (ARC-2010-045) and are according to current NIH guidelines.

<u>Left anterior descending artery occlusion and measurement of infarct size</u>

Rats were anesthetized with ketamine (80 mg/kg i.p.) and xylazine (8 mg/kg i.p.), and the rats were intubated and ventilated (VENTELITE Catalogue 557040, Harvard Apparatus). The hearts were exposed through a left thoracotomy in the fourth intercostal space. The pericardium was opened, and a 6.0 Prolene suture was tightened around the proximal left anterior descending (LAD) coronary artery. The hearts were subjected to 45 minutes ischemia followed by 3 hours reperfusion. In the rescue experiments, the duration of reperfusion was increased to 24 hours to give sufficient time for not only miR-98 expression to change, but also to modify the expression of its targets. At the onset of reperfusion, rats either received 10nM of miR-98 inhibitor (ThermoFisher, #4464088) or scramble inhibitor (ThermoFisher, #4464079) intravenously through the femoral vein. At the end of the experiment, the LAD coronary artery was retied and 3.0mL of 2% Evans blue dye (Sigma, #314-13-6) was intravenously injected through the femoral vein. The ventricles of the hearts were sliced transversely into 2 mm thick slices. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 15 min to identify the non-infarcted and infarcted areas. The myocardial ischemic area at risk (AAR) was identified

as the region lacking blue staining. The infarct size (IS) was displayed as the white area. IS was expressed as a percentage of the AAR. The AAR expressed as the percentage of total LV area. All quantifications were done on Adobe Photoshop. In a different set of rats, which were used for RNA and Western Blot analysis or histology, the rats did not receive Evans blue dye at the end of IRI.

Tissue preparation and *Fluorescence in situ hybridization (FISH)*

Rat hearts were fixed in 4% paraformaldehyde (ThermoFisher) for 24 hours, immersed in 20% sucrose (Sigma) for 24 hours, and embedded in OCT compound (Sakura Tissue-Tek). Rat cardiac tissue were cut to 10μM sections for *in situ* hybridization. Images were acquired using a Nikon A1 confocal microscope.

The ViewRNA Tissue Assay Fluorescence Kit (ThermoFisher #QVT0600C) was employed according to the manufacturer's instructions. The following ViewRNA probes were used for detection: miR-98 (VM1-34964-VCP) and rat cardiac Troponin T (tnnt2) (VC6-3229560-VT). Tissue slides were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen, #P36935) and imaged on a Nikon confocal microscope. Single transcripts of miR-98 and tnnt2 were counted and calculated as miR-98/tnnt2 ratio in 5 fields per tissue section.

MicroRNA-microarray analysis

Using non-Affymetrix single channel arrays (MiRBase 17.0 MicroRNA Array, Ocean Ridge Biosciences), we performed a microRNA-microarray screen in LV tissue of NP sham, LP sham, NP IRI, and LP IRI Sprague-Dawley rats (n=5 per group). MicroRNA-

microarray expression ratios were calculated as the power-2 exponential of the log2 differences. The acceptance criteria for microRNA expression changes were a minimum 1.5-fold change and a one-way Analysis of Variance (ANOVA) t-test p-value of <0.05. Data analysis and hierarchical clustering were performed using XLSTAT (www.xlstat.com).

Patient Characteristics and Human Plasma Sampling and RNA Isolation

We collected plasma samples from three groups of human subjects for this study: 1) healthy NP, 2) healthy LP, and 3) LP with acute MI. Troponin levels were obtained from the acute MI patients. Approval was obtained from the Institutional Review Board at UCLA and consent was taken from all patients to obtain their blood samples. Patient characteristics are displayed in **Supplemental Table 1**.

The human plasma samples (200µL) for miR detection were collected in ETA-K2 tubes and processed within 4 hours of collection. Samples were centrifuged and plasma was collected and stored in -80°C. MiR were extracted with miRNeasy serum/plasma kit (Qiagen, #217184) according to the manufacturer's protocol. MiR-39 mimic was used as Spike-In control (Qiagen, #432421090).

Cell culture, Serum Incubation, Estrogen and Progesterone stimulation, and Hypoxia/Reoxygenation

Female ARVMs and H9c2 cells were utilized for the *in vitro* experiments. We confirmed the H9c2 cells were from females by the lack of sex-determining region Y gene (Sry) in the genomic DNA of these cells **(Supplemental Figure 1).** Genomic DNA of H9c2 cells and male mouse heart (positive control) were used as a template for real time RT-PCR.

In brief, 5% Chelex solution was added to the cell samples and incubated at 100°C for 30 minutes. The tubes were then vortexed and centrifuged. The supernatant was collected for PCR. The primer sequence used for *Sry* is listed in **Supplemental Table 2**. *Sry* gene was then visualized by DNA Electrophoresis **(Supplemental Figure 1).** ATCC-formulated DMEM (Catalog #30-2002) was used to culture the cells, with the addition of 10% fetal bovine serum (FBS, catalog#26140079, ThermoFisher) and 1% antibiotic/antimycotic (catalog#15240062, ThermoFisher) for complete growth medium. The cells were rendered quiescent in serum-free DMEM for 24 hours prior to experiments.

Serum from either NP or LP rats was utilized for these experiments. Sera from multiple animals were snap frozen in liquid nitrogen, stored at -80°C, and pooled for each experiment. H9c2 cells were stimulated with 1% serum from either NP or LP rats for 24 hours, which was followed by either normoxia or hypoxia/reoxygenation (H/R). High glucose DMEM with no phenol red (Gibco, Catalog # 21063029) was used for these experiments.

B-estradiol (Sigma, catalog#E2758) and Progesterone (Sigma, catalog#P8783) were utilized for experiments. H9c2 cells were stimulated with 10nM of either B-estradiol or Progesterone for 24 hours. Glucose-free DMEM with no phenol red without serum (Life Technologies, Catalog #11966-025) was used for these experiments.

To stimulate hypoxia in H9c2 cells, cell medium was changed from normal growth medium to glucose-free DMEM with no phenol red without serum (Life Technologies, Catalog #11966-025) and cells were placed in a hypoxia chamber (UCLA Storehouse, 94% N2, 5% CO2 at 37°C, 1% O2) for 3 hours. The cells were subsequently reoxygenated by replacing the medium to regular growth medium and placing the cells back to normoxic

gas conditions (95% O2 and 5% CO2) at 37°C for 6 hours. The control cells remained under normoxic conditions at 37°C.

<u>H9c2 Cell Transfection</u>

MiR-98 mimic, miR-98 inhibitor, and corresponding scrambled controls (ThermoFisher Scientific, miR-98 mimic: #4464066, Assay ID: MC10426, miR-98 inhibitor: #4464084, Assay ID: MH10426, scramble mimic: #4464058, scramble inhibitor: #4464076) were transfected into H9c2 cells using Lipofectamine RNAiMAX (ThermoFisher, #13778075) in OptiMEM medium. Various doses of mimic and antagomir (20-100nM) were tested to determine the optimal concentration of mimic and inhibitor. The optimal dose was selected at a final concentration of 40nM for 6 hours, after which the medium was replaced with DMEM-F12+0.5% FCS+ 1% antibiotic/antimycotic. Experiments were performed 24 hours after transfection.

<u>Immunofluorescence on Fixed H9c2 Cells</u>

H9c2 cells were grown on coverslips until they reached 80% confluence, fixed in 4% paraformaldehyde (ThermoFisher) for 15 minutes, permeabilized with 10% Triton (Sigma, #9036-19-5) and blocked with either 5% normal donkey or 5% normal goat serum for 30 minutes (Jackson ImmunoResearch #017-000-121 and 005-000-121). Cleaved caspase 3 (CC3) primary antibody (Cell Signaling Technology, #9661, 1:500) was incubated overnight at 4°C. Secondary Alexa Flour antibody (1:1000, Invitrogen #A21245) against CC3 was incubated for 1 hour at room temperature and sections were mounted with Fluoromount G with DAPI (ThermoFisher, #00-4959-52). All images were acquired used

a confocal microscope (Nikon). Cells positive for CC3 as a proportion of total cell number were quantified in 5 random fields per coverslip. Experiments were performed in triplicate.

<u>RNA isolation, reverse transcription, and quantitative polymerase chain reaction (RT-qPCR)</u>

Total RNA was extracted from snap-frozen rat myocardium and H9c2 cells using Trizol Reagent (Invitrogen, ThermoFisher Scientific) according to the manufacturer's protocol. Quantitated RNA was used for generating cDNA by using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, #4368814) and qPCR was performed by using PowerUp SYBR Green Master Mix (ThermoFisher, #A25779) on a BioRad CFX Connect PCR detection system. RPLP0 was used as a reference gene. Primers are listed in **Supplemental Table 2**. For miR RT-qPCR, Taqman RT Kit (ThermoFisher, #4366596), Taqman assays (ThermoFisher, #4427975, Assay ID: 001973 and 000577) and Taqman Universal Master Mix (ThermoFisher, #4440040) were used. U6 was used as a reference gene. The relative levels of gene expression were assessed using the $2^{-\Delta\Delta Ct}$ method.

<u>Protein extraction, SDS-PAGE, and Western Blot assay</u>

Protein was extracted using RIPA lysis buffer (50mM NaCl, 50mM Tris pH 8, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, Sigma #R0278) supplemented with protease inhibitor (cOmplete Mini, 11836153001) and phosphatase inhibitor (Roche, 04906845001). Protein concentration was measured using a Bradford assay (Sigma, #B6916) and 20µg of protein was loaded per well. Proteins were diluted in 4x sample buffer (BioRad #161-0747) and separated on 10% gels by SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes (BioRad #170-4270) using semi-dry

blotting (TransBlot Turbo System, BioRad). After transfer, membranes were blocked with either 5% bovine serum albumin (Sigma #A9647, Stat3 Western Blots) or 5% non-fat milk (Pgc1-α Western Blots), and incubated with antibodies directed against p-Stat3 (1:200, Cell Signaling #9145S), Stat3 (1:200, Cell Signaling, #9139S, Pgc1-α (1:200, Novus Biologicals NBP1-04676SS), and GAPDH (1:10.000, Cell Signaling #2118). IRDye-conjugated secondary antibodies (1:10.000, LI-COR #32210, #68070, and #32214) were used for detection and blots were scanned using the LI-COR Odyssey. The intensity of the bands was quantified using Image Studio Lite Version 5.2.

Mitochondrial Reactive Oxygen Species Measurements

MitoSox Red mitochondrial superoxide indicator (Molecular Probes, #M36008) was utilized and the assay was run based on manufacturer recommendations. In brief, H9c2 cells were plated and subjected to H/R. The cells were washed with PBS and incubated with un-supplemented media containing a concentration of 5μM MitoSox Red for 45 minutes. The cells were then washed 3 times with PBS, pelleted (5 min spin at 500 ×g) and resuspended with 300μL of PBS in placed plastic culture tubes (ThermoFisher, #14–961-10A). Mitochondrial superoxide levels were then determined in the live cells via flow cytometry using an absorption/emission maxima of ~510/580 nm.

RNA Sequencing

Libraries for RNA-Sequencing were prepared with KAPA RNA Hyper Prep Kit following the manufacturer's instruction by UCLA Clinical and Translational Science Institute Core. Sequencing was performed on Illumina HiSeq3000 for a single-end 1x50 run. A data quality check was done on Illumina SAV. Demultiplexing was performed with Illumina

Bcl2fastq v2.19.1.403 software. The reads were mapped and quantified by STAR 2.7.9a (19) using the rat genome rn6. In Partek Flow (Partek® Flow® software, v7.0 Copyright ©. 2019 Partek Inc., St. Louis, MO, USA.), read counts were normalized by CPM +1.0E-4. Statistical analysis comparing the late pregnant group to the other group was performed on the gene count matrix using the DESeq2 R package (20). Genes were considered differentially expressed for an adjusted p-value below 0.05 and an absolute fold change above 1.5.

Statistical Analysis

Data distribution was tested with the Kolmogorov-Smirnov normality test. Significant outliers were tested with Grubb's test. Data are represented as mean±SEM and tested with Student's *t*-test (2 groups) or one-way ANOVA with Holm-Bonferroni post-hoc correction (>2 groups). Experiments were repeated at least four times. A p-value less than 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism version 9 software (GraphPad Software, Inc., San Diego, CA, USA).

Results

MicroRNA-microarray analysis reveals a novel miR-98 that could underlie higher vulnerability of the heart in LP to IRI

We have previously reported that the LP rodent is more vulnerable to myocardial IRI compared to the NP rodent (15). However, the underlying molecular mechanisms involved in the higher susceptibility of LP to IRI is unknown. To find novel miRs that could promote higher vulnerability to IRI in LP, we performed microRNA-microarray profiling on rat LV of four groups, NP sham, LP sham, NP IRI, and LP IRI **(Fig. 1A)**. Among the numerous miRs detected in the microRNA microarray analysis, only two miRs, miR-98

and miR-322, exhibited significant changes in LP vs. NP, and in LP IRI vs. NP IRI **(Fig. 1B).** Our selection criteria for significance were an absolute fold change greater than 1.5 (logFC >0.58) and a p-value less than 0.05. As we are interested to assess why the LP heart is more prone to IRI, we chose to focus on miR98 as it was increasing further in IRI compared to sham, compared to miR322, which showed no further change in IRI compared to sham **(Fig. 1B).** We validated by qPCR the significant upregulation of miR-98 **(Fig. 1C)** in LP rat LV compared to NP rat LV in sham, which was even further increased upon IRI in LP LV compared to NP LV. Our data shows that the upregulation of miR98 in IRI is only specific to LP, but not to NP.

Increased expression of miR-98 in LP upon IRI is associated with decreased expression of its targets Stat3 and Pgc-1a

TargetScan identified Stat3 and Pgc-1α as potential targets of miR-98. Our qPCR data validated significant downregulation of miR-98 targets Stat3 and Pgc-1α in LP rat myocardium in sham and even further downregulation upon IRI compared to its corresponding NP **(Fig. 1D, E)**. These data suggest that both Stat3 and Pgc-1α are indeed targets of miR-98 since increased expression of miR-98 in LP rat myocardium subjected to IRI is associated with decreased expression of Stat3 and Pgc-1αcompared to both NP IRI and LP sham.

Plasma miR98 expression is increased in both LP rats and humans compared to NP which is exacerbated upon IRI in rats and in LP patients with acute MI

To examine the clinical relevance of our findings, we first assessed whether miR-98 is expressed in rat plasma. Our data shows miR-98 is in fact expressed in rat plasma and
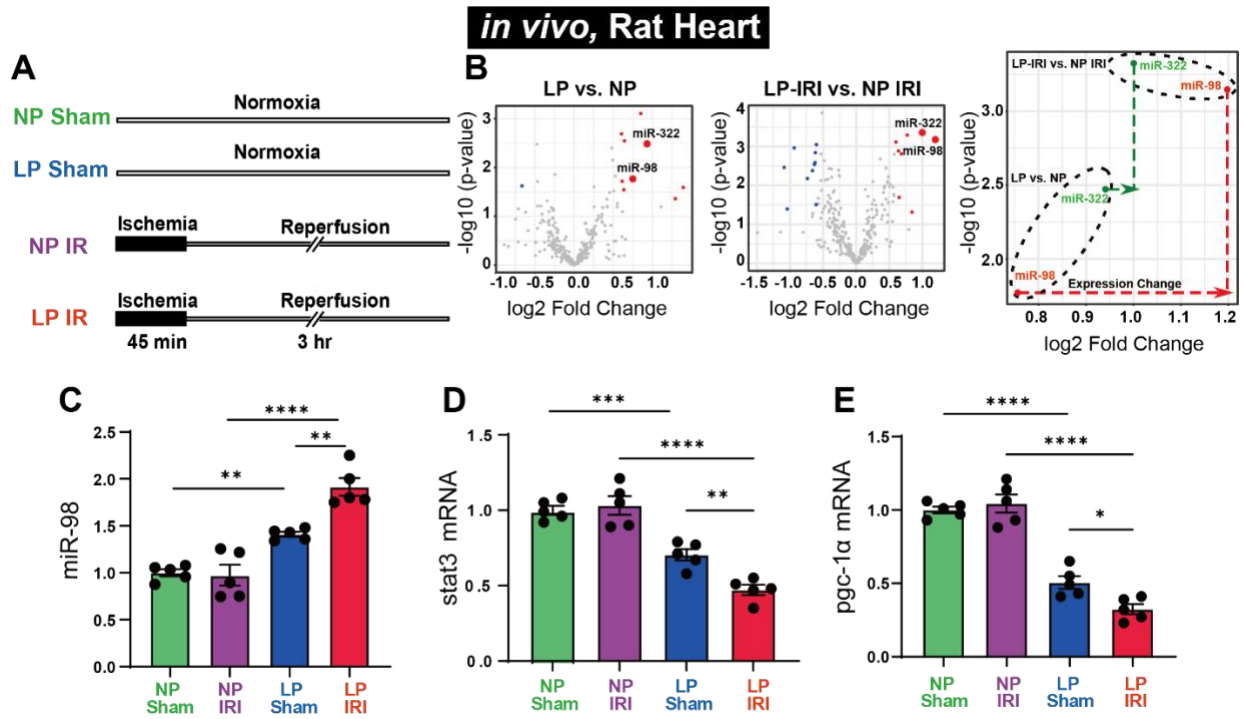
Figure 1. We identified miR-98 to be upregulated in LP rat myocardium subjected to IRI and its targets Stat3 and Pgc-1α to be downregulated. A. Experimental protocol depicting sham vs. IRI. **B.** Volcano plot on microRNA-microarray analysis. **C-E.** Validation of miR-98, Stat3, and Pgc-1α transcript expression by qPCR in LP rat myocardium in sham and upon IRI (normalized to NP sham). Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

its expression is increased in the plasma of healthy LP rats compared to NP rats and exacerbated upon IRI **(Fig. 2A).** Similar to our rat data, we also observed that plasma miR-98 expression is significantly increased in healthy LP individuals compared to healthy NP individuals **(Fig. 2B).** Strikingly, plasma miR-98 was increased even further in patients who had an acute MI during LP compared to healthy LP individuals **(Fig. 2B)**. More importantly, we observed a significant positive correlation between miR-98 plasma levels and troponin in acute MI patients (p-value: 0.0013, Pearson's r: 0.9423, n=6) **(Fig. 2C).** Patient characteristics are shown in **Supplemental Table I.** These data suggest that miR98 could serve as a novel biomarker in LP patients with acute MI.

miR-98 is expressed in cardiomyocytes in NP rats and its expression is increased in LP rats

To examine whether miR-98 is expressed in cardiomyocytes, we employed fluorescent *in situ* hybridization (FISH) in NP and LP rat hearts. Since FISH probes recognize individually labeled transcripts within a cell, co-localization of two transcripts within a cell is demonstrated by the proximity of the two transcripts and a nucleus. We found that miR-98 is expressed in cardiomyocytes since miR-98 (red) were in close proximity to cardiac specific tnnt2 transcripts (white) **(Fig. 3A).** We next assessed whether the upregulated miR-98 observed in LV of LP rats is due to increased expression of miR-98 in cardiomyocytes compared to NP rats. We found a higher miR-98/tnnt2 ratio in LP rat myocardium compared to NP rat myocardium **(Fig. 3B),** indicating enhanced miR-98 expression in cardiomyocytes in LP rats.
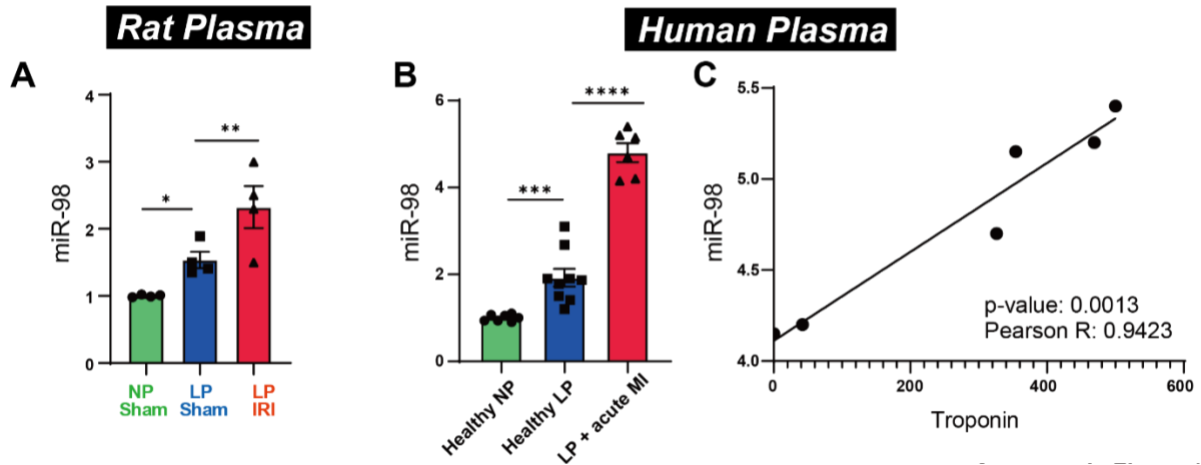
Figure 2. miR-98 could serve as a biomarker in LP. A. Expression of miR-98 transcripts in rat plasma in sham and upon IRI (normalized to NP sham). **B.** Plasma levels of miR-98 in healthy NP individuals, healthy LP individuals, and LP patients with acute MI (normalized to healthy NP). **C.** Correlation of miR-98 and troponin. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
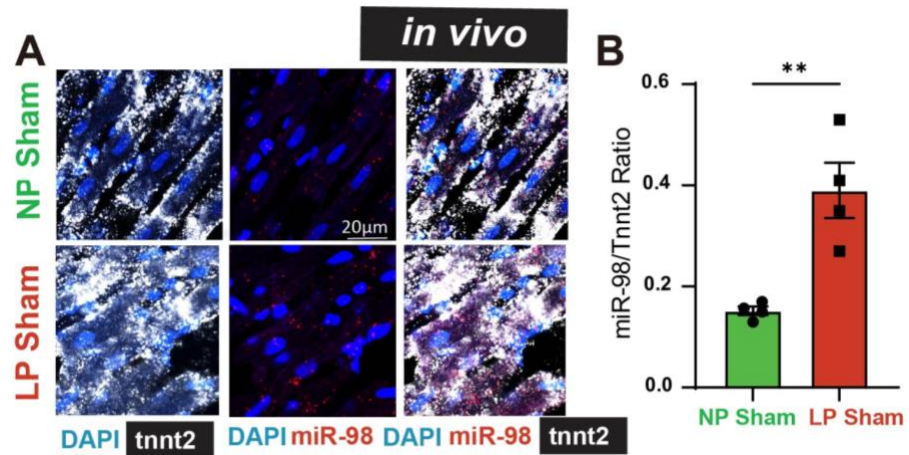
Figure 3. miR-98 is expressed in cardiomyocytes. A. miR-98 (red) expression in rat LV assessed by fluorescent *in situ* hybridization with cardiomyocyte marker tnnt2 (white) and **B.** quantification depicting higher miR-98 levels in LP sham compared to NP sham. Data presented as mean+SEM. Student's t-test, **p<0.01. CC3: cleaved caspase 3.

Serum from LP rats is sufficient to upregulate miR-98 expression, and to increase the vulnerability to H/R injury in H9c2 cells

As miR-98 is upregulated in cardiomyocytes of LP rats, in LP rat myocardium subjected to IRI, we next assessed whether we could mimic our LP model in cardiomyocytes *in vitro*.

Female ARVMs and H9c2 cells were utilized for the *in vitro* experiments. We incubated ARVMs and H9c2 cells with serum from either NP or LP rats for 24 hours, followed by either normoxia or H/R **(Fig. 4A)**. Our data revealed the expression of miR-98 was increased in ARVMs and H9c2 cells incubated with serum from LP rats compared to serum from NP rats, in normoxia **(Fig. 4B, C)**. Strikingly, H/R enhanced miR-98 expression further in cells with both NP and LP serum, however to a significantly larger extent in cells treated with LP serum compared to NP serum **(Fig. 4B, C)**. As we observed the same results with ARVMs as we did with H9c2 cells, and as ARVMs are extremely difficult to grow in culture and infect, we proceeded with H9c2 for the remainder of the *in vitro* experiments. H9c2 cells are widely used to study the effects, mechanisms, and therapeutic interventions of hypoxia/reoxygenation (H/R), which mimics the IRI setting (21,22). In line with increased miR-98 expression, expression of its targets Stat3 **(Fig. 4D)** and Pgc-1α **(Fig. 4E)** were significantly lower in H9c2 cells incubated with LP serum.

As Stat3 and Pgc-1α are known to play a protective role against inflammation, mitochondrial superoxide production, and apoptosis in the heart (23–26), we measured these parameters in H9c2 cells subjected to LP rat serum and H/R. Our data revealed upregulation of pro-inflammatory markers (TNFα and IL-1β) **(Fig. 5A, B)**, mitochondrial superoxide **(Fig. 5C)**, and apoptosis **(Fig. 5D, E)** in H9c2 cells incubated with serum from
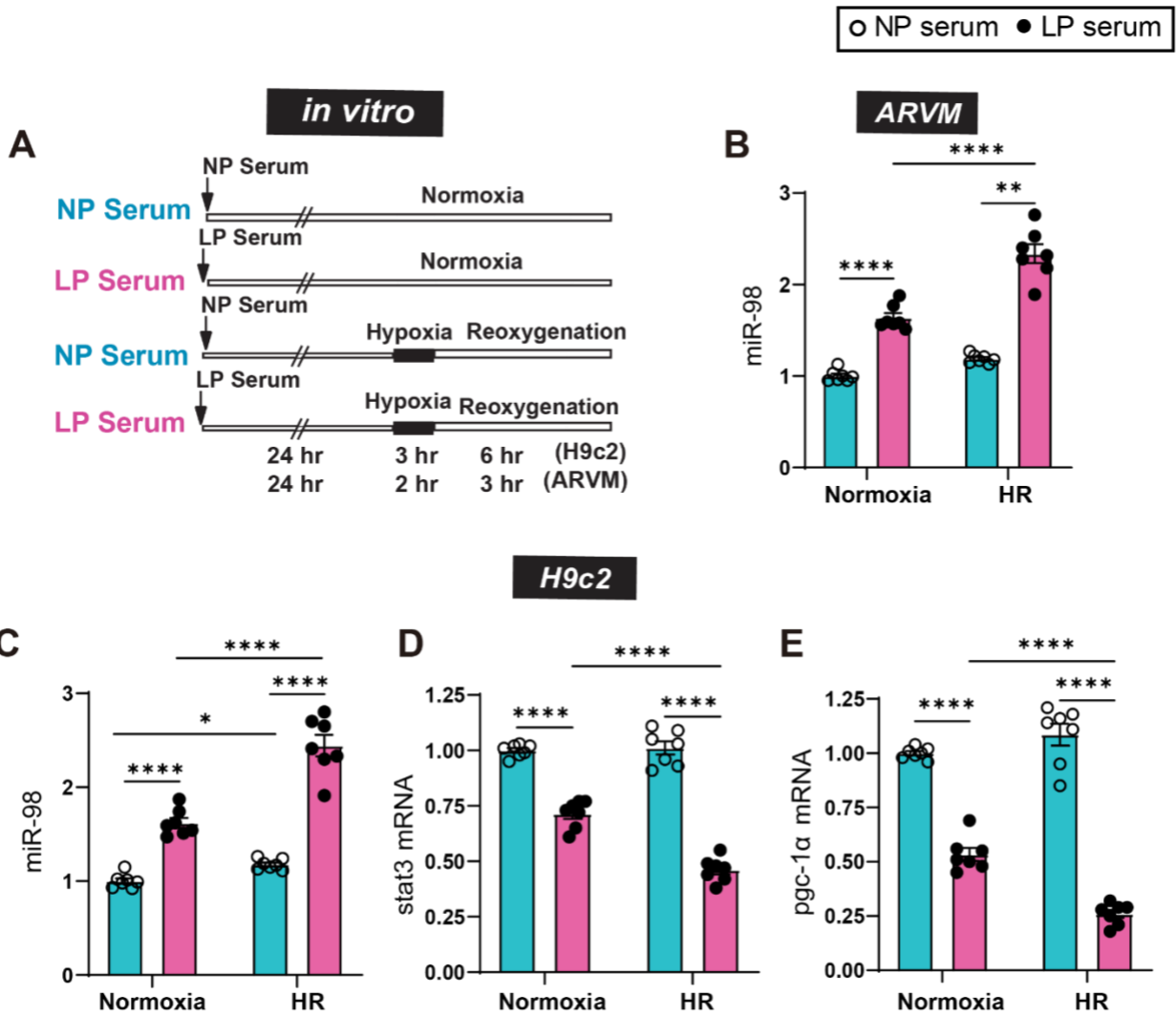
Figure 4. Serum from LP rats is sufficient to upregulate miR-98 expression and downregulate its targets Stat3 and Pgc-1α. A. Experimental protocol depicting NP vs. LP serum stimulation in the presence and absence of H/R stimulus. **B.** miR-98 transcript expression in ARVMs via qPCR (normalized to NP serum upon normoxia). **C-E.** miR-98, Stat3, and Pgc-1α transcript expression in H9c2 cells via qPCR (normalized to NP serum upon normoxia). Data presented as mean+SEM. Student's t-test and ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ****p<0.0001. ARVMs: adult rat ventricular myocytes.
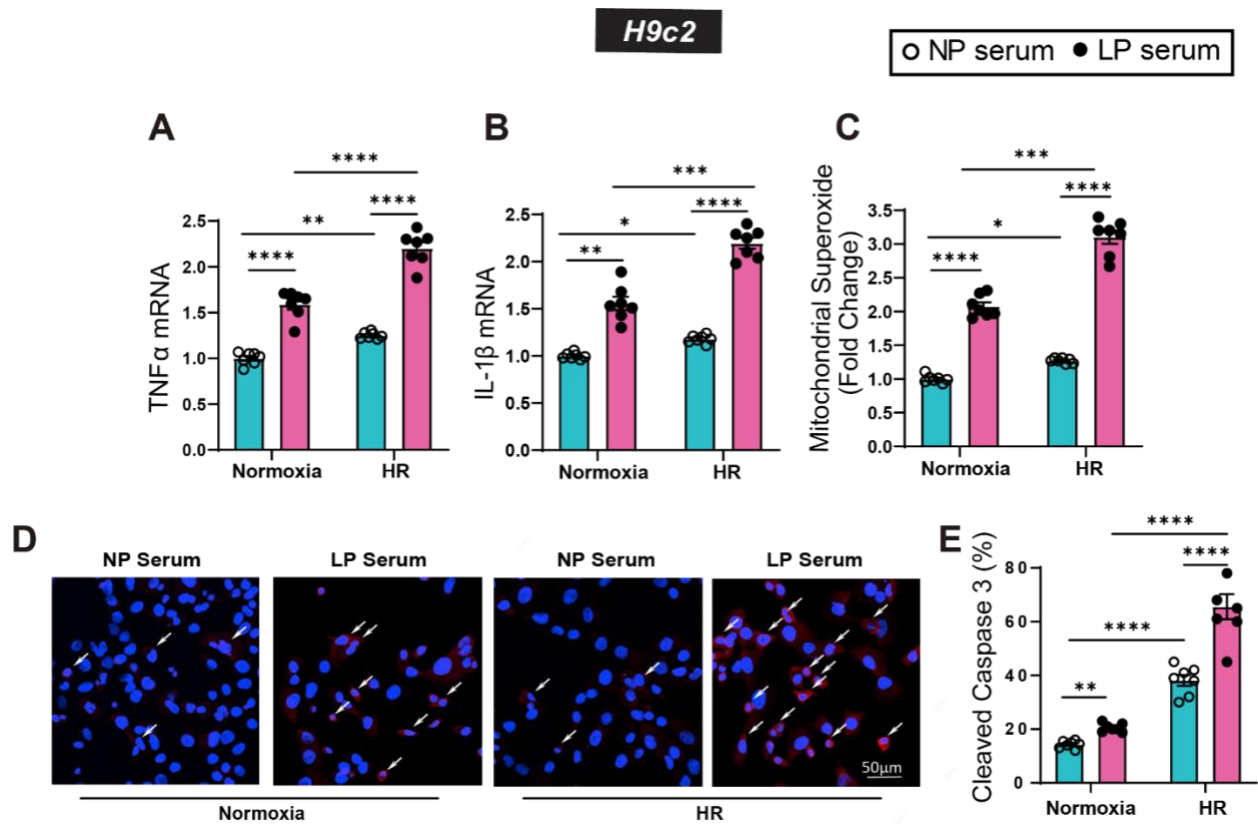
Figure 5. Serum from LP rats increases the vulnerability to H/R injury in H9c2 cells. **A,B** Expression of pro-inflammatory markers TNFα and IL-1β as assessed by qPCR (normalized to NP serum upon normoxia). **C.** Expression of mitochondrial superoxide levels (normalized to NP serum upon normoxia). **D,E.** Representative images depicting apoptosis levels as assessed by CC3 immunofluorescence staining and quantification. Data presented as mean+SEM. Student's t-test and ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CC3: cleaved caspase 3.

LP rats compared to serum from NP rats, in both the presence and absence of H/R stimulus. Strikingly, HR enhanced pro-inflammatory markers, mitochondrial superoxide levels, and apoptosis to a significantly larger extent in H9c2 cells with LP serum **(Fig. 5A-E)**.

Together, these data suggest that serum from LP is sufficient to increase expression of miR-98 in cardiomyocytes, and to induce inflammation, ROS, and apoptosis. Furthermore, H9c2 cells are vulnerable to H/R to a larger extent upon stimulation with LP serum, compared to NP serum.

Estrogen, not progesterone, is sufficient to upregulate miR-98 expression

As serum from LP rats is sufficient to upregulate miR-98 expression, and as estrogen and progesterone reach their peak during LP, we next assessed whether either estrogen or progesterone is sufficient to upregulate miR-98 expression *in vitro*.

We incubated H9c2 cells with estrogen or progesterone for 24 hours. Our data revealed the expression of miR-98 was increased in H9c2 cells in the presence of estrogen, not progesterone **(Fig. 6).** This data suggests that the increased expression of miR-98 upon LP serum stimulation is due to an increase in estrogen levels.

miR-98 regulates Stat3 and Pgc1α expression in H9c2 cells in the setting of H/R

To further examine if Stat3 and Pgc-1α are indeed targets of miR-98 *in vitro*, H9c2 cells were transfected with either miR-98 mimic or inhibitor in both the presence and absence of H/R stimulus **(Fig 7A)**. Transfection with miR-98 mimic resulted in a significant upregulation of miR-98 expression and transfection with miR-98 inhibitor resulted in the
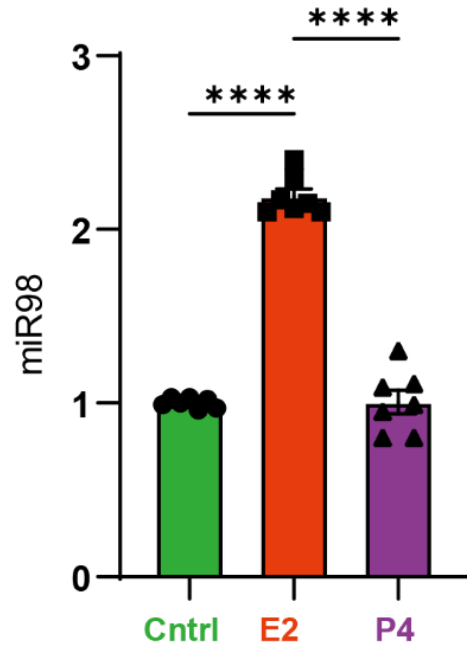
Figure 6. Estrogen, not progesterone, is sufficient to increase the expression of miR-98.
H9c2 cells were stimulated with 10nM of either E2 or P4 for 24 hours (normalized to control). Data

presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, ****$p < 0.0001$. Cntrl:

control, E2: B-estradiol, P4: progesterone.

downregulation of miR-98 expression, confirming successful overexpression and knockdown of miR-98, respectively **(Fig. 7B,E)**. In the presence of H/R stimulus, the expression of miR-98 was changed to a greater extent upon both miR-98 mimic and inhibitor **(Fig. 7B,E)**. MiR-98 overexpression resulted in a significant downregulation of its targets Stat3 and Pgc-1α gene **(Fig. 7C,D)**, while miR-98 knockdown resulted in the upregulation of Stat3 and Pgc-1α **(Fig. 7F,G)**. Similar to miR-98, the expression of Stat3 and Pgc-1α were exacerbated to a greater extent upon H/R stimulus when miR-98 expression was modulated with mimic or inhibitors **(Fig. 7C,D,F,G).** Taken together, these data suggest that Stat3 and Pgc-1α are indeed targets of miR-98 *in vitro.*

miR-98 promotes inflammation, ROS, and apoptosis in H9c2 cells in the setting of H/R

We next assessed the functional role of miR-98 on inflammation, ROS, and apoptosis in the context of H/R using H9c2 cells **(Fig. 8A)**. Our data revealed increased levels of pro-inflammatory markers TNFα and IL-1β **(Fig. 8B,C)**, and mitochondrial superoxide **(Fig. 8D)** in H9c2 cells transfected with miR-98 mimic in both the presence and absence of H/R stimulus, while transfection with miR-98 knockdown resulted in the opposite effects **(Fig. 8E-G).** Importantly, our data revealed the effects of H/R were significantly larger with miR-98 mimic or inhibitor compared to their respective scramble controls in normoxia **(Fig. 8B-G)**. Our data also revealed increased levels of apoptosis in H9c2 cells transfected with miR-98 mimic in both the presence and absence of H/R stimulus, while transfection with miR-98 knockdown resulted in the opposite effects **(Fig. 9A-C).**

Taken together, these data suggest that miR-98 promotes apoptosis, inflammation, and mitochondrial superoxide in the context of H/R in vitro.
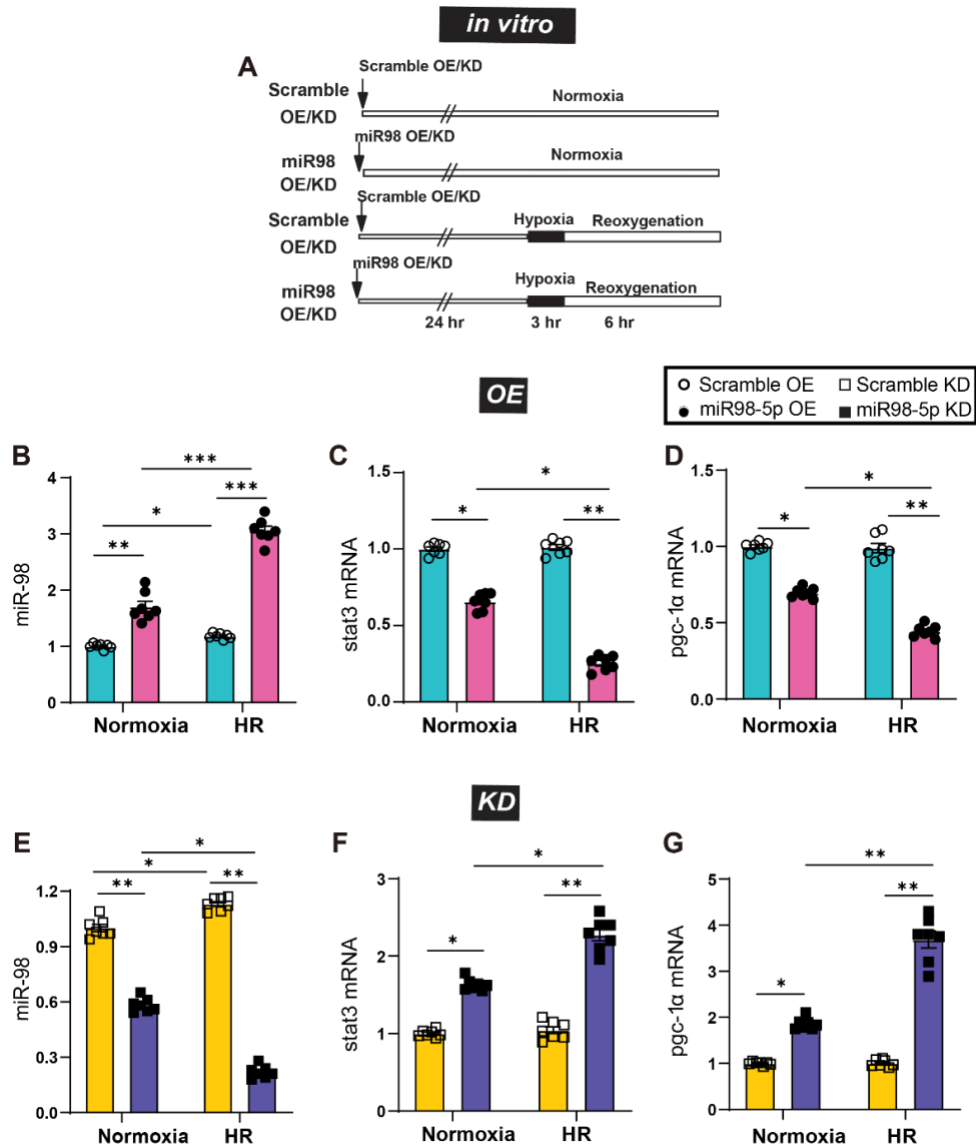
Figure 7. miR-98 targets Stat3 and Pgc1α in H9c2 cells in the setting of H/R. A. Experimental protocol depicting miR-98 vs. scramble OE/KD in the presence and absence of H/R stimulus. **B.** miR-98 transcript expression via qPCR assessing overexpression. **C,D.** Stat3 and Pgc-1α transcript expression via qPCR assessing knockdown. **E.** miR-98 transcript expression via qPCR assessing knockdown. **F,G.** Stat3 and Pgc-1α transcript expression via qPCR assessing knockdown. All data normalized to scramble OE/KD upon normoxia. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001. OE: overexpression. KD: knockdown.
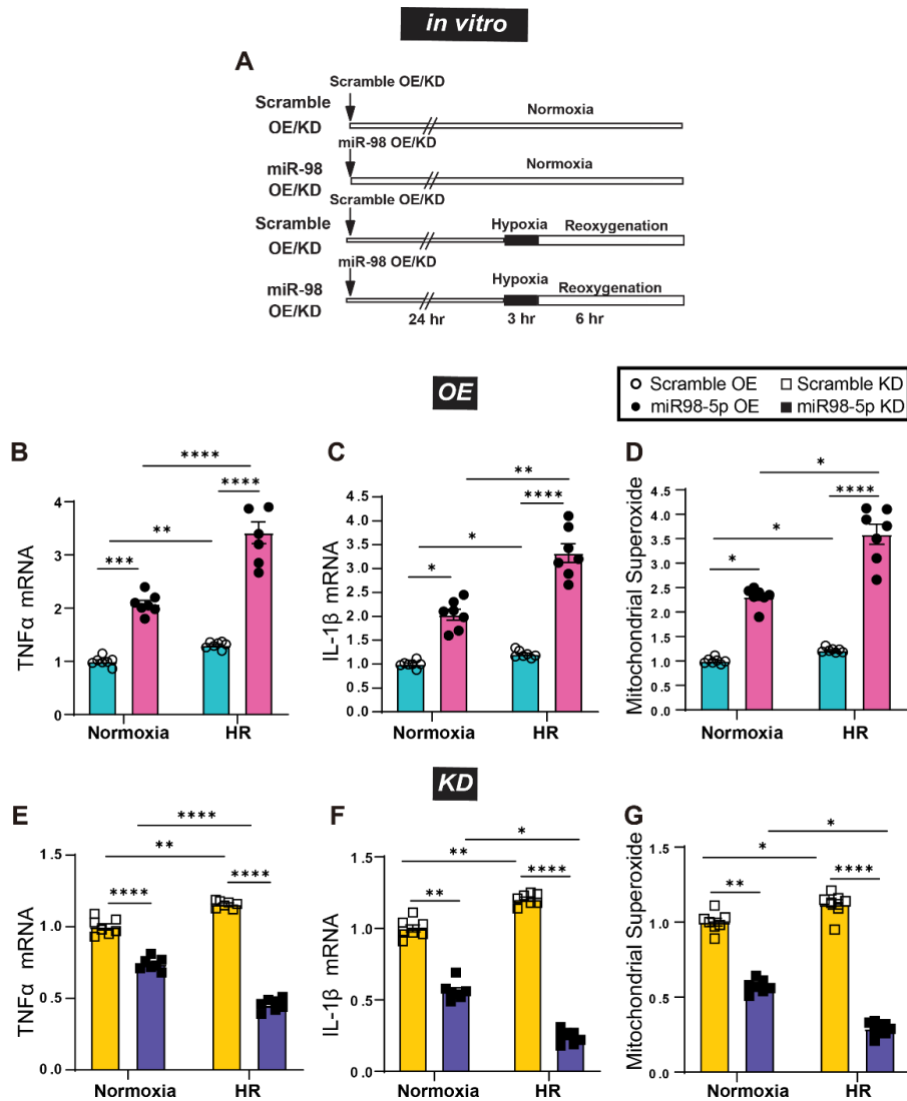
Figure 8. miR-98 promotes inflammation, ROS, and apoptosis in H9c2 cells in the setting of H/R. A. Experimental protocol depicting miR-98 vs. scramble OE/KD in the presence and absence of H/R stimulus. **B,C.** Expression of pro-inflammatory markers TNFα and IL-1β as assessed by qPCR assessing overexpression. **D.** Expression of mitochondrial superoxide levels assessing overexpression. **E,F.** Expression of pro-inflammatory markers TNFα and IL-1β as assessed by qPCR assessing knockdown. **G.** Expression of mitochondrial superoxide levels assessing knockdown. All data normalized to scramble OE/KD upon normoxia. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001. OE: overexpression. KD: knockdown.

Figure 9. miR-98 promotes apoptosis in H9c2 cells in the setting of H/R. A. Experimental protocol depicting miR-98 vs. scramble OE/KD in the presence and absence of H/R stimulus. **B,C.** Representative images depicting apoptosis levels as assessed by CC3 immunofluorescence staining and quantification. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
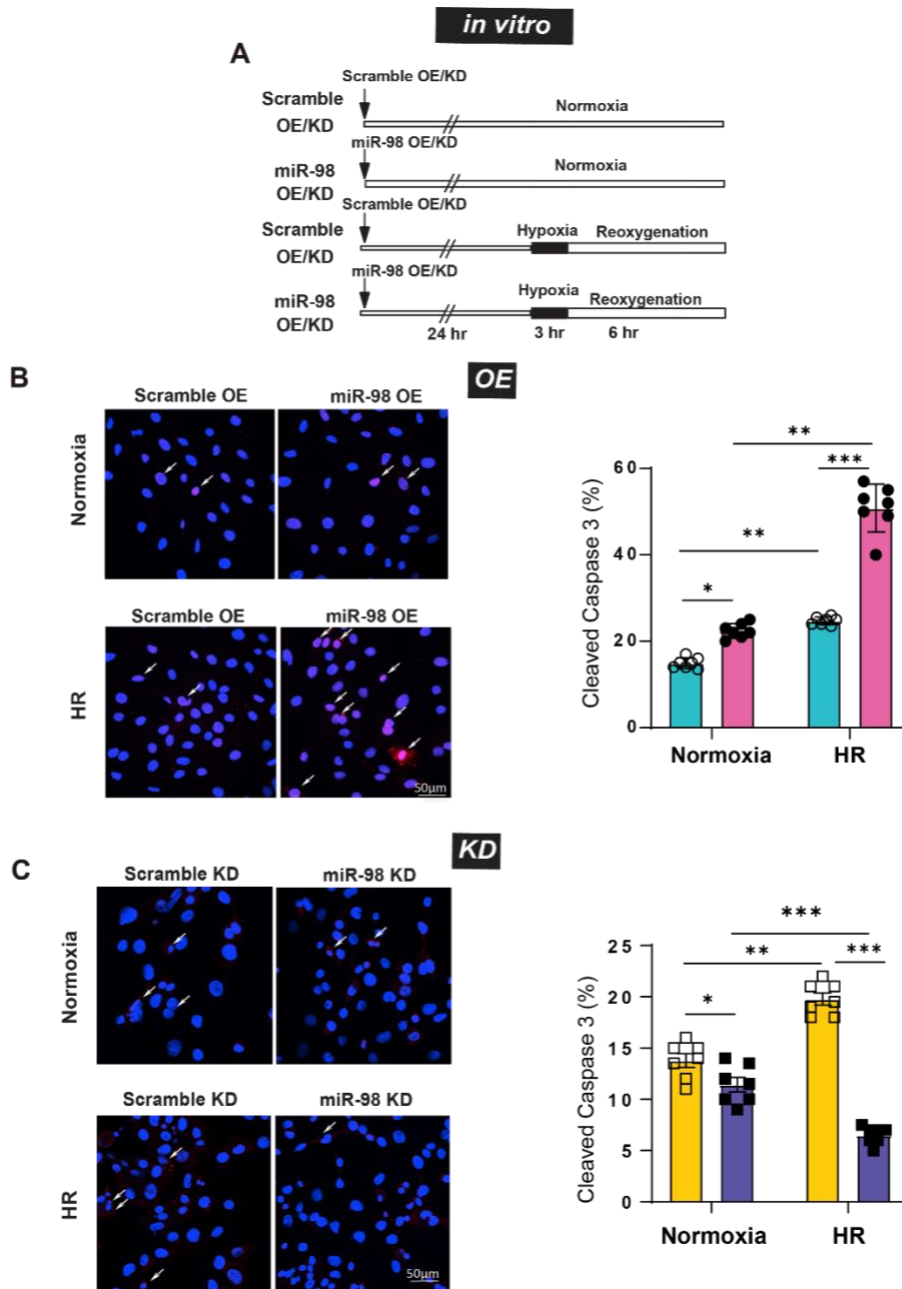
RNA-Seq analysis reveals novel downstream targets of Stat3 and Pgc-1α

miR98 targets Stat3 and Pgc-1α are transcription factor transcriptional co-activator, respectively (27,28). To examine which genes downstream of Stat3 and Pgc-1α are dysregulated in the setting of LP, H9c2 cells were incubated with LP serum followed by overexpression of Stat3, Pgc-1α, or both Stat3 and Pgc-1α **(Fig. 10A)**. As expected, overexpression of Stat3 resulted in a significant upregulation of Stat3 expression **(Fig. 10B)**, and overexpression of Pgc-1α resulted in a significant upregulation of Pgc-1α expression, confirming successful overexpression of Stat3 and Pgc-1α **(Fig. 10C)**.

We next performed RNA-Seq on the aforementioned cells. The Volcano Plot on the RNA-Seq analysis shows significant up-regulation (blue) and down-regulation (red) with the magnitude changes **(Fig. 10D-F)**. RNA-Seq data analysis revealed 185 genes that were significantly upregulated and 169 genes that were significantly downregulated in the group treated with both Stat3 and Pgc-1 α overexpression **(Fig. 10G)**.

Using gene ontology (29,30), we identified all genes commonly dysregulated that are part of the gene ontology for the inflammatory response (GO:0006954), apoptotic process (GO:0006915), and response to oxidative stress (GO:0006979) **(Fig. 11A)**. As Stat3 and Pgc-1α are mainly known to be involved in transcriptional activation (31,32), to define the most relevant genes, we focused on genes significantly upregulated by both Stat3 and Pgc-1α and we excluded all transcription factors. Furthermore, we selected genes that are known to play a protective role against oxidative stress, cell apoptosis, and inflammation. Based on these criteria we identified 5 genes Ash1l (Absent, Small, or Homeotic 1-Like) and Tnip1 (TNFAIP3 Interacting Protein 1) in the inflammatory pathway,
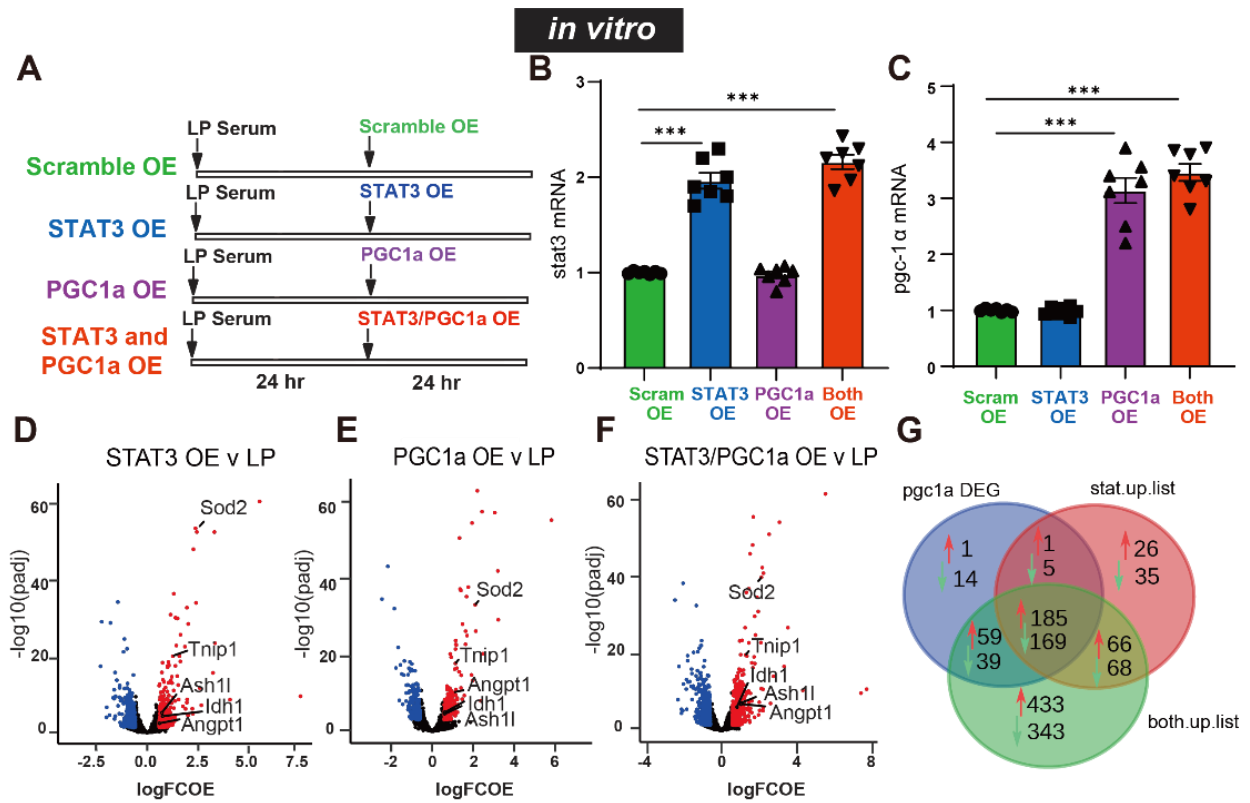
Figure 10. RNA-Seq analysis reveals novel downstream targets of Stat3 and Pgc-1α. A. Experimental protocol depicting Stat3 and Pgc-1α overexpression upon 1% LP serum stimulation in H9c2 cells. **B,C.** Validation of Stat3 and Pgc-1α transcript expression via qPCR (normalized to scramble OE). **D-F.** Volcano Plots on RNA-Seq analysis. **G.** Venn Diagram depicting 185 genes significantly upregulated and 169 genes significantly downregulated. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, ***p<0.001. OE: overexpression.

Idh1 (isocitrate dehydrogenase 1) and Sod2 (superoxide dismutase 2) in the oxidative stress pathway, and Angp1 (Angiopoietin 1) and Sod2 in the apoptotic pathway. We confirmed a significant upregulation of all 5 genes in the group treated with both Stat3 and Pgc-1α overexpression via qPCR (**Fig. 11B-F).**

Taken together, our results revealed a subset of downstream target genes of Stat3 and Pgc-1α that play a protective role against oxidative stress, cell apoptosis, and inflammation.

miR-98 inhibition at the onset of reperfusion significantly reduces myocardial infarct size in LP rats by targeting Stat3 and Pgc-1α

Our *in vitro* data suggests that miR-98 knockdown may confer protective effects against H/R injury. Thus, we examined the therapeutic role of miR-98 inhibition in LP upon IRI *in vivo*. LP rats were subjected to 45 minutes of ischemia, followed by 24 hours of reperfusion. At the onset of reperfusion, 10nM of miR-98 inhibitor or a scrambled control were injected via the femoral vein **(Fig. 12A)**. Our data revealed successful knockdown (2-fold) of miR-98 expression in the heart **(Fig. 12B)**. Most importantly, the infarct size was significantly smaller in the LP rats treated with miR-98 inhibitor compared to scramble inhibitor **(Fig. 12C,D),** despite the fact that both groups were subjected to a comparable degree of ischemic risk as there was no significant different in area at risk between the two groups **(Fig. 12E)**. Concomitant with downregulated miR98 expression, Stat3 and Pgc-1α transcript **(Fig. 12F,G)** and protein **(Fig 12H-L)** expression were significantly increased by miR-98 inhibitor.
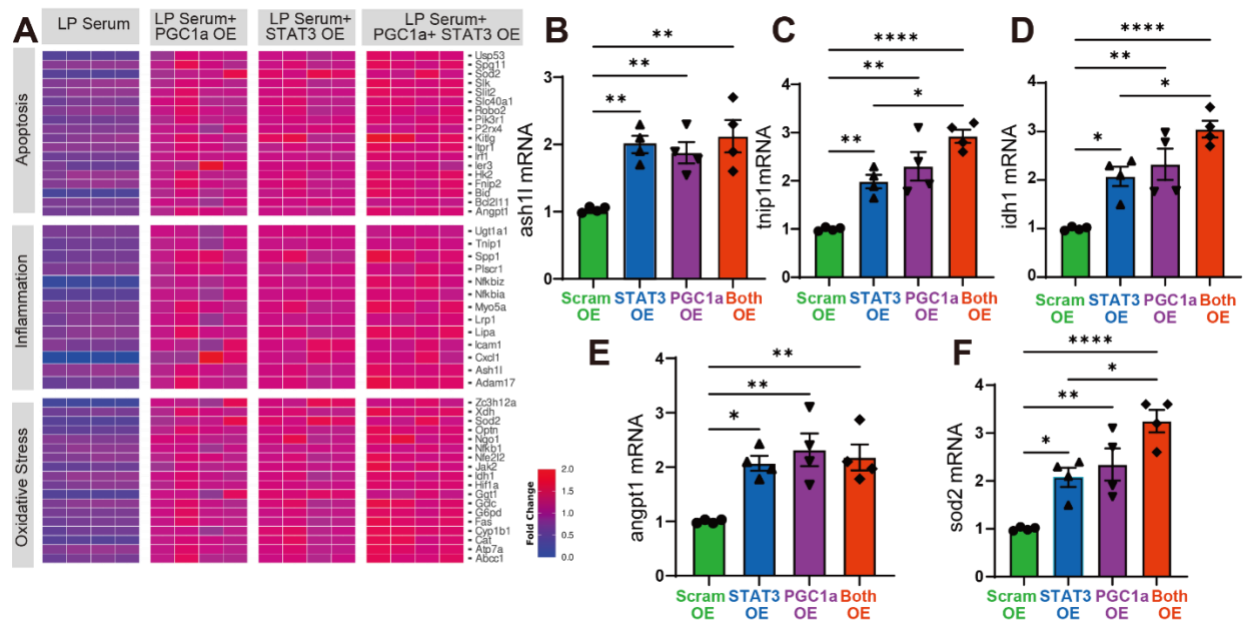
Figure 11. RNA-Seq analysis reveals novel downstream targets of Stat3 and Pgc-1α. A.
Genes commonly dysregulated that are a part of the gene ontology for the inflammatory response, apoptotic process, and oxidative stress response. **B-F.** Validation of *ash1l, tnip1, idh1, angpt1, and sod2* transcript expression via qPCR (normalized to scramble OE). **D**. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ****p<0.0001. OE: overexpression.
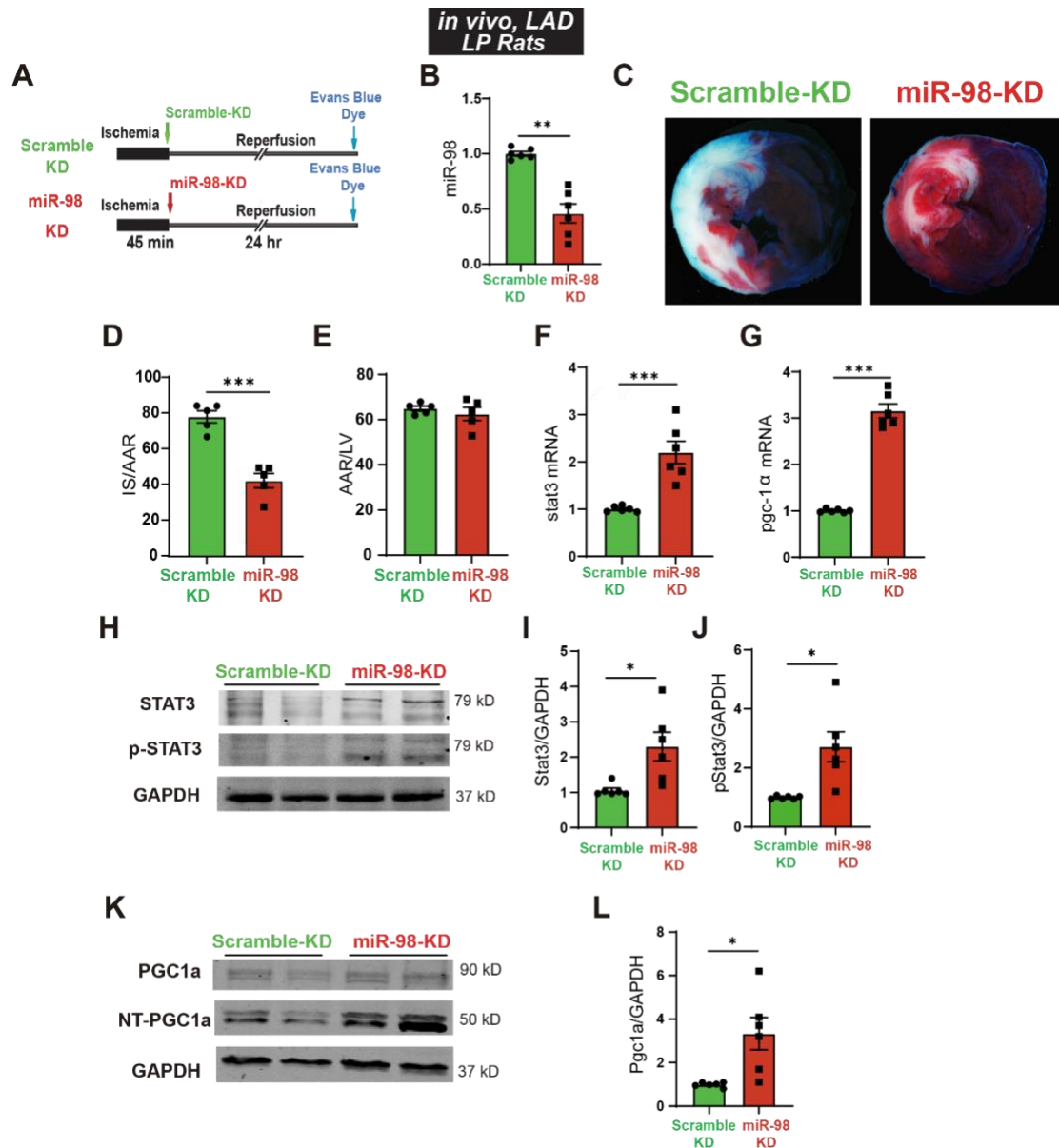
Figure 12. miR-98 inhibition at the onset of reperfusion reduces myocardial infarct size in LP rats by targeting Stat3 and Pgc-1α. A. Protocol of IRI model. Arrows indicate femoral vein injections of 10nM scramble inhibitor or miR-98 inhibitor **B.** LV miR-98 expression assessed by qPCR. **C.** Heart cross sections using TTC staining. **D.** Infarct size divided by area at risk. **E.** Area at risk divided by LV. **F,G.** Stat3 and Pgc-1α transcript in LV upon miR-98 inhibitor as measured by qPCR. **H-L.** Representative Western Blots and quantifications. Data presented as mean+SEM. Student's t-test. *p<0.05, **p<0.01, ***p<0.001, Tnnt2: cardiac troponin T. LV: left ventricle.

Accordingly, the expression of Stat3 and Pgc-1α downstream genes, Sod2, Ash1l, Tnip1, Idh1, and Angpt1 was significantly upregulated upon miR-98 inhibitor treatment **(Fig. 13F-J)**. We examined the impact of miR-98 knockdown on markers of inflammation, ROS, and apoptosis in the miR-98 inhibitor group compared to scramble inhibitor. Our data revealed decreased levels of pro-apoptotic marker Bax **(Fig. 13A)** and increased levels of anti-apoptotic marker Bcl2 **(Fig. 13B)** upon miR-98 inhibitor compared to scramble inhibitor. Furthermore, pro-inflammatory markers TNFα and IL-1β were decreased in the miR-98 knockdown group **(Fig. 13C,D)**. We also observed higher levels of ROS scavengers Sod2 and Aldh2 in the group treated with miR-98 inhibitor **(Fig. 13E,F)**.

Taken together, our data show that a single dose of miR-98 inhibitor at the onset of reperfusion reduces MI size, inflammation, apoptosis, and ROS production, and is also associated with an increase in expression of its targets Stat3 and Pgc-1α and their downstream effector genes in rats *in vivo*.
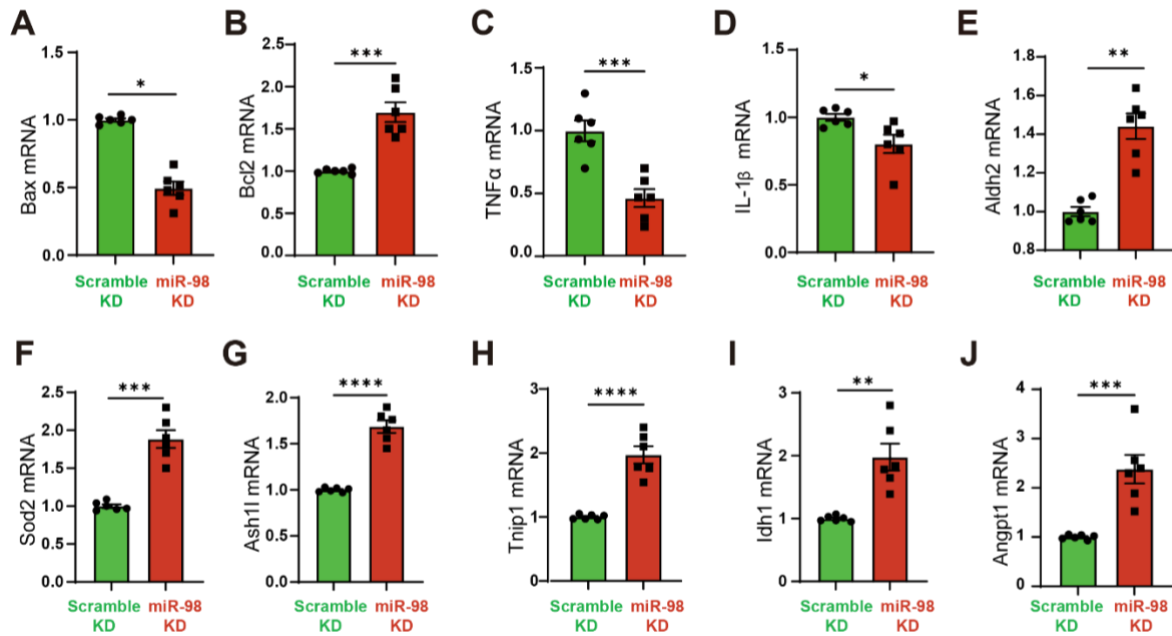
Figure 13. miR-98 inhibition at the onset of reperfusion decreases myocardial apoptosis, inflammation, and oxidative stress and increases common downstream targets of Stat3 and Pgc-1α. A-E: Expression of *sod2, ash1l, tnip1, idh1, and angpt1* in LV upon miR-98 knockdown as assessed by qPCR. **F,G**: Expression of pro-apoptotic marker Bax, and anti-apoptotic marker Bcl-2 as assessed by qPCR. **H,I**: Expression of pro-inflammatory markers TNFα and IL-1β as assessed by qPCR. **J:** Expression of ROS scavenger Aldh2 as assessed by qPCR. Data presented as mean+SEM. Student's t-test. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Discussion

Using microRNA-microarray and an experimental approach, we discovered that miR-98 expression is increased in hearts of LP rats compared to hearts of NP rats, and this increase is exacerbated after IRI. In addition, our data demonstrates that upregulation of miR98 in IRI is only specific to LP, but not to NP. Importantly, we found that miR-98 expression is upregulated in the plasma of pregnant patients with acute MI. Our data confirmed Stat3 and Pgc-1α as targets of miR98 both in vivo and in vitro. Strikingly, a single dose of miR-98 inhibitor at the onset of reperfusion in LP rats is able to reduce myocardial infarct size, inflammation, apoptosis, and ROS production *in vivo*, concomitant with enhanced expression of Stat3 and Pgc-1α and their downstream targets.

Thus far, opposing reports have been published on the role of miR-98 in the heart in the context of IRI or MI. In agreement with our data, miR-98 inhibition was shown to improve IRI-induced microvascular dysfunction in male rats *in vivo* (33). On the other hand, it was shown that overexpression of miR-98 decreased infarct size and improved cardiac function of infarcted heart in male mice *in vivo* (34). Of importance, both studies were conducted on male rodents as opposed to our study, which was conducted on LP rats. Additionally, in the former study, the coronary artery was ligated for 1 hour followed by 6 hours of reperfusion, while the latter study examined permanent ligation of the LAD consistent with a permanent ischemia model as opposed to IRI. Lastly, both studies administered the miR-98 agomir/antagomir prior to the coronary artery ligation, as opposed to the onset of reperfusion, which is a more clinically relevant method used in our study.

In the heart, miR-98 has been found to target several genes and signaling pathways. miR-98 negatively regulates MI-induced cardiomyocyte apoptosis by down-regulating Fas and caspase-3 in male mice (34). Overexpression of miR-98 has been shown to play a protective role in mice with acute coronary syndrome by targeting ADAM15, and in turn, inhibiting the activation of the p38MAPK pathway, thereby reducing the inflammatory response (35). Further, miR-98 has been shown to regulate myocardial differentiation of mesenchymal stem cells by targeting TBX5 (36). As cardiomyocyte apoptosis, oxidative stress, and inflammation are cornerstones of IRI pathophysiology (13,14), we focused on the targets of miR-98 that are known to regulate these pathways in the heart, and identified Stat3 and Pgc-1α as miR-98 targets on TargetScan (23–26). Interestingly, both Stat3 and Pgc-1α are known negative regulators of ROS production and their deletion in cardiomyocytes *in vivo* predisposes mice to peripartum cardiomyopathy (PPCM) (37,38). Stat3 has been shown to be involved in various mechanisms that contribute to cardioprotection against different heart pathologies, including IRI, MI, PPCM, and hypertrophy (27). We have previously shown that hindered Stat3 activation in LP makes the hearts of LP rats more vulnerable to IRI in LP (15). In cardiomyocytes from failing human hearts, Stat3 activation has been shown to be impeded (39). Supporting our findings, in the hearts of Stat3-KO mice, pregnancy was associated with increased oxidative stress (25). Also in line with our findings, activation of Stat3 in male mice *in vivo* protects the myocardium from IRI through decreasing apoptosis and ROS generation (27,40). In agreement with our results, Stat3 is required for the IL-10 receptor signaling pathway, which leads to the generation of anti-inflammatory response (41). Previously, miR-98 has been shown to target Stat3, as pre-treatment with melatonin protects cardiac

progenitor cells against damage caused by oxidative stress in MI via miR-98/Stat3 signaling pathways (42). Pgc-1α is a well-known master regulator of mitochondrial biogenesis, oxidative phosphorylation, and mitochondrial antioxidant defense (24). In the heart, Pgc-1α plays a key role in transcriptional control of metabolic genes (43). Pgc-1α knockout mice show deficiencies in cardiac energy reserve and function and demonstrate accelerated heart failure development (44–46). Some reports have suggested that downregulation of Pgc-1α and its transcriptional targets are a major cause of mitochondrial impairment and metabolic defects in the failing mouse heart (28,43). Supporting our findings, Pgc-1α has been shown to protect against hepatic IRI in male mice *in vivo* (47). Of importance, our study is the first to show miR-98 regulates myocardial infarct size in LP by targeting Stat3 and Pgc-1α.

We demonstrate the therapeutic potential of miR-98 inhibition in the LP rat myocardium. Systemic delivery of only one dose of miR-98 inhibitor at the onset of reperfusion to LP rats reduced infarct size, mitigated inflammation, and decreased mitochondrial oxidative stress, all hallmarks of IRI, and upregulated its targets Stat3 and Pgc1-a in the LP myocardium. We furthermore show that inhibition of miR-98 via systemic delivery significantly reduced miR-98 expression in cardiomyocytes. However, we cannot exclude that systemic miR-98 inhibition may also reduce miR-98 expression in other cardiac cells. Regardless, any non-cardiomyocyte off-target effect that systemic miR-98 inhibition delivery may have, still leads to beneficial outcomes regarding myocardial apoptosis, ROS, and inflammation, highlighting the therapeutic potential of miR-98. Our model is clinically relevant, as we have administered only one dose at the time of reperfusion, as

administering miR antagomir several times during pregnancy may have undesirable effects on both the mother and fetus.

We also report upregulated miR-98 expression in plasma of LP individuals versus NP individuals, and a further upregulation in the plasma of pregnant patients with acute MI compared to healthy LP individuals. Furthermore, we observe a positive correlation between miR-98 plasma expression and troponin in a very limited number of samples. As acute MI is a significant cause of maternal morbidity and mortality in pregnancy, identifying potential biomarkers for this condition is crucial for early diagnosis and management, and could have several important clinical implications. Since detection of cardiac events such as MI in pregnancy is often achieved only after clinical signs are already apparent, it is of utmost importance to have reliable biomarkers for early detection of pregnant individuals at high risk of cardiac events. Since the initial detection of circulating miRs in human blood, significant research attention has been devoted to investigating their potential as biomarkers (48–50). There is increasing evidence to suggest that miR-98 could potentially serve as a biomarker for several diseases. A previous study has shown that miR-98 promotes IRI-induced microvascular dysfunction and may potentially serve as a biomarker for microvascular reperfusion (33). MiR-98 downregulation has been shown to promote pancreatic ductal adenocarcinoma proliferation and metastasis, which could serve as a biomarker for predicting poor prognosis of pancreatic ductal adenocarcinoma patients (51). The role of miR-98 has also been studied in the placentas of pregnant patients. Upregulated miR-98 expression has been reported in placentas from gestational diabetes mellitus patients compared with controls (52). On the other hand, miR-98 has been shown to be downregulated in pre-

eclampsia placentas compared to healthy control (53). Additional research is necessary to establish the relationship between plasma levels of miR-98 and cardiac function parameters in LP individuals with cardiovascular complications, as well as to determine the viability of using miR-98 as a biomarker.

Taken together, our work provides new perspectives on the pathological mechanisms and potential pre-clinical therapeutic strategies for myocardial IRI in LP. There is a paucity of experimental data on the mechanisms responsible for cardiac events and the potential therapies to limit IRI in the setting of late pregnancy. Our work provides pivotal information in unraveling the specific mechanisms by which a novel miR could regulate cardiac susceptibility to IRI in late pregnancy. Furthermore, miR-98 may serve as a prognostic biomarker and/or potential therapeutic target for the treatment of pregnant individuals who are at higher risk of acute MI. It could provide insights into the pathophysiology of the disease, help identify key molecular pathways involved in the disease, and provide targets for developing novel therapies. It could enable early diagnosis, risk stratification, and monitoring of disease progression, leading to timely intervention and better management. This is a stepping-stone for the development of new therapeutics for the treatment of MI in LP.

Supplemental Table 1. Patient characteristics

	Healthy NP	Healthy LP	LP + acute MI
Age	33.17±3.65	32.1±1.16	32.3±2.186
Pre-pregnancy BMI	23.1±1.5	NA	31.37±0.86
Late-pregnancy BMI	NA	25.31±0.54	38.02±4.05
cHTN	0/6 (0%)	0/10 (0%)	2/6 (33%)
Type II Diabetes	0/6 (0%)	0/10 (0%)	1/6 (16.67%)
Gravidity	NA	1.4±0.3	2.5±0.56
Parity	NA	0.2±0.13	1.5±0.56
Pregnancy Outcome	NA	8/10 (80%)=VD 2/10 (20%)= C-section	5/6 (83%)=VD 1/6 (16.7%)=C-section
Troponin	NA	NA	<0.04 - >500

Supplemental Table 2. Rat primer sequences

Mouse primer	Forward	Reverse
Adh2	ctgatggaggggtggacttt	ttggggacggcatctttact
Angpt1	tgatggactgggaagggaac	cacaggcatcaaaccaccaa
Ash1l	agccagaccaggaaagagtc	gtagtttcctgcctgaccct
Bax	tcatgaagacaggggccttt	ctgcagctccatgttgttgt
Bcl-2	cttcagggatggggtgaact	atcaaacagaggtcgcatgc
Idh1	tggtgatgtgcagtcagact	aatgggattggtggacgtct
IL-1β	gggatgatgacgacctgcta	tgtcgttgcttgtctctcct
Pgc-1α	ctcgcggaggtttctgaatg	tgtaacgcctggtctctgtt
Rplp0	tgggctttgtgttcaccaag	cccaccttgtctccagtctt
Sod2	ctgctggggattgatgtgtg	ctacaaaacacccaccacgg
Sry	cacactatcatatacggacag	tggacagtaagtaggttagc
Stat3	tcagtgagagcagcaaggaa	tttccgaatgcctcctcctt
Tnfα	agctcaccagaccctgagaa	tcccagcaatcgttaccttc

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Supplemental Figure I. Lack of expression of *Sry* gene in female H9c2 cells
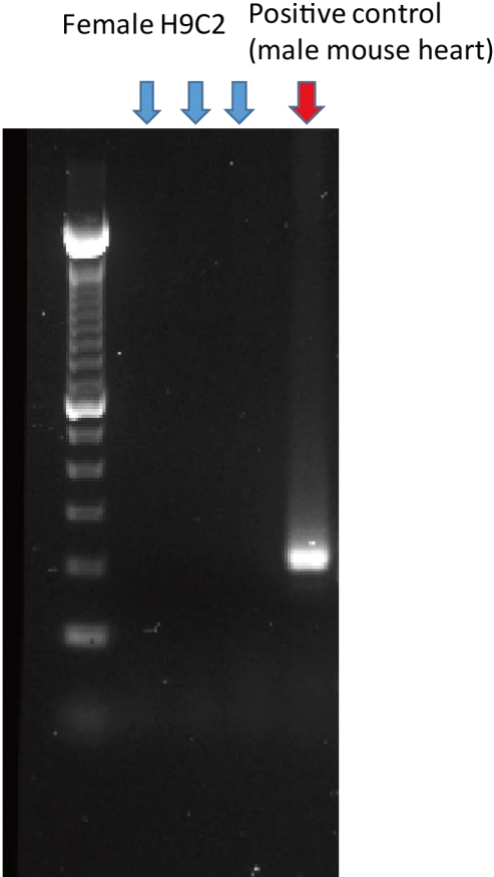
Lack of expression of *Sry* gene in female H9c2 cells versus healthy male mouse heart.

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Chapter 3: Role of microRNA-122/PKM2 Axis in the Intralipid-Mediated Cardioprotection against ischemia/reperfusion injury in Late Pregnancy

Abstract

Background

In the United States, cardiovascular disease is the leading cause of maternal mortality and is responsible for >33% of pregnancy-related deaths. Our lab has previously demonstrated that administration of intralipid (ITLD) immediately at the onset of reperfusion protects the late pregnant (LP) heart against IRI by reducing infarct size. However, the underlying molecular mechanisms are unknown.

Methods

We performed microRNA (miR) microarray analysis on the left ventricle (LV) of NP and LP rats in sham and upon IRI as well as LP rats upon IRI who were administered ITLD. Female H9c2 rat cardiomyoblast cells were transfected with miR mimics and inhibitors, treated with ITLD, and subjected to hypoxia/reoxygenation. We administered miR inhibitor intravenously at the onset of reperfusion. Plasma was collected from healthy NP and LP humans, and LP patients with acute MI.

Results

Our microRNA-microarray analysis revealed downregulation of microRNA-122-5p (miR-122) expression in LV of LP IRI compared to NP IRI, and a drastic upregulation of miR-122 in LP IRI who received ITLD at the onset of reperfusion compared to LP IRI, which we validated experimentally. In humans, plasma miR-122 levels were significantly lower in healthy LP compared to healthy NP individuals and even lower in LP patients with acute MI. The expression of miR-122 target Pkm2 was increased in LP IRI compared to NP IRI, and decreased in LP IRI that received ITLD at the onset of reperfusion compared to LP

IRI. Administration of ITLD *in vitro* in female H9c2 cells increased the expression of miR-122, decreased Pkm2 expression, and reduced apoptosis and oxidative stress levels. Our data also suggests that the cardioprotective role of ITLD is mainly mediated by miR-122. Overexpression of miR-122 *in vitro* in female H9c2 cells subjected to hypoxia/reoxygenation decreased Pkm2 levels and promoted apoptosis and oxidative stress markers. A single dose of miR-122 mimic in LP rats at the onset of reperfusion significantly reduced infarct size, apoptosis, and oxidative stress, and was associated with upregulation of Pkm2.

Conclusions

We show the protective effects of ITLD, which is mainly mediated through miR-122 by decreasing cardiomyocyte apoptosis and oxidative stress via its target Pkm2 in the context of late pregnancy. MiR-122 could be a novel cardio-protective strategy or biomarker in late pregnancy.

Introduction

The relative risk of myocardial infarction (MI) during pregnancy has been shown to be approximately 3 to 4 fold higher than the estimated age-specific rates of MI in the reproductive age group (1). MI has been reported to occur more frequently and carry a worse prognosis during the third trimester of pregnancy for unclear reasons (2). Furthermore, detection of cardiac events such as MI in pregnancy is sometimes achieved only after clinical signs are already apparent, necessitating the urgency of understanding the molecular underpinnings of the disease. There is a paucity of experimental data on the mechanisms responsible for cardiac events and the potential therapies to limit IRI in the setting of late pregnancy. Micro*RNAs* (miRs) are small, highly conserved non-coding RNAs involved in the regulation of gene expression through translational repression or degradation of target mRNAs (3). Many studies have shown the involvement of miRs in the heart in the context of IRI (4–6). However, the impact of miR on pregnancy-related cardiovascular conditions lacks sufficient research (6).

Intralipid (ITLD) has been in clinical use for almost 4 decades and is known to cross the placental barrier and does not have any harmful effects for the mother or the fetus. Our lab discovered that administration of one bolus of ITLD at reperfusion reduces the infarct size by ~60% in LP rat subjected to ischemia reperfusion injury (IRI). However, the mechanisms responsible for the protective role of ITLD remain to be elucidated.

In summary, to unravel the cardioprotective effects of ITLD, we employed a combination of bioinformatics and experimental methods in rat models, we discovered microRNA-122-5p (miR-122) that reduces cardiomyocytes apoptosis and mitochondrial oxidative stress

via its target Pkm2 in LP in IRI. Moreover, in a pre-clinical *in vivo* LP IRI rat model, we show the therapeutic potential of miR-122 overexpression at the onset of reperfusion.

Materials and Methods

Animals

Non-pregnant (NP) and timed late-pregnant (LP) female rats (Sprague-Dawley, 2-3 months old, day 20-21 of pregnancy) were purchased from The Jackson Laboratory. The institutional Animal Research Committee approved all animal procedures (ARC-2010-045) and are according to current NIH guidelines.

Left anterior descending artery occlusion and measurement of infarct size

Rats were administered ketamine (80 mg/kg i.p.) and xylazine (8 mg/kg i.p.) for anesthesia. The rats were intubated and ventilated using the (VENTELITE Catalogue 557040, Harvard Apparatus). A left thoracotomy was performed in the fourth intercostal space to expose the hearts. The pericardium was incised, and a 6.0 Prolene suture was used to constrict the proximal left anterior descending (LAD) coronary artery. The hearts underwent 45 minutes of ischemia, following by 3 hours of reperfusion. In the rescue experiments, we extended the reperfusion period to 24 hours to allow ample time for both the alteration of miR-122 expression and the delivery of miR-122 mimic to cardiomyocytes, resulting in the modification of its target gene expression. At the onset of reperfusion, rats either received 10nM of miR-122 mimic (ThermoFisher, #4464066) or scramble mimic (ThermoFisher, #4464061) intravenously through the femoral vein. At the conclusion of the experiment, the LAD was retied and a 3.0mL intravenous injection of 2% Evans blue dye (Sigma, #314-13-6) was administered through the femoral vein. The

ventricles of the hearts were sliced and incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C for 15 min to identify the non-infarcted and infarcted areas. The myocardial ischemic area at risk (AAR), infarct size (IS), and total left ventricle (LV) were identified and quantifications were done on Adobe Photoshop. In an alternative group of rats utilized for RNA and Western Blot analysis, Evans blue dye was not administered at the end of IRI. Western blot analysis, Evans blue dye.

MicroRNA-microarray analysis

The non-Affymetrix single channel arrays (MiRBase 17.0 MicroRNA Array, Ocean Ridge Biosciences) was utilized to perform a microRNA-microarray screen in LV tissue of Sprague-Dawley rats from the following five groups: NP sham, LP sham, NP IRI, LP IRI, and LP IRI who were administered ITLD at the onset of reperfusion (n=5 per group). MicroRNA-microarray expression ratios were calculated as the power-2 exponential of the log2 differences. MicroRNA expression changes were considered significant if they met the acceptance criteria of a minimum 1.5-fold change and a one-way Analysis of Variance (ANOVA) t-test p-value of <0.05. Data analysis and hierarchical clustering were performed using XLSTAT (www.xlstat.com).

Patient Characteristics and Human Plasma Sampling and RNA Isolation

Plasma samples from three groups of human subjects were collected: 1) healthy NP, 2) healthy LP, and 3) LP who had an acute MI during pregnancy. Troponin levels was obtained from the patients with acute MI. Approval was obtained from the Institutional Review Board at UCLA. Consent was taken from all patients to obtain their blood samples.

The collection of human plasma samples (200μL) for miRNA detection was conducted using ETA-K2 tubes and processed within a 4-hour timeframe from collection. After centrifugation, the samples were subjected to plasma collection, which was then stored at -80°C. MiR were extracted with miRNeasy serum/plasma kit (Qiagen, #217184) according to the manufacturer's protocol. MiR-39 mimic was used as Spike-In control (Qiagen, # 432421090).

H9c2 cell culture, Hypoxia/Reoxygenation, and ITLD stimulation

H9c2 cells were confirmed to be a pure population of female cells. To induce hypoxia in female H9c2 cells, the cell medium was replaced with low-nutrient DMEM (Life Technologies, Catalog #11966-025) without growth factors and the cells were incubated in a hypoxia chamber (UCLA Storehouse, 94% N2, 5% CO2 at 37°C, 1% O2) for 3 hours. Afterward, the cells were reoxygenated by returning the medium to regular growth medium and placing the cells in normoxic gas conditions (95% O2 and 5% CO2) at 37°C for a duration of 6 hours. The control cells were maintained under normoxic conditions at 37°C. To stimulate H9c2 cells with ITLD, various time-points of ITLD stimulation were tested. The optimal time-point was selected as 45 minutes. Cells were treated with ITLD (1%) or PBS for 45 minutes.

H9c2 Cell Transfection

H9c2 cells were transfected with miR-122 mimic, miR-122 inhibitor and corresponding scramble controls (ThermoFisher Scientific miR-122 mimic: 4464066, miR-122 inhibitor: #4464084, Assay ID: MH11012, scramble mimic: #4464058, scramble inhibitor: #4464076) using Lipofectamine RNAiMAX (ThermoFisher, #13778075) in OptiMEM

medium. Various doses of mimic and antagomir (20-100nM) were tested to determine the optimal concentration of mimic and inhibitor.

The transfection was carried out at a final concentration of 40nM for 6 hours. Following transfection, the medium was replaced with DMEM-F12 supplemented with 0.5% FCS and 1% antibiotic/antimyocotic. All experiments were conducted 24 hours after transfection.

Immunofluorescence on Fixed H9c2 Cells

H9c2 cells were cultured on coverslips until they reached 80% confluence. Subsequently, they were fixed with 4% paraformaldehyde (ThermoFisher) for 15 minutes, permeabilized using 10% Triton (Sigma, #9036-19-5), and blocked with either 5% normal donkey or 5% normal goat serum for 30 minutes (Jackson ImmunoResearch #017-000-121 and 005-000-121). The primary antibody for cleaved caspase 3 (CC3) from Cell Signaling Technology (#9661, 1:500) was then incubated overnight at 4°C. Following this, a secondary Alexa Fluor antibody (1:1000, Invitrogen #A21245) against CC3 was applied for 1 hour at room temperature, and sections were mounted with Fluoromount G containing DAPI (ThermoFisher, #00-4959-52). Confocal microscopy (Nikon) was utilized for image acquisition. The quantification of cells positive for CC3, relative to the total cell number, was performed in 5 randomly chosen fields per coverslip.

RNA isolation, reverse transcription, and quantitative polymerase chain reaction (RT-qPCR)

RNA was isolated from snap-frozen rat myocardium and H9c2 cells by employing Trizol Reagent (Invitrogen, ThermoFisher Scientific) in accordance with the manufacturer's

instructions. The quantified RNA served as the template for cDNA synthesis, utilizing the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, #4368814). Subsequent qPCR was conducted using PowerUp SYBR Green Master Mix (ThermoFisher, #A25779) on a BioRad CFX Connect PCR detection system. RPLP0 was employed as a reference gene, and the primer details can be found in Supplemental Table II. For miRNA RT-qPCR, the Taqman RT Kit (ThermoFisher, #4366596), Taqman assays (ThermoFisher, #4427975, Assay ID: 001973 and 000577), and Taqman Universal Master Mix (ThermoFisher, #4440040) were utilized. U6 was used as the reference gene. The relative expression levels of genes were determined using the 2−ΔΔCt method.

Protein extraction, SDS-PAGE, and Western Blot assay

Protein extraction was carried out with RIPA lysis buffer (Sigma #R0278), comprising 50mM NaCl, 50mM Tris pH 8, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS, supplemented with protease inhibitor (cOmplete Mini, 11836153001) and phosphatase inhibitor (Roche, 04906845001). The protein concentration was determined using a Bradford assay (Sigma, #B6916), and 20µg of protein was loaded per well. Proteins were diluted in 4x sample buffer (BioRad #161-0747) and subjected to separation on 10% gels via SDS-PAGE. Subsequently, proteins were transferred to nitrocellulose membranes (BioRad #170-4270) using semi-dry blotting (TransBlot Turbo System, BioRad).

Following transfer, membranes were blocked with 5% bovine serum albumin (Sigma #A9647). Incubation with antibodies targeting Pkm2 (1:500, Cell Signaling #4053S) and GAPDH (1:10.000, Cell Signaling #2118) followed. Detection was achieved using IRDye-conjugated secondary antibodies (1:10.000, LI-COR #32210, #68070), and blots were

scanned with the LI-COR Odyssey. Band intensities were quantified using Image Studio Lite Version 5.2.

Mitochondrial Reactive Oxygen Species Measurements

The MitoSox Red mitochondrial superoxide indicator (Molecular Probes, #M36008) was employed, and the assay was conducted in accordance with the manufacturer's guidelines. Briefly, H9c2 cells were seeded and exposed to hypoxia/reoxygenation (H/R). Following this, the cells were rinsed with PBS and exposed to unsupplemented media containing 5μM MitoSox Red for 45 minutes. Subsequently, the cells underwent three PBS washes, were centrifuged (5 minutes at 500 ×g), and then suspended in 300μL of PBS in plastic culture tubes (ThermoFisher, #14–961-10A). The levels of mitochondrial superoxide were assessed in live cells using flow cytometry, with absorption/emission maxima of approximately 510/580 nm.

Statistical Analysis

The normality of the data distribution was assessed using the Kolmogorov-Smirnov normality test. Significant outliers were tested with Grubb's test. Data are represented as mean±SEM and analyzed using either Student's *t*-test (for 2 groups) or one-way ANOVA with Holm-Bonferroni post-hoc correction (for more than 2 groups). All experiments were repeated at least three times. Statistical significance was defined as a p-value less than 0.05. Graphpad Prism version 9 software (GraphPad Software, Inc., San Diego, CA, USA) was utilized for all data analysis.

Results

MicroRNA-microarray analysis reveals miR-122 is upregulated in LP IRI that received ITLD at the onset of reperfusion compared to LP IRI

Our lab has previously demonstrated that cardiac vulnerability to IRI drastically increases in late pregnancy and administration of intralipid immediately prior to reperfusion protects the LP heart against IRI by reducing infarct size [6,8]. However, the underlying molecular mechanisms involved in the cardioprotective role of ITLD is unknown. In order to unravel the cardioprotective effects of ITLD, we employed microRNA-microarray profiling on five groups of rat LV: NP sham, LP sham, NP IRI (45 minutes ischemia, 3 hours reperfusion), LP IRI, and LP IRI that were administered 20% ITLD at the onset of reperfusion **(Fig 1A)**. MicroRNA profiling analysis revealed various microRNAs to be differentially expressed in the LP rat LV subjected to IRI with the administration of ITLD **(Fig 1B)**. Of interest was miR-122, as its expression was not only downregulated in LP IRI compared to NP IRI (~4 fold), but was drastically upregulated in LP IRI that were administered ITLD at the onset of reperfusion compared to LP IRI (~50 fold). We validated by qPCR the significant downregulation of miR-122 in LP IRI compared to NP IRI, and a significant upregulation in LP IRI that were administered a bolus of ITLD at the onset of reperfusion compared to LP IRI **(Fig 1C).**

Increased expression of miR-122 in LP IRI upon administration of ITLD is associated with decreased expression of its target Pkm2, as well as decreased apoptosis and oxidative stress markers

TargetScan identified pyruvate kinase isozyme M2 (Pkm2) as a potential target of miR-122. Our qPCR data validated significant upregulation of Pkm2 in LP IRI compared to NP IRI and a significant downregulation in LP IRI that were administered ITLD at the onset of reperfusion compared to LP IRI **(Fig. 1D)**. This data suggests that Pkm2 is indeed a target of miR-122.

Concomitantly with enhanced miR-122 and downregulated Pkm2 in LP IRI upon ITLD administration, our data revealed decreased expression of pro-apoptotic marker Bax **(Fig. 2A)** and increased expression of anti-apoptotic marker Bcl-2 **(Fig 2B)** by qPCR. Expression of reactive oxygen species (ROS) scavengers Sod2 and Aldh2 were increased in LP rat myocardium subjected to IRI that were administered ITLD **(Fig. 2C,D)**.

Together, these data show increased expression of miR-122 and decreased expression of its target Pkm2 in LP rat myocardium subjected to IRI that were administered ITLD compared to LP rat myocardium subjected to IRI. Furthermore, increased expression of miR-122 in LP IRI that were administered ITLD was associated with decreased levels of apoptosis and ROS compared to LP IRI.

miR-122 expression is decreased in the plasma of healthy LP individuals and decreased further in LP patients with acute MI

To assess the clinical significance of our results, we assessed miR-122 expression in the plasma of healthy NP individuals, healthy LP individuals, and in LP patients who had an acute MI during pregnancy. Our data revealed that plasma miR-122 expression is significantly decreased in healthy LP individuals compared to healthy NP individuals. Strikingly, plasma miR-122 was decreased even further in LP patients suffering from
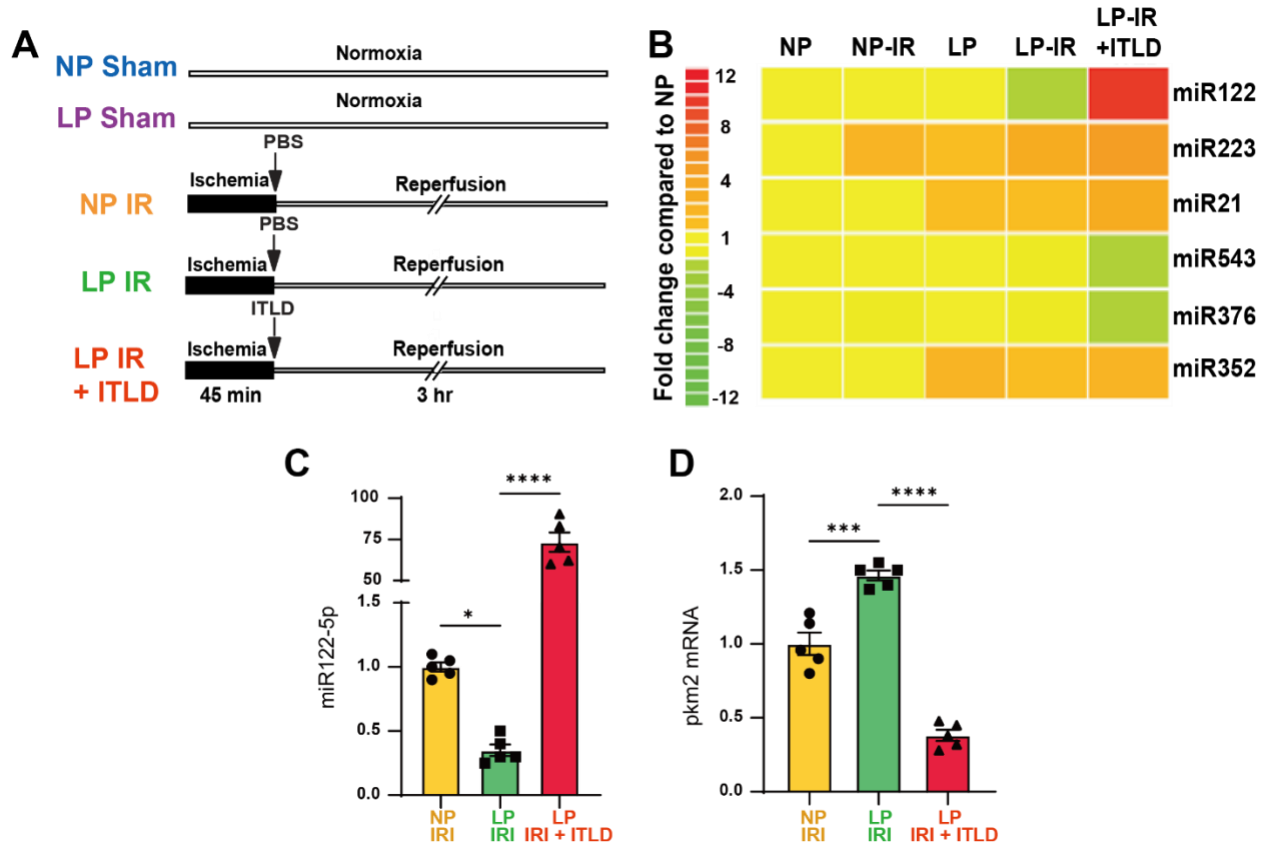
Figure 1. We identified miR-122 to be upregulated in LP rat myocardium subjected to IRI which received one bolus of ITLD at the onset of reperfusion. A. Experimental protocol. **B.** Heatmap of microRNA-microarray analysis. **C-D.** Validation of miR-122 and Pkm2 transcript expression by qPCR in LP rat myocardium (normalized to NP IRI). Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, ***p<0.001, ****p<0.0001. NP: non-pregnant. IRI: ischemia-reperfusion injury.
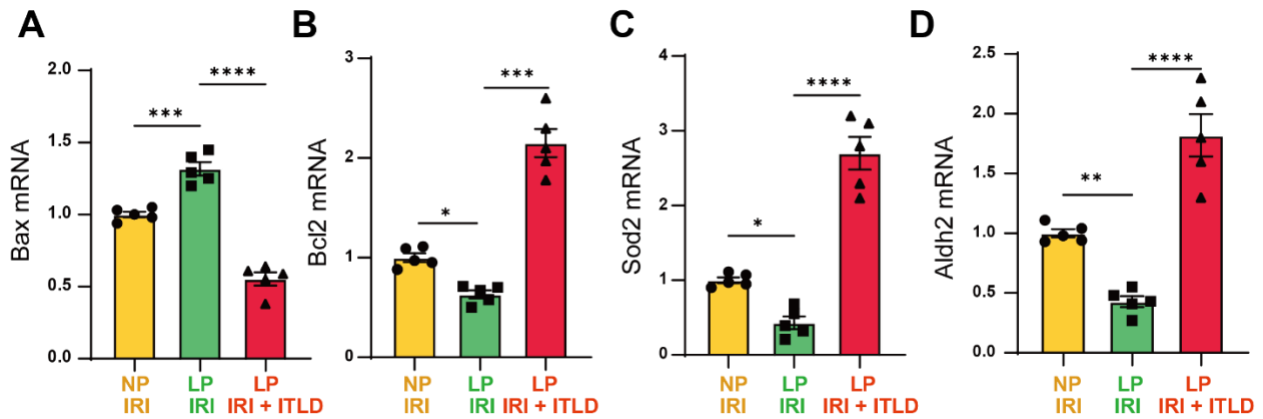
Figure 2. Decreased apoptosis and ROS in LP rat myocardium subjected to IRI which received one bolus of ITLD at the onset of reperfusion. A,B. Expression of pro-apoptotic marker Bax and anti-apoptotic marker Bcl2 as assessed by qPCR (normalized to NP IRI). **C,D.** Expression of ROS scavengers Sod2 and Aldh2 (normalized to NP IRI). Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ROS: reactive oxygen species. NP: non-pregnant. IRI: ischemia-reperfusion injury.

acute MI **(Fig. 3A)**. Consistent with our data, we observed a negative correlation between miR-122 plasma expression and troponin in acute MI patients (p-value: 0.015, Pearson's r: 0.807, n=6) **(Fig. 3B).** This data suggests miR-122 in blood samples of LP women may potentially serve as a biomarker for cardiovascular complications during LP, as lower levels of miR-122 indicate heightened risk of acute MI.

ITLD stimulation is sufficient to enhance miR-122 expression, decrease Pkm2 expression, and decrease apoptosis and oxidative stress markers

Female H9c2 cells were utilized for the *in vitro* experiments. We treated H9c2 cells with ITLD for 45 minutes **(Fig. 4A)**. Our data revealed the expression of miR-122 was increased in female H9c2 cells treated with ITLD compared to PBS **(Fig. 4B)**. In line with increased miR-122 expression, expression of its target Pkm2 was significantly lower in H9c2 cells treated with ITLD compared to PBS **(Fig. 4C)**. Furthermore, upon ITLD treatment, we observed lower levels of apoptosis **(Fig. 4D,E)** and ROS **(Fig. 4F,G).** This data suggests that ITLD treatment is sufficient to upregulate miR-122 and exert its cardioprotective effects.

miR-122 overexpression in the absence of intralipid is sufficient to decrease apoptosis and ROS in H9c2 cells in the setting of H/R

As ITLD treatment was sufficient to increase miR-122 expression and exert cardioprotective effects, we next assessed whether miR-122 overexpression in the presence of H/R would exert similar effects. We transfected H9c2 cells with either scramble overexpression or miR-122 overexpression for 24 hours, followed by 3 hours hypoxia and 6 hours reoxygenation **(Fig. 5A).** miR-122 expression was increased upon
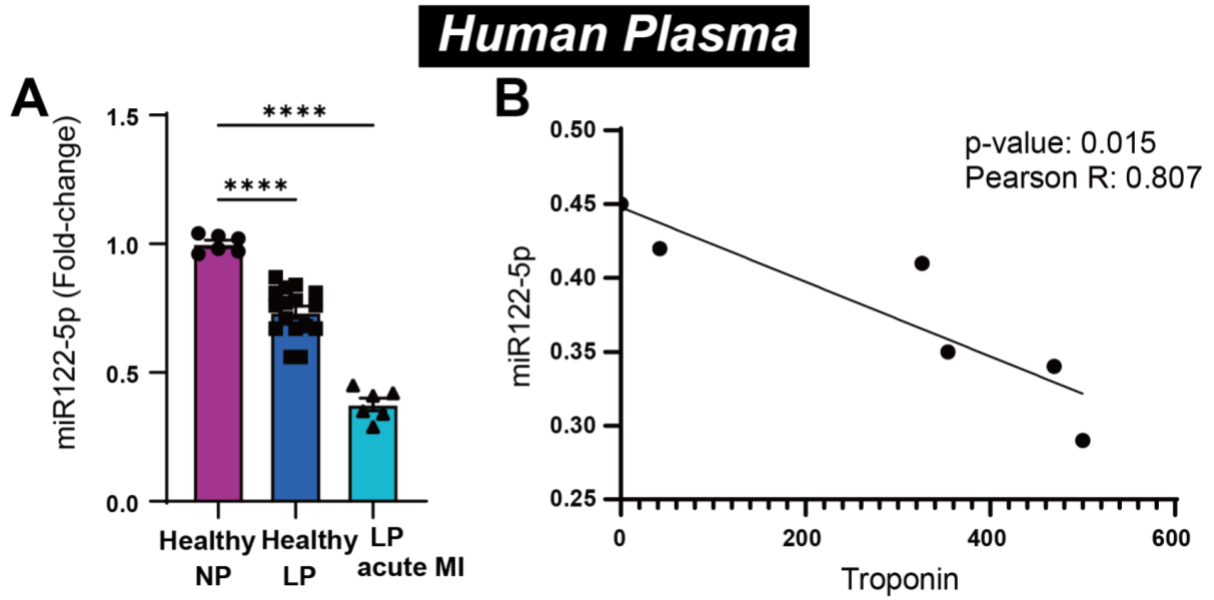
Figure 3. miR-122 could serve as a biomarker. A. Plasma levels of miR-122 in healthy NP individuals, healthy LP individuals, and LP patients with acute MI (normalized to healthy NP). **B.** Correlation of miR-122 and troponin. Data presented as mean+SEM. ANOVA with Holm-Bonferroni post-hoc correction, ****$p < 0.0001$.
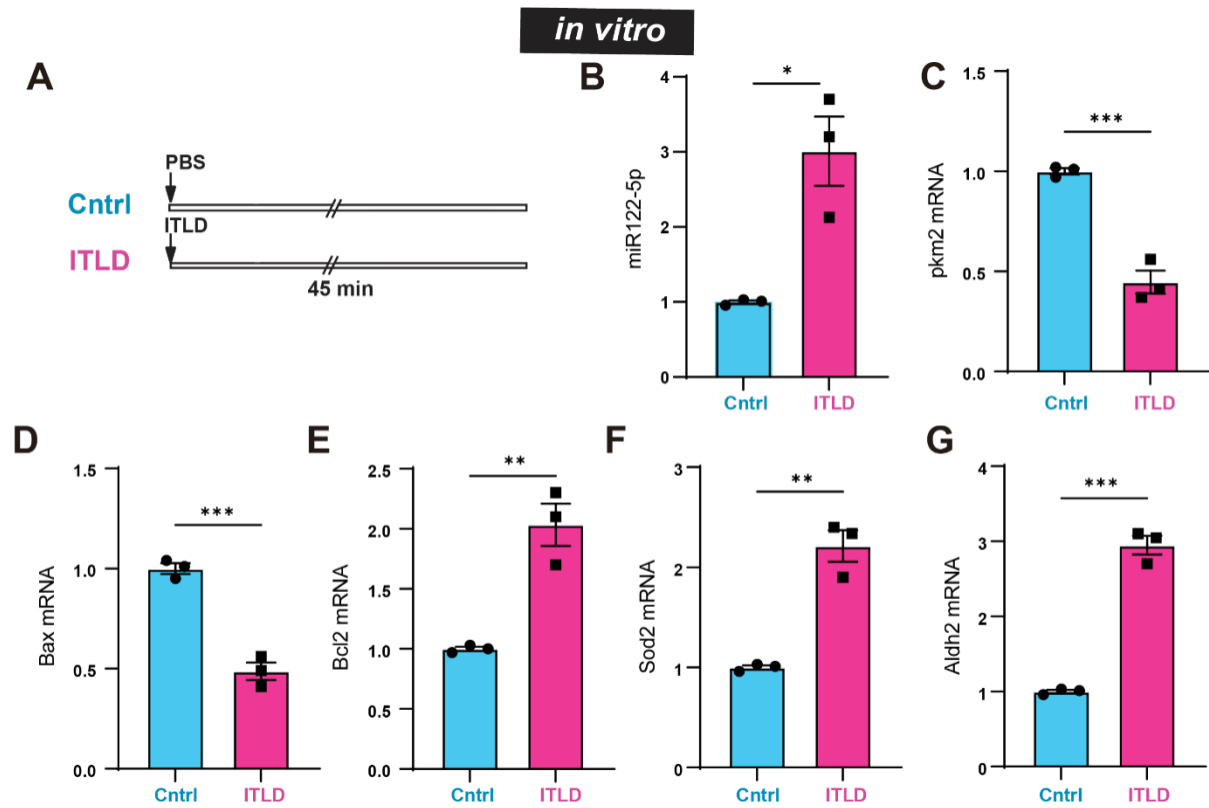
Figure 4. ITLD stimulation is sufficient to enhance miR-122 expression, decrease Pkm2 expression, and decrease apoptosis and oxidative stress markers. A. Experimental protocol. **B,C.** miR-122 and Pkm2 transcript expression via qPCR. **D,E.** Expression of pro-apoptotic marker Bax and anti-apoptotic marker Bcl2. **F,G.** Expression of mitochondrial superoxide levels as depicted by ROS scavengers. Data presented as mean+SEM. Student's t-test, *p<0.05, **p<0.01, ***p<0.001. ROS: reactive oxygen species.

miR-122 overexpression, suggesting the success of the experiment **(Fig. 5B).** In line with increased miR-122 expression, the expression of Pkm2 transcript **(Fig. 5C)** and protein **(Fig. 5D,E)** were decreased.

Our data also revealed decreased levels of pro-apoptotic marker Bax **(Fig. 6A)** and increased anti-apoptotic marker Bcl2 **(Fig. 6B)** upon miR-122 overexpression compared to scramble overexpression. Furthermore, we observed lower levels of ROS, as indicated by high levels of ROS scavengers Sod2 and Aldh2 in the group treated with miR-122 overexpression **(Fig. 6C,D).** Lastly, we observed lower levels of cleaved caspase 3 (CC3), a marker of apoptosis, in the miR-122 overexpression group **(Fig. 6E,F).** Taken together, this data suggests that miR-122 overexpression in the presence of H/R exerts similar cardioprotective effects as ITLD stimulation.

Cardioprotective action of ITLD is abolished when miR-122 is knocked Down

To assess whether the ITLD-mediated protection is mainly mediated through miR-122, we transfected H9C2 cells with either scramble inhibitor or miR-122 inhibitor for 24 hours, followed by treatment with either PBS or ITLD for 45 minutes **(Fig. 7A).** Our data confirmed successful miR-122 knockdown, since miR-122 expression was significantly decreased **(Fig. 7B).** Furthermore, upon miR-122 knockdown in the presence of ITLD, ITLD could not increase miR-122 expression **(Fig. 7B),** suggesting the opposing effects of ITLD and miR-122 knockdown. The expression of miR-122 target Pkm2 was the opposite as miR-122 in all conditions **(Fig. 7C).** Lastly, in the presence of miR-122 inhibitor, ITLD no longer had its protective effects against apoptosis and ROS **(Fig. 7D-G).** This data suggests miR-122 is, at least in part, mediating the protective effects of ITLD.
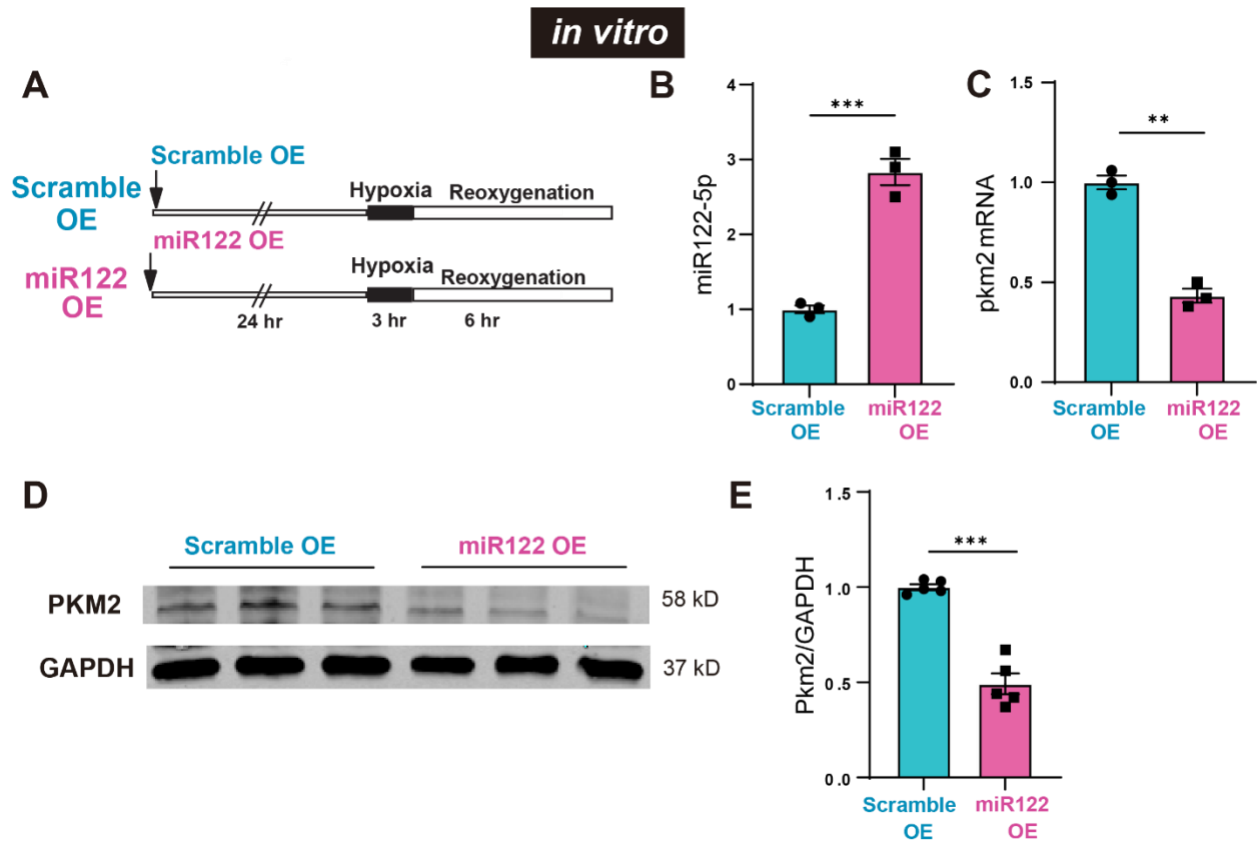
Figure 5. A. miR-122 overexpression decreases pkm2 transcript and protein levels H9c2 cells in the setting of H/R. A. Experimental protocol. **B,C.** miR-122 and Pkm2 transcript expression via qPCR. **D,E.** Western blot depicting Pkm2 protein expression and quantification. Data presented as mean+SEM. Student's t-test, **p<0.01, ***p<0.001.
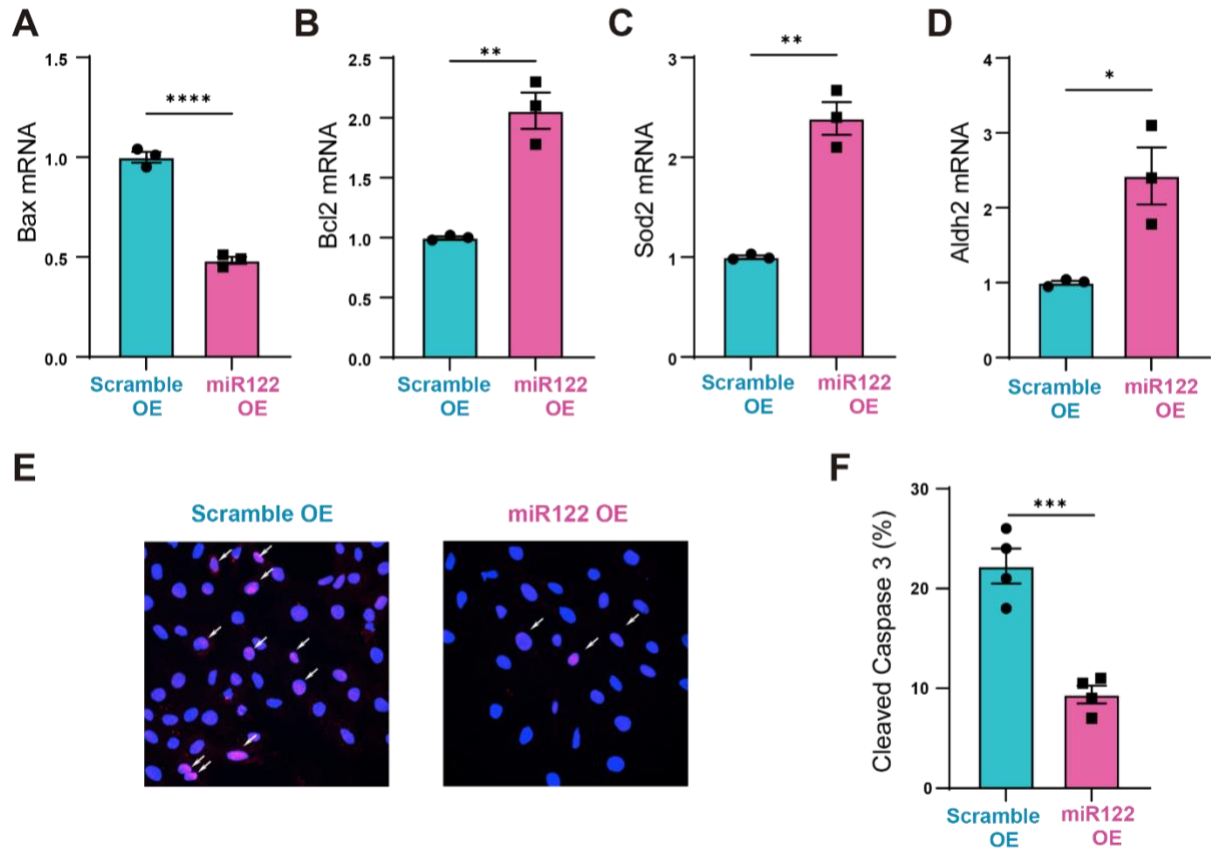
Figure 6. miR-122 overexpression decreases apoptosis and ROS in H9c2 cells in the setting of H/R. A,B. Expression of pro-apoptotic marker Bax and anti-apoptotic marker Bcl2. **C,D.** Expression of mitochondrial superoxide levels as depicted by ROS scavengers. **E,F.** Representative images depicting apoptosis levels as assessed by CC3 immunofluorescence staining and quantification. Data presented as mean+SEM. Student's t-test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. ROS: reactive oxygen species. CC3: cleaved caspase 3.

Figure 7. Knockdown of miR-122 inhibits ITLD protection. A. Experimental protocol. **B,C.** miR-122 and Pkm2 transcript expression via qPCR. **D,E.** Expression of pro-apoptotic marker Bax and anti-apoptotic marker Bcl2. **F,G.** Expression of mitochondrial superoxide levels as depicted by ROS scavengers. All data normalized to scramble KD + cntrl. Data presented as mean+SEM. Student's t-test, $p < 0.05$, $p < 0.01$, $p < 0.001$, $p < 0.0001$. ROS: reactive oxygen species. KD: knockdown. Cntrl: control.

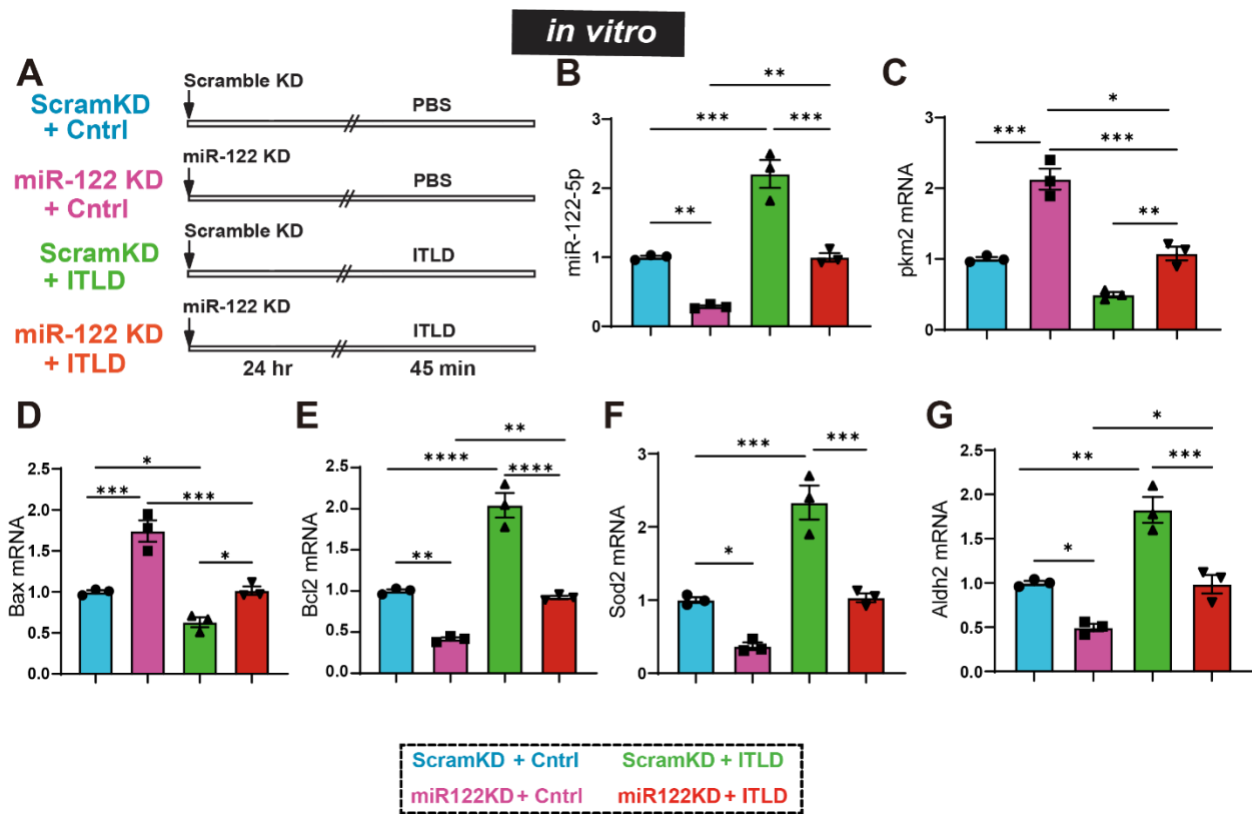***miR-122 overexpression at the onset of reperfusion significantly reduces myocardial infarct size in LP rats by targeting Pkm2***

Our *in vitro* data suggests that miR-122 overexpression in the absence of intralipid protects against H/R injury and the cardioprotective action of ITLD is mainly mediated through miR122. Thus, we examined the therapeutic role of miR-122 overexpression in LP upon IRI *in vivo*. LP rats were subjected to 45 minutes of ischemia, followed by 24 hours of reperfusion. At the onset of reperfusion, 10nM miR-122 overexpression or a scrambled control were injected via the femoral vein **(Fig. 8A)**. Our data revealed successful overexpression (4-fold) of miR-122 expression in the heart **(Fig. 8B)**. Despite the fact that both groups were subjected to a comparable degree of ischemic risk as there was no significant different in area at risk between the two groups **(Fig. 8C, D)**, the infarct size was significantly smaller in the LP rats treated with miR-122 overexpression compared to scramble control **(Fig. 8C,E).**

Upregulation of miR122 expression leads to downregulation of its target Pkm2 transcript **(Fig. 8F)** and protein **(Fig 8G,H)** expression. We also examined the impact of miR-122 overexpression on markers of apoptosis and ROS. Our data revealed decreased levels of pro-apoptotic marker Bax **(Fig. 9A)** and increased levels of anti-apoptotic marker Bcl2 **(Fig. 9B)** upon miR-122 overexpression compared to scramble control. We also observed lower levels of ROS as indicated by higher ROS scavengers Sod2 and Aldh2 in the group treated with miR-122 overexpression **(Fig. 9C,D).**

Taken together, our data show that a single dose of miR-122 overexpression at the onset of reperfusion reduces myocardial infarct size, apoptosis, and ROS production, and is also associated with a decrease in expression of its target Pkm2 in rat LVs *in vivo*.
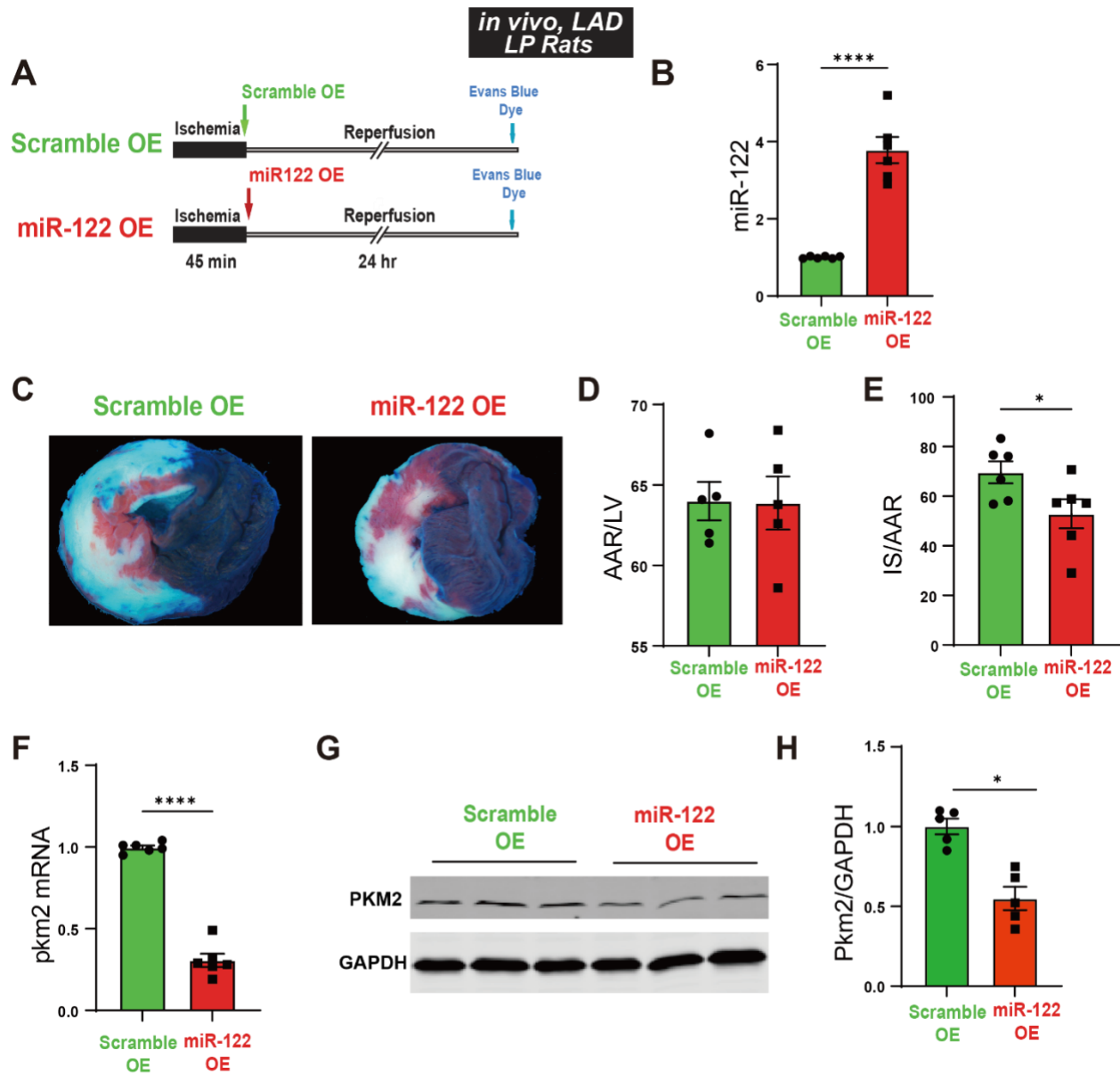
Figure 8. A. miR-122 overexpression at the onset of reperfusion reduces myocardial infarct size in LP rats by targeting Pkm2. A. Protocol of IRI model. Arrows indicate femoral vein injections of 10nM scramble overexpression or miR-122 overexpression. **B.** LV miR-122 expression assessed by qPCR. **C.** Heart cross sections using TTC staining. **D.** Area at risk divided by LV. **E.** Infarct size divided by area at risk. **F.** Pkm2 transcript as measured by qPCR. **G,H.** representative western blots and quantifications. Data presented as mean+SEM. Student's t-test. *p<0.05, ****p<0.0001. LV: left ventricle.
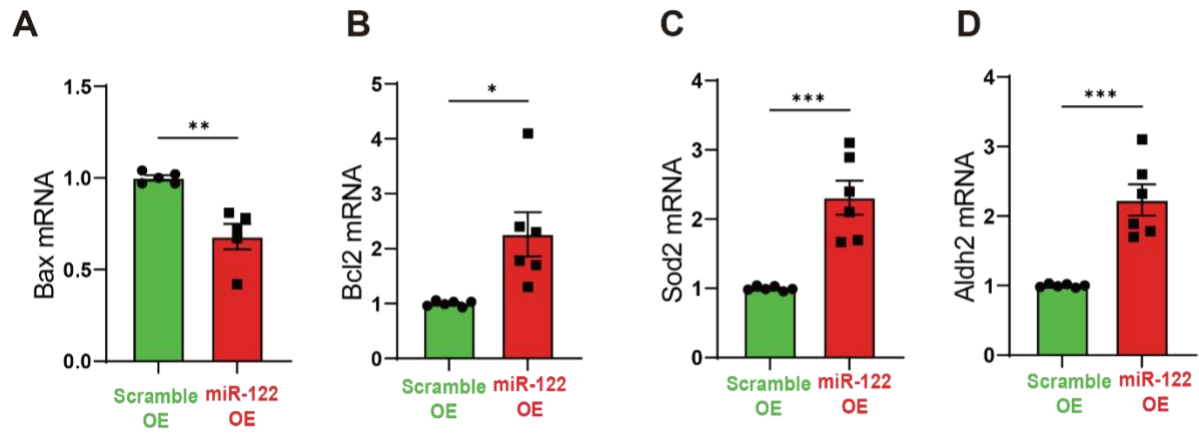
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Figure 9. A. miR-122 overexpression at the onset of reperfusion decreases myocardial apoptosis and oxidative stress. A,B. Expression of pro-apoptotic marker Bax, and anti-apoptotic marker Bcl-2 as assessed by qPCR. **C,D.** Expression of ROS scavenger Aldh2 as assessed by qPCR. Data presented as mean+SEM. Student's t-test. *p<0.05, **p<0.01, ***p<0.001.

Discussion

Previously, our group has shown that ITLD administration at the onset of myocardial reperfusion reduces infarct size in LP rats by inhibiting mPTP opening via Cav2/STAT3/GSK-3β pathway (8). In this dissertation, we identified a novel mechanism for ITLD-induced cardioprotection from myocardial IRI during LP. We demonstrate that the cardioprotective effects of ITLD in IRI of LP rats by suppressing cardiomyocyte apoptosis and ROS production is mainly mediated through miR-122. Previous reports have shown parenteral administration of lipid emulsions to be effective in reducing IRI in numerous organs, including intestine (9), brain (10), and heart (11). As use of ITLD in the treatment of local anesthetic cardiotoxicity has been adopted in standard clinical practice (12), much of the research has focused on the protective properties of ITLD in cardiovascular diseases, particularly in myocardial IRI. Several studies using rabbit, porcine, and rat models for myocardial IRI have shown that ITLD administration at the onset of reperfusion may improve post-ischemic cardiac function and myocardial contractility (13,14). However, the underlying mechanisms for ITLD-induced cardioprotection, particularly in pregnancy, are not fully established. Our results suggest that the upregulation of miR-122 may be a crucial mechanism for mediating the protective effects of ITLD in IRI during LP.

Our findings revealed a significant association between miR-122 overexpression and reduced cardiomyocyte apoptosis and oxidative stress in both our *in vivo* rat model of IRI in pregnancy and *in vitro* model of H/R, respectively. To our knowledge, this is the first study exploring the role of miR-122 in IRI during pregnancy. While research on the role of miR-122 in the heart remains scarce, its function in the liver has been investigated

more extensively. This is due to the great degree of tissue specificity, as miR-122 is known to be a highly liver-enriched miRNA, accounting for more than 70% of all miRNAs expressed in the liver (15). Increasing evidence indicates that miR-122 plays an important role not only in the regulation of liver functions integral to cholesterol and lipid metabolism, but also in the pathogenesis of various liver diseases ranging from steatohepatitis and fibrosis to hepatocellular carcinoma (HCC).[10–12] Particularly, the role of miR-122 as a tumor suppressor in HCC has been well-established (16,17) with miR-122 overexpression-induced apoptosis possibly being one of the underlying mechanisms (18).

Although the biological functions of miR-122 in the heart remain largely unidentified, emerging evidence points to miR-122 playing an important role in the patho-mechanism of numerous cardiovascular diseases (19). Recent studies suggest that miR-122 may be involved in the progression of cardiovascular fibrosis with miR-122 overexpression being associated with increased inflammation, oxidative stress, and apoptosis in addition to fibrosis (20). Similarly, two previous studies on the role of miR-122 in myocardial IRI have found that the downregulation of miR-122 may have cardioprotective effects while, contrary to the results of our study, miR-122 overexpression was associated with increased cardiomyocyte apoptosis (21,22). However, in the former study, the myocardial IRI model was solely performed *in vitro* on H9c2 rat cardiomyocytes (21). Although the second study validated its results *in vivo* by demonstrating that the inhibition of miR-122 led to reduced infarct size in an experimental rat model, male rats were employed and the LAD was permanently ligated consistent with a permanent ischemia model as opposed to a IRI model (22).

We found that circulating miR-122 in blood samples of LP women may potentially serve as a biomarker for cardiovascular complications during LP, as plasma levels of miR-122 were decreased in LP women with acute MI compared to healthy LP women. Ever since circulating miRNAs were first detected in human blood, much research has been focused on miRNAs serving as potential biomarkers for various diseases (23–25) particularly since miRNAs remain very stable in plasma and are therefore easily quantified (23). Research on the potential function of miR-122 as a cardiovascular biomarker remains sparse with the evidence thus far being inconsistent. While some clinical studies report an association between elevated circulating miR-122 levels and incident MI (26,27) others show an association between decreased expression of circulating miR-122 and MI (28). However, the two former studies did not specify whether blood samples were obtained before or after coronary revascularization, while the latter study reporting decreased circulating miR-122 levels after MI stated that the majority of blood samples were obtained after coronary revascularization via percutaneous coronary intervention (PCI). No studies on circulating miR-122 levels were conducted on pregnant women, although one study revealed significantly decreased circulating miR-122 levels in women with premature acute coronary syndrome and a history of preeclampsia during pregnancy (29).

Our results suggest that decreased Pkm2 expression by miR-122 has significant cardioprotective effects in myocardial IRI in LP rats. Previously, Pkm2 has been reported as a target of miR-122 mostly in neoplastic diseases such as hepatocellular carcinoma, gallbladder carcinoma, and colorectal cancer (30–32). Pkm2 is one of four isoforms of pyruvate kinase (PK) which catalyzes the final and irreversible reaction of glycolysis, the conversion of phosphoenolpyruvate into pyruvate (33). Out of all isoforms, only Pkm2 is

heavily implicated in playing an important role in cell proliferation and viability, as high expression of Pkm2 has been observed not only in tumor cells, but also during tissue repair and embryogenesis (34,35). However, there is a marked shift in cardiac PK isoenzyme expression post embryonic development from Pkm2 in the fetal heart towards Pkm1 in the adult heart (35). Interestingly, recent data suggests that while Pkm2 expression in the adult heart is generally low, its expression increases in various cardiac pathologies such as heart transplantation and MI (36–38). While previous findings point towards Pkm2 playing an integral part in the pathogenesis of various cardiovascular diseases therefore presenting as a possible novel therapeutic target, the mechanisms remain largely unclear. Thus far, there is no consensus on whether the upregulation of Pkm2, as suggested by our results, or its downregulation may have cardioprotective effects (36). To our knowledge, the role of Pkm2 in pregnancy-related cardiovascular complications such as myocardial IRI has not been previously studied.

In support of our findings, a previous study in a male rat experimental model of myocardial IRI has demonstrated that the inhibition of Pkm2 leads to a reduction of STAT3 phosphorylation, myocardial apoptosis, and macrophage-mediated inflammation ultimately resulting in improved cardiac function (39). On the other hand, upregulated Pkm2 expression in male and female mice after MI resulted in improved cardiac function and survival, which in turn led to cardiomyocyte proliferation and a reduction of oxidative stress (37). This discrepancy with our data could be explained by both the protocol and the species used in this study, as it was conducted on mice and employed a permanent MI model. Additionally, it is worth noting that our model utilizes pregnancy, which is another distinguishing factor from this study.

In summary, our work provides new perspectives on the potential pre-clinical therapeutic strategies of miR122 overexpression for protecting myocardial IRI in LP. miR-122 may serve as a prognostic biomarker for the treatment of pregnant individuals who are at higher risk of acute MI. It could also offer understanding into the disease's pathophysiology, aid in pinpointing crucial molecular pathways implicated in the disease, and suggest targets for developing novel therapies. It has the potential to facilitate early detection and monitoring disease advancement, ultimately resulting in prompt intervention and improved disease management. This represents a foundational step toward creating new therapeutics for treating MI in LP.

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Chapter 4: Conclusions and Future Directions

Maternal mortality rates continue to be a significant concern, with pregnancy-related MI contributing to a substantial portion of maternal deaths. The risk of experiencing an MI during pregnancy is notably elevated, being three to four times higher compared to NP individuals of similar age within the reproductive age bracket (1–3). Our lab has previously demonstrated that cardiac vulnerability to myocardial IRI drastically increases in LP, and administration of ITLD immediately prior to reperfusion protects the LP heart against IRI by reducing infarct size (4,5). However, the exact underlying mechanisms were unknown. The work included in this dissertation offers compelling insights into the heightened susceptibility of the LP heart to IRI and offers novel perspectives on therapeutic interventions for protecting maternal cardiac function during LP, with a particular focus on the potential of microRNAs as biomarkers to aid in early detection and intervention strategies.

We employed a rat model of IRI and microRNA-microarray profiling to investigate the mechanism behind the heightened susceptibility of the heart in LP to IRI. We identified miR-98 which its expression was significantly higher in LP sham compared to NP sham, and even higher in LP IRI. Our data also demonstrate that miR98 promotes cardiomyocyte apoptosis, mitochondrial oxidative stress, and inflammation through its targets Stat3 and Pgc-1α. Similar to hearts, in rat plasma samples, miR-98 levels were significantly higher in LP sham compared to NP sham, and even higher in LP IRI. Supporting the clinical relevance of our experimental rat model, in human plasma samples, we also found miR-98 levels were significantly higher in healthy LP compared to healthy NP individuals. Interestingly, in LP patients who had an acute MI during

pregnancy, plasma miR98 expression was significantly higher than healthy LP and it was correlated with troponin levels **(Chapter 2).**

Using FISH on rat heart tissue sections, we confirmed miR-98 is expressed in cardiomyocytes in the heart. Next, we found that serum from LP rats is sufficient to upregulate miR-98 expression in female H9c2 cells, and to increase the vulnerability to H/R injury. As both estrogen and progesterone reach their peak levels during LP, we stimulated H9c2 cells with either estrogen or progesterone for 24 hours and found that estrogen, but not progesterone, is sufficient to upregulate miR-98 expression.

Next, we examined which genes downstream of Stat3 and Pgc-1α are dysregulated in the setting of LP. We performed RNA-Seq on cells that were incubated with LP serum followed by overexpression of Stat3, Pgc-1α, or both Stat3 and Pgc-1α. We identified five novel downstream genes, Ash1l, Tnip1, Idh1, Sod2, and Angp1 to be the common downstream genes of Stat3 and Pgc-1α which play a role against oxidative stress, cell apoptosis, and inflammation **(Chapter 2).**

More importantly, we found that *in vivo* inhibition of miR-98 in LP rats at the onset of reperfusion was sufficient to protect the heart against IRI in a preclinical rat model. Our data shows that a single dose of miR-98 inhibitor reduces MI size, inflammation, apoptosis, and ROS production, and is also associated with an increase in expression of its targets Stat3 and Pgc-1α and their downstream effector genes in rats *in vivo* **(Chapter 2).**

Taken together, using microRNA-microarray profiling and an experimental approach of myocardial IRI, we discovered that miR-98 expression is increased in hearts of LP rats

compared to hearts of NP rats, and this increase is exacerbated after IRI. In addition, our data demonstrates that upregulation of miR98 in IRI is only specific to LP, but not to NP, further confirming the role of estrogen. Importantly, we found that miR-98 expression is upregulated in the plasma of pregnant patients with acute MI, and there is a positive correlation between plasma miR-98 and troponin levels in pregnant patients. Estrogen stimulation was able to increase the expression of miR-98 in H9c2 cells. Our data confirmed Stat3 and Pgc-1α as targets of miR98 both *in vivo* and *in vitro*. We also discovered a subset of common downstream targets of Stat3 and Pgc-1α that play a protective role against myocardial apoptosis, inflammation, and ROS production, namely angpt1, sod2, idh1, ash1l, tnip1. Strikingly, a single dose of miR-98 inhibitor at the onset of reperfusion in LP rats can reduce myocardial infarct size, inflammation, apoptosis, and ROS production *in vivo*, concomitant with enhanced expression of Stat3 and Pgc-1α and their downstream targets. Thus, miR-98 could serve as a novel cardio-protective strategy or biomarker in LP **(Figure 1).**

We next studied the molecular mechanism underlying therapeutic role of ITLD in attenuating myocardial IRI in LP. Our lab has previously demonstrated that administration of ITLD immediately prior to reperfusion protects the LP heart against IRI by reducing infarct size (5). However, the underlying molecular mechanisms of ITLP-induced cardioprotection against IRI in LP are unknown. We utilized miR microarray analysis on the LV of 5 groups of rats NP and LP in sham and upon IRI as well as LP rats that were received ITLD at the onset of reperfusion. Our analysis revealed downregulation of miR-122 expression in LV of LP IRI compared to NP IRI, and a drastic upregulation of miR-122 in LP IRI that received ITLD at the onset of reperfusion compared to LP IRI, which
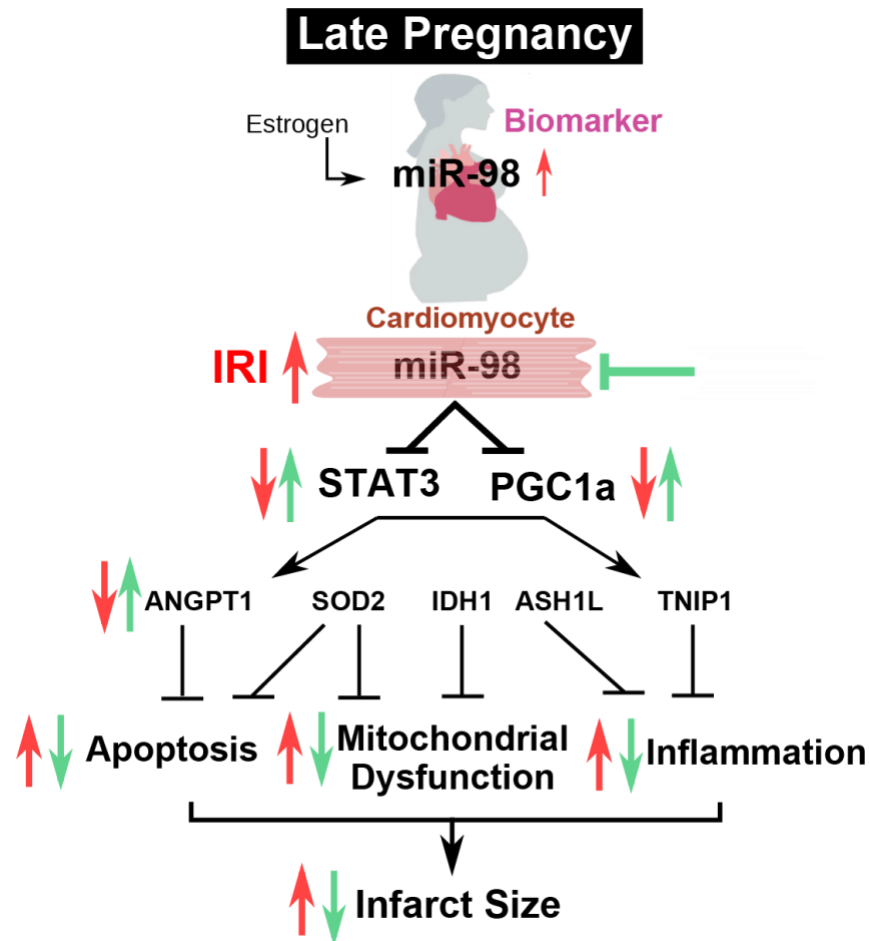
Figure 1. Schematic of the proposed mechanism. miR-98 expression is increased in healthy LP in the heart compared to NP, and IRI leads to further upregulation of miR-98 expression in LP compared to NP. Increased expression of miR-98 in the heart leads to decreased expression of its targets Stat3 and Pgc-1α, and their downstream effector genes, *angpt1, sod2, idh1, ash1l, tnip1*, resulting in increased apoptosis, mitochondrial dysfunction, and inflammation, and ultimately increased infarct size (red arrows). Inhibition of miR-98 protects the heart by upregulating Stat3 and Pgc-1α and their targets, leading to decreased apoptosis, mitochondrial dysfunction, and inflammation and ultimately, decreased infarct size (green arrows). MiR-98 may serve as a prognostic biomarker and/or potential therapeutic target for the treatment of pregnant individuals who are at higher risk of acute MI.

we validated experimentally. We also identified Pkm2 as the potential target of miR-122 and validated it experimentally since the expression of Pkm2 was increased in LP IRI compared to NP IRI, and decreased in LP IRI that received ITLD at the onset of reperfusion compared to LP IRI. Our data also showed that miR-122 reduces cardiomyocytes apoptosis and mitochondrial oxidative stress both *in vivo* and *in vitro* via its target Pkm2 in LP in IRI. In humans, plasma miR-122 levels were significantly lower in healthy LP compared to healthy NP individuals and even lower in LP patients with acute MI. We also found a negative correlation between plasma miR-122 and troponin levels in human patients. We excitingly also found that overexpression of miR-122 at the onset of reperfusion in a pre-clinical *in vivo* LP IRI rat model was sufficient to protect the heart against IRI by targeting Pkm2 **(Chapter 3).**

Taken together, using microRNA-microarray profiling and an experimental model of in-vivo and in-vitro IRI, we discovered that miR-122 expression is decreased in IRI in LP, which in turn upregulates its target PKM2, resulting in increased apoptosis and mitochondrial dysfunction, ultimately leading to a significantly larger infarct size. ITLD protects the heart by increasing miR-122 expression, which in turn downregulates its target PKM2, resulting in decreased apoptosis and mitochondrial dysfunction, ultimately leading to a significantly smaller infarct size. Strikingly, a single dose of miR-122 mimic at the onset of reperfusion in LP rats is able to reduce myocardial infarct size, apoptosis, and ROS production *in vivo*, concomitant with reduced expression of Pkm2. Thus, we show miR-122 could serve as a novel cardioprotective strategy and a protective biomarker in LP **(Figure 2).**
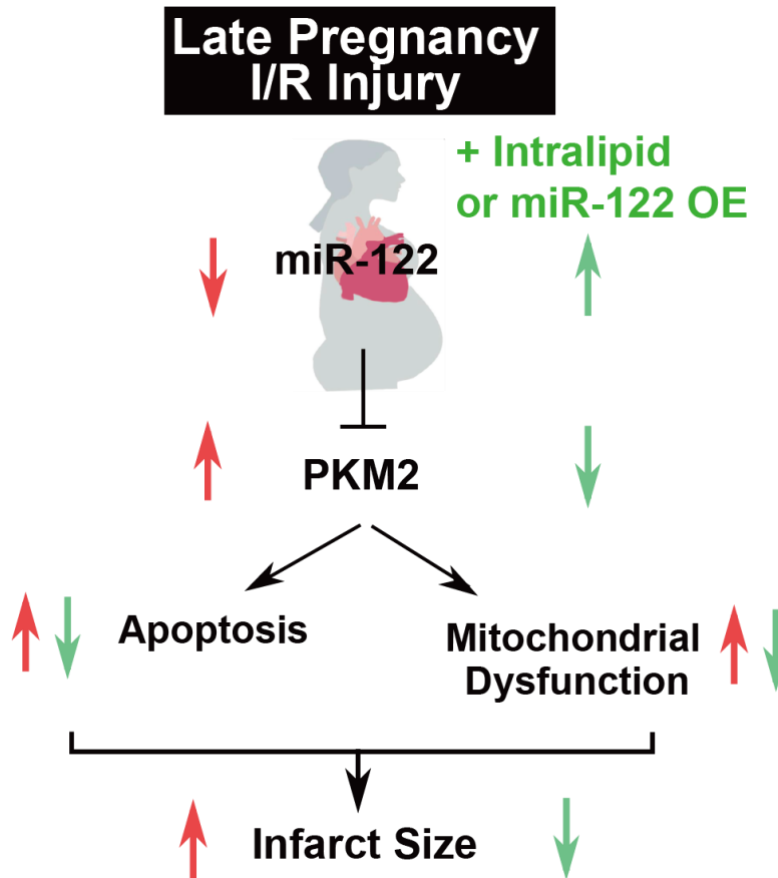
Figure 2. Schematic of the proposed mechanism. miR-122 expression is decreased in IRI in LP, which in turn upregulates its target PKM2, resulting in increased apoptosis and mitochondrial dysfunction, ultimately resulting in increased infarct size (red arrows). ITLD protects the heart by increasing miR-122 expression, which in turn downregulates its target PKM2, resulting in decreased apoptosis and mitochondrial dysfunction, ultimately resulting in decreased infarct size (green arrows). ITLD: intralipid.

Future Directions

While this dissertation sheds light on the intricate roles of miR-98 and miR-122 in the susceptibility and protection against IRI in LP respectively, several limitations and avenues for future research deserve attention.

Examining in which cell types miR98 is expressed in the heart is and whether this expression is altered upon IRI will provide valuable insights into the multifaceted roles of miR-98 in the pathophysiology of MI, enhancing our understanding of its therapeutic potential and paving the way for the development of targeted interventions to mitigate IRI in pregnancy. We have already established that miR-98 is expressed in cardiomyocytes through FISH, it is important to assess in which other cardiac cell types miR98 is expressed, i.e. cardiac fibroblasts, endothelial cells, and/or smooth muscle cells. FISH should be performed with two probes: probe for miR-98 as well as marker for each respective cell type: cardiac fibroblasts (DDR2$^+$), endothelial cells (PECAM1$^+$), or smooth muscle cells (SMTN$^+$). The role of inflammation is well-established in IRI (6,7), and macrophages are known to play a critical role in the immune response, particularly in the context of tissue injury and repair (8). In addition, macrophages have been reported to affect several cardiomyocyte processes (9,10) and are recruited by stressed cardiomyocytes, which give off a number of signals to regulate macrophage number and inflammatory phenotype (11). Future studies should expand this investigation by examining whether miR-98 is also expressed in macrophages using CD68 probe .

If miR98 is expressed in macrophages, future work should assess the role of miR98 in interactions of cardiomyocytes and macrophages using co-culture methods. For these experiments, adult rat ventricular myocytes (ARVMs) should be utilized as they better

mimic the physiological characteristics and responses of mature cardiac tissue. Although most *in vitro* data presented in this dissertation were on H9c2 cells, we show in Chapter 2 that incubation of ARVMs with serum from either NP or LP rats for 24 hours is able to upregulate miR98 in normoxia and H/R, but to a further extent in H/R. Migration assay should be performed using co-culture of cardiomyocytes and bone-marrow derived macrophages using transwell **(Figure 3).** In the transwell plates, cardiomyocytes isolated from ARVMs will be plated in the lower chamber, and bone-marrow derived macrophages will be plated in the higher chamber. MiR98 will then be overexpressed or knocked down in either cardiomyocytes or macrophages and the cells will be incubated with either NP serum or LP serum followed by either normoxia or H/R (8 total conditions as shown in Figure 3). Apoptosis, inflammation, oxidative stress, and mitochondrial respiration and dysfunction should be measured. Furthermore, miR-98 levels in the media should be measured by qPCR to assess whether the crosstalk effects are mediated by secreted miR-98.

Additionally, future work should also investigate in which cell types in the heart miR-122 is expressed. Thus, similar experiments as proposed above should be conducted to assess miR-122 expression in different cell types such as cardiomyocytes, macrophages, endothelial cells, cardiac fibroblasts, and smooth muscle cells using FISH. If miR-122 is in fact expressed in both cardiomyocytes and macrophages, similar transwell experiments as proposed above should be utilized **(Figure 3).** Here, ITLD should also be proposed as an additional experimental group. Such experiments would provide valuable insights into the tissue-specific roles of miR-122 and enhance our understanding of its contributions to pregnancy-related cardiovascular pathologies.
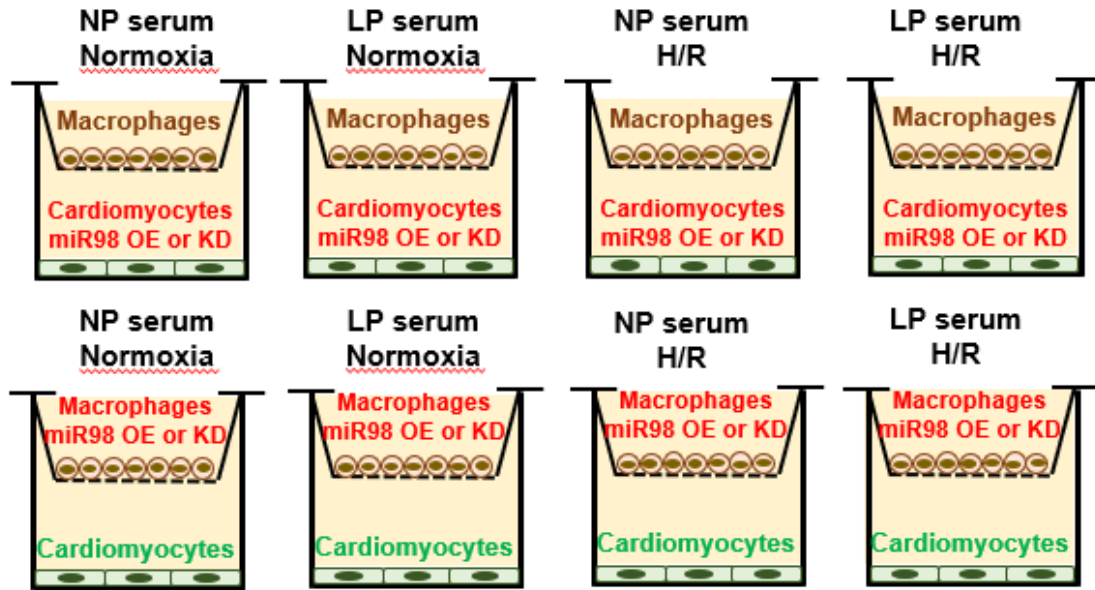
Figure 3. Experimental setup for co-culture experiments to assess the effects of

miR98, pregnancy and H/R on cardiomyocytes and macrophage cross-talk.

Moreover, future work should incorporate single-nucleus sequencing to assess to which extent cardiomyocytes, macrophages, or other cell types are responsible for the effects that pregnancy and miR98 antagomir or miR122 mimic therapies have on myocardial IRI, as well as obtain transcriptional signatures of cell death, oxidative stress, and inflammation in these cell populations. This technique will aid in identifying different cell clusters and discriminate between miR98 antagomir and control samples, or miR122 mimics and their corresponding control samples within the cell clusters. The single-nucleus sequencing results should be validated by immunofluorescence and FISH. Integrating single-nucleus sequencing could provide a more holistic understanding of the complex interplay between miRs and other determinants of IRI in pregnancy.

To further examine the role of increased expression of miR98 specifically in cardiomyocytes in the higher vulnerability of the heart in LP to IRI, future investigations should employ cardiomyocyte-specific miR-98 knockout mice. This will be accomplished by acquiring miR-98 floxed LacZ mice and breeding them with α-myosin heavy chain (α-MHC) Cre mice. If the infarct size in these KO animals are significantly smaller compared to wildtype animals, it will be evident that the increased expression of miR-98 in cardiomyocytes in LP is the cause of the more vulnerability of the heart to IRI. If, however, the infarct size is partially decreased or not changed, then other cell types also play a role in the higher vulnerability of LP to IRI. Furthermore, these cardiomyocyte-specific miR-98 knockout mice will also be useful in assessing whether the cardiomyocytes in the heart is the source of miR-98 in the plasma. We have already observed higher miR-98 plasma levels in LP sham compared to NP sham **(Chapter 2).** However, if miR-98 plasma levels in cardiomyocyte-specific miR-98 knockout LP sham mice is not higher than

cardiomyocyte-specific miR-98 knockout NP sham mice, then the source of the plasma miR-98 is cardiomyocytes. If, on the other hand, higher miR-98 levels in LP sham persist in the plasma despite cardiomyocyte-specific knockout of miR-98, this is indicative that miR-98 does not originate from cardiomyocytes. In such a scenario, it is possible that miR-98 may be sourced from other cell types in the heart such as endothelial cells, which often serve as both culprits and sensors in various physiological processes, or from other organs. To test this hypothesis, knocking out miR-98 in endothelial cells and assessing miR-98 expression in the plasma is necessary. Similar experiments using cardiomyocyte-specific miR-122 overexpression mice should be performed to examine the role of miR122 overexpression only in cardiomyocytes in protecting the heart in LP and source of miR122 in the plasma.

Furthermore, it is essential to recognize that our *in vivo* rat studies did not examine the long-term adverse effects of miR interventions on the mother nor the fetus, which could be a limitation of our study. However, given that we administered only one dose of mimic or antagomir one day before delivery, it is unlikely for the single dose to have any negative or detrimental effects on fetal development. Nevertheless, it is imperative to conduct further investigations to thoroughly assess potential toxicity, mortality, and overall health of the fetus. Gross examination conducted previously revealed no adverse effects on the fetus at the administered dose. Before proceeding to clinical trials, however, it is crucial to ensure that the intervention has no negative off-target effects on liver or kidney functions. In addition, understanding the potential impacts of miR interventions on fetal development and health is crucial for comprehensively assessing their safety and efficacy in pregnant individuals. Future research endeavors should include thorough evaluations

of the fetal outcomes following miR-targeted interventions to provide a more comprehensive understanding of their implications for maternal and fetal health.

Additionally, translating the findings of this study into clinical applications is crucial. Conducting translational studies to validate the diagnostic and therapeutic potential of targeting miR-98 and miR-122 in pregnancy-related IRI is imperative. While there are currently no miR-based therapies targeted for IRI in LP, therapies targeting miRs have progressed to clinical trials for addressing a broad spectrum of illnesses (12–15). It is important to note that special consideration should be warranted when enrolling pregnant women in clinical trials as they are considered a vulnerable population. Concerns may arise regarding the safety of experimental treatments for both the pregnant individual and the developing fetus. Given the critical developmental stages during LP, there is a heightened risk of adverse effects on maternal health and fetal well-being. Addressing these concerns and ensuring the safety of both mother and fetus would be paramount in designing and implementing clinical trials involving miR. Exploring the safety and efficacy of interventions targeting these miRs in clinical trials could pave the way for personalized therapeutic strategies.

In conclusion, while this dissertation lays a foundation for understanding the roles of miR-98 and miR-122 in pregnancy-associated IRI, addressing the aforementioned limitations and pursuing future research directions are critical for advancing our knowledge and ultimately improving the management of this condition in pregnant individuals.

Final remarks from the author

Maternal mortality rates remain a pressing issue, with pregnancy-related MI contributing significantly to the overall number of maternal fatalities. Investigating innovative therapeutic approaches is crucial to enhance patient survival rates. It has been an honor to delve into the study of this illness, offer innovative perspectives on its origins, and provide a glimmer of hope to those impacted by this disease. My utmost aspiration is for the preclinical therapies outlined in this research to undergo clinical trials successfully, ensuring that this work extends far beyond the confines of this dissertation. Lastly, I trust that this dissertation acts as a catalyst, inspiring researchers to delve into the study of MI during pregnancy.

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