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Role of TLRs, Hippo-YAP1 Signaling, and microRNAs in Cardiac Repair and Regeneration of Damaged myocardium During Ischemic Injury

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Role of TLRs, Hippo-YAP1 Signaling, and microRNAs in Cardiac Repair and Regeneration of Damaged myocardium During Ischemic Injury

A dissertation presented to the faculty of the Departments of Surgery and Biomedical Science East Tennessee State University In partial fulfillment of the requirements for the degree Doctor of Philosophy in Biomedical Science

by Xiaohui Wang August 2017

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ABSTRACT

Role of TLRs, Hippo-YAP1 Signaling, and MicroRNAs in Cardiac Repair and Regeneration of Damaged myocardium During Ischemic Injury

by

Xiaohui Wang

Cardiovascular disease is a leading cause of death in the United States. Toll-like receptor (TLR)-mediated pathways have been demonstrated to play a role in myocardial ischemia/reperfusion (I/R) injury. We and others have shown that PI3K/Akt signaling is involved in regulating cellular survival and protecting the myocardium from I/R induced injury. In this dissertation, we provide compelling evidence that miR-125b serves to "fine tune" TLR mediated NF-κB responses by repressing TNF-α and TRAF6 expression. We constructed lentiviral expressing miR-125b, delivered it into the myocardium. The data showed that delivery of lentivirus expressing miR-125b significantly reduces myocardial infarct size and improves cardiac function in I/R hearts. Mechanistic studies demonstrated that miR-125b negatively regulates TLR mediated NF-κB activation pathway by repressing TNF-α and TRAF6 expression in the myocardium.

We also observed that transfection of the myocardium with lentivirus expressing miR-214 markedly attenuates I/R induced myocardial infarct size and cardiac dysfunction. We demonstrated that miR-214 activates PI3K/Akt signaling by targeting PTEN expression in the myocardium.

We also investigated the role of TLR3 in neonatal heart repair and regeneration following myocardial infarction (MI). Wild type (WT) neonatal mice showed fully cardiac
functional recovery and small infarct size, while TLR3 deficient mice exhibited impaired cardiac functional recovery and large infarct area after MI. Poly (I:C), a TLR3 ligand, administration significantly enhances glycolysis, YAP1 activation and the proliferation of WT neonatal cardiomyocytes. 2-deoxyglucose (2-DG), a glycolysis inhibitor treatment abolished cardiac functional recovery and YAP1 activation in neonatal mice after MI. In vitro either inhibition of glycolysis by 2-DG or inhibition of YAP1 activation prevents Poly (I:C) induced YAP1 activation and neonatal cardiomyocyte proliferation. Importantly, YAP1 activation increases miR-152 expression, leading to cardiomyocyte proliferation through suppression P27kip1 and DNMT1 expression.

We conclude that microRNAs play an important role in TLR modulation induced protection against myocardial I/R injury by increasing the activation of PI3K/Akt signaling pathway, decreasing TLR/NF-κB mediated inflammatory response, and suppressing activation of apoptotic signaling following myocardial I/R injury.

In addition, TLR3 is an essential for neonatal heart repair and regeneration after myocardial infarction. TLR3 modulation could be a novel strategy for heart regeneration and repair.
DEDICATION

I would like to dedicate my dissertation work to my family and friends whose support and encouragement has made this achievement possible. Without the constant love, support, and encouragement of my beloved wife Hongxia, my precious daughter Celina, my parents Jingxin and Xuemei, I would not have been able to complete my Ph.D. They all contributed in significant ways to make my dream become a reality and I am forever grateful.
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CHAPTER 1

INTRODUCTION

Myocardial Ischemia/Reperfusion Injury

The heart is the first functional organ to be develop during embryogenesis and one of the most important organs in our body. The heart functions as a pump which drives blood throughout the body, carrying all the vital nutrients and oxygen, and assists in the removal of waste products that the body does not need. The heart, like all other tissues in our body, requires nutrients and oxygen through the coronary circulation. Coronary heart disease is the most common type of heart disease, which is caused by the severe impairment of coronary blood supply from atherosclerotic plaque buildup. The impairment of coronary circulation can damage or destroy part of the cardiac tissue also called myocardial ischemic injury.

Heart attack caused by coronary artery disease is the leading killer of both men and women in the United States. On average, more than 2,150 Americans die from heart disease each day, which is about 1 death every 40 seconds (Benjamin et al. 2017). In ischemic patients, the most efficient approach to reduce the myocardial ischemic injury and improve outcomes is to restore blood flow in a timely and effective manner. However, the process of restoring blood flow to the ischemic myocardium can also induce cardiomyocyte death and further damage, known as myocardial reperfusion injury (Braunwald and Kloner 1985; Piper et al. 1998; Yellon and Hausenloy 2007; Hausenloy and Yellon 2013). It has been reported that up to 50% of the final infarct size is caused by myocardial reperfusion injury (Yellon and Hausenloy 2007). Experimental studies have identified some of the major contributors to myocardial reperfusion induced
injury including robust ROS production, increased mitochondrial permeability, and calcium overload (Brown et al. 1988; Ambrosio et al. 1993; Liu et al. 1993; Liu et al. 1994; Levraut et al. 2003; Weiss et al. 2003; Honda et al. 2005). However, at present, no effective therapeutic treatment exists for myocardial reperfusion injury.

Pathophysiological Mechanisms of Myocardial Ischemia and Reperfusion Injury

The deprivation of nutrients and oxygen to the heart tissue caused by acute occlusion of the coronary artery results in a series of abrupt metabolic and biochemical changes within the myocardium (Yellon and Hausenloy 2007). Unlike the neonatal heart, more than 90% of the energy in adult cardiomyocytes is produced by oxidative phosphorylation in mitochondria, a process which is necessary for myocardial contractile function (Harris and Das 1991). During myocardial ischemia, oxygen deprivation will prevent mitochondrial oxidative phosphorylation and ATP production (Lesnefsky et al. 1997). In addition, oxygen deficiency causes cardiomyocyte metabolism to switch from oxidative phosphorylation to anaerobic glycolysis. This leads to lactate accumulation, a decrease in pH, and intracellular acidosis (Buja 2005; Sanada et al. 2011; Kalogeris et al. 2012; Kalogeris et al. 2016). Furthermore, ATP depletion and intracellular acidosis will impair the activity of Na⁺/H⁺ exchanger (NHE), Na⁺/K⁺-ATPase and Ca²⁺-ATPase, and trigger Ca²⁺ overloading (Buja 2005; Sanada et al. 2011; Kalogeris et al. 2012; Kalogeris et al. 2016).

During reperfusion, the intracellular accumulation of H⁺ produced by anaerobic glycolysis will be transported into the extracellular space causing an exacerbation of Ca²⁺ overload (Liu et al. 1994; Buja 2005; Kalogeris et al. 2016). Meanwhile, the
increase in mitochondrial membrane potential will result in robust ROS generation (Barry 1987; Lesnefsky et al. 1997; Levraut et al. 2003; Kalogeris et al. 2016). ROS overproduction will cause damage to cellular proteins, DNA, and lipids (Levraut et al. 2003; Weiss et al. 2003; Genova et al. 2004).

Figure 1.1 Pathophysiologic Mechanisms of Myocardial Ischemia/Reperfusion injury. During myocardial ischemia, the derivation oxygen supply decreases ATP production, resulting in increased anaerobic glycolysis. The increased anaerobic glycolysis leads to lactate accumulation, a decrease in intracellular pH, Ca2+ overload and increased ROS production. Myocardial ischemia also leads to myocardial necrosis and inflammatory responses which contribute to myocardial infarction and cardiac remodeling. Myocardial reperfusion will further increase ROS production, calcium overload and mitochondrial mediated cellular apoptosis. Massive functional cardiomyocytes loss and inflammatory cytokine production will trigger left ventricular remodeling, leading to left ventricular dilation and heart failure.
Animal studies have demonstrated that the detrimental effects of the robust ROS production and intracellular Ca\(^{2+}\) overloaded will induce cardiomyocyte apoptosis and necrosis following myocardial ischemic injury (Barry 1987; Lesnefsky et al. 1997; Levraut et al. 2003; Kalogeris et al. 2016).

Excessive production of inflammatory cytokines (IL-1\(\beta\), TNF-\(\alpha\), IL-6) triggered by DAMPs released from necrotic cells during ischemia/reperfusion also contribute to I/R induced contractile dysfunction, fibrosis, hypertrophy, and cell death (Kukielka et al. 1995; Seta et al. 1996; Kapadia et al. 1998; Arslan et al. 2011; Timmers et al. 2012; Lin and Knowlton 2014; Vilahur and Badimon 2014). In addition, accumulation and activation of neutrophils and macrophages in the infarcted myocardial tissue will further exaggerate I/R induced myocardial injury and cardiac dysfunction (Kukielka et al. 1995; Seta et al. 1996; Kapadia et al. 1998; Ao et al. 2009; Arslan et al. 2010; Gao et al. 2011; Lin and Knowlton 2014; Vilahur and Badimon 2014). The massive death of functional cardiomyocytes in conjunction with the dysregulated inflammatory response in the infarct area will trigger left ventricular remodeling, collagen deposition, cardiac hypertrophy and dilation (Kleinbongard et al. 2011; Sanada et al. 2011; Kalogeris et al. 2016; Moe and Marin-Garcia 2016). Uncontrolled left ventricular remodeling and fibrotic scar size enlargement will ultimately result in heart failure.

**Toll-Like Receptors (TLRs)**

It is well established that acute and chronic inflammatory responses are critical mediators for tissue damage which play a crucial role in the functional deterioration of the heart following myocardial I/R injury (Lange and Schreiner 1994; Aderem and Ulevitch 2000; Thomas et al. 2003; Oyama et al. 2004; Ao et al. 2009; Chao 2009; Ha

It is well known that TLRs recognize nucleotides, peptides, and proteins and play a critical role in innate immune responses (Jin and Lee 2008; Ha et al. 2011). At present, there are 11 human and 13 mouse TLRs that have been identified. TLRs are located at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6), and in intracellular endosomes (TLR3, TLR7, TLR8, TLR9, TLR11, TLR13). The cell surface TLRs that recognize lipopeptides include TLR1, TLR2, TLR4, and TLR6 (Jin and Lee 2008; Ha et al. 2011). The major ligands for TLR2 include multiple glycolipids, lipopeptides, lipoproteins, lipoteichoic acid from bacteria, beta-glucan from fungi, and HSP70 form the host cells (Tsan and Gao 2004; Jin and Lee 2008; Ha et al. 2011). TLR4 recognizes specific components (LPS) from gram-negative bacteria, several heat shock proteins, fibrinogen, heparin sulfate fragments, and hyaluronic acid fragments from host cells (Tsan and Gao 2004; Jin and Lee 2008; Ha et al. 2011). TLR5 is highly expressed by monocytes/macrophages and dendritic cells which can recognize bacterial flagellin (Tsan and Gao 2004; Jin and Lee 2008; Ha et al. 2011).

The intracellular TLRs recognize nucleic acids, particularly viral and bacterial DNA, as well as endogenous nucleic acids. For example, TLR3 detects double stranded viral RNA, synthetic dsRNA, and the RNAs released from damaged cells (Tsan and Gao 2004; Jin and Lee 2008; Ha et al. 2011). TLR7 mainly recognizes signal strand
RNA from RNA viruses (Tsan and Gao 2004; Jin and Lee 2008; Ha et al. 2011), while TLR9 recognizes bacterial and viral unmethylated DNA (Tsan and Gao 2004; Jin and Lee 2008; Ha et al. 2011). The most important adaptor protein of the TLR mediated pathways is myeloid-differentiation primary response protein 88 (MyD88). The signaling pathways which act through this adaptor are termed MyD88 dependent pathways (Wesche et al. 1997; Dunne et al. 2003; Akira and Takeda 2004; Ha et al. 2011). All of the TLRs except TLR3, use the adaptor protein MyD88 to recruit IRAK4 and activate other IRAK family members, such as IRAK1 and IRAK2 (Wesche et al. 1997; Kanakaraj et al. 1998; Suzuki et al. 2002; Dunne et al. 2003; Akira and Takeda 2004; Ha et al. 2011). The activated IRAK1/2 kinase complex then phosphorylates and activates the TNF receptor associated factor 6 (TRAF6). TRAF6 acts as an E3 ubiquitin protein ligase which in turn polyubiquinates and activates transforming growth factor-beta activated kinase1 (TAK1). The activated TAK1 phosphorylates IκB kinases (IKK-α/IKK-β), and then phosphorylates and promotes IκB degradation. This allows NF-κB to be translocated into the nucleus and regulate pro-inflammatory gene expression (Akira and Takeda 2004; Ha et al. 2011; Chen 2012; Walsh et al. 2015).

Another important adaptor protein of TLR mediated signaling pathway is TIR-domain-containing adapter-inducing interferon-β (TRIF). Both TLR3 and TLR4 utilize the TRIF dependent pathway to activate the interferon (IFN) regulatory factor 3 and NF-κB signaling pathway. Unlike TLR3, TLR4 requires the adaptor TRAM for activating TRIF (Akira and Takeda 2004; Moynagh 2005; Ha et al. 2011). TRIF can interact with RIP1 for NF-κB activation and use TBK1 for IRF3 activation (Akira and Takeda 2004; Moynagh 2005; Ha et al. 2011).
Role of TLRs in Myocardial Ischemia/Reperfusion Injury

Increasing evidence suggests that TLRs can be activated by various endogenous ligands, also called danger-associated molecular patterns (DAMPs) which are released by damaged tissues. DAMPs can induce an overwhelming inflammatory response via activation of NF-κB and/or IRF3 dependent pathways. Recent studies have shown that both NF-κB and IRF3 mediated signaling pathways play critical roles in extending ischemic myocardial injury, cardiac remodeling and the progression of heart failure (Thomas et al. 2003; Oyama et al. 2004; Tsan and Gao 2004; Ao et al. 2009; Chao 2009; Arslan et al. 2010; Gao et al. 2011; Kleinbongard et al. 2011; Ha et al. 2011). NF-κB activation and overwhelming cytokine production are hallmark indicators for the activation of immune activation following myocardial I/R injury. Previous studies have shown that inhibition of NF-κB activation protects against I/R induced myocardial injury and cardiac dysfunction (Tsan and Gao 2004; Chao 2009; Ha et al. 2011). In addition, numerous studies have demonstrated that deficiency or inhibition of either TLR2, or TLR3, or TLR4 significantly attenuates I/R induced myocardial injury by reducing acute inflammatory responses, pro-inflammatory cytokine production, immune cell infiltration and cardiomyocyte apoptosis (Oyama et al. 2004; Ao et al. 2009; Chao 2009; Arslan et al. 2010; Ha et al. 2011; Lu et al. 2014). However, TLR5 deficiency exacerbates I/R induced myocardial oxidative stress, cardiac injury and inflammatory response (Parapanov et al. 2015).

In contrast with the results obtained from deficiency of TLR2, or TLR3, or TLR4, modulation of TLRs by specific ligands, such as Pam3CSK4 for TLR2, LPS for TLR4, and CPG-DNA for TLR9 have been shown to exert a protective effect against I/R
induced myocardial injury (Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013). Interestingly, activation of the PI3K/Akt signaling pathway and decreasing of the inflammatory response have been reported to be involved in the underlying cardio-protective effects of TLR modulation (Hua et al. 2007; Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013). Why does TLR deficiency and TLR modulation have protective effects on myocardial I/R injury and are the underlying mechanisms similar? It is easy to understand that TLR deficiency attenuates I/R induced acute inflammatory response, cytokine production, immune cell infiltration, and myocardial cell apoptosis. The question is, why does an activation of TLRs reduce inflammatory responses in myocardial I/R injury. Currently cardiac protection mediated by TLR modulation is explained as preconditioning. However, preconditioning cannot fully explain the cardioprotective effects of TLR modulation on myocardial I/R injury. It is possible that there is a negative-feedback regulatory mechanism to control TLR/NF-κB signaling pathway during TLR modulation. Indeed, our previous studies have shown that TLR modulation significantly increases the expression of miR-146a, which could serve to “fine tune” TLR mediated NF-κB signaling pathway activity. Increased expression of miR-146a significantly attenuated I/R induced myocardial injury and cardiac dysfunction by targeting IRAK1 and TRAF6 (Wang et al. 2013). In order to investigate the mechanisms by which TLR modulation attenuates I/R induced inflammatory response and tissue damage, we focused on the role of microRNAs.
MicroRNAs

MicroRNAs (miRNAs) are endogenous, small, single stranded non-coding RNAs (about 22nt) which play an important role in posttranscriptional gene regulation by inhibiting or promoting mRNA degradation in mammals, plants, fungi and some viruses (Bartel 2004). The first miRNA discovered, lin-4, was found to regulate lin-14 expression through an unexpected cellular regulatory mechanism by targeting the 3'UTR region of lin-14 in *C.elegans* in 1993 (Lee et al. 1993). Only when let-7 was discovered in the early 2000s were microRNAs recognized as a class of biological regulators. Let-7 is necessary for larval development of *C.elegans* (Reinhart et al. 2000). Since then, thousands of miRNAs have been identified in mammals, plants, fungi and some viruses (Griffiths-Jones et al. 2008). Plant miRNAs usually display perfectly or near perfectly matched nucleotide complements with their intended mRNA targets. There are similar small interfering RNAs (siRNAs) which cause repression of their target gene through cleavage of the target mRNAs. Unlike plant miRNAs, mammalian miRNAs have a large number of mismatches with their target mRNA binding sites (Millar and Waterhouse 2005). That explains why a given mammalian miRNA may have hundreds of different target mRNAs, and a given target mRNA might be regulated by multiple miRNAs.

The biogenesis of miRNAs is a multistep process that occurs in both the nucleus and cytoplasm. Mature miRNAs are about 18-24 nucleotides of small non-coding RNAs which are initially transcribed by RNA polymerase II in the nucleus as long primary transcripts, called primary miRNAs (pri-miRNAs). The expression of miRNAs is controlled by RNA Pol II –associated transcription factors such as P53, NF-κB, and YAP/TAZ which positively or negatively regulate miRNA expression (Taganov et al.
Epigenetic modification such as histones acetylation and DNA methylation are also involved in miRNA gene regulation (Saito and Jones 2006; Gatto et al. 2010).

Following transcription, the pri-miRNAs can fold to form a stem-loop hairpin structure. They are then cropped by the double-stranded RNA-specific ribonuclease complex, including the RNase III Drosha, DiGeorge syndrome critical region 8 (DGCR8) and other cofactors in the nucleus, to yielded specific hairpin shaped pre-miRNAs (also called hairpin precursors) (Lee et al. 2003; Kim 2005; Han et al. 2006).

The excised hairpin pre-miRNAs are rapidly exported out of the nucleus by nuclear export factor exportin 5 (Exportin 5)/RanGTP and additionally processed by dicer, a second RNase III endonuclease, into ∼22 long nucleotide duplex in the cytoplasm (Yi et al. 2003). Both strands of the duplex have the potential to act as a functional miRNA, however, only one strand is selectively incorporated into the RNA-induced silencing complex (RISC). The selected strand serves as the functional mature miRNA which recognizes its targets by the seed region and promotes target mRNA degradation or translational inhibition (Kim 2005; Gregory et al. 2005; Behm-Ansmant et al. 2006).

Role of microRNAs in Myocardial Ischemia/Reperfusion Injury

MicroRNAs have been demonstrated to be involved in almost all biological processes including tissue development, homeostasis, aging, cell differentiation/dedifferentiation, proliferation, apoptosis, migration, and cellular metabolism (Ambros 2003; Miska 2005; Wienholds and Plasterk 2005). Therefore, it is not surprising that more than 90% of human genes are regulated by miRNAs (Miranda
et al. 2006). Profiles of miRNA expression have suggested that a large number of miRNAs are highly expressed in heart tissue and are considered to be novel regulators in cardiac physiological and pathophysiological processes, including heart development and aging, myocardial hypertrophy, ventricular arrhythmias, myocardial infarction, cardiac remolding, and heart failure (Callis and Wang 2008; Wang 2010; Barwari et al. 2016; Samanta et al. 2016; Yan and Jiao 2016). Such evidence suggests that miRNAs are attractive potential therapeutic targets for multiple heart diseases.

Numerous studies have demonstrated that TLR/NF-κB mediated inflammatory responses contribute to myocardial I/R injury, cardiac remodeling and the progression of heart failure (Seta et al. 1996; Kapadia et al. 1998; Chao 2009; Kleinbongard et al. 2011; Ha et al. 2011). We have reported that miR-146a overexpression protects the heart from I/R injury by targeting IRAK1 and TRAF6 expression and then negatively regulating the TLR/NF-κB signaling pathway (Wang et al. 2013).

It is well known that myocardial apoptosis and necrosis contribute to a loss of functional cardiomyocytes during myocardial I/R injury (Levraut et al. 2003; Chiong et al. 2011; Moe and Marin-Garcia 2016). Therefore, preventing cardiomyocyte death or increasing cell survival is an effective intervention to restore impaired cardiac function and prevent later stage heart failure (Levraut et al. 2003; Chiong et al. 2011; Moe and Marin-Garcia 2016). Several microRNAs have been reported to be important for the regulation of cell death by targeting apoptotic and/or necrotic signaling pathways. For example, inhibition of miR-15 family members by anti-miRs significantly reduces infarct size, improves cardiac function and prevents cardiac remodeling following ischemic injury. Mechanistic studies suggest that inhibition of the miR-15 family causes an
increases in the expression of anti-apoptotic proteins (Bcl2 and SIRT1) and subsequent reduction of cardiomyocyte apoptosis during myocardial ischemic injury (Hullinger et al. 2012). MiR-320 is another proapoptotic miRNA, and inhibition of its expression has been shown to exert a protective effect against myocardial I/R injury. *In vitro* studies have demonstrated that miRNA-320 overexpression promotes cardiomyocyte death by targeting a well-known cellular protective protein named heat shock protein 20 (Ren et al. 2009). In addition, various miRNAs including miR-34, miR-140, and miR-92a serve as pro-apoptotic miRNAs to promote cardiomyocyte death (Iekushi et al. 2012; Hinkel et al. 2013; Liu et al. 2015).

In contrast to pro-apoptotic miRNAs, various miRNAs have been reported to serve as protective regulators in response to myocardial ischemic injury. MiR-21 protects against oxidative stress induced damage of cardiomyocytes and I/R induced myocardial injury by targeting programmed cell death 4 (PDCD4) and PI3K/AKT negative regulator (PTEN) (Dong et al. 2009). Bcl2-like protein 11 (Bim1) is a pro-apoptotic protein of the Bcl2 family that promotes mitochondrial mediated apoptosis by inhibiting Bcl2 anti-apoptotic function. It can directly interact with Bax in the mitochondrial outer membrane, and lead to mitochondrial membrane potential disruption, cytochrome c release, and apoptosis. Bim1 has been identified as a target of miR-24, another important antiapoptotic miRNA. Increased expression of miR-24 has been reported to significantly reduce infarct size and improve cardiac functional recovery following myocardial ischemic injury (Fiedler et al. 2011). MiR-103/107 is a cardiac protective miRNA cluster that has been shown to attenuate myocardial I/R injury by targeting FADD. In addition, various miRNAs including miR-210, miR-146a, miR-494, miR-150, etc. have been
shown to have protective effects on I/R induced death of cardiomyocytes and tissue damage (Wang et al. 2010; Wang et al. 2013; Tang et al. 2015; Ke et al. 2016).

In the first study of this dissertation, we have reported that increased expression of miR-125b significantly attenuates I/R induced infarct size and improves cardiac function by reducing cardiomyocyte cell death and decreasing acute inflammatory response following myocardial I/R injury. The protective effect is attributed to targeting of the pro-apoptotic proteins BAK1 and P53, and TNF receptor associated factor-6 (TRAF6).

**MicroRNAs and TLR Signaling Pathway**

As mentioned above, Toll like receptors are important for detecting invading pathogens, endogenous DAMPs released from damaged cells, and initiating inflammatory responses (Lange and Schreiner 1994; Seta et al. 1996; Chao 2009; Ha et al. 2011). Importantly, dysregulated immune function is involved in the majority of human diseases. TLR mediated signaling serves as a double-edged sword in human health, thus TLR activity needs to be tightly regulated. It is well established that TLR mediated signaling plays an important and complex role in myocardial ischemic injury (Ao et al. 2009; Chao 2009; Arslan et al. 2010; Ha et al. 2011). Recent studies have shown that either modulation or inhibition of TLRs induces protective effects on myocardial I/R injury.

Growing evidence suggests that miRNAs are important immunomodulators for the regulation of immune responses by targeting their molecules and promoting mRNA degradation or translational inhibition (O’Neill et al. 2011; Virtue et al. 2012; Wang et al. 2013; Kozloski et al. 2016; Mundy-Bosse et al. 2016). Several studies have demonstrated that the expression of multiple miRNAs is regulated by NF-κB, the major
transcription factor in the TLR mediated signaling pathway (O’Neill et al. 2011; Virtue et al. 2012; Wang et al. 2013). We and others have been reported that either TLR2 or TLR4 modulation significantly increases the expression of miR-146a through an NF-κB dependent mechanism. MiR-146a has been identified as a negative regulator in TLR mediated inflammatory responses by targeting IL-1R-associated kinase 1 (IRAK1) and TNFR-associated factor 6 (TRAF6), both of which are the key downstream molecules in TLR/NF-κB signaling pathway (Wang et al. 2013). Increased expression of miR-146a in the myocardium significantly attenuates I/R induced acute inflammatory responses and myocardial injury (Wang et al. 2013). Myeloid differentiation primary response gene 88 (Myd88) is the most important adapter protein of TLR signaling pathway and is used by almost all TLRs except TLR3. It has been reported that MyD88 is a direct target of miR-155 (Tang et al. 2010). MiR-155 also targets TAK1-binding protein 2 (TAB2) which is an important signaling molecule downstream of TRAF6 (Imaizumi et al. 2010).

Pro-inflammatory transcription factors are important for TLR mediated immune responses which can be directly regulated by miRNAs. For example, NF-κB has been reported to be regulated by a subset of miRNAs, including miR-9, miR-181 and miR-183 (O’Neill et al. 2011; Virtue et al. 2012; Sha et al. 2014; Sun et al. 2014; Kozloski et al. 2016). In addition, numerous studies have reported that TLR/NF-κB induced microRNAs can in turn feedback to negatively regulate TLR signaling by directly targeting TLRs (miR-223, let-7e) (Chen et al. 2007; Wang et al. 2015), TLR signaling regulators (miR-126, miR-146a, miR-149, miR-199 and miR-203) (Mattes et al. 2009; Primo et al. 2012; Callegari et al. 2013; Wang et al. 2013; Xu et al. 2014b), and inflammatory cytokines (miR-98, miR-125b, miR-211 and miR-579) (Kim et al. 2012;
Venza et al. 2015; Song et al. 2017). Collectively, these findings suggest that miRNAs “fine tune” TLR signaling and could be used to target and regulate TLR mediated immune responses during myocardial I/R injury.

**PI3K/Akt Signaling Pathway**

Phosphoinositide-3 kinase (PI3K) mediated Akt signaling (PI3K/Akt) signaling is an evolutionally conserved intracellular signaling pathway and is involved in pathologic and pathophysiologic processes including cardiac hypertrophy, cellular metabolism, cell proliferation, survival/apoptosis, and differentiation and migration (Oudit et al. 2004; Aoyagi and Matsui 2011; Martini et al. 2014; Dibble and Cantley 2015; Lien et al. 2016; Mayer and Arteaga 2016; Yu and Cui 2016; Manning and Toker 2017). It has been found that the dysregulation of PI3K/Akt signaling pathway is associated with developmental and overgrowth syndromes, cancer, diabetes, inflammatory and autoimmune diseases, neurological disorders and cardiovascular disease (Oudit et al. 2004; Wu and Mohan 2009; Aoyagi and Matsui 2011; Foster et al. 2012; Maillet et al. 2013; Martini et al. 2014; Heras-Sandoval et al. 2014; Dibble and Cantley 2015; Mayer and Arteaga 2016; Manning and Toker 2017).

The PI3K family is divided into three distinct classes based on their molecular structure and substrate specificity: Class I PI3Ks, Class II PI3Ks and Class III PI3K. It has been demonstrated that PI3Ks can be activated by RTK (receptor tyrosin kinase) and G protein coupled receptors (GPCRs) (Rodriguez-Viciana et al. 1994; Vanhaesebroeck et al. 2010; Howes et al. 2003).
Figure 1.2 PI3K/Akt signaling pathway. PI3K kinases can be activated by RTK, IGF-1R and G protein coupled receptors (GPCRs). The activated PI3K complexes phosphorylate PIP2 to PIP3 which then promotes an interaction of PDK1 and Akt, which activates Akt. PTEN acts as a negative regulator of PI3K/Akt signaling by dephosphorylating PIP3 to PIP2. The activated Akt modulates multiple substrates which are important for cell survival, apoptosis, cell cycle, metabolism, angiogenesis, and immune response.

Once the PI3Ks are activated, they promote PIP3 generation from the main precursor, PIP2. The increased production of PIP3 significantly promotes an interaction of PDK1 and Akt, resulting in the phosphorylation and activation of Akt (Guilherme et al. 1996; Alessi et al. 1997; Franke et al. 1997; Carver et al. 2000).
Akt/PKB is a serine/threonine protein kinase which contains three distinct isoforms: Akt1, Akt2 and Akt3 (Cohen, Jr. 2013; Yu et al. 2015b; Manning and Toker 2017). All three isoforms of Akt are expressed and show distinct functions in the heart (Yu et al. 2015b). Akt1 plays an important role in somatic cell growth and its deficiency significantly reduces the proliferation of cardiomyocytes and heart size (Matsui et al. 2001; Cohen, Jr. 2013; Yu et al. 2015b). Alternatively, Akt2 regulates glucose metabolism. Deficiency of Akt2 results in the development of severe insulin resistance and diabetes (DeBosch et al. 2006; Cohen, Jr. 2013). Akt3 is mainly expressed in the brain and is present at a lower level in the myocardium. However, cardiac specific overexpression of Akt3 results in maladaptive hypertrophy (Taniyama et al. 2005; Cohen, Jr. 2013).

At present, over 100 Akt downstream target substrates have been identified including transcriptional factors, metabolic enzymes, cell cycle regulators, protein kinases and many others (Manning and Cantley 2007; Manning and Toker 2017). Activation of PI3K/Akt signaling promotes cell proliferation, survival and growth by inhibiting the activity of pro-apoptotic factors and cell cycle inhibitors such as BAD, BIM, PUMA, MDM2, FOXO, P21CIP1, p27kip1 and others (Parcellier et al. 2008; Miyamoto et al. 2009; Abeyrathna and Su 2015a; Lin et al. 2015). PI3K/Akt signaling also contributes to cellular metabolic changes by directly or indirectly interacting with metabolic regulatory molecules, such as FOXO proteins, the mTOR signaling pathway, GSK-3β, glucose transporter 4 (GLUT4), PGC1a and PFK1 (Li et al. 2007; Miyamoto et al. 2009; Kim et al. 2015; Houddane et al. 2017).
Role of PI3K/Akt Signaling in Myocardial I/R Injury and Regeneration

Abundant evidence has suggested that the activation of PI3K/Akt signaling protects the heart from I/R induced injury and cardiac dysfunction by increasing cardiomyocyte survival and preventing overwhelming inflammatory responses (Howes et al. 2003; Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013). Mitochondrial dependent cardiomyocyte apoptosis contributes to myocardial I/R induced injury. Bcl2 family members, including anti-apoptotic factors (Bcl2, Bcl-w, Bcl-XL, Mcl-1 and others) and pro-apoptotic factors (Bax, Bak, Bad, Bim, Puma and others) belong to a group of proteins that play a critical role in the regulation of mitochondria mediated apoptosis (Gustafsson and Gottlieb 2008; Chiong et al. 2011). It has been reported the activity of pro-apoptotic factors such as Bad and Bim1 are tightly controlled by PI3K/Akt signaling through phospho-modification, and their phosphorylation prevents mitochondria mediated apoptosis (Murriel et al. 2004; Uchiyama et al. 2004; Lee et al. 2014). In addition, activation of PI3K/Akt signaling protects the myocardium from I/R induced injury by increasing mitochondrial fusion and elongation, promoting cell survival, proliferation, and reducing cardiomyocyte apoptosis or necrosis (Ong et al. 2015).

It has been shown that certain growth factors, i.e. insulin, TLRs ligands, and miRNAs, can protect the myocardium from I/R induced injury by positively upregulating PI3K/Akt signaling pathway (Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013). Our previous studies have shown that modulation of either TLR2, or TLR4 or TLR9 by their specific ligands induces protection against myocardial I/R injury through activation of PI3K/Akt signaling (Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al.
2013). Inhibition of either PI3K or Akt abolishes the cardioprotective effects of TLR modulation on I/R induced injury (Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013).

Activation of PI3K/Akt signaling also plays a central role in cardiac repair and regeneration following ischemic injury by inducing angiogenesis, cardiomyocyte proliferation, and cardiac progenitor cells activation (Haider et al. 2008; Meloni et al. 2010; Siragusa et al. 2010; Dharaneeeswaran et al. 2014; D’Uva et al. 2015; Lin et al. 2015; Li and Hirsch 2015). Angiogenesis is an essential process for restoring blood flow to the infarcted area, and serves as a key process to decrease cardiomyocyte apoptosis and necrosis, fibrotic scar expansion, as well as left ventricular dilation and heart failure. Furthermore, new blood vessel formation is also critical for successful cardiac regeneration (Siragusa et al. 2010; Dharaneeeswaran et al. 2014; Porrello and Olson 2014; Li and Hirsch 2015; Mandic et al. 2016; Oka et al. 2016). Activation of PI3K/Akt signaling plays a critical role in regulating angiogenesis by promoting endothelial or endothelial progenitor cell proliferation and migration, and by increasing the secretion of VEGF, IGF, HIF-1α and eNOS (Ma and Han 2005; Konoplyannikov et al. 2013; Abeyrathna and Su 2015b).

PI3K/Akt signaling is also involved in heart regeneration following myocardial ischemic injury by cross-talking with the Hippo-YAP signaling pathway. This crosstalk downregulates the expression of the cell cycle inhibitor p27kip1 and allows for cell cycle re-entry (Lin et al. 2015). Activation of PI3K/Akt signaling is also involved in therapeutic treatment of ischemic heart disease by bone marrow derived stem cells (Jackson et al. 2001; Kocher et al. 2001). Increased expression of Akt in bone marrow derived stem
cells significantly reduces infarct size, decreases fibrotic scar development, and restores impaired cardiac function following ischemic injury (Mangi et al. 2003).

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a highly-conserved phosphatase that is widely expressed in many type cells including neurons, immune cells, endothelial cells, fibroblasts and cardiomyocytes (Mocanu and Yellon 2007; Tamguney and Stokoe 2007; Worby and Dixon 2014). It is well known that PTEN is the most important negative regulator of PI3K/Akt signaling by dephosphorylating PIP3 to PIP2. Therefore, PTEN is recognized as an important switch in the balance between cell proliferation and differentiation, as well as cell survival and death (Mocanu and Yellon 2007; Tamguney and Stokoe 2007; Worby and Dixon 2014). Inactivation or inhibition of PTEN significantly attenuates the myocardial damage and cardiac dysfunction caused by I/R injury (Mocanu and Yellon 2007; Ruan et al. 2009; Keyes et al. 2010). However, PTEN mutation or long term inhibition will cause over activation of Akt and possible tumorigenesis and cardiac hypertrophy (Mocanu and Yellon 2007). Recent studies have shown that cardiac protective miRNAs, such as miR-21, miR-93, and miR-494 protect the myocardium from I/R injury by targeting PTEN (Roy et al. 2009; Wang et al. 2010; Ke et al. 2016).

Our previous studies have revealed cross talk between the TLR/NF-κB pathway and PI3K/Akt signaling (Ha et al. 2008; Ha et al. 2010; Cao et al. 2013) during myocardial I/R injury. TLR modulation significantly enhances the expression of miR-146a, miR-214 and miR-486 in the myocardium (Wang et al. 2013; Wang et al. 2016). Furthermore, PTEN has been identified as a direct target of miR-214 and miR-486. The results presented in this dissertation demonstrate that cardiac specific overexpression
of miR-214 significantly promotes Akt activation and protects the heart from I/R induced injury by targeting PTEN. Thus, this evidence suggests that microRNAs play key roles in TLR modulation induced cardiac protection through their targeting of PTEN and activation of PI3K/Akt signaling pathway.

**Hippo-YAP Signaling Pathway**

The Hippo-YAP pathway is an evolutionary conserved signaling pathway which regulates cell differentiation, proliferation, apoptosis and plays a critical role in controlling organ size (Zhao et al. 2011; Yu et al. 2015a; Meng et al. 2016; Wang et al. 2017). The key components in the Hippo-YAP signaling pathway were initially identified by genetic screening in *Drosophila* and were found to also be highly conserved in mammals (Meng et al. 2016). The Hippo-YAP signaling pathway includes core kinase complexes MST1/2 and LATS1/2 as well as adaptor proteins SAV1 and MOB1 (Hergovich and Hemmings 2009; Avruch et al. 2012; Liu et al. 2012; Meng et al. 2016). The activity of LATS1/2 kinases is regulated by MST1/2 Kinases and SAV1 complex through their phospho-modification. However, recent studies have revealed that AMPK and MAP4K family kinases also regulate LAST1/2 activation in parallel to MST1/2 kinases. YAP and TAZ are major downstream effectors of the Hippo-YAP signaling pathway. Activated LATS1/2 interacts with an adaptor protein MOB1 to further phosphorylate and inactivate the co-transcriptional factor YAP1/TAZ. This phosphorylation decreases YAP/TAZ transcriptional activity by promoting E3 ubiquitin ligase mediated degradation through a ubiquitin proteasome system or by promoting its interaction with protein 14-3-3 and cytoplasmic sequestration (Hergovich and
Hemmings 2009; Avruch et al. 2012; Liu et al. 2012; Meng et al. 2016). In addition, YAP/TAZ can also be phosphorylated by multiple protein kinases such as Src family tyrosine kinases, JNK and cyclin-dependent kinase 1 (CDK1) (Yang et al. 2013; Codelia et al. 2014).

**Figure 1.3 Hippo-YAP signaling pathway.** The MST1/2 activity will be regulated by multiple upstream signals including the GPCR, tight junction, growth factors, RASSF and NF2. MST1/2 Kinases and SAV1 complex phosphorylate and activate LATS1/2 and Mob1 which then phosphorylate YAP/TAZ. After phosphorylated, YAP/TAZ are sequestered in the cytoplasm with protein 14-3-3 or subjected to be ubiquitinated and degraded by β-TrCP. Unphosphorylated YAP/TAZ will translocate to the nucleus with TEADs to induce gene expression involving in the promotion of cell proliferation and inhibition of apoptosis.

When the core kinase complexes (MST1/2 and LATS1/2) are inactivated, YAP/TAZ will translocate into the nucleus and interact with multiple transcription factors, including TEADs, RUNX1/2, ERBB4, FOXO1, SMADs and others to regulate expression of various genes associated with cell proliferation, differentiation, survival, metabolism,
and migration (Komuro et al. 2003; Zhao et al. 2008; Shao et al. 2014; Grannas et al. 2015; Passaniti et al. 2017).

In addition, the Hippo-YAP signaling pathway can be regulated by multiple extracellular and intracellular signals, including cell polarization and adhesion, cell contact, G-protein couple receptors and growth-factor-triggered RTK signaling, cellular stress such as hypoxia, oxidative stress, and cellular metabolic stress (Yu et al. 2012; Aragona et al. 2013; Shao et al. 2014; Elbediwy et al. 2016). Furthermore, recent studies have reported that there is a cross talk between the Hippo-YAP signaling pathway and the PI3K/Akt/mTOR, WNT/β-catenin, Notch and MAPKs signaling pathways (Yu et al. 2012; Xu et al. 2014a; Lin et al. 2015; Chen et al. 2017; Kim et al. 2017; Luo 2017). In this dissertation, we provide compelling evidence that activation of TLR3 significantly stimulates YAP1 activation through cellular glycolytic metabolism.

Heart Regeneration

Heart attack caused by coronary artery disease (CAD), also known as ischemic heart disease remains the leading cause of morbidity and mortality in the United States (Benjamin et al. 2017). In general, most heart attacks (myocardial infarctions) are unpredictable and 25% of these patients will develop heart failure (Koudstaal et al. 2016). At present, advanced medical treatments can significantly increase the survival rates for patients with heart attack. However, the rate of heart failure results in these patients is not reduced. This is due to, at least in part, to the limited ability of the adult heart to regenerate and repair damaged heart tissue following heart attack. At present, there is no effective treatment for heart failure patients (Houyel et al. 2017). Heart transplantation is one approach for the treatment of heart failure, but the number of
suitable donors is very limited and the overall success rate is less than desired. Consequently, approaches focused on regenerating damaged heart tissue is an attractive and exciting potential therapeutic approach for heart failure patients. To date, a number of cardiac regenerative strategies has been developed which include stem cell based therapies, stimulation of endogenous cardiac progenitor cell differentiation, direct reprogramming of fibroblasts to functional cardiomyocytes, and induced proliferation of pre-existing cardiomyocytes (Senyo et al. 2014; Batty et al. 2016; Duelen and Sampaolesi 2017; Oh 2017).

Stem cell based therapies have shown benefits in patients with heart failure by enhancing regeneration and repair of damaged heart tissue and restoring cardiac function (Goichberg et al. 2014; Hou et al. 2016; Golpanian et al. 2016; Duelen and Sampaolesi 2017; Oh 2017). Several types of stem cells, including bone marrow derived mesenchymal stem cells, hematopoietic stem cells, endothelial progenitor cells, adult stem cells from adipose or heart tissue, embryonic stem cells and iPSCs have been employed for the clinical studies (Orlic et al. 2001; Beltrami et al. 2003; Murry et al. 2004; Jujo et al. 2008; Bu et al. 2009; Leri 2009; Loffredo et al. 2011; Schlueter and Brand 2012; Chow et al. 2013; Goichberg et al. 2014; Fisher et al. 2016; Golpanian et al. 2016; Duelen and Sampaolesi 2017). The underlying mechanisms of stem cell based therapies include their direct transdifferentiation into functional cardiomyocytes to replace damaged tissue. The stem cells can also secrete paracrine factors that promote angiogenesis, increase survival and proliferation of pre-existing cardiomyocytes, stimulate the differentiation of resident cardiac progenitor cells, and modulate immune response around damaged areas of heart tissue (Orlic et al. 2001; Beltrami et al. 2003;
Abundant studies have demonstrated that cardiac progenitor cells (CPCs) can directly differentiate into functional cardiomyocytes, thus allowing for the regeneration and repair of damaged tissue and functional recovery following ischemic injury (Beltrami et al. 2003; Bu et al. 2009; Leri 2009; Schlueter and Brand 2012; Goichberg et al. 2014). CPCs are a group of endogenous cardiac stem cells that are distributed throughout mammalian neonatal and adult hearts. CPCs based cardiac stem cell therapy for damaged heart tissue can overcome the multiple shortcomings that are present in therapies using extra-stem cells derived from other organs such as the adipose tissue, blood, and bone marrow. Intra-myocardial transplantation studies have shown that the CPCs have a higher protective effect and regenerative potential which can benefit cardiac repair and regeneration following myocardial ischemic injury. The potential mechanisms include directly transdifferentiation into functional cardiomyocytes and endothelial cells, and release of multiple immunomodulatory, anti-apoptotic and proangiogenic factors (Beltrami et al. 2003; Bu et al. 2009; Leri 2009; Schlueter and Brand 2012; Goichberg et al. 2014). Recently, several CPC populations have been isolated and identified based on their differentiation potential and the specific surface markers such as c-kit⁺, Islet-1⁺, SCA-1⁺, WT-1⁺, CDCs, and SP cells (Mayfield et al. 2014; Le and Chong 2016). However, the numbers of resident CPCs in adult heart are very limited compared with the numbers in the neonatal heart (Beltrami et al. 2003; Hesse et al. 2014; Le and Chong 2016). Unlike adult hearts, the hearts of neonatal mice
have a high regenerative capacity which can allow for complete regeneration and repair of damaged heart tissue. It is possible that the high regenerative capacity of neonatal hearts may due to the high levels of resident CPCs.

Direct reprogramming of fibroblasts into functional cardiomyocytes is another interesting regenerative strategy for replacing lost cardiomyocytes (Inagawa et al. 2012; Nam et al. 2013; Batty et al. 2016; Kurotsu et al. 2017). Ieda M et al has reported there are three cardiac specific transcription factors (TBX5, GATA4 and MEF2C) that can directly reprogram fibroblasts into cardiomyocyte-like cells (Ieda et al. 2010). Subsequent studies demonstrated that a new transcriptional factor (HAND2), inhibition of Notch signaling, or activation of Akt can dramatically accelerate the reprogramming process of fibroblasts into cardiomyocyte-like cells (Nam et al. 2013; Zhou et al. 2015; Abad et al. 2017). In addition, the combination of miR-1, miR-133, miR-208, and miR-499 has also been shown to initiate fibroblast reprogramming into functional cardiomyocytes (Jayawardena et al. 2012; Muraoka et al. 2014). Although stem cell based therapies have potential benefits to repair and regenerate the damaged heart tissue, they are still many limitations to their use in clinical practice.

It is well known that the adult mammalian cardiomyocytes are terminally differentiated cells that irreversibly lose their ability to proliferate (Poss et al. 2002; Laflamme and Murry 2011; Witman et al. 2011). This limitation explains why the adult mammalian heart fails to replace damaged heart tissue, and instead exhibits fibrotic scarring and hypertrophic remodeling in response to ischemic injury. Interestingly, recent evidence indicates that adult mammalian cardiomyocytes can be generated from pre-existing cardiomyocytes (Senyo et al. 2013; Ali et al. 2014). In a $^{14}$C based study, it has
been shown that the annual turnover rate of cardiomyocytes is about 1% at 25 years of age and 0.45% at 75 years of age (Bergmann et al. 2009). This evidence suggests that about 50% of adult human cardiomyocytes are exchanged at a low rate during a normal life span. However, the limited functional recovery in humans following myocardial injury suggests that the capacity of myocardial regeneration is nevertheless limited and fails to fully restore the normal cardiac function. This is likely due to the limited capability of cardiomyocytes to proliferate in the adult human heart following myocardial ischemic injury.

Although the regenerative capacity of mammalian adult heart is very limited, several studies have demonstrated that endogenous cardiac regenerative processes can be facilitated by multiple signaling molecules, including miRNAs, the activation of Hippo-YAP, PI3K/Akt and Wnt/beta-catenin signaling pathways (Buikema et al. 2013; Chen et al. 2013; Heallen et al. 2013; Mahmoud et al. 2013; Porrello et al. 2013; Xin et al. 2013; Lin et al. 2015).

**TLR Mediated Immune Response in Myocardial Repair and Regeneration**

TLR mediated inflammatory responses, triggered by danger associated molecular patterns (DAMPs) that are released upon myocardial I/R injury, play a detrimental role in extending ischemic myocardial injury, cardiac remodeling, and the progression of heart failure (Lange and Schreiner 1994; Kapadia et al. 1998; Thomas et al. 2003; Oyama et al. 2004; Kleinbongard et al. 2011; Ha et al. 2011). However, increasing evidence indicates that TLR mediated inflammatory response, activation and infiltration of immune cells have complex and critical roles in the tissue homeostasis, cardiac tissue repair and regeneration (Lin et al. 2012; Grote et al. 2013; Aurora et al.
2014; Carvalho et al. 2014; Kulkarni et al. 2014; Epelman et al. 2015; Frangogiannis
2015; Nelson et al. 2015; Tonkin et al. 2015; Leor et al. 2016; Natarajan et al. 2016;
Pandolfi et al. 2016; Vannella and Wynn 2017). The underlying cellular and molecular
mechanisms by which TLR-mediated signaling involved in cardiac repair and
regeneration include activation and recruitment of immune cells, elimination of damaged
cells, cytokine and growth factor production, activation of cardiac progenitor cells,
stimulation of angiogenesis, and promotion of pre-existing cardiomyocyte survival and
proliferation (Lin et al. 2012; Grote et al. 2013; Aurora et al. 2014; Carvalho et al. 2014;
Kulkarni et al. 2014; Epelman et al. 2015; Frangogiannis 2015; Nelson et al. 2015;
and Wynn 2017).

Unlike embryonic development, cardiac repair and regeneration can be initiated
and triggered by ischemic injury. Recent studies have shown that depletion of
macrophages decreased growth factor production (FGF, VEGF, TGF-β) and
angiogenesis, impaired fibroblast activation and fibrotic scar formation, and led to
severe reparative defects and heart rupture after myocardial ischemic injury (Aurora et
al. 2014; Epelman et al. 2015; Frangogiannis 2015; Tonkin et al. 2015; Leor et al.
2016). In neonatal mice, macrophages are recognized as necessary for heart
regeneration by stimulating preexisting cardiomyocyte proliferation and
neovascularization in responses to ischemic injury (Aurora et al. 2014; Frangogiannis
2015; Leor et al. 2016). In the neonatal heart, there are two different types of
macrophages: embryo-derived macrophages (MHC-IIlowCCR2−) and monocyte derived
macrophages (MHC-IIlowCCR2+) (Aurora et al. 2014; Lavine et al. 2014; Dutta et al.
2015; Frangogiannis 2015; Leor et al. 2016). Only MHC-II\textsuperscript{low}CCR2\textsuperscript{−} macrophages can selectively expand in sufficient amounts to promote neonatal cardiomyocyte proliferation following myocardial ischemic injury. However, in the injured adult heart the numbers of monocytes and MHC-II\textsuperscript{high}CCR2\textsuperscript{+} monocyte-derived macrophages are significantly increased, rather than MHC-II\textsuperscript{low}CCR2\textsuperscript{−} macrophages (Lavine et al. 2014; Dutta et al. 2015; Leor et al. 2016). This evidence suggests that immune response involved in heart repair and regeneration depend on multiple factors, including age, species, and immune cell types.

Consistent with the above observations, it has been reported that cardiomyocyte proliferation and heart regeneration are significantly inhibited by immunosuppressive compounds, such as dexamethasone treatment or downregulation of inflammatory cytokines, such as IL-6, IL1β, Ccl3 and Cxcl5 (Lavine et al. 2014; Han et al. 2015; O'Meara et al. 2015; Gay et al. 2016; Leor et al. 2016).

Since TLRs are major receptors for the induction of innate immune and inflammatory responses, TLR mediated signaling pathway could play an important role for cardiac repair and regeneration following ischemic injury. Recent studies have shown that TLR3 mediated immune signaling contributes to somatic cell nuclear reprogramming and de-differentiation (Lee et al. 2012; Sayed et al. 2015). In addition, dsRNA mediated TLR3 activation plays a critical role in skin regeneration (Nelson et al. 2015; Natarajan et al. 2016). However, the role of TLR3 in cardiac repair and regeneration has not been investigated. In this dissertation, we provide compelling evidence that TLR3 plays an important role in neonatal heart repair and regeneration following myocardial ischemic injury. The underlying cellular and molecular mechanisms
involve the promotion of glycolytic metabolism, the activation of Hippo-YAP signaling pathway, and the expression of microRNAs.

Glycolytic Metabolism in Heart Regeneration

Glycolysis is the ten step metabolic pathway that converts glucose into pyruvate, adenosine triphosphate (ATP), and reduced nicotinamide adenine dinucleotide (NADH) (Jones and Bianchi 2015). A surge of studies indicated that tumor cells or other rapidly proliferating cells exhibit high levels of glycolysis during proliferation, and suggested that glycolytic metabolism is vital for cell proliferation (Vander Heiden et al. 2009; Krisher and Prather 2012). Glycolytic metabolism provides the majority of chemical precursors required for the synthesis of nucleotides, amino acids and lipids which are necessary building blocks for cell growth and proliferation (Vander Heiden et al. 2009; Krisher and Prather 2012; Donnelly and Finlay 2015). In addition, glycolytic metabolism also plays crucial roles in regulating signal transduction and gene expression. It also influences epigenetic modifications by altering DNA methylation/demethylation and affects protein posttranslational modifications such as acetylation and glycosylation (Donohoe and Bultman 2012; Hirschey et al. 2015; Arts et al. 2016b; Jaworski et al. 2016).

It is well established that glycolysis is necessary for the reprogramming of somatic cells to induced pluripotent stem cells (Panopoulos et al. 2012; Zhang et al. 2012; Shyh-Chang et al. 2013). Moreover, metabolic rewiring is involved in immune cells activation, differentiation and dendritic cell maturation (Cheng et al. 2014; Everts et al. 2014; O’Neill 2014; Arts et al. 2016b). In addition, metabolic reprogramming is necessary for β-glucan induced innate immune memory which is termed as trained
immunity (Cheng et al. 2014; Arts et al. 2016a). Collectively, glycolytic metabolism is an important modulator for cell fate decision which is particularly important for tissue repair and regeneration.

Recent studies have suggested that glycolytic metabolism is tightly linked to multiple cellular signaling pathways associated with cell proliferation and differentiation. These signaling include the Hippo-YAP, PI3K/Akt/mTOR, Wnt/beta-catenin, and TLR signaling pathways (Everts et al. 2014; O'Neill 2014; Courtnay et al. 2015; Enzo et al. 2015; Mo et al. 2015; Karner and Long 2017). Aerobic glycolysis increases YAP/TAZ transcriptional activity. On the other hand, glucose starvation or the glycolysis inhibitor 2-DG induces cellular energy stress which increases AMPK dependent LATS activation and inhibits YAP/TAZ activation (DeRan et al. 2014; Enzo et al. 2015; Mo et al. 2015). In addition, administration of methylglyoxal, a metabolite of glycolysis, increases YAP activation and nuclear translocation by increasing Hsp90 glycation and LATS1 degradation (Nokin et al. 2016).

A hypoxic environment has been reported to be beneficial for promoting cardiomyocyte proliferation and heart regeneration. The mechanisms involve reducing oxidative induced DNA damage and cell cycle arrest. This observation suggests that glycolysis may be involved in hypoxia induced cardiomyocyte proliferation and heart regeneration (Kimura et al. 2015; Nakada et al. 2017). During neonatal heart development, glycolysis is the predominate metabolism for neonatal cardiomyocyte proliferation. However, mitochondrial fatty acid β-oxidation (FAO) and oxidative phosphorylation (OXPHOS) is the primary energy source for terminally differentiated mature cardiomyocytes (Lopaschuk et al. 1991; Kimura et al. 2015). This suggests that
cellular metabolism especially glycolytic metabolism may play an important role in cardiomyocyte proliferation and heart regeneration.

However, the role of glycolysis in neonatal cardiomyocyte proliferation and regeneration has not yet been defined. Importantly, we found that treatment of cardiomyocytes with TLR3 ligands promotes metabolic switching from oxidative phosphorylation to glycolysis. In this dissertation, we demonstrated that TLR3 is essential for neonatal heart regeneration and explained the underlying mechanisms involving glycolytic metabolism, TLR3 mediated YAP1 activation, miRNA expression and cardiomyocytes proliferation.

**MicroRNAs and Heart Regeneration**

Growing evidences suggest that microRNAs play an important role in embryonic and post-natal heart development and maturation (Callis and Wang 2008; Wang 2010; Yan and Jiao 2016). Specific knockout of miRNAs processing enzymes (Dicer or DGCR8) in cardiac progenitor cells leads to heart failure and embryonic lethal, suggesting that miRNAs are necessary for cardiac embryonic development (Chen et al. 2008; Chapnik et al. 2012). MiR-1 and miR-133 are muscle specific miRNAs and are expressed at high levels in cardiomyocytes (Liu et al. 2007). MiR-1 serves as an important regulator for heart development and its deficiency leads to embryonic lethality due to stunted cardiomyocyte maturation, pericardial edema and cardiac dysfunction (Sayed et al. 2007; Zhao et al. 2007; Callis and Wang 2008; Wystub et al. 2013). In contrast, cardiac specific expression of miR-1 results in heart development arrest (Zhao et al. 2007; Callis and Wang 2008; Wystub et al. 2013). MiR-133 also plays an
important role in the heart development and maturation by regulating cardiomyocyte proliferation and maintaining cardiac progenitor cells (Wystub et al. 2013; Izarra et al. 2014). Collectively, these studies suggest that miRNAs are critical regulators of heart development and postnatal maturation.

Importantly, miRNAs are not only involved in the progression of heart development, but also served as important regulators in cardiac regeneration. For example, Inhibition of miR-34a improves the cardiac regeneration after ischemic injury by targeting BCL2, CyclinD1 and Sirt1, leading to cardiomyocyte proliferation and survival (Yang et al. 2015). The tumor suppressor miRNAs (miR-15 and miR-195) are highly upregulated during heart development and have been shown to be important for postnatal cardiomyocyte cell cycle arrest (Porrello et al. 2011a; Porrello et al. 2013). Overexpression of miR-195 leads to premature cell cycle arrest in the developing heart and results in ventricular septal defects (Porrello et al. 2011a). In contrast, inhibition of both miRs significantly promotes cardiomyocyte proliferation and heart regeneration (Porrello et al. 2011a; Porrello et al. 2013). Overexpression of miR-590-3P and miR-199-3p has been reported to significantly induce cardiomyocyte proliferation and heart regeneration in adult mice (Eulalio et al. 2012). In addition, cardiac specific overexpression of miR-17-92 protects heart from myocardial ischemic injury and significantly promotes cardiomyocyte proliferation in adult hearts (Chen et al. 2013). MiR-302-367 cluster is highly expressed during early cardiac development and also contributes to adult cardiomyocyte dedifferentiation and proliferation by targeting several components of Hippo-YAP signaling pathway, MST1, LATS2 and MOB1 (Tian et al. 2015).
These observations indicate that the delivery of miRNAs into damaged heart tissue or endogenous modulation of specific miRNAs expression could be a useful approach to stimulate cardiomyocyte proliferation, cardiac regeneration and heart functional recovery following ischemic injury.

**Hippo-YAP Signaling Pathway and Heart Regeneration**

The Hippo-YAP signaling pathway is highly conserved between *Drosophila* and mammals and plays an important role in controlling organ size and tissue homeostasis (Zhao et al. 2011; Yu et al. 2015a). Mutation of the Hippo-YAP signaling pathway kinases (HPO and WTS), and upstream regulators (mer, kibra, ft. ets) or Yki overexpression results in overgrowth of wings, eyes and other appendages in *Drosophila* (Justice et al. 1995; Wu et al. 2003; Lai et al. 2005; Harvey et al. 2003; Zhao et al. 2011; Yu et al. 2015a). In mammals, liver specific knockout of Hippo-YAP pathway kinases or related adaptor factors (Mob1, Sav1, MST1/2, or LATS1/2), or tissue specific YAP1 overexpression leads to liver enlargement and liver tumor development by increasing cell proliferation and reducing apoptosis (Lai et al. 2005; Song et al. 2010; Zhao et al. 2011; Yimlamai et al. 2014; Yu et al. 2015a; Patel et al. 2017).

Embryonic deletion of SAV1, MST1/2, or LATS1/2 or YAP1 overexpression leads to cardiomyocyte hyper-proliferation and heart enlargement (Xin et al. 2011; Zhao et al. 2011; Heallen et al. 2013; Yu et al. 2015a). In contrast, heart specific YAP1 or YAP1/TAZ deletion results in heart hypoplasia. In adult mouse hearts, high levels of YAP1 activation could enhance cardiomyocyte proliferation and heart regeneration following heart ischemic injury (Xin et al. 2011; Zhao et al. 2011; Heallen et al. 2013; Xin et al. 2013; Lin et al. 2015; Yu et al. 2015a). The role of Hippo-YAP signaling in
cardiomyocyte proliferation and heart regeneration has been clearly established, but the mechanisms by which YAP/TAZ regulate cardiomyocyte proliferation has not yet been completely defined. It is well established that miRNAs play a critical role in myocardial/reperfusion injury by regulating cellular proliferation, migration, differentiation, survival, apoptosis, as well as tissue remodeling, inflammatory responses, angiogenesis, fibrosis as we mentioned before. Recent evidence has shown that miRNAs also play an important role in neonatal and adult myocardial regeneration by regulating cardiomyocyte proliferation. In this dissertation, we defined that miR-152 expression was significantly increased following TLR3 ligand (Poly I:C administration) through a YAP1 dependent mechanism. We demonstrated that miR-152 is an important potential target in TLR3 mediated YAP1 mediated cardiomyocyte proliferation and heart regeneration.

Questions to be Answered in this Dissertation

As mentioned in the introduction section, the TLR mediated NF-κB signaling pathway plays a deleterious role in myocardial I/R injury (Chao 2009; Ha et al. 2011). Our previous studies demonstrated that deficiency of either TLR3 or TLR4 significantly protects the myocardium from acute I/R induced injury by decreasing acute inflammatory response and increasing activation of PI3K/Akt signaling (Hua et al. 2007; Lu et al. 2014). Interestingly, we and others also found that modulation of TLRs by their specific ligands, such as Pam3CSK4 for TLR2, Poly I:C for TLR3 and LPS for TLR4 resulted in the protection against I/R induced myocardial injury (Ha et al. 2008; Ha et al. 2010; Cao et al. 2013). Mechanistic studies suggest that modulation of TLRs
significantly decreases acute inflammatory response and promotes activation of PI3K/Akt signaling in the myocardium subjected to I/R injury (Ha et al. 2008; Ha et al. 2010; Cao et al. 2013). The question is, why does a deficiency or activation of specific TLRs (TLR2, TLR3, and TLR4) result in a similar protective effect on the myocardium in response to I/R challenge? As we mentioned above, miRNAs are the fine tuners of TLR signaling pathway (O'Neill et al. 2011). We demonstrated that treatment with TLR2 ligand, Pam3CSK4, increases the expression of miR-146a serves as a negative regulator for TLR mediated pathway by targeting IRAK1 and TRAF6 (Wang et al. 2013). Indeed, delivery of lentivirus expressing miR-146a into the myocardium significantly attenuates I/R-induced NF-κB activation by targeting IRAK1 and TRAF6 and thus deceasing inflammatory cytokine production and myocardial injury (Wang et al. 2013). These results have answered the question of why TLR modulation attenuates I/R injury trigged by acute inflammatory responses and cytokine production.

Our previous studies also showed that the cardioprotective effect of TLR modulation is mediated by activation of the PI3K/Akt signaling pathway. To address how modulation of TLRs activates PI3K/Akt signaling, we examined several miRNAs and observed that TLR2 modulation significantly enhances the expression of miR-214 and miR-486 (Wang et al. 2016). Both miR-214 and miR-486 directly target PTEN, leading to an activation of the PI3K/Akt signaling pathway. In this dissertation, we provide compelling evidence to show that miR-214 plays an important role in mediating the crosstalk between the TLR/NF-κB and PI3K/Akt signaling pathways during myocardial I/R challenge. We also demonstrated that increased expression of miR-214 activates PI3K/Akt signaling pathway in the myocardium and protects it from I/R induced injury. In
addition, we found that miR-125b also serves as a protective effect in I/R induced myocardial injury. It directly targets TRAF6 and TNF-α and therefore downregulates the TLR mediated NF-κB pathway. In addition, increased expression of miR-125b markedly reduces the expression of the tumor suppressor protein p53 and mitochondrial related pro-apoptotic protein BAK1 in the myocardium following myocardial I/R injury.

Heart failure is the leading cause of death in the United States and throughout the world (Benjamin et al. 2017). At present, there is no effective treatment approaches for heart failure patients (Houyel et al. 2017). In our previous studies, we found that there is a crosstalk between the TLR/NF-κB and PI3K/Akt signaling pathways during myocardium I/R injury. As mentioned in the introduction, activation of TLRs and PI3K/Akt signaling contribute to the activation of Hippo-YAP signaling pathway which plays a critical role in cardiomyocyte proliferation and heart regeneration. In addition, TLR3 mediated innate immune responses play a fundamental role in cell reprogramming and skin regeneration (Lin et al. 2012; Nelson et al. 2015). However, the role of TLR3 in heart regeneration has not been investigated. Therefore, we explored the role of TLR3 in neonatal heart repair and regeneration following myocardial infarction (MI). Unlike adult hearts, neonatal mouse hearts have a high regenerative capacity (Porrello et al. 2011b). Therefore, we employed one day old neonatal mice as our heart regeneration model and determined whether TLR3 is necessary for neonatal heart regeneration and functional recovery following myocardial infarction. In this dissertation, we demonstrated that TLR3 is required for neonatal heart repair and regeneration after myocardial ischemic injury. We then investigated the mechanisms by
which TLR3 is an essential for neonatal heart repair and regeneration following myocardial infarction.

Glycolysis is predominant metabolism for energy production in zebrafish and neonatal hearts and is a necessary for somatic cell reprogramming and dedifferentiation (Lopaschuk et al. 1991; Zhang et al. 2012). Recent studies have reported that TLR3 ligand, Poly I:C, promotes the metabolic reprogramming from oxidative phosphorylation to glycolysis (Cheng et al. 2014; Everts et al. 2014; O'Neill 2014). We investigated the role of TLR3 mediated glycolysis increasing in neonatal cardiomyocyte proliferation and heart regeneration.

As mentioned in the introduction, YAP1 is an important downstream effector of the Hippo signaling pathway and plays a critical role in cardiomyocyte proliferation and heart regeneration (von et al. 2012; Xin et al. 2013; Lin et al. 2015). In this dissertation, we found that stimulation of TLR3 by its ligand significantly increases YAP1 activation and its nuclear translocation. YAP1 is necessary for TLR3 induced cardiomyocytes proliferation. In addition, we demonstrated that TLR3 modulation enhanced YAP1 activation is mediated by glycolytic dependent mechanism.

Several research groups have report the role of YAP1 in cardiomyocyte proliferation (von et al. 2012; Xin et al. 2013; Lin et al. 2015). However, the mechanism by which YAP1 activation regulates cardiomyocyte proliferation and heart regeneration remains elusive. YAP1 is a con-transcriptional factor that interacts with its DNA binding partner TEAD and regulates miRNA biogenesis and expression (Chaulk et al. 2014). Interestingly, we observed that stimulation of TLR3 significantly increases the
expression of miR-152 which is positively correlated with the YAP1 activation. We then addressed several important questions: what is the role of YAP1 in TLR3 mediated miR-152 expression? Does miR-152 contribute to TLR3 mediated YAP1 dependent cardiomyocyte proliferation? What is the mechanism by which miR-152 regulates cardiomyocyte proliferation? The results presented in this dissertation have answered the above questions.

Overall, the studies in this dissertation focus on the role of TLRs, microRNAs, and the PI3K/Akt and Hippo-YAP signaling pathways in myocardial I/R injury and neonatal heart repair and regeneration following myocardial infarction. We provide compelling evidence to demonstrate that miRNAs and PI3K/Akt, as well as Hippo-Yap signaling pathway, regulate innate immune and inflammatory responses, cell survival and proliferation. This could provide novel strategies for therapeutic treatments in patients with cardiac ischemic injury and heart failure.
CHAPTER 2
MICRORNA-125B PROTECTS AGAINST MYOCARDIAL ISCHEMIA/REPERFUSION INJURY VIA TARGETING P53-MEDIATED APOPTOTIC SIGNALING AND TRAF6

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Running head: Attenuation of myocardial ischemic injury by microRNA-125b

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Introduction

It has well been documented that activation of NF-κB mediated by Toll-like receptor/interleukin-1 receptor (TLR/IL-1R) contributes to myocardial ischemia/reperfusion (I/R) injury(1-4). Inhibition of NF-κB binding activity has been shown to protect against myocardial I/R injury(2-6). The protective effects involve inhibition of innate immune and inflammatory responses and attenuated I/R-induced cardiac myocyte apoptosis(2-6). However, the mechanisms by which inhibition of NF-κB binding activity decreases innate immune and inflammatory responses and attenuates cardiac myocyte apoptosis during myocardial I/R are still unclear.

Recent studies have demonstrated that activation of NF-κB regulates microRNA expression which, in turn, negatively regulate NF-κB binding activity(7, 8), thereby decreases in the innate immune and inflammatory responses(9-11). MicroRNAs (miRs) are 21 to 23 nucleotide non-coding RNA molecules and have been identified as novel regulators of gene expression at the post-transcriptional level by binding to target messenger RNAs(9-14). MicroRNAs have been demonstrated to play a critical role in the negative regulation of innate immune and inflammatory responses by regulation of NF-κB binding activity(9-11). Importantly, recent studies have shown that NF-κB activation regulates the expression of microRNAs(7, 8), including miR-146, miR-155 and miR-21, etc., while these miRs, in turn, down regulate NF-κB binding activity. miR-21 has been demonstrated to play a protective role in myocardial I/R injury(15). We have reported that increased expression of miR-146a significantly decreases myocardial infarct size and attenuates I/R-induced cardiac dysfunction via down-regulation of NF-κB activation by targeting IRAK1 and TRAF6(16). Collectively, the data
suggest that the miRs that regulate TLR/IL-R1 mediated NF-κB activation may be a new approach for management and treatment of myocardial I/R injury.

MiR-125b is a homolog of lin-4, which is the first miR discovered and an important regulator of developmental timing in C. elegans(17). Recent studies have shown that activation of NF-κB decreases the expression of miR-125b(18, 19). Tili et al reported that treatment of Raw 264.7 cell with LPS, a TLR4 ligand, suppresses the expression of miR-125b(18), while miR-125b suppresses TNF-α expression by targeting the 3'-untranslated region of TNF-α mRNA(18, 19). MiR-125b has been reported to play a role in down-regulation of apoptosis by repressing p53 and Bak-1(20, 21). p53 is a tumor suppressor protein which plays a critical role in regulating cell cycle and apoptosis in response to hypoxia and ischemic stress(22, 23). Inhibition of p53-mediated apoptotic signaling significantly reduces I/R-induced myocardial injury(24). We have reported that increased expression of miR-125b in macrophages attenuates hypoxia/reoxygenation induced cell injury(25). However, whether miR-125b serves as a protective role in myocardial I/R injury in vivo has not been investigated. miR-125b has been shown to target TNFα(26) and inhibit p53-mediated apoptotic signaling(27), therefore it is possible that miR-125b serves a protective role in myocardial I/R injury.

In the present study, we examined the role of miR-125b in myocardial I/R injury. We observed that increased expression of miR-125b in the myocardium significantly decreases myocardial infarct size and prevents I/R-induced cardiac dysfunction. The
mechanisms involve the inhibition of I/R-induced activation of NF-κB and the prevention of I/R-activated p53 mediated apoptotic signaling in the myocardium.

Materials and Methods

Animals
Male wild type (WT) C57BL/6J mice were obtained from Jackson Laboratory. The experiments outlined in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). The animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

gPCR assay of microRNAs (miRs)
miRs were isolated using the mirVanaTM miR isolation kit (Ambion)(16, 25)

Construction of miR-125b into lentivirus expressing system
MiR-125b, mature sequence mmu-miR-125b-5p (MIMAT0000136), was constructed into lentivirus expression vector using a lentivirus expressing system (Invitrogen corporation) as described previously(16, 25) (Supplement Data, Methods section).

Transgenic mice
Transgenic mice (Tg) with overexpression of miR-125b were developed with C57BL/6J background (Supplemental data, Methods section).
**In vitro experiments**

The H9C2 rat cardiomyoblasts were obtained from the American Type Culture Collection (Rockville, MD) and was maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented 10% fetal bovine serum (FBS) under 5% CO$_2$ at 37°C(31). The cells were plated in 6 well plates at 1 x 10$^5$ cells/well. The cells were transfected with lentivirus expressing miR-125b (LmiR-125b) or lentivirus expressing vector that served as control (LmiR-con). The lentivirus expressing vector contains a non-sense miR sequence that allows formation of a pre-miRNA hairpin predicated not to target any known vertebrate gene (Invitrogen Corporation). Stably transfected cells were selected using a Blasticidin resistant marker. The cells were subjected to hypoxia for 2 h followed by reoxygenation (H/R)(25) for 24 h. The cells that were not subjected to H/R served as control (normoxia). There were 3 independent experiments in each group. The cells were harvested at 24 h for isolation of cellular protein.

In separate experiments, adult cardiac myocytes were isolated from 9 male mice as described previously(28). The cells were transfected with miR-125b, miR-scrambled control (miR-con) or anti-miR-125b, respectively carried by exosomes that were isolated from bone marrow stromal cells (BMSCs)(29) (Supplement data, Methods section). The cardiac myocytes were subjected to hypoxia (2 hrs) followed by reoxygenation for 24 hrs. Cardiac myocytes were harvested for analysis of the effect of miR-125b on H/R-induced cardiac myocyte injury.
**microRNA microarray**

Cardiac myocytes were isolated from 3 adult mice (28) and subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs) (Supplement data). The cells were harvested and total RNA was isolated for microRNA microarray analysis (Supplement data, Methods section).

**In vivo transfection of lentivirus expressing miR-125b (LmiR-125b) into mouse hearts**

Lentivirus expressing miR-125 or miR-125b mimics were delivered into the myocardium of mice as described previously (16, 30) (Supplemental data, Methods section).

**Induction of myocardial I/R injury**

Myocardial I/R injury was induced seven days after transfection of LmiR-125b or Lmi-con as described previously (2, 3, 31) (Supplemental data, Methods section).

**In situ apoptosis assay**

Myocardial apoptosis was examined as described previously (2, 3, 31, 32) using the *in situ* cell death detection kit (Roche, USA). Three slides from each block were evaluated for percentage of apoptotic cells and four fields on each slide were examined at the border areas using a defined rectangular field area with 20x magnification. A total of 100 nuclei were accounted. Numbers of apoptotic cardiac myocytes are presented as the percentage of total cells counted.
Measurement of cell viability and LDH activity

Cell viability was assessed by measuring mitochondrial dehydrogenase activity using the MTT assay kit (Sigma). Cell injury was assessed by measurement of lactate dehydrogenase (LDH) activity in culture medium using a commercial kit (Cytotoxicity Detection Kit (Sigma)).

Western Blot

Western blot was performed as described previously(2, 3, 31). The primary antibodies (anti-Fas, anti-p-53, anti-Bax, Bak-1, and TRAF6) and peroxidase-conjugated secondary antibody were purchased from Cell Signaling Technology, Inc. The signals were quantified using the G:Box gel imaging system by Syngene (Syngene, USA, Fredrick, MD).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated from heart samples as previously described(2, 3, 31). NF-κB binding activity was measured using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA).

Caspase-activity

Caspase-3/7 and -8 activities in heart tissues were measured as described previously(33) using a Caspase-Glo assay kit (Promega).
ELISA for cytokine assay

The levels of cytokines (TNFα and IL-1β) were measured by ELISA using OptEIA cytokine kits according to instructions provided by the manufacture (BD Biosciences).

Infiltration of neutrophils into the myocardium

Neutrophil accumulation in heart tissues was examined by staining with anti-neutrophil marker antibody (NIMP-R14, Santa Cruz Biotechnology) as described previously (34) (Supplemental data, Methods section).

Statistical analysis

The data is expressed as mean ± SD. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey’s procedure for multiple-range tests was performed. P< 0.05 was considered to be significant.

Results

Hypoxia/reoxygenation decreased miR-125b expression via NF-κB activation in H9C2 cardiomyoblasts.

We examined the effect of hypoxia/reoxygenation (H/R) on the expression of miR-125b in H9C2 cardiomyoblasts. As shown in Figure 2.1A, hypoxia (2 hrs) followed by reoxygenation (24 hrs) resulted in decreases in the levels of miR-125b by 33% compared with the normoxic control. To investigate whether miR-125b expression is regulated by NF-κB activation, we measured NF-κB binding activity following H/R. 

Figure 2.1B shows that H/R induced increases in NF-κB binding activity by 27%.
compared with the normoxic control. Treatment of the cells with an antioxidant, pyrrolidine dithiocarbamate (PDTC), which has been shown to inhibit NF-κB activation (35), significantly increases the expression of miR-125b in normoxic control and in H/R cells, when compared with untreated normoxia and H/R group, respectively. Administration of PDTC also significantly decreased NF-κB binding activity following H/R.

Figure 2. 1  H/R decreases miR-125b expression and increased NF-κB binding activity in H9C2 cells. H9C2 cells were treated with or without PDTC 15 min prior to hypoxia (2 h) followed by reoxygenation (24 h). Cells were harvested. miRs were isolated from harvested cells and miR-125b expression was examined by quantitative polymerase chain reaction (qPCR). Nuclear proteins were isolated for analysis of NF-κB-binding activity. H/R decreases the expression of miR-125b (A, $P = 0.001$) and increases NF-κB-binding activity (B, $P < 0.001$). LPS treatment decreases the expression of miR-125b (C, $P = 0.006$). H9C2 cells were treated with PDTC or NAC 15 min before LPS stimulation (24 h). The levels of miR-125b were measured by qPCR. There were three independent experiments in each group. *$P < 0.05$ compared with indicated groups.
Figure 2.1C shows that LPS stimulation significantly induced decreases in miR-125b expression in H9C2 cells. However, treatment of the cells with antioxidants, PDTC or N-acetyl cysteine (NAC), prevented LPS-mediated suppression of miR-125b expression. Collectively, the data suggests that miR-125b expression during H/R is regulated by NF-κB activation.

We also analyzed the effect of H/R on microRNA expression in adult cardiac myocytes. MicroRNA array showed that 43 miRNAs were differentially expressed in the cardiac myocytes after H/R at a false discovery rate of 0.05, when compared with non-H/R cells (normoxia) (supplemental data, Tables 1 and 2).

Increased expression of miR-125b attenuated hypoxia/reoxygenation-induced cell injury in H9C2 cardiomyoblasts.

To determine whether miR-125b plays a role in the protection against H/R-induced cell injury, we generated stably transfected H9C2 cells with LmiR-125b or LmiR-control (LmiR-Con). The stably transfected cells were subjected to hypoxia (2 h) followed by reoxygenation (24 h). Untransfected H9C2 cells served as control. As shown in Figure 2.2A, transfection of H9C2 cells with LmiR-125b significantly increased the levels of miR-125b in the cells. Figure 2.2B shows that H/R induced increases in LDH activity by 5.0 fold compared with non-H/R (normoxia) groups. However, H/R-induced LDH activity in H9C2 cells was significantly attenuated by LmiR-125b transfection. LmiR-Con transfection did not affect H/R-induced LDH activity in H9C2 cells. Figure 2.2C shows that H/R markedly decreased cell viability (51%) compared with untreated normoxic cells. However, H/R-induced decrease in cell viability was significantly attenuated by
LmiR-125b transfection when compared with untreated H/R cells. Transfection of LmiR-con did not alter H/R-decreased cell viability.

Similarly, increased expression of miR-125b in isolated adult cardiac myocytes significantly attenuated H/R-induced cell injury (Figure 2.2D, E and F). In addition, inhibition of miR-125b expression in adult cardiac myocytes increased the susceptible to H/R-induced cell injury (Figures 2.2D, E and F).

We also examined the effect of LmiR-125b transfection on H/R-induced caspase-3 and -8 activities. Figure 2.2G shows that H/R induced caspase-3/7 activity by 79.8% and caspase-8 by 44.2%, when compared with normoxic cells. However, increased expression of miR-125b markedly attenuated H/R-increased caspase-3/7 and -8 activities. There was no significant difference in caspase-3/7 and -8 activities between LmiR-Con H/R cells and untreated H/R cells. The data suggests that increased expression of miR-125b plays a protective role in H/R-induced cellular injury.
Figure 2.2. Increased expression of miR-125b attenuates H/R-induced cell injury and cell death in H9C2 cardiomyoblasts and adult cardiac myocytes. Stably transfected H9C2 cells with LmiR-125b or LmiR-con were subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs). H9C2 cells that were not transfected served as control. (A) Increased levels of miR-125b after transfection of cells with LmiR-125b. Overexpression of miR-125b decreased LDH activity (B, p=<0.001), increased cell viability (C, p=<0.001), and attenuated caspase-3/7 and caspase-8 activities (G, p=0.003) following H/R. There were 3 independent experiments in each group. * p<0.05 compared with indicated groups. # p<0.05 compared with control H/R group. Cardiac myocytes were isolated from adult male mice and transfected with miR-125b mimics, anti-miR-125b mimics, or miR-scrambled control (miR-con) that were carried by BMSC-derived exosomes, respectively. Untransfected cells served as control. Twenty-four hrs after transfection, the cells were subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs). (D) Transfection of cardiac myocytes with BMSC-derived exosomes that loaded with miR-125b mimics significantly increased the levels of miR-125b in cardiac myocytes. Increased expression of miR-125b attenuated H/R-increased LDH activity (E) and H/R-decreased cell viability (F). Inhibition of miR-125b expression by transfection of anti-miR-125b mimics increased the susceptibility to H/R-induced cell injury (E) and H/R-decreased cell viability (F). There were 3 independent experiments in each group. * p <0.05 compared with indicated groups. # p<0.05 compared with control H/R group.
MiR-125b suppresses p53 and Bak-1 expression in H9C2 cardiomyoblasts.

To understand the mechanisms by which overexpression of miR-125b attenuated H/R-induced caspase-3/7 and -8 activities, we examined the effect of miR-125b on p53 and Bak-1 expression in H9C2 cardiomyoblasts in the presence and absence of H/R.
Figure 2.3. Increased expression of miR-125b decreased p53 and Bak-1 expression in H9C2 cells and adult cardiac myocytes. LmiR-125b or LmiR-con stably transfected H9C2 cells were subjected to hypoxia (2 h) followed by reoxygenation (24 h). Untransfected H9C2 cells served as control. Overexpression of miR125b suppresses the expression of p53 (A, p=<0.001) and Bak-1 (B, p=<0.001) expression. (C, p=0.003) The supernatants were harvested for analysis of TNFα. There were 3 independent experiments in each group. * p<0.05 compared with indicated groups. #, & p<0.05 compared with normoxic groups.

Cardiac myocytes were isolated from adult male mice and transfected with miR-125b mimics, anti-miR-125b mimics, or miR-scrambled controls (miR-con) that were carried by BMSC-derived exosomes, respectively. Untransfected cells served as control. Twenty-four hrs after transfection, the cells were subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs). Increased expression of miR-125b inhibited expression of p53 (C) and Bak1 (D) in the presence and absence of H/R. Inhibition of miR-125b expression by transfection of anti-miR-125b mimics increased the expression of p53 (C) and Bak1 (D) in the presence and absence of H/R. There were 3 independent experiments in each group. * p< 0.05 compared with indicated groups. # p <0.05 compared with non-transfected control groups.

Figure 2.3 shows that the levels of p53 (A) and Bak-1 (B) were markedly lower in LmiR-125b transfected normoxic cells than in normoxic control cells. H/R increased the levels of p53 (64.7%) and Bak-1 (91.3%) compared with normoxic control cells. The levels of p53 and Bak-1 in LmiR-125b transfected cells were also increased following H/R stimulation. However, the levels of p53 and Bak-1 in LmiR-125b H/R cells were significantly lower by 49.3% and 42.5% compared with untransfected H/R cells and
were comparable with normoxic control group. LmiR-Con transfection did not alter H/R-induced increases in p53 and Bak-1 expression.

Increased expression of miR-125b in isolated adult cardiac myocytes inhibited H/R-increased expression of p53 and Bak1. In contrast, inhibition of miR-125b expression in cardiac myocytes increased expression of p53 and Bak1 expression (Figure 2.3C and D).

H/R also induced increases in TNFα production in the cardiomyoblasts compared with normoxic control (Figure 2.3E). TNFα interacts with TNF receptors (TNFRs), resulting in activation of extrinsic apoptotic signaling(37). However, increased expression of miR125b prevents H/R-induced increases in TNFα production (Figure 2.3C).

In vivo increased expression of miR-125b decreased infarct size and improved cardiac function following myocardial I/R.

To examine whether increased expression of miR-125b will induce protection against myocardial I/R injury, we transfected mouse hearts with LmiR-125b or LmiR-Con before the hearts were subjected to I/R. Figure 2.4A shows that the green fluorescent protein (GFP) that is carried by LmiR-125b or LmiR-Con is mainly expressed in the myocardium. qPCR data showed the levels of miR-125b in LmiR-125b transfected hearts were significantly increased by 8.2 fold compared with LmiR-Con transfected hearts (Fig. 2.4B). Figure 2.4C shows that I/R induced significant injury as denoted by infarct size in untreated hearts. In contrast, infarct size was significantly reduced (60%) following I/R in LmiR-125b transfected mice compared with the
untreated I/R group. LmiR-Con transfection did not alter I/R-induced myocardial infarct size.

Figure 2.4. LmiR-125b transfection protects the myocardium from I/R injury. Mouse hearts were transfected with either LmiR-125b or LmiR-con, respectively. Seven days after transfection, hearts were harvested and sectioned. (A) Green fluorescent protein expression was viewed using a fluorescent microscope (Green) and GFP
expression was confirmed by staining with anti-GFP antibody (Red). (B) Increased expression of miR-125b in the myocardium 7 days after LmiR-125b transfection. (C) Increased expression of miR125b by transfection of LmiR-125b for 7 days reduced myocardial infarct size (p=<0.001). The infarct area (white) and the area at risk (red + white) from each section were measured using an image analyzer. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and are presented in the graphs. Photographs of representative heart sections are shown above. (D) Increased expression of miR-125b by transfection of LmiR-125b for 7 days attenuated I/R-induced cardiac dysfunction (p=<0.001). Cardiac function was examined by echocardiography before (Baseline), 3 and 7 days after I/R. There were 6-8 mice in each group. * p<0.05 compared with indicated groups.

We also examined the effect of increased expression of miR-125b on cardiac function following myocardial I/R. As shown in Figure 2.4D, ejection fraction (EF%) and fractional shortening (%FS) in untreated I/R hearts were significantly reduced by 39.6% and 44.6% on day 3 and by 26.8% and 32.4% on day 7 after myocardial I/R compared with baseline. However, I/R-induced cardiac dysfunction was prevented by LmiR-125b transfection. EF% and %FS values in LmiR-125b transfected mice were not significantly decreased at 3 and 7 days after myocardial I/R compared with LmiR-125b baseline. Transfection of miR-Con did not alter I/R-induced cardiac dysfunction.

I/R-induced myocardial apoptosis was attenuated by LmiR-125b transfection.

Cardiac myocyte apoptosis contributes to myocardial I/R injury(38). We examined the effect of increased expression of miR-125b on I/R-induced myocardial apoptosis. Figure 2.5A shows that I/R markedly induced myocardial apoptosis compared with sham control. In LmiR-125b transfected mice, I/R-induced myocardial apoptosis was significantly attenuated, when compared with the untreated I/R group (12.0 ± 2.87% vs. 28.5 ± 1.72%). LmiR-Con transfection did not affect I/R-induced myocardial apoptosis.
Figure 2.5B shows that I/R increased caspase-3/7 (31.4%) and caspase-8 (40.5%) activities in the myocardium compared with sham control. However, increased expression of miR-125b prevented I/R-induced caspase-3/7 and -8 activities compared with the I/R group. There was no significant difference in caspase-3/7 and -8 activities between LmiR-Con I/R mice and the untreated I/R group.
Figure 2.5 Transfection of LmiR-125b attenuates I/R-induced myocardial apoptosis. Mice were transfected with LmiR-125b or LmiR-control for 7 days before the hearts were subjected to myocardial ischemia (45 min) followed by reperfusion (4 hrs). (A) Myocardial apoptosis were examined by the TUNEL assay in the heart sections. DAPI stains nucleus (blue color) and TUNEL positive cells show green fluorescence. The bar graph shows the percent apoptotic cells (p<0.001). (B) Increased expression of miR-125b attenuated I/R-induced caspase-3/7 and -8 activities in the myocardium (p=0.002). (C) Increased expression of miR-125b prevents I/R-increased p53, Bak-1, Bax, and FasL levels in the myocardium (p<0.001). There were 5 mice in each group. * P<0.05 compared with indicated groups. # p< 0.05 compared with untreated I/R group.
Transfection of LmiR-125b prevented the increase in p53, Bak-1, Bax and Fas levels in the myocardium following I/R.

To determine the mechanisms by which increased expression of miR-125b attenuated I/R-induced myocardial apoptosis, we examined the levels of pro-apoptotic effectors including p53, Bak-1, Bax, and Fas in the myocardium. As shown in Figure 2.5C, I/R increased the levels of p53 by 94%, Bak-1 by 72%, Bax by 90%, and Fas by 64.5%, respectively, compared with sham control. LmiR-Con transfection did not alter the levels of pro-apoptotic effectors in the myocardium. However, increased expression of miR-125b prevented the increases in p53, Bak-1, Bax, and Fas levels in the myocardium following I/R. Both p53 and Bak-1 levels in LmiR-125b transfected hearts were markedly lower than in the untreated sham control.

Increased expression of miR-125b decreased TRAF6 expression and attenuated neutrophil infiltration in the myocardium.

Activation of TLR-mediated NF-κB signaling contributes to myocardial I/R injury by promoting the inflammatory responses(2-4). We examined the effect of LmiR-125b transfection on NF-κB activation during myocardial I/R. TRAF6 is an important effector in TLR-mediated NF-κB activation pathway(36). Figure 2.6A shows that the levels of TRAF6 in LmiR-125b transfected sham and I/R hearts were significantly lower than in untransfected sham and I/R groups. Transfection of LmiR-Con did not affect myocardial TRAF6 levels in the presence and absence of I/R. I/R significantly induced NF-κB binding activity compared with the sham control (Figure 2.6B) In contrast, increased expression of miR-125b prevented I/R-induced NF-κB binding activity. There was no
significant difference in NF-κB binding activity between the untransfected I/R group and LmiR-Con transfected I/R mice. In addition, we have observed that increased expression of miR-125b significantly attenuated I/R-induced neutrophil infiltration into the myocardium (supplemental data, Figure 2.S1).

Figure 2.6. LmiR125b transfection decreases TRAF6 expression and prevents I/R-induced NF-κB binding activity. Mice were transfected with LmiR-125b or LmiR-control for 7 days before the hearts were subjected to myocardial ischemia (45 min) followed by reperfusion (4 h). Increased expression of miR125b suppresses TRAF6 expression (A, p=<0.001) and NF-κB binding activity (B, p=<0.001). There were 5 mice in each group. * p<0.05 compared with indicated groups. # p<0.05 compared with control sham or control I/R group.

Transgenic mice with overexpression of miR-125b protect against myocardial I/R injury.

To confirm our observation, we developed transgenic mice (Tg) with overexpression of miR-125b. Figure 2.7A shows increased expression of miR-125b in the myocardium of Tg mice. Tg and WT mice were subjected to I/R. Figure 2.7B shows that I/R markedly induced myocardial infarct size in wild type (WT) mice.
However, infarct size in Tg mice were significantly reduced by 50% compared with WT I/R mice. In contrast, inhibition of miR-125b by transfection of anti-miR-125b into the myocardium of WT mice resulted in susceptible to I/R-induced injury. Infarct size was markedly greater than in untreated I/R group (Figure 2.7B). Tg mice also showed prevention of cardiac dysfunction following I/R. As shown in Figure 2.7C, both EF% and %FS values were significantly decreased by 42.6% and 48.8% in WT mice after myocardial I/R. However, I/R-induced cardiac dysfunction was prevented in Tg I/R mice.
Figure 2.7. Reduced myocardial infarct size and attenuated cardiac dysfunction in the transgenic mice with overexpression of miR-125b. (A) Increased levels of miR-125b in the myocardium of transgenic mice (Tg) mice. (B) Tg mice show decreases in myocardial infarct size. Tg and WT mice that were treated with and without anti-miR-125b or anti-miR-scrambled control were subjected to ischemia (45 min) followed by reperfusion (24 hrs). The hearts were harvested and infarct size was analyzed. Ratios of risk area vs. left ventricle area (RA/LV) and infarct area vs. risk area (IA/RA) were calculated and are presented in the graphs. (C) Tg mice show the prevention of I/R-induced cardiac dysfunction (p=<0.001). Cardiac function was examined by echocardiography before (Baseline) and 24 h after I/R. There were 5 mice in each group. * p<0.05 compared with indicated groups.

Discussion

The present study demonstrates that increased expression of miR-125b in the myocardium significantly decreased myocardial infarct size and prevented I/R-induced cardiac dysfunction. To the best of our knowledge, this is the first report that miR-125b exerts a protective role in myocardial I/R injury. Inhibition of miR-125b resulted in significant susceptibility to I/R-induced myocardial injury. The mechanisms by which miR-125b protects against myocardial I/R injury involve the prevention of I/R-induced NF-κB activation and p53-mediated apoptotic signaling. Our data suggest that modulation of miR-125b may be a useful strategy for the induction of cardioprotection.

We have previously reported that scavenger receptor type A (SR-A) deficiency attenuates myocardial I/R injury(25). Interestingly, we have observed that the levels of miR-125b in SR-A deficient mice are significantly greater than in wild type mice(25). Transfection of macrophages with miR-125b mimics attenuated H/R-induced cell injury(25), indicating that miR-125b may play a protective role in myocardial I/R. Indeed, we demonstrate in the present study that increased expression of miR-125b
attenuates myocardial I/R injury and prevents I/R-induced cardiac dysfunction. In contrast, inhibition of miR-125b expression resulted more injury of the myocardium following I/R. MiR-125b is highly conserved among mammals, vertebrates and nematodes(8). MiR-125b is expressed in several organs including brain, heart, lung, spleen, and skeletal muscle(39). Recent studies have reported that miR-125b expression is regulated by NF-κB activation(40), while miR-125b acts as a negative regulator of the NF-κB pathway by reducing the levels of tumor necrosis factor(18, 41) and by enhancing the stability of the NF-κB inhibitor NKIRAS2 (KBRAS2)(41). We have observed that hypoxia followed by reoxygenation decreased the expression of miR-125b and increased NF-κB binding activity in H9C2 cells. Inhibition of NF-κB binding activity by antioxidant, PDTC, which has been reported to inhibit NF-κB activation(35), prevents H/R-induced decreases in miR-125b expression. The data indicates that H/R-induced decreases in the expression of miR-125b are mediated, in part, by NF-κB activation.

However, our in vivo data shows that increased expression of miR-125b in the myocardium significantly prevents I/R-induced myocardial NF-κB binding activity. Importantly, we have observed that the expression of TRAF6 was suppressed by transfection of LmiR-125b. TRAF6 plays a crucial role in the induction of inflammatory response via activation of IκB kinases (IKKs), leading to NF-κB nuclear translocation and activation(36, 42). NF-κB activation regulates inflammatory cytokine expression(36, 42). We have observed that transfection of H9C2 cells with LmiR-125b prevents H/R-induced increases in TNFα production. Our observation is consistent with previous
reports showing that TNF-α mRNA is the target for miR-125b(18, 19). Androulidaki et al (19) and Tili et al (18) reported that LPS, a TLR4 ligand, suppresses macrophage expression of miR-125b, while miR-125b negatively regulates TNFα expression(18, 19). Collectively, our data supports the concept that NF-κB activation regulates miR-125b expression during H/R, while increased expression of miR-125b negatively regulates NF-κB activation. Therefore, inhibition of NF-κB activation by targeting TRAF6 in the myocardium could be an important mechanism for miR-125b protection against myocardial I/R injury. In addition, increased expression of miR-125b also down-regulated systemic inflammatory responses following myocardial I/R. We also observed that transfection of LmiR-125b significantly attenuated I/R-induced infiltration of neutrophils into the myocardium.

Myocardial apoptosis contributes to myocardial I/R injury(38). We have observed that increased expression of miR-125b significantly attenuated I/R-induced myocardial apoptosis. The mechanisms by which miR-125b attenuated I/R-induced myocardial apoptosis involve suppression of p53-mediated apoptotic signaling in the myocardium following myocardial I/R. p53 is a tumor suppressor protein that regulates and interacts with the apoptotic protein Bax. Bax acts as an antagonist against anti-apoptotic Bcl2, resulting in increases in mitochondrial membrane permeability and the release of cytochrome c(22, 23). In addition, apoptotic lipid products serve as chemokines which promote infiltration of inflammatory cells into the myocardium during myocardial I/R(22, 23). Therefore, p53 is a critical pro-apoptotic effector for myocardial apoptosis during myocardial I/R injury(24). Inhibition of p53 expression is an important approach for
attenuation of myocardial I/R injury (24). Our \textit{in vitro} data showed that increased expression of miR-125b in H9C2 cells and adult cardiac myocytes suppressed the expression of p53 and Bak-1 in both non-H/R and H/R cells, suggesting that miR-125b targets both p53 and Bak-1 in cardiomyoblasts. \textit{In vivo} data demonstrated that transfection of the myocardium with LmiR-125b prevents I/R-induced increases in the expression of p53 and Bak-1 in the myocardium. In addition, increased expression of miR-125b also prevents I/R-induced increases in Fas levels and caspase-3/7 and -8 activities in the myocardium. The data indicate that anti-apoptotic properties of miR-125b include inhibition of both extrinsic and intrinsic apoptotic signaling pathways during myocardial I/R.

In summary, we demonstrated in the present study that miR-125b plays a significant role in the protection against myocardial I/R injury. The mechanisms involve the inhibition of NF-κB activation as well as TNF-α production and the prevention of p53-mediated apoptotic signaling following myocardial I/R. Our data suggest that miR-125b is a target for the induction of protection against myocardial I/R injury.

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\textbf{Disclosure:} None
References


Materials and methods

qPCR assay of microRNAs (miRs):

MiRs were isolated using the mirVanaTM miR isolation kit (Ambion)(16, 25). Quantitative real-time (qPCR) was conducted using a 4800 Real time PCR machine (Bio-Rad)(25). miR-125b levels were quantified by qPCR using specific Taqman assays for miR (Applied Biosystems, USA). Specific primers for miR-125b were obtained from Applied Biosystems (Primer identification numbers: 000449 for hsa-miR-125b and 001973 for snRU6). miR-125b levels were quantified with the 2(-ΔΔct) relative quantification method that was normalized to the U6 small nucleolar RNA (snRU6).

Construction of miR-125b into lentivirus expressing system.

MiR-125b, mature sequence mmu-miR-125b-5p (MIMAT0000136), was constructed into lentivirus expression vector using a lentivirus expressing system (Invitrogen corporation) as described previously(16, 25). The oligonucleotides for pre-miR-125b (up strand:
5’TGCTGGGCTAGTCCCTGAGACCCTAACTTTGAGGTATTTTAGTAACATCACAAGTCAGGTTCCTTGGGACCTAGGC-3’ and down strand:
5’CCTGGCCTAGGTCCCAAGAACCTGACTTGTGATGTTACTAAAATACCTCACAAGTGAGGTTCCTCAGGGACTAGGC-3’), which contains mature miR-125b-5p, miR-125b-3p and stem loop, were synthesized at Integrated DNA Technologies. Five specific nucleotides indicated by red color were added to create a four nucleotide 5’ overhang which is compatible with the overhang in linearized pcDNA (pcDNATM6.2-GW/
EmGFP-miR). After ligation of pre-miR-125b with pcDNA6.2-GW/ EmGFP-miR, the pcDNA6.2-GW/EmGFP-miR cassette was subsequently transferred to pDONR221TM and finally pLenti6/V5-DEST by two sequential Gateway BP and LR recombinations. The construct was verified by sequencing. The viral particles were produced by third generation packaging in 293FT cells and Lentiviral stocks were concentrated using ultracentrifugation.

**Transgenic mice:**

Transgenic mice (Tg) with overexpression of miR-125b were developed with C57BL/6J background. Briefly, the pcDNA6.2-GW/EmGFP-miR125b vector was digested by NruI. Two fragments (3.5kb and 2.5kb) were obtained. The fragment of 3.5 kb was further digested with Pvu II and two fragments (2.1kb and 1.1kb) were obtained. The 2.1kb fragment was isolated, purified and used for microinjection. The Tg mice were identified by PCR to confirm that the miR-125b fragments have been incorporated into genome. PCR Primer sequence: F1: TTCAAGACCCGCAACAAC and R1: CAGCATACAGCCCTCAGCAA (312 bp). F2: ATGGGCGTGGATAGCGTTTG and R2: TCGGGCATGGCGACTTGA (567 bp). For examination of miR-125b expression in transgenic mice, the specific primer sequences are: F: gtccctgagaccctaacttg; R: cttccgtgtttcagctagcc. There was no production in WT mice, but 218 bp were generated from transgenic mice. Tg mice exhibit the normal phenotype and normal cardiac function.
Isolation of cardiac myocytes.

Cardiac myocytes were isolated from adult mice as described previously(28). Briefly, the mice were injected with heparin (50 IU) and anesthetized with isoflurane in 100% O2 as described in the Materials and Methods. The heart was removed and immediately placed in a 60 mm dish containing 10 ml of perfusion buffer at room temperature. After removing extraneous tissues, the heart was cannulated via the ascending aorta, and mounted on a modified Langendorff perfusion apparatus. The heart was perfused with perfusion buffer (NaCl, 113 mM; KCl, 4.7 mM; KH2PO4, 0.6 mM; Na2HPO4, 0.6 mM; MgSO4, 1.2 mM; NaHCO3, 12 mM; KHCO3, Taurine, 30 mM; HEPES, 10mM; Bleb, 0.01mM; Glucose, 5.5mM) for 2 min before the perfusion buffer was switched to the digestion buffer (Collagenase II, 1.6mg/ml in perfusion buffer). After 3 min of perfusion, the digestion buffer was supplemented with 15 µl of 100 mM cacl2, and perfusion was continued for an additional 10 min. After removing the atria and aorta, the ventricles were cut into small pieces and incubated in the digestion buffer for 3 min to allow further digestion. The suspension was then added with 5 ml stopping buffer (perfusion buffer plus 10%FBS and 0.1 mM CaCl2) and pipetted up and down, till no big pieces. The cells were pelleted at 100 g for 2 min and re-suspended in 10 ml stopping buffer. The cell suspension was added with 100 µl of ATP (200 mM). The Ca2+ concentration in the cell suspension was gradually increased through several steps. The cells were then pelleted at 100 g for 2 min and resuspended in plating media (10% FBS, 2 mM glutamine, 100 u/ml penicillin, 10 uM Bleb and 2 mM ATP in HMEM medium). The cells were incubated on plates that were precocated with matrigel for 1 hour,
followed by incubation with culture media (1mg/ml BSA, 2 mM glutamine, 100u/ml penicillin, 10uM Bleb and 0.1% ITS in HMEM medium).

Preparation of exosomes containing miR-125b.

Bone marrow stromal cells (BMSCs) were isolated from C57BL/6J mice as described previously(29). Briefly, the femurs and tibias were isolated from mice and flushed with complete medium constituted of EMEM-LG (Sigma, St., Louis, MO), 10% fetal calf serum (HyClone, ThermoFisher Scientific Waltham, MA), glutamine (2 mM) and penicillin/streptomycin (50 U/ml and 50 mg/ml, Sigma), respectively, supplemented with heparin at a final concentration of 5 U/ml. The cells were washed twice in a medium without heparin, plated in a Petri dish at a density of 2 x 10^6 cells/cm^2 and incubated at 37°C with 5% CO₂. After three days, non-adherent cells were removed by two to three washes with PBS and adherent cells further cultured in complete medium at 37°C with 5% CO₂. The medium was changed every other day. Cells at the 4th -7th generation were transfected with 40 nmol/L microRNA mimics for miR-125b (MC10148, Ambion), anti-miR-125b (AM10148, Ambion) or Cy3™ dye labeled miR-scrambled control (AM17010, Ambion), using Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacture's protocol. Twenty-four hours after transfection, supernatants were harvested for exosomes isolation using Exoquick-TC™ Exosome Precipitation Solution (SBI) according to the manufacturer's protocol.
Transfection of cardiac myocytes with Exosomes containing miR-125b.

Adult cardiac myocytes were incubated on plates that were pre-coated with Matrigel and they were maintained in culture medium. Exosomes that were isolated from bone marrow cells (BMSC-derived exosomes) were added into the cultured adult cardiac myocytes (2 µg/ml). Transfection efficiency was confirmed by the fluorescence of Cy3™ dye labeled miR-scrambled control. The miR-125b levels were assessed by TaqMan MicroRNA assay.

In vivo transfection of lentivirus expressing miR-125b (LmiR-125b) into mouse hearts.

Mice were intubated and anesthetized with mechanical ventilation using 5% isoflurane. The anesthesia was maintained by inhalation of 1.5-2% isoflurane in 100% oxygen. An incision was made in the middle of the neck and the right common carotid artery was carefully isolated. A micro-catheter was introduced into the isolated common carotid artery and positioned into the aortic root through an arteriotomy site in the external carotid artery(16). One hundred microliter of LmiR-125b (1x10⁷ PFU) or LmiR-con was injected through the micro-catheter. The micro-catheter was gently removed and the common carotid artery was tightened before the skin was closed. Seven days after transfection, the hearts were harvested and transfection efficiency was evaluated by examining the green fluorescent protein (GFP) expression and the expression of miR125b in the heart tissues.
In vivo delivery of anti-miR-125b mimics.

Anti-miR-125b or anti-miR-scrambled control was with Lipofectamine RNAiMAX according to the protocol provided by the manufacturer (Invitrogen). Mice were anesthetized with isoflurane and the hearts were explored. A 30 G needle with microsyringe was inserted into the left ventricular wall in the direction from the base to the apex, penetrating about 1mm deep. Five microgram of anti-miR-125b or anti-miR-scrambled control that were mixed with Lipofectamine RNAiMAX was injected into the left anterior ventricular wall. After injection, the needle was removed from the left ventricular wall and the chest was closed in layers. The animals were allowed to recover.

Induction of myocardial I/R injury.

Myocardial I/R injury was induced as described previously(2, 3, 28). Briefly, the mice were anesthetized by 5.0% isoflurane, intubated and ventilated using a rodent ventilator. Anesthesia was maintained by inhalation of 1.5% to 2% isoflurane driven by 100% oxygen flow. Body temperature was regulated at 37°C by surface water heating. The hearts were exposed and the left anterior descending (LAD) coronary artery was ligated with an 8-0 silk ligature. After completion of 45 min of occlusion, the coronary artery was reperfused by releasing the knot of suture. During reperfusion, cardiac function was measured by echocardiography as described previously(28, 29). After reperfusion for the time indicated, the mice were euthanized by CO₂ inhalation and the hearts were harvested. Infarct size was evaluated by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described previously(2, 3, 28).
**MicroRNA microarray.**

The isolated adult cardiac myocytes were subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs) as described previously(16). The cells that were not subjected to H/R served as control. The cells were harvested for total RNA preparation using RNAzo kit (Molecular Research Center INC). RNA samples were sent to Ocean Ridge Biosciences (ORB, Palm Beach Gardens, FL) for analysis using custom multi-species microarrays containing 1281 probes covering 1279 mouse mature microRNAs present in miRBase version 19. The sensitivity of the microarray is such that it could detect as low as 20 amoles of synthetic microRNA being hybridized along with each sample. There were 3 replicates in each group.

**Myocardial infiltration of neutrophils.**

Neutrophil accumulation in heart tissues was examined by staining with anti-neutrophil marker antibody (NIMP-R14, Santa Cruz Biotechnology) as described previously(24). Three slides from each block were evaluated and three different areas of each section were evaluated. The results are expressed as the numbers of neutrophils/field (40x).
Figure 2.S1. Increased \textit{in vivo} expression of miR-125b attenuated I/R-induced neutrophil infiltration into the myocardium. Mouse hearts were transfected with either LmiR-125b or LmiR-Con. Seven days after transfection, the hearts were subjected to myocardial ischemia (45 min) followed by reperfusion (4 hrs). Hearts were harvested and sectioned for immunohistochemical staining of neutrophils with anti-neutrophil antibody. The bar graph shows the numbers of neutrophils in examined fields. N=3/group. * p<0.05 compared with indicated groups.

Tables 1 and 2: Effect of hypoxia/reoxygenation on microRNA expression in the isolated adult cardiac myocytes. Cardiac myocytes were isolated from adult male mice and subjected to hypoxia (2 hrs) followed by reoxygenation (24 hrs). Non-H/R cells
(normoxia) served as control. The cells were harvested for the isolation of total RNA. MicroRNA microarray was performed and analyzed by Ocean Ridge Biosciences for analysis using custom multi-species microarrays containing 1281 probes covering 1279 mouse mature microRNAs present in in miRBase version 19. There were 3 replicates in each group. After quality-control, background subtraction, transformation and normalization of probe intensity, 43 miRNAs were differentially expressed in the cardiac myocytes after H/R at a false discovery rate of 0.05, when compared with non-H/R cells (normoxia). Among the 43 miRNAs, 18 were elevated and 25 were down regulated after H/R. In addition, 28 miRNAs which are highly expressed in adult cardiac myocytes showed saturation such as miR-1, miR-24 and miR-125b (Table 2).
CHAPTER 3

MICRORNA-214 PROTECTS AGAINST HYPOXIA/REOXYGENATION INDUCED CELL DAMAGE AND MYOCARDIAL ISCHEMIA/REPERFUSION INJURY VIA SUPPRESSION OF PTEN AND BIM1 EXPRESSION

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Running head: microRNA-214 decreases myocardial I/R injury

Key words: microRNA-214, myocardial ischemia/reperfusion injury, myocardial apoptosis, PTEN, Bim1.
Abstract

Background

Myocardial apoptosis plays an important role in myocardial ischemia/reperfusion (I/R) injury. Activation of PI3K/Akt signaling protects the myocardium from I/R injury. This study investigated the role of miR-214 in hypoxia/reoxygenation (H/R)-induced cell damage in vitro and myocardial I/R injury in vivo.

Methods and Results

H9C2 cardiomyoblasts were transfected with lentivirus expressing miR-214 (LmiR-214) or lentivirus expressing scrambled miR-control (LmiR-control) respectively, to establish cell lines of LmiR-214 and LmiR-control. The cells were subjected to hypoxia for 4 h followed by reoxygenation for 24 h. Transfection of LmiR-214 suppresses PTEN expression, significantly increases the levels of Akt phosphorylation, markedly attenuates LDH release, and enhances the viability of the cells subjected to H/R. In vivo transfection of mouse hearts with LmiR-214 significantly attenuates I/R induced cardiac dysfunction and reduces I/R-induced myocardial infarct size. LmiR-214 transfection significantly attenuates I/R-induced myocardial apoptosis and caspase-3/7 and caspase-8 activity. Increased expression of miR-214 by transfection of LmiR-214 suppresses PTEN expression, increases the levels of phosphorylated Akt, represses Bim1 expression and induces Bad phosphorylation in the myocardium. In addition, in vitro data shows transfection of miR-214 mimics to H9C2 cells suppresses the expression and translocation of Bim1 from cytosol to mitochondria and induces Bad phosphorylation.
Conclusions

Our in vitro and in vivo data suggests that miR-214 protects cells from H/R induced damage and attenuates I/R induced myocardial injury. The mechanisms involve activation of PI3K/Akt signaling by targeting PTEN expression, induction of Bad phosphorylation, and suppression of Bim1 expression, resulting in decreases in I/R-induced myocardial apoptosis.

Introduction

MicroRNAs (miRs) are 21 to 23 nucleotide non-protein-coding RNA molecules, which have been identified as novel regulators of gene expression at the post-transcriptional level by binding to target messenger RNAs (mRNAs). Recently published data indicates that miRs, such as miR-1/106, miR-125b, miR-146a, miR-223, miR-21, miR-144/145, miR-320, miR-494, and miR-92a, are involved in ischemic heart disease[1-8]. miR-214 has been reported to protect cardiac myocytes from H$_2$O$_2$-induced injury[9]. Recently, Aurora et al [10] have shown that deficiency of miR-214 resulted in severe myocardial ischemia/reperfusion (I/R) injury and increased fibrosis progression as well as cardiac myocyte apoptosis. These authors demonstrated that miR-214 targets sodium-calcium exchanger-1, thus influencing cardiac myocyte calcium trafficking following myocardial I/R injury[10].

It is well known that myocardial apoptosis contributes to myocardial I/R injury[11]. Bad is a pro-apoptotic protein, a member of the Bcl-2 family and induces apoptosis by inhibiting the anti-apoptotic effects of Bcl-2 and Bcl-X, thereby allowing the pro-apoptotic proteins, Bak and Bax to aggregate and induce release of cytochrome c, followed by
activation of caspase-mediated apoptotic signaling[12]. Phosphorylation of Bad by activated Akt prevents the interaction of Bad with Bcl-2 and Bcl-X[13]. Bcl-2 homology domain 3 (BH3)-only Pro-Protein Bim1 is an another pro-apoptotic protein and plays an important role in Bax/Bak mediated cytochrome c release and apoptosis[14]. Bim expression is regulated by activated FOXO3 (Forkhead box transcription factor, class O) which is controlled by activated phosphatidylinositol 3-kinase (PI3K)/Akt signaling[15]. In addition, activated Akt phosphorylates Bim1 at Ser87, resulting in blocking the pro-apoptotic effect of Bim1[16].

Toll like receptor (TLR)-mediated innate immune and inflammatory responses have been demonstrated to play a critical role in myocardial ischemia/reperfusion (I/R) injury[17]. We have previously reported that administration of TLR ligands to mice induced protection against myocardial I/R injury[18-20]. The protective mechanisms involve the activation of PI3K/Akt signaling[18-21] which plays an important role in regulating cellular proliferation and survival[22,23]. Phosphatase and tensin homolog (PTEN) is a tumor suppressor lipid protein phosphatase which negatively regulates PI3K/Akt signaling activation[24] by dephosphorylating PIP3 at its 3’ inositol position, resulting in decreased translocation of Akt to cellular membranes and subsequent down-regulation of PI3K/Akt activation[25]. Recent studies have shown PTEN plays a critical role in mitochondrial dependent apoptosis[26].

In the present study, we demonstrated that increased expression of miR-214 by transfection of the myocardium with lentivirus expressing miR-214 (LmiR-214) protects hearts from I/R injury. The mechanisms involve suppression of PTEN expression,
leading to activation of PI3K/Akt signaling, induction of Bad phosphorylation, and targeting Bim1 expression, resulting in attenuation of I/R-induced myocardial apoptosis.

**Results**

**Increased miR-214 levels suppressed PTEN expression and increased Akt phosphorylation in H9C2 cardiomyoblasts.**

We have previously reported that either TLR4 deficiency or TLR2 modulation by Pam3CSK4 significantly attenuates I/R induced myocardial injury via activating PI3K/Akt dependent mechanism[20]. Interestingly, we observed that the levels of myocardial miR-214 were markedly greater in either TLR4 deficient mice or Pam3CSK4 treated mice compared with untreated group (Figure 3.1A). Our previous studies have shown that TLR2 modulation can significantly attenuate I/R induced myocardium injury by activating PI3K/Akt signaling. PTEN, a negative regulator of PI3K/Akt signaling is a potential target of miR-214. To investigate the underlying mechanisms by which TLR2 modulation regulates the miR-214 expression, H9C2 cardiomyoblasts were treated with TLR2 specific ligand Pam3CSK4. As shown in Figure 3.1B, the levels of miR-214 in Pam3CSK4 treated cells are significantly increased. The levels of phosphorylated Akt are also significantly increased following Pam3CSK4 treatment (Figure 3.1C) which is consistent with our previous studies. However, PI3K inhibition with LY294002 (LY) significantly prevented Pam3CSK4 induced Akt phosphorylation but did not alter Pam3CSK4 induced increases in miR-214 expression. To determine whether NF-kB signaling involves Pam3CSK4 induced miR-214 expression, we treated cells with NF-kB specific inhibitor, JSH-23 and observed that JSH-23 treatment significantly prevented
Pam3CSK4 induced increases in the expression of miR-214 (Figure3.1B). Collectively, these data suggest the TLR2 ligand induced increases in the expression of miR-214 is mediated through NF-κB activation pathway.

To examine whether increased expression of miR-214 will activate PI3K/Akt signaling, we transfected H9C2 cells with lentivirus expressing miR-214 (LmiR-214) or LmiR-control respectively, before the cells were subjected to hypoxia (2 h) followed by reoxygenation (H/R). Figures 3.1D and E show that LmiR-214 transfection markedly increases the levels of Akt phosphorylation and suppresses PTEN expression in the presence or absence of H/R. The data suggests that miR-214 targets PTEN expression, resulting in activation of PI3K/Akt signaling.

Increased expression of miR-214 attenuates H/R induced cell injury and increases survival in H9C2 cardiomyoblasts.

Activation of PI3K/Akt signaling plays an important role in protection against H/R-induced cell injury[27,28]. We examined whether transfection of LmiR-214 will protect the H9C2 cells from H/R-induced injury. Figure 3.1F shows that H/R significantly increased LDH activity by 5.6-fold compared with the control cells (normoxia). In contrast, transfection of cells with LmiR-214 markedly attenuates H/R-induced LDH activity by 54%, when compared with untransfected H/R group. H/R also significantly decreased cell viability by 47% compared with normoxia group (Figure 3.1G). However, the viability in LmiR-214 transfected cells that were subjected to H/R was significant greater by 75% compared with H/R group. Transfection of LmiR-control did not affect H/R-induced cell injury and death in H9C2 cells.
Figure 3.1. TLR4 deficiency or TLR2 ligand, Pam3CSK4 treatment increases the expression of miR-214 in the myocardium. TLR4 deficient (TLR4\(^{-/-}\)) mice (n=3) or wild type (WT) mice were treated with and without Pam3CSK4 (50 µg/25 g body weight) and then subjected to myocardial ischemia (45 min) followed by reperfusion (4 h). Sham operation served as sham control. Hearts were harvested and microRNAs were isolated.
for qPCR measurement of miR-214 (n=3-4/group). (B-C) NF-κB activation is required for Pam3CSK4 induced miR-214 expression. Myoblast H9C2 cells were treated with PamsCSK4 in the presence of LY294002 or JSH23 for 24 hrs. The cells were harvested for microRNA preparation for qPCR measurement of miR-214 expression (B) and for Western blot analysis of Akt phosphorylation (C). There were 3 replicates in each group. * p<0.05 compared with indicated groups. (D-G) H9C2 cells were transfected with lentivirus expressing miR-214 (LmiR-214) or lentivirus expressing miR-control (LmiR-control) respectively. The cells were subjected to hypoxia (4 h) followed by reoxygenation (24 h). The levels of Akt phosphorylation (D) and PTEN expression (E) were assessed by Western blot (n=3). LDH activity (F) in the supernatants was measured by a commercially available kit and cell viability (G) was measured by MTT assay (n=3-5). *p<0.05 compared with indicated groups. # p<0.05 compared with respective control.

LmiR-214 transfection attenuates cardiac dysfunction and reduces infarct size after myocardial I/R.

Our in vitro data shows that increased expression of miR-214 markedly activates PI3K/Akt signaling and protects H9C2 cells from H/R-induced injury. We evaluated whether in vivo transfection of LmiR-214 will protect the heart from myocardial I/R injury. Mouse hearts were transfected with LmiR-214 through the right carotid artery[1,2]. LmiR-control served as vector control. Figure 3.2D shows that seven days after transfection, the levels of miR-214 in the myocardium were significantly increased by 3.7-fold compared with control. We also examined whether increased expression of miR-214 will protect against myocardial I/R-induced injury. The hearts were subjected to ischemia (45 min) followed by reperfusion up to 7 days. As shown in Figures 3.2A and B, I/R significantly decreased cardiac function. The values for ejection fraction (EF%) and fractional shortening (FS%) were markedly reduced by 38.1% and 44.5% on day 3 and by 24.6% and 32.1% on day 7 respectively, after myocardial I/R injury compared with sham control. In contrast, LmiR-214 transfection attenuated I/R-induced cardiac
dysfunction. The values for EF% and FS% in LmiR-214 transfected hearts were significantly greater than in the untransfected I/R group. Figure 3.2C shows that transfection of LmiR-214 into the hearts markedly reduced infarct size by 52.1% compared with the untransfected I/R group. Transfection of LmiR-control into the myocardium did not alter I/R-induced decreases in the values of EF% and FS% and myocardial infarct size.

LmiR-214 transfection suppresses PTEN expression and increases Akt phosphorylation in the myocardium.

PTEN is a negative regulator of PI3K/Akt signaling[29,30]. We examined the effect of LmiR-214 transfection on PTEN expression and Akt phosphorylation in the myocardium following I/R. Figure 3.2E shows that I/R did not affect PTEN expression but enhanced the levels of Akt phosphorylation compared with sham control. However, LmiR-214 transfection significantly suppressed the expression of PTEN and further increased Akt phosphorylation levels in both sham and I/R groups when compared with untransfected respective controls. In the LmiR-214 transfected group, the levels of phosphorylated Akt were increased by 34.1% and PTEN decreased by 42.4%, when compared with untransfected I/R hearts. Transfection of LmiR-control did not alter the levels of Akt phosphorylation and PTEN expression in the myocardium of both sham and I/R groups.
Figure 3.2. Transfection of lentivirus expressing miR-214 into the myocardium improved cardiac function and decreased infarct size following myocardial I/R injury. Mouse hearts were transfected with LmiR-214 through the right common carotid artery (n=8/group). Seven days after transfection, hearts were subjected to ischemia (45 min) followed by reperfusion for up to 7 days. (A and B) Cardiac function was measured by echocardiography 3 and 7 days after myocardial I/R. (C) Hearts were harvested 24 h after reperfusion for TTC staining infarct size. (D) The level of miR-214 was increased following LmiR-214 transfection. (E) LmiR-214 transfection suppressed PTEN expression and increased Akt phosphorylation levels. n=6/group. * P<0.05 compared with indicated group. # p<0.05 compared with respective control.
Increased expression of miR-214 attenuates I/R induced myocardial apoptosis.

It is well known that myocardial apoptosis contributes to cardiac dysfunction after myocardial I/R injury[31]. We examined the effect of LmiR-214 transfection on myocardial apoptosis following myocardial I/R injury. Figure 3.3A shows that I/R increased Tunnel positive myocardial apoptotic cells by 29.8% compared with sham control. In contrast, transfection of LmiR-214 into the myocardium significantly decreased I/R-induced myocardial apoptosis by 60.9%, when compared with the untransfected I/R group. Activation of caspase-3/7 and caspase-8 have been considered as specific markers for apoptosis[32,33]. Figures 3.3B and C show that I/R-induced an increase in capase-7 by 30% and caspase-8 by 40.4%, when compared with sham control. However, increased expression of miR-214 prevents I/R-induced myocardial caspase-3/7 and caspase-8 activities (Figures 3.3B and C). Transfection of LmiR-control did not alter I/R-induced myocardial apoptosis.
Figure 3.3. Increased expression of miR-214 attenuates I/R-induced myocardial apoptosis. LmiR-214 or LmiR-control was transfected into the myocardium of mice via the right common carotid artery 7 days before the hearts were subjected to ischemia (45 min) followed by reperfusion (24 h). Hearts were harvested and sectioned for TUNEL assay of myocardial apoptosis (A). Caspase-3/7 (B) and Caspase-8 (C) activities were measured by ELISA kits. N=4-6, *p<0.05 compared with indicated group.

LmiR-214 transfection increases Bad phosphorylation and suppresses the expression of Bim1 in the myocardium.

Figure 3.4. Increased expression of miR-214 suppressed Bim expression and prevents Bad phosphorylation in the myocardium following myocardial I/R. LmiR-214 or LmiR-control was transfected into the myocardium of mice via the right common carotid artery 7 days before the hearts were subjected to ischemia (45 min) followed by reperfusion (24 h). The hearts were harvested for isolation of cellular proteins. The levels of phosphorylated Bad (A) and Bim1 (B) were examined by Western blot. N=4-6, *p<0.05 compared with indicated group.

Bad is a pro-apoptotic protein which interacts with Bcl2, resulting in blocking Bcl2 anti-apoptotic function[34]. When Bad is phosphorylated, Bcl2 will release from Bad/Bcl2 complex and functions in an anti-apoptotic role[13,34,35]. Figure 3.4A shows
that transfection of LmiR-214 markedly increased the levels of phosphorylated Bad in both sham and I/R groups compared with untransfected respective controls. Bim1 is a mitochondrial pro-apoptotic factor which will translocate from the cytosol to mitochondria, resulting in cardiomyocyte apoptosis during I/R[14;36]. As shown in Figure 3.4B, I/R decreased the cytosolic levels of Bim1 compared with sham control. However, transfection of LmiR-214 further decreases the cytosolic levels of Bim1 in both sham and I/R groups, indicating increased expression of miR-214 may suppress the expression and translocation of Bim1 from cytosol to mitochondria. Transfection of LmiR-control did not alter I/R induced changes of Bad phosphorylation and Bim1 expression.

LmiR-214 suppresses the translocation of Bim1 from cytosol to mitochondria in myocardioblasts following H/R.

To examine the effect of miR-214 on Bim1 translocation from cytosol to mitochondria, we transfected H9C2 cells with miR-214 mimics or scrambled miR-control respectively, before the cells were subjected to hypoxia/reoxygenation (H/R). Figures 3.5A and B show that H/R induces translocation of Bim1 from the cytosol to the mitochondria as evidenced by a significant decrease in the cytosolic levels of Bim1 (A), but a markedly enhanced amount of Bim1 in mitochondrial extracts (B). In contrast, miR-214 markedly suppresses the expression of Bim1 in the cytosol and decreases the levels of Bim1 in the mitochondria following H/R challenge. MiR-214 transfection also significantly increased Bad phosphorylation in the presence and absence of H/R compared with untransfected control groups (Figure 3.5C). Transfection of scrambled miR-control did not induce Bad phosphorylation in the presence and absence of H/R.
addition, H/R markedly decreased mitochondrial membrane potential which was significantly attenuated by LmiR-214 transfection (Figure 3.5D).

Figure 3.5. Increased expression of miR-214 suppressed the expression and mitochondrial translocation of Bim1 and increased the levels of phosphorylated Bad in
cardiomyoblasts H9C2 cells. H9C2 cells were transfected with miR-214 mimics. miR-control mimics served as control. The cells were subjected to hypoxia (4 h) followed by reoxygenation (24 h). The cells were harvested for isolation of mitochondria. The levels of Bim1 (A and B) and phosphorylated Bad (C) were examined by Western blot. n=3/group. (D) Mitochondrial membrane potential was measured by JC-1 Dye (n=6-8).

* p<0.05 compared with indicated groups.  # p<0.05 compared with respective control.

**Discussion**

In the present study, we demonstrated that increased expression of miR-214 in the myocardium significantly attenuates I/R-induced cardiac dysfunction and myocardial infarct size. The mechanisms involve an anti-apoptotic effect via activation of PI3K/Akt signaling and suppression of Bim expression. Specifically, we observed that miR-214 suppresses PTEN expression, leading to activation of PI3K/Akt signaling. It is well known that activated Akt phosphorylates Bad [13], thereby blocking the pro-apoptotic effect of Bad. In addition, miR-214 represses the expression of pro-apoptotic protein Bim1 [10] and its translocation from cytosol to the mitochondria, thus preventing I/R induced apoptosis.

We have previously reported that there is an interaction between TLR-mediated pathway and PI3K/Akt signaling during myocardial I/R injury[20,37]. Activation of PI3K/Akt signaling has been reported to protect the myocardium from I/R injury and promotes cell survival following H/R[27,28,37,38]. However, the mechanisms by which stimulation of TLRs induces activation of PI3K/Akt signaling are unclear. Interestingly, we observed that miR-214 expression in the myocardium was significantly increased by a TLR2 ligand, Pam3CSK4, in the presence and absence of I/R. Since Pam3CSK4 induced activation of PI3K/Akt signaling[20], we examined whether increased miR-214
expression would activate PI3K/Akt signaling. Indeed, our data show that increased expression of miR-214 by transfection of LmiR-214 into H9C2 cells suppresses PTEN expression and increases the levels of Akt phosphorylation in the presence and absence of H/R challenge. LmiR-214 transfection also markedly attenuates H/R-induced cell injury as evidenced by decreased LDH release after the cells were subjected to H/R. In addition, cell viability was markedly improved by increased expression of miR-214. Collectively, the *in vitro* data suggests that miR-214 has a protective effect on H/R-induced cell injury via suppression of PTEN, leading to activation of PI3K/Akt signaling.

Next, we investigated whether increased expression of miR-214 in the myocardium would protect the hearts from I/R-induced injury. We constructed LmiR-214 and transfected it into the myocardium through the right carotid artery seven days before the hearts were subjected to myocardial I/R[1,2]. The *in vivo* data shows that LmiR-214 transfection prevents I/R-induced cardiac dysfunction and reduced I/R-induced myocardial infarct size. Our data is consistent with the report by Aurora *et al* [10] showing that deficiency of miR-214 resulted in more severe cardiac dysfunction and myocardial I/R injury, indicating that miR-214 is essential for cardioprotection against I/R injury. To understand the mechanisms by which miR-214 protects against myocardial I/R injury, Aurora *et al* [10] observed that miR-214 targets sodium-calcium exchanger-1, thereby influencing calcium trafficking in cardiac myocytes after myocardial I/R injury[10]. We found that miR-214 significantly suppresses PTEN expression, leading to Akt phosphorylation in the myocardium. Previous studies have demonstrated that activation of PI3K/Akt signaling plays a critical role in reduced I/R-induced injury and
attenuated I/R-induced cardiac dysfunction[18-21]. Our data indicates that activation of PI3K/Akt signaling by miR-214 may be an important mechanism for protection against I/R injury.

We observed that increased expression of miR-214 significantly attenuates I/R-induced myocardial apoptosis. Apoptosis has been demonstrated to contribute to I/R-induced myocardial damage and cardiac dysfunction[31]. Apoptosis is the process of programmed cell death which is predominately dependent on mitochondrial function[39]. Bcl2 family members play a critical role in regulating mitochondrial mediated apoptosis through controlling the permeabilization of the mitochondrial membrane[39]. Bad is a pro-apoptotic protein that promotes activation of the programmed cell death pathway through formation of heterodimerizes with Bcl2 or Bcl-XL to inhibit their anti-apoptotic functions[35]. Our in vitro and in vivo data show that increased miR-214 expression significantly enhances Bad phosphorylation, thereby blocking Bad’s pro-apoptotic effect. It has been reported that Bad is phosphorylated by a variety of kinases including Akt and P70S60 kinase[13,34] with subsequent loss of its pro-apoptotic action by binding with 14-3-3 protein[35,40]. Our findings indicate that targeting PTEN expression by mir-214, resulting in activation of Akt could be responsible for Bad phosphorylation.

We also found that increased expression of miR-214 represses the expression of Bim1 and prevents its translocation from the cytosol to mitochondria. Bim1 is another pro-apoptotic protein of Bcl-2 family and triggers apoptosis by inhibiting Bcl2 anti-apoptotic function and/or direct activation of Bax[14], leading to cytochrome c release and activation of apoptotic signaling[39]. Therefore, suppression of Bim1 expression and preventing its translocation from the cytosol to the mitochondria are necessary for
preventing apoptosis during myocardial I/R injury[36]. Aurora et al reported that the expression of Bim1 in the myocardium was significantly increased in miR-214 deficient mice 7 days after myocardial I/R injury, indicating that miR-214 may target Bim[10]. We demonstrated in the present study that increased in vivo expression of miR-214 by LmiR-214 transfection suppresses the expression of Bim1 in the myocardium following I/R injury and that in vitro enhanced miR-214 levels prevent the translocation of Bim1 from the cytosol to the mitochondria. Collectively, targeting Bim1 expression and preventing its translocation from cytosol to mitochondria could be an important protective mechanism of miR-214 in myocardial I/R injury.

In summary, our data demonstrated that miR-214 plays a protective role in myocardial I/R injury. The mechanisms involve an anti-apoptotic effect through suppression of PTEN expression, leading to activation of PI3K/Akt signaling which suppresses Bad activity via its phosphorylation. In addition, miR-214 suppresses the expression and translocation of Bim1 from the cytosol to the mitochondria. MiR-214 could be a target for the induction of protection against myocardial I/R injury. Future studies should search for which natural conditions will induce miR-214 expression.

Materials and Methods

Animals

Male C57BL/6J mice were obtained from Jackson Laboratory and maintained in the Division of Laboratory Animal Resources, East Tennessee State University (ETSU). The experiments outlined in this article conform to the Guide for the Care and Use of
Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). All aspects of the animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

**Induction of myocardial I/R injury**

Myocardial I/R injury was induced as described previously[1,2,18,20,21]. Briefly, the mice were anesthetized by 5.0% isoflurane, intubated and ventilated using a rodent ventilator. Anesthesia was maintained by inhalation of 1.5% to 2% isoflurane driven by 100% oxygen flow. Body temperature was regulated at 37°C by surface water heating. The hearts were exposed and the left anterior descending (LAD) coronary artery was ligated with an 8-0 silk ligature. After completion of 45 min of occlusion, the coronary artery was reperfused by releasing the suture knot. After reperfusion for the time indicated, the mice were euthanized by CO2 inhalation and the hearts were harvested. Infarct size was evaluated by triphenyltetrazolium chloride (TTC, Sigma-Aldrich) staining as described previously[1,2,18,20,21].

**Echocardiography**

Transthoracic two-dimensional M-mode echocardiogram and pulsed wave Doppler spectral tracings were obtained using a Toshiba Aplio 80 Imaging System (Tochigi, Japan) equipped with a 12-MHz linear transducer as described previously[1,2,19,20]. Ejection fraction (EF) and percent fractional shortening (FS) were calculated as described previously[1,2,19,20].
In situ apoptosis assay

Measurement of cardiac myocyte apoptosis was performed as described previously[1,2,18-21] using the in situ cell death detection kit, fluorescein (Roche, USA) according to instructions of the manufacturer.

Measurement of cell viability and mitochondrial membrane potential

Cell viability was assessed by measuring mitochondrial dehydrogenase activity using the MTT assay kit (Sigma). Cell injury was assessed by measurement of lactate dehydrogenase (LDH) activity in culture medium using a commercial kit (Cytotoxicity Detection Kit, Sigma). Mitochondrial membrane potential was evaluated by the fluorescence ratio of JC-1 aggregates (red) to monomers (green).

Real time PCR assay of miRNAs

miRs were isolated from heart tissues or cultured cells using the miRNAs isolation kit (RNAzol®RT, MRC) in accordance with the manufacturer’s protocol. Quantitative real-time (qPCR) was conducted using a 4800 Real-time PCR machine (Bio-Rad). MicroRNA levels were quantified by qPCR using specific Taqman assays for miR (Applied Biosystems, USA) and Taqman Universal Master Mix (Applied Biosystems). Specific primers for miR-214 were obtained from Applied Biosystems. MicroRNA-214 levels were quantified with the 2^(-ΔΔCt) relative quantification method that was normalized to the snRU6.
Construction of lentivirus expressing miR-214

MiR-214 was constructed into a lentivirus expressing vector using a lentivirus expressing system (Invitrogen Corporation) as described previously[1,2]. Briefly, the oligonucleotides for miR-214 were synthesized at Integrated DNA Technologies, annealed and ligated into pcDNATM6.2-GW/EmGFP-miR. The pcDNATM6.2-GW/EmGFP-miR cassette was subsequently transferred to pDONR221TM and finally pLenti6/V5-DEST by two sequential Gateway BP and LR recombinations. The construct was verified by sequencing. The viral particles were produced by third generation packaging in 293FT cells and lentiviral stocks were concentrated using ultracentrifugation.

Transfection of lentivirus expressing miRNA

We transfected mouse hearts with lentivirus expressing miR-214 (LmiR-214) or lentivirus expressing miR-control (LmiR-control) via the right common carotid artery as described previously[1,2]. Briefly, mice were intubated and anesthetized with mechanical ventilation using 5% isoflurane. The anesthesia was maintained by inhalation of 1.5-2% isoflurane in 100% oxygen. An incision was made in the middle of the neck and the right common carotid artery was carefully isolated. A micro-catheter was introduced into the isolated common carotid artery and positioned into the aortic root through an arteriotomy site in the external carotid artery [1,2]. One hundred microliters of LmiR-214 (1x10^7 PFU) or LmiR-Con was injected through the micro-catheter. The micro-catheter was gently removed and the common carotid artery was tightened before the skin was closed. Seven days after transfection, the hearts were
harvested and transfection efficiency was evaluated by examining the green fluorescent protein (GFP) expression and the expression of miR-214 in the heart tissues.

**In vitro experiments**

The H9C2 cardiomyoblasts stably expressing miR-214 or miR-con were generated by transfection of LmiR-214 or LmiR-con and selection with Blasticidin (Invitrogen). Transient expression of miR-214 in H9C2 cells was accomplished by transfection of miR-214 mimics (40 nM) or miR-control mimics (40 nM). The cells were subjected to hypoxia/reoxygenation as described previously. Briefly, the medium was changed to hypoxia-equilibrated medium immediately before the cells were incubated at 37°C with 5% CO2 and 0.1% O2 in a hypoxia chamber (Pro-Ox Model C21, BioSpherix Ltd, Redfield NY) for 2 h followed by reoxygenation in an incubator with 5% CO2. The cells were not subjected to H/R served as control (normoxia). The cells were harvested and cellular proteins were isolated for Western blot analysis.

**Mitochondrial Isolation and Western Blot**

Mitochondria was isolated from H9C2 cells using a mitochondrial isolation kit (Thermo Scientific) according to the manufacturer’s protocol. Western blots were performed as described previously. The membranes were incubated with appropriate primary antibodies respectively, including anti-PTEN, anti-p-Akt, anti-Akt, anti-Bim, anti-p-Bad, anti-COX4 (Cell Signaling Technology, Inc, Danvers, MA), respectively, followed by incubation with peroxidase-conjugated second antibodies (Cell Signaling Technology, Inc.) and examination with the ECL system (Amersham...
Pharmacia, Piscataway, NJ). The signals were quantified using a G:Box gel imaging system by Syngene (Syngene, USA, Frederick, MD).

**Caspase-3/7 and caspase-8 activities Assay**

Caspase-3/7 and caspase-8 activity were measured using a Caspase-Glo assay kit (Promega) according to the manufacturer’s protocol as described previously[20].

**Statistical analysis**

Data are expressed as mean ± SD. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey’s procedure for multiple-range tests was performed. The overall survival probabilities were analyzed using the log-rank test. P< 0.05 was considered to be significant.

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None

**CONFLICT OF INTEREST**

The authors have no conflicts of interest to declare.

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References


CHAPTER 4

TLR3 MEDIATES REPAIR AND REGENERATION OF DAMAGED NEONATAL HEART THROUGH GLYCOLYSIS DEPENDENT YAP1 REGULATED MIR-152 EXPRESSION

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Running head: TLR3 is required for neonatal heart regeneration

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Abstract

The present study investigated whether TLR3 is required for neonatal heart repair and regeneration following myocardial infarction (MI). TLR3 deficient neonatal mice exhibited impaired cardiac functional recovery and a larger infarct size, while wild type neonatal mice showed cardiac functional recovery and small infarct size after MI. The data suggests that TLR3 is essential for the regeneration and repair of damaged neonatal myocardium. In vitro treatment of neonatal cardiomyocytes with a TLR3 ligand, Poly (I:C), significantly enhances glycolytic metabolism, YAP1 activation and proliferation of cardiomyocytes which were prevented by a glycolysis inhibitor, 2-deoxyglucose (2-DG). Administration of 2-DG to neonatal mice abolished cardiac functional recovery and YAP1 activation after MI, suggesting that TLR3 mediated regeneration and repair of the damaged neonatal myocardium is through glycolytic dependent YAP1 activation. Inhibition of YAP1 activation abolished Poly (I:C) induced proliferation of neonatal cardiomyocytes. Interestingly, activation of YAP1 increases the expression of miR-152 which represses the expression of cell cycle inhibitory proteins, P27kip1 and DNMT1, leading to cardiomyocyte proliferation. We conclude that TLR3 is required for neonatal heart regeneration and repair after MI. The mechanisms involve glycolytic dependent YAP1 activation, resulting in miR-152 expression which targets DNMT1/p27kip1.
Introduction

Ischemic heart disease remains the major cause of death in the United States(1). The adult heart has limited capacity to regenerate and repair damaged myocardium induced by ischemia/reperfusion (I/R) injury. The mammalian heart, including humans, contains pre-existing cardiomyocytes which have the capability of proliferation and regeneration(2-4). The mechanisms involve multiple signaling molecules, including Hippo-YAP, PI3K/Akt and Wnt/beta-catenin as well as microRNAs(2, 5-12). Although the pre-existing cardiomyocytes in the adult heart have the capability to proliferate, the ability to repair and regenerate the damaged adult heart seems to be extremely limited(2-4).

Interestingly, the hearts of zebrafish and the neonatal mouse have the ability to repair and regenerate damaged myocardium(13-15). However, neonatal mouse hearts lose the capacity for proliferation and regeneration seven days after birth(14). This positively correlates with the changes in cardiomyocyte metabolism from glycolysis to oxidative phosphorylation. It is well known that more than 90% of the energy in adult cardiomyocytes is generated by mitochondrial oxidative phosphorylation(16), suggesting that glycolysis could play an important role in the proliferation of cardiomyocytes. Indeed, glycolytic metabolism is predominant in zebrafish and neonatal cardiomyocytes(17, 18) and is essential for somatic cell reprogramming and differentiation(19-22). Therefore, enhanced glycolytic metabolism could be an important approach for induction of cardiomyocyte proliferation.
Toll like receptor (TLR) ligands have been reported to promote metabolic reprogramming from oxidative phosphorylation to glycolysis which is necessary for activation of immune cells and for trained innate immunity (23-25). TLRs are well known as pattern-recognition receptors that play a critical role in the induction of innate immunity, inflammatory responses(26, 27) and myocardial ischemia/reperfusion (I/R) injury(28). Recent studies have reported that TLR-mediated signaling involves cell reprogramming and tissue regeneration(29-31). However, the mechanisms remain elusive.

YAP and TAZ are major downstream effectors of the Hippo signaling pathway which play critical roles in controlling organ size(32). The Hippo pathway is comprised of core kinase complexes including mammalian STE20-like protein kinase 1 and 2 (MST1/2), large tumor suppressor 1 and 2 (LATS1/2), and the adaptor proteins SAV1 and MOB1. Activation of MST1/2 phosphorylates LATS1/2 which suppress YAP/TAZ transcriptional activity by phosphorylation(32, 33). YAP1 and TAZ have been demonstrated to regulate cardiomyocyte proliferation and regeneration(8, 9, 11, 12, 33, 34). However, the mechanisms by which YAP/TAZ regulates cardiomyocyte proliferation and heart regeneration have not been elucidated.

MicroRNAs (miRs) are 21 to 23 nucleotide non-coding RNAs that can regulate up to 90% of human gene expression(35). Several microRNAs have been reported to be involved in the protection against myocardial I/R injury(36, 37) and in the regulation of heart regeneration(5, 10, 38-40). Interestingly, miR-152 has been reported to target cell
cycle inhibition proteins (41, 42). However, the role of miR-152 in the cardiomyocyte proliferation has not been investigated.

In the present study, we demonstrated that TLR3 is required for neonatal damaged heart regeneration. TLR3 activation induces glycolysis dependent YAP1 activation which regulates the expression of miR-152, leading to regulation of neonatal cardiomyocyte proliferation by targeting cell cycle inhibitory proteins DNMT1/p27kip1.

Results

TLR3 deficiency impairs neonatal heart regeneration after MI.

Neonatal mouse heart has a high regenerative capacity (13, 14). To investigate whether TLR3 could involve neonatal heart regeneration and repair, one day old wild type (WT) or TLR3 deficient (TLR3−/−) mice were subjected to myocardial infarction (MI) by permanent ligation of LAD. Cardiac function was examined by echocardiography and cardiomyocyte proliferation was evaluated by 5-ethynyl-2′-deoxyuridine (EdU) incorporation 21 days after MI. As shown in Figure 4.1A, WT neonatal mice show a smaller infarct size, while TLR3−/− neonatal hearts exhibit larger scarring compared with WT MI mice. EdU positive staining of cardiomyocytes in WT neonatal MI heart tissues are significantly greater (52.9%) than that in TLR3−/− neonatal MI heart tissues. In addition, the values of cardiac function in WT neonatal MI mice are compatible with WT sham control. In contrast, the values of cardiac function in TLR3−/− neonatal MI mice are significantly lower than that in TLR3−/− sham control and WT MI mice (Fig. 4.1B). The data suggest that TLR3 is necessary for neonatal heart regeneration and repair after MI.
Figure 4.1. TLR3 deficiency reduced neonatal cardiomyocyte proliferation (A) and impaired the cardiac functional recovery (B) following MI. TLR3 activation increases glycolysis and glycolytic capacity in isolated neonatal cardiomyocytes (C). Glycolysis is necessary for TLR3 mediated cardiomyocyte proliferation (D) and neonatal heart functional recovery after MI (E). n=4-8/group. *p<0.05 compared with indicated groups.
**TLR3 activation enhances glycolysis and promotes cardiomyocyte proliferation.**

TLR ligands have been reported to induce glycolytic re-programming through PI3K/AKT/Hif1α dependent signaling in immune cells(23-25). To investigate whether activation of TLR3 with Poly (I:C) would enhance glycolysis in cardiomyocytes, we isolated neonatal cardiomyocytes from one day old WT mice, treated them with Poly (I:C) for 12 hours, and analyzed glycolytic metabolism. As shown in **Figure 4.1C**, Poly (I:C) treatment markedly enhances glycolysis and glycolytic capacity compared with untreated controls. Poly (I:C) treatment also significantly increases the proliferation rate of neonatal cardiomyocytes as evidenced by increased Edu staining of neonatal cardiomyocytes (**Fig. 4.1D**). Interestingly, treatment of neonatal cardiomyocytes with 2-deoxy-D-glucose (2-DG), an inhibitor for hexokinase 2 which is an initial step in glycolysis, prevents Poly (I:C) induced neonatal cardiomyocyte proliferation (**Fig. 4.1D**). Importantly, *in vivo* administration of 2-DG to one day old (P1) WT neonatal mice immediately after induction of MI significantly impairs cardiac functional recovery, when compared with untreated WT neonatal hearts 21 days after MI (**Fig. 4.1E**). The data suggests that glycolytic metabolism plays a critical role in TLR3-mediated neonatal cardiomyocyte proliferation and neonatal heart regeneration and repair after MI.

**TLR3 mediates YAP1 activation via a glycolytic dependent mechanism.**

Our data shows that Poly (I:C) induced proliferation of neonatal cardiomyocytes is mediated via glycolysis. To address how enhanced glycolysis by Poly (I:C) promotes neonatal cardiomyocyte proliferation, we investigate whether YAP1/TAZ activation involves TLR3-mediated neonatal heart regeneration and repair following MI. YAP1/TAZ
are two main downstream effectors of Hippo signaling which plays a critical role in cell proliferation and tissue regeneration\(^8, 11, 34, 43, 44\). **Figures 4.2A** show that the levels of YAP1/TAZ in cytosol and nuclei were markedly increased in WT MI hearts compared with WT sham control. In TLR3\(^{-/-}\) MI mice, however, the levels of YAP1/TAZ in the cytosol and nuclei did not significantly change compared with TLR3\(^{-/-}\) sham control and were markedly lower than that in WT MI mice. The data suggests that TLR3 is an essential for YAP1-/TAZ activation after MI.

We then examined whether Poly (I:C) induced activation of YAP1 is mediated by glycolysis. As shown in **Figure 4.2B/C**, Poly (I:C) treatment significantly increases the levels of YAP1 in both cytosol and nuclei in WT cardiomyocytes, but not in TLR3\(^{-/-}\) cardiomyocytes (**Fig. 4.2B**). However, inhibition of glycolysis by 2-DG prevented Poly I:C-induced activation of YAP1 (**Fig. 4.2C**), suggesting that TLR3-mediated YAP1 activation is through glycolytic dependent mechanism.
Figure 4.2. TLR3 deficiency suppressed MI induced YAP1/TAZ expression and nucleus translocation (A) following MI. TLR3 ligand Poly I:C treatment increases the level of YAP1 in the cytosol and promotes YAP1 nuclear translocation in WT cardiomyocytes, but not in TLR3 deficiency cardiomyocytes (B). Glycolysis inhibition with 2-DG prevents Poly I:C-induced YAP1 activation and nuclear translocation (C). n=3-6/group. *p<0.05 compared with indicated groups.

YAP1 activation is needed for TLR3 mediated cardiomyocyte proliferation.

To determine the role of YAP/TAZ in TLR3-mediated cardiomyocyte proliferation, we analyzed the expression pattern of YAP/TAZ in the myocardium at the different stages of neonatal heart maturation. As shown in Figure 4.3A, the levels of YAP1/TAZ expression are the highest in P1 and P3 neonatal hearts, gradually reduced on P7, and remained low on P14, and p21 which were positively correlated with a loss of regenerative capacity in damaged neonatal hearts. To conform that YAP1 plays an important role in cardiomyocyte proliferation, we transfected neonatal cardiomyocytes
with activated YAP1 (AAV-YAP^{S127A}) to induce overexpression of YAP1. AAV-Luci served as a vector control. Figures 4.3B and C show that activated YAP1 transfection significantly promotes neonatal cardiomyocyte proliferation. Inhibition of YAP1 with specific siRNA for YAP1 (Fig. 4.3D) or YAP1 inhibitor, verteporfin (Fig. 4.3D) markedly suppresses Poly (I:C)-induced YAP1 expression, YAP1 nuclear translocation (Fig. 4.3E-G) and proliferation of neonatal cardiomyocytes. Verteporfin was a more effective inhibitor of YAP1 than siRNA. However, by using both approaches we were able to confirm that activation of YAP1 is necessary for Poly (I:C)-induced proliferation of neonatal cardiomyocytes.
Figure 4.3. YAP1 activation is required for TLR3 mediated cardiomyocyte proliferation. The expression of YAP/TAZ is gradually downregulated during post-neonatal heart development (A). AAV virus mediated activated YAP1 overexpression (B) promotes neonatal cardiomyocyte proliferation (C). YAP1 inhibition with specific siRNA for YAP1 or YAP inhibitor, verteporfin (VP) markedly suppresses Poly I:C (PIC)-induced neonatal cardiomyocyte proliferation (D), YAP1 expression in cytosol and nuclear translocation(E-G). n=3-8/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.

The TLR3 activation suppresses YAP1 phosphorylation, leading to YAP1 activation.

Our data shows that YAP1 activation is involved in TLR3 mediated neonatal cardiomyocyte proliferation. To investigate whether Poly (I:C) induces YAP1 expression at transcriptional or post-transcriptional levels, we analyzed mRNA levels of YAP/TAZ in neonatal cardiomyocytes treated with Poly (I:C).
**Figure 4.4A** shows that Poly (I:C) treatment did not alter the levels of YAP/TAZ mRNAs, indicating that TLR3 meditated YAP1 activation is through post-translational modification.

Figure 4.4. TLR3 activation reduces YAP1 phosphorylation, leading to YAP1 activation. TLR3 activation has no effect on YAP1 or TAZ mRNA expression (A). Poly (I:C) treatment significantly reduced the phosphorylation level of LATS1 (B), MOB1 and YAP1(C). n=3-5/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.

YAP1 contains two main sites for the phosphorylation of S127 and S397(32). Phosphorylation of S127 promotes YAP1 binding with protein 14-3-3, thus preventing its translocation into the nucleus(32, 45). Phosphorylation of S397 facilitates YAP1 degradation(32, 46). Therefore, dephosphorylation of YAP1 will lead to activation and nuclear translocation. As shown in **Figure 4.4C**, Poly (I:C) treatment significantly
decreases the levels of phosphorylated YAP1 at both S127 and S397 in a time dependent manner, and markedly increases total YAP1 levels. The data suggests that Poly (I:C) induces YAP1 activation by suppressing YAP1 phosphorylation.

Suppression of YAP1 phosphorylation by Poly (I:C) is mediated through dephosphorylation of LATS1 and MOB1.

LATS1/2 phosphorylates YAP1 at both the S127 and S397 sites, resulting in inactivation or degradation of YAP1(32, 45, 46). MOB1 is an adaptor protein of Hippo signaling and interacts with LATS1/2 to promote YAP1 phosphorylation(47). In contrast, decreased LATS1 and MOB1 phosphorylation will release their inhibitory effect on YAP/TAZ activation(32, 47). We examined the effect of Poly (I:C) treatment on LATS1 and MOB1 phosphorylation in neonatal cardiomyocytes. Figures 4.4B and C show that Poly (I:C) treatment markedly reduced the levels of LATS1 and MOB1 phosphorylation in a time dependent manner, indicating that Poly (I:C)-induced YAP1 activation is mediated through induction of LATS1 and MOB1 dephosphorylation.
Figure 4.5. PP1α is involved in Poly (I:C) induced LATS1 and MOB1 pephosphorylation, YAP1 activation, and neonatal cardiomyocyte proliferation. Poly (I:C) treatment increased the interaction of PP1 with LATS1 and PP1 with MOB1 (A). PP1 inhibitor Okadaic acid (OA) treatment increased phosphorylation of LATS, YAP1 and MOB1 and prevented Poly (I:C) induced decreases in the levels of LATS1, YAP1 and MOB1 phosphorylation. (B) PP1 inhibitor OA treatment prevented Poly (I:C) induced YAP1 nuclear translocation (C) and neonatal cardiomyocyte proliferation (D). n=3-8/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.
TLR3 activation induces an interaction between PP1a with LATS1 and MOB1

Protein phosphorylase 1 (PP1) plays an important role in the induction of protein dephosphorylation (48, 49). We investigated whether Poly (I:C) could induce an interaction between PP1a and LATS1, resulting in LATS1 and the downstream effector YAP1 dephosphorylation in the neonatal cardiomyocytes. As shown in Figure 4.5, Poly (I:C) treatment significantly promotes the interaction between PP1a and LATS1 (Fig. 4.5A) as evidenced by showing high levels of LATS1 in the PP1a immuneprecipitates. Poly (I:C) treatment also markedly promotes the interaction of PP1a with MOB1 (Fig. 4.5A). The data indicates that PP1a is involved in Poly (I:C) induced decreases in the levels of LATS1 and MOB1 phosphorylation.

To confirm our observation, we treated neonatal cardiomyocytes with a PP1a inhibitor, Okadaic acid (50) and observed that PP1a inhibition abolished Poly (I:C) induced decreases in the levels of phosphorylated LATS1 as well as MOB1. PP1a inhibition also increased YAP1 phosphorylation, resulting in decreases in the levels of YAP1 (Fig. 4.5B). In addition, PP1a inhibition markedly attenuated Poly (I:C) induced YAP1 nuclear translocation and neonatal cardiomyocyte proliferation (Fig. 4.5C/D). The data suggests that Poly (I:C) induced YAP1 activation and nuclear translocation are mediated by promoting the interaction of PP1a with LATS1 and MOB1, resulting in inactivation of both LATS1 and MOB1 through their dephosphorylation.
Activation of TLR3 induces glycolysis mediated PP1a dependent LATS1 and YAP1 dephosphorylation.

Our above data indicates that PP1a and LATS1 interaction contributes to Poly (I:C) induced YAP1 activation via its dephosphorylation (Fig. 4.5). We also observed that Poly (I:C) induced YAP1 activation is mediated through glycolytic dependent mechanism (Fig. 4.2B/C). To investigate whether glycolysis plays a role in Poly (I:C) induced the interaction of PP1a with LATS1 and decreased levels of phosphorylated LATS1 and YAP1, we treated neonatal cardiomyocytes with 2-DG in the presence or absence of Poly (I:C) and examined the interaction of PP1a with LATS1 and the levels of LATS1 and YAP1 phosphorylation. As shown in Figure 4.6A, Poly (I:C) treatment significantly strengthened the interaction between PP1a and LATS1. However, inhibition of glycolysis by 2-DG prevented Poly I:C-induced interaction of PP1a with LATS1, suggesting that Poly I:C-induced interaction of PP1a with LATS1 is mediated through glycolytic metabolism. To further conform the role of glycolysis in Poly I:C-induced dephosphorylation of LATS1 and YAP1, we treated neonatal cardiomyocytes with 2-DG and examined the levels of LATS1, MOB1 and YAP1 phosphorylation. As shown in Figure 4.6B, 2-DG treatment alone markedly increased the levels of LATS1 and YAP1 phosphorylation (Figs. 4.6B and C). Importantly, 2-DG administration also abolished Poly (I:C) suppressed phosphorylation of LATS1, thereby increasing YAP1 phosphorylation. The data suggests that glycolysis is involved in Poly (I:C) induced decreases in the levels of LATS1 phosphorylation by promoting the interaction between PP1a and LATS1, leading to YAP1 activation. We observed that 2-DG treatment did not alter Poly (I:C) induced decreases in the levels of MOB1 phosphorylation (Fig. 4.6C),
indicating that MOB1 dephosphorylation induced by Poly (I:C) could be mediated through glycolytic independent mechanism.

Figure 4.6. Poly (I:C) induced glycolysis mediated PP1a dependent LATS1 and YAP1 dephosphorylation. Glycolysis inhibitor, 2-DG treatment prevents Poly I:C-induced interaction of LATS1 with PP1 (A) and decreases in the levels of phosphorylated LATS and YAP1 (B and C). 2-DG treatment did not alter Poly (I:C) decreased MOB1 phosphorylation (C). n=3-5/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.
TLR3 modulates AMPK phosphorylation and YAP1 activation via a glycolytic dependent mechanism.

AMPK is abundantly expressed in the heart and plays an important role in the regulation of cellular metabolism(51). AMPK activity is regulated by glycolysis and involves energy stress-induced inactivation of YAP1(52). Since Poly (I:C) enhances glycolysis in neonatal cardiomyocytes, we examined whether Poly (I:C) would regulate AMPK activation via glycolysis. As shown in Figure 4.7A, following Poly (I:C) treatment, the levels of phosphorylated AMPK are gradually reduced in a time dependent manner. Administration of 2-DG abolished Poly (I:C)-suppressed AMPK phosphorylation and significantly increase the levels of phosphorylated AMPK (Fig. 4.7B). The data indicates that Poly (I:C) decreases AMPK phosphorylation via glycolysis.

To examine whether AMPK would play a role in the regulation of YAP1 activation, we treated neonatal cardiomyocytes with an AMPK specific activator, metformin and examined YAP1 activation as well as the proliferation of neonatal cardiomyocytes in the presence and absence of Poly (I:C). As shown in Figure 4.7C, treatment of the cells with metformin abolished Poly (I:C) induced decreases in AMPK phosphorylation. Importantly, metformin treatment also abolished Poly (I:C) induced dephosphorylation of YAP1 (Fig. 4.7D), YAP1 nuclear translocation (Fig. 4.7E), and neonatal cardiomyocyte proliferation (Fig. 4.7F). The data suggests that AMPK is an upstream of YAP1 and that activation of AMPK negatively regulates YAP1 activation and nuclear translocation as well as neonatal cardiomyocyte proliferation.
Figure 4.7. Effect of AMPK on TLR3 mediated YAP1 activation. TLR3 activation significantly reduced the phosphorylation level of AMPK (A). 2-DG treatment prevented TLR3 mediated dephosphorylation of AMPK (B). Treatment of neonatal cardiomyocytes with AMPK activator metformin prevents TLR3 mediated dephosphorylation of AMPK and YAP1 (C and D). Treatment with the AMPK activator, metformin, prevented TLR3 mediated YAP1 nuclear translocation (E). Metformin prevented TLR3 mediated neonatal cardiomyocyte proliferation (F). n=3-6/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.

Activation of YAP1 regulates miR-152 expression in neonatal cardiomyocytes. To investigate the mechanisms by which activated YAP1 induces neonatal cardiomyocyte proliferation, we examined the role of microRNA-152 (miR-152) in TLR3 mediated YAP1 dependent neonatal cardiomyocyte proliferation. It is well known that miR-152 targets cell cycle entry proteins p27kip1 and DNA methyltransferase1 (DNMT1) which are important proteins in the regulation of cell proliferation(41, 42). As shown in Figure 4.8A, miR-152 levels are the highest in P1 neonatal hearts but gradually reduced in P3 and P7 neonatal hearts (Fig. 4.8A). In vitro treatment of neonatal cardiomyocytes with Poly (I:C) significantly increases expression of miR-152 (Fig. 4.8B). However, inhibition of YAP1 with a YAP1 inhibitor (VP) prevented Poly (I:C) induced increases in miR-152 expression (Fig. 4.8B). In contrast, increased YAP1 activation by transfection of neonatal cardiomyocytes with AAV-YAP1 markedly increases the levels of miR-152 expression (Fig. 4.8C). The data suggests that activation of YAP1 regulates the expression of miR-152 in neonatal cardiomyocytes.
Figure 4.8. TLR3 mediated miR-152 expression is YAP1 dependent which is involved in TLR3 mediated cardiomyocyte proliferation. The expression of miR-152 gradually decreased during postnatal heart development (A). YAP1 is necessary for TLR3 mediated miR-152 upregulation (B and C). miR-152 overexpression promotes cardiomyocyte proliferation (D) and inhibits p27kip1 and DNMT1 expression (E). Anti-miR-152 treatment partially reduces TLR3 mediated cardiomyocyte proliferation (F). n=3-6/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.
MiR-152 contributes to TLR3 mediated cardiomyocyte proliferation.

To investigate the role of miR-152 in TLR3 mediated neonatal cardiomyocyte proliferation, we transfected neonatal cardiomyocytes with miR-152 mimics or anti-miR-152 mimics, respectively (Fig. 4.S1) and examined the proliferation of neonatal cardiomyocytes. Figure 4.8D shows that delivery of miR-152 mimics significantly promotes the proliferation of neonatal cardiomyocytes as evidenced by incorporation of Edu into the neonatal cardiomyocytes. MiR-152 mimic transfection markedly suppresses the expression of p27kip and DNMT1 in the neonatal cardiomyocytes (Fig. 4.8E). P27kip1 is a cyclin-dependent kinase inhibitor while DNMT1 plays a critical role in regulation of the cell cycle(53, 54). Suppression of p27kip1 and DNMT1 will promote cell cycle entry, leading to proliferation. However, suppression of miR-152 expression by transfection of cells with anti-miR-152 significantly attenuated Poly (I:C) induced cell proliferation (Fig. 4.8F). The data suggests that an increased level of miR-152 promotes neonatal cardiomyocyte proliferation via suppression of p27kip and DNMT1 expression.

Discussion.

The present study demonstrated that TLR3 is needed for regeneration and repair damaged neonatal hearts following myocardial infarction. Mechanisms involve glycolytic dependent YAP1 activation which up-regulates the expression of miR-152, leading to activation of cell cycle by suppressing p27kip1 and DNMT1 expression. There are several novel findings in the present studies. First, we provided compelling evidence showing TLR3 is essential for neonatal heart regeneration and repair of infarcted myocardium. Second, we demonstrated that activation of TLR3 enhances glycolysis,
leading to YAP1 activation. Third, we observed that TLR3 mediated glycolysis also induces AMPK dephosphorylation, resulting in YAP1 activation, indicating that AMPK negatively regulates YAP activation and nuclear translocation. Finally, we demonstrated that activation of YAP1 increases the expression of miR-152 which targets cell cycle inhibitory proteins p27kip1 and DNMT1, resulting in promoting cell proliferation (Fig. 9).

Figure 4.9. Illustration of TLR3 mediated YAP1 activation and miR-152 expression in cardiomyocyte proliferation. Activation of TLR3 increases glycolysis, resulting in inactivation of LATS and AMPK by reducing their phosphorylation. Inactivated LATS and AMPK lead to activation of YAP1 which, as a co-transcriptional factor regulates miR-152 expression. miR-152 suppresses p27kip1 and DNMT1 expression, promoting cell proliferation.

Toll-like receptors (TLRs) are conserved pattern-recognition receptors that play a critical role in the induction of innate and inflammatory responses(26, 27) and involve
the pathophysiology of myocardial ischemia/reperfusion (I/R) injury(28). We have previously reported that TLR3 contributes to acute and early I/R-induced adult myocardial injury(28). Recent studies have shown that TLR3-mediated signaling is involved in cell reprogramming and tissue regeneration(29-31), indicating that TLR3 may be involved in damaged heart repair and regeneration in the late stage of myocardial ischemic injury. It is well known that adult heart has extremely limited capability for regeneration and repair of damaged myocardium(2-4). Therefore, neonatal heart is an optional model for investing the role of TLR3 in damaged heart repair and regeneration. The role of macrophages in neonatal heart regeneration has been well demonstrated(55). Therefore, the present study focused on the role of TLR3 in cardiac myocyte proliferation in damaged heart repair and regeneration. We observed that TLR3 is required for neonatal heart repair and regeneration after MI. Specifically, we observed that TLR3 deficiency impairs neonatal heart repair and regeneration after MI. At present, we do not understand what endogenous ligands might activate TLR3 for repair and regeneration in the damaged neonatal heart. We observed that the TLR3 ligand, Poly (I:C), significantly induced proliferation of neonatal cardiomyocytes via glycolytic dependent mechanisms. Recent studies have shown that activation of TLRs involves re-programming in immune cells via glycolytic metabolism(23-25) which is necessary for cell proliferation by supplying most of the intermediates for lipid production, nucleotide and amino acid synthesis. In contrast to non-proliferating adult cardiomyocytes, glycolysis is predominant in neonatal cardiomyocytes(17, 18). We observed that TLR3 activation markedly enhanced glycolysis in neonatal cardiomyocytes and demonstrated, for the first time to our knowledge, that glycolysis
plays a critical role in the proliferation of neonatal cardiomyocytes \textit{in vitro} and heart repair and regeneration \textit{in vivo}.

Activation of YAP1 has been shown to play an important role in mediating cell proliferation and differentiation\cite{32, 33}. We observed that administration of Poly (I:C) significantly increased the activation of YAP1 in neonatal cardiomyocytes, indicating that Poly (I:C) induced neonatal cardiomyocyte proliferation could be mediated by activation of YAP1. Indeed, we observed that the levels of YAP1 are markedly greater in P1 and P3 neonatal hearts, but gradually decreased in the hearts of P7, P14 and P21 neonatal mice, which is consistent with the capability of the neonatal heart to regenerate after ischemic injury. Reduced YAP1 levels in the post-neonatal heart may also be associated with the loss of regenerative capacity in adult heart. To investigate why neonatal hearts contain higher levels of YAP1, we examined whether glycolysis would regulate the expression of YAP1 in neonatal cardiomyocytes. We found that suppression of glycolysis by 2-DG significantly reduced the expression of YAP1 and prevented Poly (I:C) induced increases in the levels of YAP1 in the neonatal cardiomyocytes. This finding, suggests that glycolysis regulates YAP1 expression in neonatal cardiomyocytes.

We observed that TLR3 activation does not alter YAP/TAZ mRNA levels in neonatal cardiomyocytes, indicating that TLR3 regulates YAP1 activation at the post transcriptional level. It is well known that increased phosphorylation of LATS1 and its adaptor protein MOB1 will result in YAP1 phosphorylation and degradation\cite{32, 47}. The
present study showed that Poly (I:C) treatment significantly induced decreases in the levels of LATS1 and MOB1 phosphorylation. Importantly, inhibition of glycolysis by 2-DG abolished Poly (I:C) induced de-phosphorylation of LATS1 but not MOB1. This data suggests that Poly (I:C) induced decreases in the levels of phosphorylated MOB1 may be mediated through a glycolytic independent mechanism.

PP1 is a protein phosphatase which has been reported to interact with LATS1, resulting in inactivation of LATS1/2 by de-phosphorylation of LATS1 or direct dephosphorylation of YAP1/TAZ, leading to their activation(48, 49, 52). We observed that Poly (I:C) treatment significantly promotes the interaction of PP1a with MOB1 as evidenced by showing that MOB1 appears in the immunoprecipitates by anti-PP1a. Poly (I:C) administration also induces an interaction between PP1a and LATS1. Thus Poly (I:C) induced de-phosphorylation of MOB1 and LATS1/2 could be through an interaction of PP1 with both molecules. To confirm our observation, we treated neonatal cardiomyocytes with a PP1a inhibitor, okadaic acid, and observed that inhibition of PP1a significantly induced phosphorylation of MOB1 and LATS1 and abolished Poly (I:C) induced decreases in the levels of phosphorylated MOB1 and LATS1. The data suggests that Poly (I:C) induced YAP1 activation promotes the co-association of PP1a with MOB1 and LATS1, resulting in inactivation of both MOB1 and LATS1. Indeed, PP1a inhibition by okadaic acid abolished Poly (I:C) induced neonatal cardiomyocyte proliferation.
Activation of AMPK signaling has been reported to suppress YAP1 activation through phosphorylation of LATS1(52, 56). In the present study, we observed that TLR3 activation induces enhanced glycolysis in neonatal cardiomyocytes. AMPK has been reported to be involved in glycolytic metabolism(51). We examined whether AMPK might be involved in Poly (I:C) induced proliferation of neonatal cardiomyocytes. To the best of our knowledge, this is the first report that TLR3 activation decreases AMPK phosphorylation. This effect was abolished by 2-DG administration, suggesting that Poly (I:C) induced de-phosphorylation of AMPK is mediated by glycolysis. To confirm the role of AMPK in Poly (I:C) induced YAP activation and the proliferation of neonatal cardiomyocytes, we treated neonatal cardiomyocytes with metformin, an AMPK activator and observed that increased phosphorylation of AMPK by metformin significantly abolished Poly (I:C) induced YAP1 activation and nuclear translocation as well as proliferation of neonatal cardiomyocytes. Our findings suggest that, in addition to inactivation of LATS1 leading to activation of YAP1 through glycolysis, Poly (I:C) also induces de-phosphorylation of AMPK via glycolysis resulting in YAP1 activation and nuclear translocation in neonatal cardiomyocyte proliferation.

It is well known that activation of YAP1 promotes cell proliferation and differentiation(32, 33). However, the mechanisms by which activated YAP1 regulates cell proliferation and heart regeneration remain elusive. YAP1 is a co-transcriptional factor which can regulate microRNAs biogenesis and expression with its DNA binding partner TEAD(57, 58). To address this issue, we investigated whether activated YAP1/TAZ could regulate the expression of proteins, such as P27kip1 and DNMT1
(DNA methyltransferase 1), controlling cell cycle entry(53, 54). P27kip1 is a cyclin-dependent kinase inhibitor by binding and inhibiting cyclin/CDKs(53). DNA methylation in the promoter region of cell cycle related genes is associated with gene repression and prevents cell proliferation. DNMT1 is gradually upregulated during post-neonatal heart development and inhibition of DNMT1 markedly increases cardiomyocyte proliferation(54). Therefore, it is possible that targeting both P27kip1 and DNMT1 could promote cell proliferation. We demonstrated that activation of YAP1 by Poly (I:C) significantly increases the expression of miR-152 which represses the expression of P27kip1 and DNMT1, resulting in proliferation of neonatal cardiomyocytes. DNMT1 has been reported to be gradually upregulated during post-neonatal heart development(59). Meanwhile, the miR-152 expression is downregulated which is associated with losing repair and regenerative capacity of damaged hearts.

In summary, we demonstrated that TLR3 is necessary for the proliferation of neonatal cardiomyocytes and repair and regeneration of ischemic injured hearts. The mechanisms involve glycolysis dependent YAP1 activation via PP1a mediated suppression of MOB1 and LATS1 and through AMPK inactivation. Activated YAP1 increases the expression of miR-152 which targets DNMT1/p27kip1, leading to promoting cell proliferation. Activation of TLR3 could be a novel strategy for the treatment of ischemic heart injury.
Materials and Methods:

Animals

TLR3 deficient (TLR3−/−) and wild type C57BL/6 mice were obtained from Jackson Laboratory (Indianapolis, IN). The mice were maintained in the Division of Laboratory Animal Resources at East Tennessee State University. The experiments outlined in this manuscript conform to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication, 8th Edition, 2011). The animal care and experimental protocols were approved by the ETSU Committee on Animal Care.

Induction of myocardial MI injury

One day old (P1) mice were subjected to myocardial infarction (MI) as described previously(55). Briefly, the neonatal mice will be anesthetized by hypothermia on ice for 3-5 min. The chest was swabbed with betadine and opened at the fourth intercostal space. 8-0 silk ligature will be used for LAD permanent ligation. The ribs will be sutured together and the chest wall incision closed. The pups will be warmed immediately after surgery by hand. Once all the surgeries were completed, the blood and skin of the pups were cleaned with mixed stuff from mother’s cage before the pups were sent back to mother’s cage. Cardiac function was assessed by echocardiography for up to 21 days after induction of MI as described previously(36, 37). Ejection fraction (EF%) and percent fractional shortening (FS%) were calculated(36, 37).
Isolation of neonatal cardiomyocytes

Neonatal cardiomyocytes were isolated from one day old (P1) WT mouse hearts as described previously(7). Briefly, hearts were harvested from P1 neonatal mice and cut into small pieces followed by digestion in dissociation buffer (116 mM NaCl, 20 mM Hepes, 0.8 mM Na₂HPO₄, 5.6 mM glucose, 5.4 mM KCl, and 0.8 mM MgSO₄, pH 7.35) containing 0.6 mg/ml of pancreatin and 0.4 mg/ml Collagenase Type II for 10 min. The supernatant was removed and digestion buffer was added. After several repeated digestions with 10 min for each step, the cell suspension was added with fetal bovine serum followed by centrifugation for 5 min at 100g. The cells were seeded onto uncoated dishes for 2-4 hours. The supernatant was recollected and plated into gelatin pre-coated plates.

Cardiomyocyte proliferation

The proliferation of neonatal cardiomyocytes was measured by EdU incorporation and anti-Ph3 staining(7, 9, 11). EdU incorporation was examined by Click-iT EdU imaging kit (Life Technologies) according to the manufacture’s protocol. Nuclei were stained with DAPI and cardiomyocytes were stained with a monoclonal anti-actin (Abcam, Cambridge, MA). The proliferation rate was calculated by dividing EdU⁺ cardiomyocytes by the total number of cardiomyocytes(7, 9, 11).

qPCR assay

mRNAs and microRNAs were isolated from heart tissues or cultured cells using the miRNAs isolation kit (RNAzol®RT, MRC) in accordance with the manufacture’s
protocol. Quantitative real-time (qPCR) was conducted using a 4800 Real-time PCR machine (Bio-Rad). mRNAs and microRNAs levels were quantified by qPCR using specific Taqman assays (Applied Biosystems, USA) and Taqman Universal Master Mix (Applied Biosystems). The levels of miR-152 and mRNA levels of YAP1 or TAZ were quantified with the 2 (-ΔΔct) relative quantification method that was normalized to the snRU6 or β-Actin (Applied Biosystems, USA).

**In vitro experiments**

The isolated neonatal cardiomyocytes were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented 10% fetal bovine serum under 5% CO2 at 37°C. To determine the role of TLR3 in the metabolism and proliferation of neonatal cardiomyocytes, the cells were treated with TLR3 specific ligand, Poly (I:C) (1 µg/ml). Cell proliferation was assessed by EdU incorporation (10umol/L)(7). The glycolytic capacity (ECAR) was examined with a Seahorse system. To examine the role of glycolysis and protein phosphotase 1 (PP1a) in TLR3-mediated YAP1 activation and cardiomyocyte proliferation, the isolated cardiomyocytes were treated with the glycolytic inhibitor, 2-DG (5 mmol/L) or PP1a inhibitor, okadaic acid, (0.5 mmol/L) respectively, before the cells were treated Poly (I:C) at 1 µg/ml. To determine whether YAP1 or miR-152 is necessary for TLR3-mediated cardiomyocyte proliferation, the isolated cardiomyocytes were treated with YAP1 inhibitor verteporfin (VP, 1 mmol/L) or transfected with siRNA-Con (80 nmol/L), siRNA-YAP1 (80 nmol/L), microRNA-Con mimics (40 nmol/L), miR-152 mimics (40 nmol/L), anti-miR-Con mimics (60 nmol/L), anti-miR-152 mimics (60 nmol/L), respectively prior to Poly (I:C) treatment. To
determine whether AMPK was involved in TLR3 mediated YAP1 activation and cardiomyocyte proliferation, the AMPK activator Metformin (1 mmol/L) was used prior to Poly (I:C) treatment.

**AAV virus packaging**

pAAV.cTnT::3Flag-hYAP and pAAV.cTnT::Luciferase vectors were kindly provided by Dr. William T. Pu (Harvard Stem Cell Institute). AAV virus was packaged in 293T cells using AAV-DJ Helper Free Packaging System (Cell Biolabs, Inc). The hYAP vector contains a S-127-A mutation resulting in a constitutive active form. The AAV virus were purified and concentrated by CsCl gradient centrifugation. The AAV virus titer was determined by AAVpro Titration Kit (Takara).

**Immunoprecipitation**

Approximately 200 µg of cellular proteins were subjected to immunoprecipitation with 2 µg of antibody to PP1a (Santa Cruz Biotechnology, CA) followed by the addition of 15 µl of protein A-agarose beads (Santa Cruz Biotechnology) as previously described(60). The precipitates were washed four times with lysis buffer and subjected to immunoblotting with the appropriate antibodies.

**Immunoblotting**

Immunoblotting was performed as described previously(36, 37). The primary antibodies (p-LATS1, LATS1, p-YAP1 (S127), p-YAP1 (S397), YAP1, p-MOB1, MOB1, p-AMPK, AMPK, p27kip1, DNMT1) and peroxidase-conjugated secondary antibody
were purchased from Cell Signaling Technology, Inc. The PP1a antibody was purchased from Santa Cruz Biotechnology. The signals were quantified using the G:Box gel imaging system by Syngene (Syngene, Fredric, MD).

**Statistical analysis**

Data are expressed as mean ± SD. Comparisons of data between groups were made using one-way analysis of variance (ANOVA), and Tukey’s procedure for multiple-range tests was performed. P< 0.05 was considered to be significant.

**Author contributions:** XHW and CFL conceived the project, designed the experiments, analyzed and interpreted data, and wrote the manuscript. XHW, TZH and YPH performed experiments. LL, RK and DW contributed to the scientific discussion and data interpretation. JK provided support for statistical analysis.

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**Disclosures:**

None.
References


Figure 4. S1. miR-152 is involved in TLR3 mediated cardiomyocyte proliferation. Transfection of MiR-152 mimics significantly increased miR-152 expression in isolated neonatal cardiomyocytes. n=3-4/group. *p<0.05 compared with indicated groups. #P < 0.05 compared with the control group.
Myocardial I/R injury is one of the most common forms of cardiovascular disease and the major cause of morbidity and mortality globally (Benjamin et al. 2017). It has been well established that the acute inflammatory response triggered by I/R injury exacerbates myocardial injury and leads to heart failure (Lange and Schreiner 1994; Kapadia et al. 1998; Chao 2009; Ha et al. 2011). Previous studies demonstrated that attenuation of I/R induced inflammatory responses significantly decreases I/R induced myocardial injury and cardiac dysfunction (Ha et al. 2008; Chao 2009; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013). We have reported that miR-146a overexpression protects the myocardium from I/R induced injury by negatively regulating TLR-mediated NF-κB signaling pathway through suppressing the expression of IRAK1 and TRAF6 (Wang et al. 2013). Interestingly, TRAF6 is a potential target of miR-125b based on the microRNA target prediction databases. In addition, miR-125b also plays an important role in apoptotic signaling by targeting tumor suppressor protein p53 and mitochondrial related pro-apoptotic protein BAK1. Activation of cellular apoptotic and necrotic signaling contribute to I/R induced myocardial injury and cardiac remodeling. Inhibition of the tumor suppressor gene p53 or pro-apoptotic protein BAK1, BAX, or Bim1 mediated apoptotic signaling markedly attenuates I/R induced myocardial injury and cardiac dysfunction (Lesnefsky et al. 1997; Levraut et al. 2003; Fiedler et al. 2011). Our microRNA array data indicates that miR-125b has a high level in adult cardiomyocytes. However, the role of miR-125b in I/R induced myocardial injury remains elusive. In chapter 2, we demonstrated that cardiac specific increases in the expression of miR-
125b significantly reduce infarct size and cardiac dysfunction after myocardial I/R injury. The underlying mechanisms include the inhibition of TLR-NF-κB mediated inflammatory response by suppressing TRAF6. In addition, increased expression of miR-125b significantly attenuates I/R induced myocardial cell apoptosis by targeting P53, Bax and BAK1. This study suggests that miR-125b could serve as a potential therapeutic target for inhibiting TLR-NF-κB signaling mediated inflammatory response, preventing P53, Bax and Bak1 mediated apoptotic signaling and ultimately protect against myocardial ischemia/reperfusion injury.

As mentioned in the introduction, the cardio-protective effect of TLR modulation is mediated by PI3K/Akt dependent mechanism (Hua et al. 2007; Ha et al. 2008; Ha et al. 2010; Ha et al. 2011; Cao et al. 2013). Although abundant studies have proposed that there has a crosstalk between TLR and PI3K/Akt signaling pathway, however, how TLR mediated pathway regulates PI3K/Akt signaling pathway remains elusive. We observed that TLR2 modulation by its ligands significantly attenuates I/R induced myocardial injury by increasing the activation of PI3K/Akt signaling (Ha et al. 2010). Importantly, we found that TLR2 ligand significantly increases the expression of miR-214 (Wang et al. 2016). MiR-214 directly targets PTEN which is an important negative regulator of PI3K/Akt signaling. In chapter 3, we investigated the role of miR-214 in I/R induced myocardial injury and observed that increased expression of miR-214 protects cardiac myoblasts H9C2 from hypoxia/reoxygenation induced cell injury in vitro and attenuates I/R induced myocardial infarct size and cardiac dysfunction in vivo. Both in vitro and in vivo data demonstrate that increased expression of miR-214 promotes activation of the PI3K/Akt signaling by suppressing PTEN expression.
When put together as a whole, our studies demonstrated that microRNAs play an important role in TLR modulation induced protection against myocardial I/R injury by increasing the activation of PI3K/Akt signaling pathway, decreasing TLR/NF-κB mediated inflammatory response, and suppressing activation of apoptotic signaling following myocardial I/R injury. The information generated from this dissertation could be beneficial for the development of new strategies for the treatment of patients with heart attack and heart failure.

Adult patients have limited cardiac functional recovery following myocardial infarction mainly due to loss and/or insufficient regeneration of cardiomyocytes. Interestingly, recent studies have shown that mammalian adult hearts can regenerate from pre-existing cardiomyocytes and exhibit a limited capacity of regeneration (Chen et al. 2013; Heallen et al. 2013; Senyo et al. 2013; Xin et al. 2013; Ali et al. 2014; Lin et al. 2015). TLR mediated immune response has an important role in tissue repair and regeneration (Grote et al. 2013; Carvalho et al. 2014; Kulkarni et al. 2014; Nelson et al. 2015; Natarajan et al. 2016). Recent studies have shown that TLR3 mediated signaling is required for somatic cell nuclear reprogramming and dsRNA mediated TLR3 activation plays an important role in skin regeneration (Lee et al. 2012; Nelson et al. 2015; Sayed et al. 2015; Natarajan et al. 2016). In the last study of this dissertation, we demonstrated for the first time to our knowledge, TLR3 is required for the neonatal heart repair and regeneration following myocardial infarction.

Mechanistic studies indicated that TLR3 mediated YAP1 activation plays an important role in neonatal cardiomyocyte proliferation and heart regeneration. YAP1 is a major downstream effector of the Hippo pathway which has been reported to regulate
cardiomyocyte proliferation and heart regeneration (Heallen et al. 2013; Xin et al. 2013; Lin et al. 2015). It is well known that glycolysis is predominant in zebrafish and neonatal heart which providing the most necessary building blocks for cell growth and proliferation (Vander Heiden et al. 2009; Krisher and Prather 2012; Donnelly and Finlay 2015). Glycolytic metabolism also plays a fundamental role in somatic cell reprogramming and tissue regeneration (Krisher and Prather 2012; Panopoulos et al. 2012; Zhang et al. 2012; Jones and Bianchi 2015). In addition, we also observed the level of YAP1 was high in P1 (one day old) and P3 neonatal mice. However, the levels were gradually decreased from P7 to P21 which were the periods of the metabolic switching from glycolysis to oxidative phosphorylation in cardiomyocyte. Various studies have demonstrated that glycolytic metabolism contributes to YAP1 transcriptional activity (DeRan et al. 2014; Enzo et al. 2015; Mo et al. 2015). Interestingly, we observed that TLR3 modulation by its specific ligand poly I:C significantly increased glycolysis and glycolytic capacity. Our in vitro studies also demonstrated that glycolysis increasing is necessary for TLR3 mediated YAP1 activation and cardiomyocyte proliferation. In vivo suppression of glycolysis significantly impaired neonatal cardiac repair and regeneration following MI injury in wild type mice, suggesting that glycolytic metabolism plays a critical role in TLR3 mediated YAP1 activation and neonatal heart regeneration.

YAP1 plays a fundamental role in cardiomyocytes proliferation and heart regeneration (Heallen et al. 2013; Xin et al. 2013; Lin et al. 2015). However, the mechanisms by which activated YAP1 regulates heart regeneration remain elusive. We found that TLR3 modulation significantly increases miR-152 expression which was YAP1 dependent. Meanwhile, our study also demonstrates that miR-152 involved in
TLR3 mediated cardiomyocytes proliferation by targeting the cell cycle inhibitory protein P27kip1 and DNMT1.

This study demonstrated for the first time to our knowledge, that TLR3 is necessary for neonatal heart repair and regeneration following ischemic injury. The underlying mechanisms including TLR3 induced glycolysis increasing and YAP1 activation. Moreover, YAP1 dependent miR-152 is also involved in the TLR3 mediated proliferative process by targeting p27kip1 and DNMT1. It is possible that TLR3 modulation could be a novel strategy for the treatment of heart failure patients by triggering heart regeneration and repair.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AMPK</td>
<td>-AMP-activated protein kinase</td>
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<tr>
<td>ANOVA</td>
<td>-Analysis of variance</td>
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<tr>
<td>AP-1</td>
<td>- Activator protein-1</td>
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<tr>
<td>ATP</td>
<td>-Adenosine triphosphate</td>
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<tr>
<td>Bak-1</td>
<td>-Bcl-2 homologous antagonist killer</td>
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<tr>
<td>Bax</td>
<td>-Bcl-2-associated X protein</td>
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<tr>
<td>BMSCs</td>
<td>-Bone marrow stromal cells</td>
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<tr>
<td>CDK1</td>
<td>-Cyclin-dependent kinase 1</td>
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<tr>
<td>CPCs</td>
<td>-Cardiac progenitor cells</td>
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<tr>
<td>CpG DNA</td>
<td>-Unmethylated CpG dinucleotides</td>
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<tr>
<td>DAMPS</td>
<td>-Damage associated molecular patterns</td>
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<tr>
<td>DGCR8</td>
<td>-Drosha, DiGeorge syndrome critical region 8</td>
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<tr>
<td>DNMT1</td>
<td>-DNA methyltransferase1</td>
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<tr>
<td>dsRNA</td>
<td>-Double stranded RNA</td>
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<tr>
<td>ECAR</td>
<td>-Extracellular acidification rate</td>
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<tr>
<td>EdU</td>
<td>-5-ethynyl-2'-deoxyuridine</td>
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<tr>
<td>EF</td>
<td>-Ejection fraction</td>
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<tr>
<td>EMSA</td>
<td>-Electrophoretic mobility shift assay</td>
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<tr>
<td>ER</td>
<td>-Endoplasmic reticulum</td>
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<tr>
<td>ERBB4</td>
<td>-Receptor tyrosine-protein kinase erbB-4</td>
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<tr>
<td>FBS</td>
<td>-Fetal bovine serum</td>
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<tr>
<td>FS</td>
<td>-Fraction shortening</td>
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FOXO3  -Forkhead box transcription factor
GAPDH  -Glyceraldehyde 3-phosphate dehydrogenase
GFP    -Green fluorescent protein
HMGB1  -High-mobility group protein B1
H/R    -Hypoxia/Reoxygenation
HIF-1α -Hypoxia-inducible factor 1-alpha
I/R    -Ischemia/reperfusion
IA     -Ischemic area
IFN    -Interferon
IHC    -Immunohistochemistry
IKK    -IκB kinase
IL-1β  -Interleukin-1β
IRAK   -Interleukin-1 receptor-associated kinase
IRF    -Interferon regulatory factor
IRF3   -IFN regulatory factor 3
LAD    -Left anterior descending
LATS1/2 -Large tumor suppressor 1 and 2
LDH    -Lactate dehydrogenase
LPS    -Lipopolysaccharide
LV     -Left ventricle
LY     -LY294002
MI     -Myocardial infarction
MiRNAs -MicroRNAs
MOB1   -Mps one binder kinase activator-like 1A
mPTP  -Mitochondrial permeability transition pore
MyD88  -Myeloid differentiation primary response gene (88)
MST1/2 -Mammalian STE20-like protein kinase 1 and 2
MTT    -3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC    -N-acetylcysteine
NF-κB -Nuclear factor kappa-B
OA     -Okadaic acid
PFK1   -Phosphofructokinase 1
PAMPS  -Pathogen associated molecular patterns
PBS    -Phosphate buffered saline
PDCD4  -Programmed cell death 4
PDK1   -Phosphoinositide-dependent kinase-1
PDTC   -Pyrrolidine dithiocarbamate
PGC1a  -Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PP1    -Protein phosphorylase 1
PI3K   -Phosphoinositide 3-kinase
PIP2   -Phosphatidylinositol 4, 5-bisphosphate or PtdIns(4,5)P2
PIP3   -Phosphatidylinositol (3, 4, 5)-triphosphate
Poly I:C -Polyinosine-polycytidylic acid
PRRS   -Pattern recognition receptors
PTEN   -Phosphatase and tensin homolog
PUMA   -p53 upregulated modulator of apoptosis
p53    -Tumor protein p53
qPCR   -Quantitative real-time
RA - Risk area
RIP1 - Receptor-interacting serine/threonine-protein kinase 1
RISC - RNA-induced silencing complex
RUNX1/2 - Runt-related transcription factor 1/2
ROS - Reactive oxygen species
SAV1 - Protein salvador homolog 1
SD - Standard error
SEM - Standard error of the mean
SR-A - Scavenger receptor type A
ssRNA - Single stranded RNA
TAB2 - TAK1-binding protein 2
TAZ - WW domain-containing transcription regulator protein 1
TBST - Tris buffered saline tween 20
TEADs - Transcriptional enhancer factor TEF-s
Tg - Transgenic mice
TNFα - Tumor necrosis factor alpha
TIR domain - Toll/interleukin-1 receptor homology domain
TRAF6 - TNFR-associated factor 6
TRAM - TRIF-related adaptor molecule
TRIF - TIR-domain-containing adapter-inducing interferon-β
TLRs - Toll-like receptors
TNF-α - Tumor necrosis factor-α
TRAF6 - TNF receptor associated factor 6
TRIF - TIR-domain-containing adapter-inducing interferon-β
TTC - Triphenyl tetrazolium chloride
TUNEL - Terminal deoxynucleotidyl transferase dUTP nick end labeling
WT - Wild type
VEGF - Vascular endothelial growth factor
VP - Verteporfin
YAP1 - Yes-associated protein 1
2-DG - 2-deoxyglucose
mg - Milligram
ml - Milliliter
pg - Picogram
µg - Microgram
µl - Microliter
µM - Micromolar
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