J Comp Physiol B (2016) 186:229–241
DOI 10.1007/s00360-015-0951-3

ORIGINAL PAPER

MicroRNA regulation in heart and skeletal muscle over the freeze–thaw cycle in the freeze tolerant wood frog

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Received: 25 September 2015 / Revised: 9 November 2015 / Accepted: 25 November 2015 / Published online: 11 December 2015

Abstract The North American wood frog, *Rana sylvatica*, is one of just a few anuran species that tolerates whole body freezing during the winter and has been intensely studied to identify the biochemical adaptations that support freeze tolerance. Among these adaptations is the altered expression of many genes, making freeze-responsive changes to gene regulatory mechanisms a topic of interest. The present study focuses on the potential involvement of microRNAs as one such regulatory mechanism and aims to better understand freeze/thaw stress-induced microRNA responses in the freeze-tolerant wood frog. Using quantitative PCR, relative levels of 53 microRNAs were measured in heart and skeletal muscle of control, 24 h frozen, and 8 h thawed frogs. MicroRNAs showed tissue specific expression patterns: 21 microRNAs decreased in the heart during thawing, whereas 16 microRNAs increased during freezing stress in skeletal muscle. These findings suggest that select genes may be activated and suppressed in heart and skeletal muscle, respectively, in response to freezing. Bioinformatics analysis using the DIANA miRPath program (v.2.0) predicted that the differentially expressed microRNAs may collectively regulate tissue-specific cellular pathways to promote survival of wood frogs undergoing freezing and thawing.

Keywords Hypometabolism · Non-coding RNA · *Rana sylvatica* · Cryoprotection

Communicated by I. D. Hume.

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Abbreviations

ARVC	Arrythmogenic right ventricular cardiomyopathy
cDNA	Complementary DNA
DIANA	DNA intelligent analysis
HCM	Hypertrophic cardiomyopathy
KEGG	Kyoto encyclopedia of genes and genomes
MAPK	Mitogen-activated protein kinase
miRNA	MicroRNA
mRNA	Messenger RNA
p-bodies	Processing bodies
qPCR	Quantitative polymerase chain reaction
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
rRNA	Ribosomal RNA

Introduction

The wood frog, *Rana sylvatica,* is one of several amphibians known to tolerate whole body freezing as an adaptation for survival when environmental temperatures plummet below 0 °C during the winter months. The wood frog has been studied for over 30 years as the main model animal of vertebrate freezing survival (Storey and Storey 1984, 1992, 2013). These frogs are distributed over a large geographical range, spanning the Piedmont of western Georgia, USA all the way to the treeline north of the Arctic Circle in Canada and Alaska (Martof and Humphries 1959). Throughout its range, wood frogs tolerate freezing as a winter survival mechanism. Frogs indigenous to subarctic regions can survive freezing down to about −16 °C whereas populations found in southern Canada and the American Midwest can

tolerate freezing temperatures of −3 to −6 °C (Costanzo et al. 2015; Larson et al. 2014).

Wood frogs have adopted various biochemical and physiological adaptations that allow them to tolerate the freezing of 65–70 % of total body water that accumulates in extracellular and extraorgan ice masses (Storey and Storey 1996, 2013). In addition to a need to manage ice formation, frozen frogs also need to endure the interruption of oxygen delivery to their tissues (since heart beat and blood flow cease) as well as strong dehydration and shrinkage of their cells when water is drawn out of cells to freeze in extracellular compartments. Thus, the wood frog has evolved various adaptations that allow it to effectively combat prolonged ischemia/anoxia and extreme cellular dehydration (Storey 2004; Storey and Storey 1992, 2013). One crucial mechanism utilized by the wood frog is the accumulation of high amounts of glucose that act as a cryoprotectant; glucose levels can increase ~100-fold in heart and ~20-fold in skeletal muscle when compared to unfrozen controls (Storey and Storey 1984). In addition, urea concentrations are elevated; this osmolyte is well known to accumulate under dehydration stress in amphibians and helps to retard the loss of body fluids that would otherwise lead to injury/death caused by reduced blood volume, increased blood viscosity, and circulatory impairment (Costanzo and Lee 2005). Adaptations to these stresss (freezing, dehydration, anoxia) involve complex biochemical changes and alteration of signal transduction pathways at the transcriptional, post-transcriptional, translational, and post-translational levels (Cowan and Storey 2003).

During freezing, wood frogs undergo prolonged cessation of physiological functions including heart beat and skeletal muscle movement (Layne and First 1991). Cardiac muscle requires the combinatorial actions of multiple signaling pathways to fulfill its physiological function (Sadoshima and Izumo 1993); it is under the control of the autonomic nervous system, and acts on involuntary stimulus (Robinson et al. 1966). As the frog transitions from an unfrozen to a frozen state, the heart undergoes significant biochemical changes and contractile activity is halted. Skeletal muscles are similarly regulated by multiple signaling pathways, but are functionally distinguished from cardiac muscles and fulfill the role of voluntary movement (Bassel-Duby and Olson 2006). Cardiac hypertrophy is a compensatory mechanism that occurs when insufficient oxygen and fuels are transported to the heart (Taegtmeyer et al. 2004; Bugger et al. 2010; Aubert et al. 2013). Conversely, skeletal muscle atrophy is induced by extended periods of disuse (Hunter et al. 2002), disuse being a condition that wood frogs must endure during prolonged freezing. Although neither cardiac hypertrophy nor skeletal muscle atrophy has been studied in the wood frog, it is of interest to understand how the relevant molecular pathways are regulated in stress-tolerant animal models. Several molecular pathways involved in the maintenance of muscle mass have been well characterized such as the PI3K/Akt pathway and MAPK signaling pathway (Glass 2005, 2010). Heart and skeletal muscle need to be maintained throughout the frozen period and then transitioned back into fulfilling their normal physiological functions when the frog thaws. Given the differences in function between these two types of muscle, it is important to understand the tissue-specific regulation of cellular processes involved in a freeze–thaw cycle.

MicroRNAs (miRNAs) are short, non-encoding, single stranded RNA molecules that function to regulate gene expression at the post-transcriptional level in most eukaryotic organisms (Bartel 2004). They are initially transcribed as longer pri-miRNAs containing secondary hairpin structures that are subsequently processed by the enzymes Drosha and DCGR8 into pre-miRNAs (Bartel 2004). Pre-miRNA molecules are then transported from the nucleus into the cytoplasm where they are processed by Dicer, which produces a single stranded RNA molecule (18–25 nt long) to be combined with other proteins such as Ago2 into a RNA-induced silencing complex (RISC) (Bartel 2004). RISC targets specific mRNA sequences by interacting with them at the 3′ untranslated region and inhibits their expression in one of three ways (He and Hannon 2004). Repression mediated by miRNAs depends on the degree of base complementarity that exists between the miRNA and target mRNA (Bartel 2004). MicroRNAs can reduce gene expression by initiating a signal cascade that targets mRNA transcripts for degradation, inhibiting translation by disrupting assembly of ribosomal initiation factors, or sequestering target mRNAs in cytoplasmic loci, such as P-bodies or stress granules (Liu et al. 2005). It is well documented that one miRNA can regulate the expression of many targets, and conversely, any specific gene transcript can be regulated by multiple miRNAs (Bartel 2004). Thus, miRNAs are crucial regulators of cellular signaling and stress responses which warrant further study in the freeze-tolerant wood frog.

In this regard, miRNAs may serve as a rapid and reversible mechanism to help reprioritize ATP usage for mRNA translation and dynamically regulate cellular processes under stress conditions (Biggar and Storey 2011). A growing number of studies in different stress-tolerant animals have suggested that microRNAs are important regulators of global metabolic rate depression and of specific coping strategies to deal with the consequences to cells of anoxia, dehydration, or hibernation (Kornfeld et al. 2012; Wu et al. 2013; Biggar and Storey 2014; Wu et al. 2014). To date, only 5 miRNAs species have been characterized in the freeze-tolerant wood frog (Biggar et al. 2009; Zhang and Storey 2013). Further studies on miRNAs would broaden our understanding of their role in the adaptation to freezing stress.

The present study evaluates the expression of 53 miRNAs in the heart and skeletal muscle of wood frogs. Relative levels of conserved miRNAs were quantified by employing a stem-loop miRNA amplification protocol. Tissue-specific expression of several miRNAs was identified suggesting a role for these non-coding RNAs in the maintenance and regulation of muscle tissues during freezing. Furthermore, bioinformatics resources were used to predict molecular pathways targeted by miRNA action in heart and skeletal muscle.

Materials and methods

Animal preparation

Adult male wood frogs (*R. sylvatica*) were collected in early spring from breeding ponds in the Ottawa (Canada) area. Frogs were washed in a tetracycline bath and then placed in plastic boxes containing moist sphagnum moss. Animals were then acclimated at 5 °C for at least 1 week before use. Control animals were directly sampled from these conditions. To freeze frogs, groups of 8–12 animals were placed in a plastic container lined with damp paper toweling. A thermistor was placed inside the box to measure air temperature and then the container was placed in an incubator set to −4 °C for 45 min. This time/temperature has been well-established as sufficient to chill the frogs below their supercooling point (about −2 °C) and trigger freezing. The incubation temperature was then raised and maintained at −2.5 °C for 24 h of freezing. For thawing recovery, frogs were given the 24 h freeze treatment and were then transferred in their containers to an incubator set at 5 °C to thaw for 8 h. Animals were euthanized by pithing, and heart and hind leg thigh muscle were quickly excised, blotted free of blood, and frozen in liquid nitrogen. Tissues were kept frozen at −80 °C until use. All animal protocols for care, experimentation and euthanasia had the prior approval of the Carleton University Animal Care Committee in accordance with the guidelines established by the Canadian Council on Animal Care.

Extraction of total RNA

Total RNA was extracted from frozen tissue as previously described (Luu and Storey 2015). RNA quality was assessed based on the ratio of absorbance at 260 and 280 nm using a spectrophotometer. In addition, RNA integrity was determined by running a 1 % agarose gel to visualize both 18S and 25S ribosomal RNA bands.

MicroRNA polyadenylation and cDNA synthesis

Polyadenylation and cDNA synthesis was performed with total RNA samples, as previously described (Biggar et al. 2014). MicroRNAs were polyadenylated using a polymerase tailing kit (Epi-Bio, cat.no. PAP5104H) following the manufacturer's guidelines. Polyadenylated RNA samples were then subjected to cDNA synthesis using a modified reverse transcription protocol (Biggar et al. 2014). Instead of an oligo-dT primer used in most protocols, a stem-loop adapter primer was used (Appendix 1). Serial dilutions of the resulting cDNA were produced and stored at −20 °C until further use.

Relative microRNA expression by quantitative PCR

MicroRNA expression was assessed by quantitative PCR (qPCR) on a Bio-Rad MyiQ2 Detection System (BioRad, Hercules, CA, USA) using a sense primer specific to the individual miRNA and a universal reverse primer (Appendix 1), as previously described (Biggar et al. 2014). Reagents were prepared and reactions were performed as previously described (Pellissier et al. 2006). Amplification was performed first with an incubation at 95 °C for 3 min, followed by a two-step cycling of 95 °C for 10 s and 60 °C for 30 s for 60 repeats. A melting curve analysis was performed for each miRNA for quality control. Reference gene stability was determined as previously described (Schmittgen and Livak 2008).

Bioinformatics: DIANA-miRPath microRNA analysis

DNA Intelligent Analysis (DIANA) miRPath v2.0 is a bioinformatics program that predicts gene mRNA transcript targets that are complementary to different miRNAs and was used to identify putative gene targets of the differentially expressed miRNAs identified in wood frog tissues (Vlachos et al. 2012). These gene targets were then analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) to determine cellular pathways that may be affected during freezing and thawing.

Statistics

For qPCR analysis, the comparative ΔΔCt method was used to quantify relative expression of miRNA expression. The stable reference genes identified and used in this study were *5S rRNA* (heart) and *sno96A* (skeletal muscle). Data are expressed as relative mean expression (mean ± SEM, $n = 4$ independent samples from different animals at each sampling point) with controls values set to a mean of 1.0.

Statistical analysis of the data was carried out using one-way ANOVA, followed by the post hoc Holm-Sidak test, where $p < 0.05$ represents a significant change (SigmaPlot 12.0 statistical package).

Results

MicroRNA expression during freezing and thawing

A modified stem-loop procedure (Biggar et al. 2014) was used to amplify 53 highly conserved microRNAs. This protocol allowed us to examine microRNA expression in wood frogs, *R. sylvatica*, a species that is not yet genome-sequenced. The effect of freezing (24 h at −2.5 °C and subsequent thawing (8 h at 5 °C after a 24 h freeze) on microRNA expression in wood frog heart and skeletal muscle was examined. Notably, wood frogs achieve maximum ice content of about 65 % of total body water well within the 24 h time at −2.5 °C whereas after 8 h thawing at 5 °C frogs have regained physiological parameters including heartbeat, breathing, skeletal muscle tone, and normal posture.

Cardiac muscle of *R. sylvatica* showed significant changes (with respect to values for 5 °C acclimated control frogs) in 21 out of the 53 miRNAs measured (Fig. 1). Of these, rsy-miR-145 was unique in showing a significant increase of 1.30-fold during freezing. Seven miRNAs (rsy-miR-208, rsy-miR-27b, rsy-miR-221, rsy-miR-490, rsy-miR-499, rsy-miR-727, and rsy-miR-130a-1) showed the opposite response, significantly reduced expression during freezing (to 42–70 % of control). However, the most prominent response was a strong reduction in miRNA expression during thawing. Twenty out of 21 miRNAs exhibited decreased expression after 8 h of thawing as compared with controls ($p < 0.05$); for example, rsy-miR-145 levels were reduced to 32 % of control values after thawing. Only rsy-miR-221 levels were not significantly different from controls after thawing.

Wood frog skeletal muscle exhibited a different trend of miRNA expression in response to freezing and thawing. Nineteen miRNAs showed stress-responsive changes in their expression levels, and contrary to the findings in heart, 16 out of these 19 miRNAs showed significantly increased expression (by 1.47-fold to 3.11-fold) during freezing with respect to controls (Fig. 2). Of these, five miRNAs (rsy-miR-130a-1, rsy-miR-1805, rsy-miR-221, rsy-miR-455, and rsy-miR-726) that showed increased expression during freezing also sustained elevated levels (1.65-fold to 1.94-fold higher than control) during thawing. Expression levels of rsy-miR-219-1 increased 1.68-fold during thawing but showed no significant change from control during freezing. In addition, rsy-miR-19b and rsy-miR-190 showed significant decreases in the frozen and thawed conditions, respectively, as compared to controls. Overall, a general increase in miRNA expression in response to freezing stress was observed in the skeletal muscle of the wood frog.

Predicted targets for differentially expressed microRNA

The DIANA miRPath application was used to predict cellular pathways that may be regulated by sets of differentially expressed miRNAs in response to freeze/thaw stress in heart and skeletal muscle of the wood frog. Given a list of miRNAs, this application performs target enrichment and identifies potentially regulated KEGG pathways. For frog cardiac muscle, 18 of the miRNAs that showed a decrease in their expression levels during 8 h thawing were recognized by the program (Table 1). The program identified (1) actin cytoskeleton, (2) arrhythmogenic

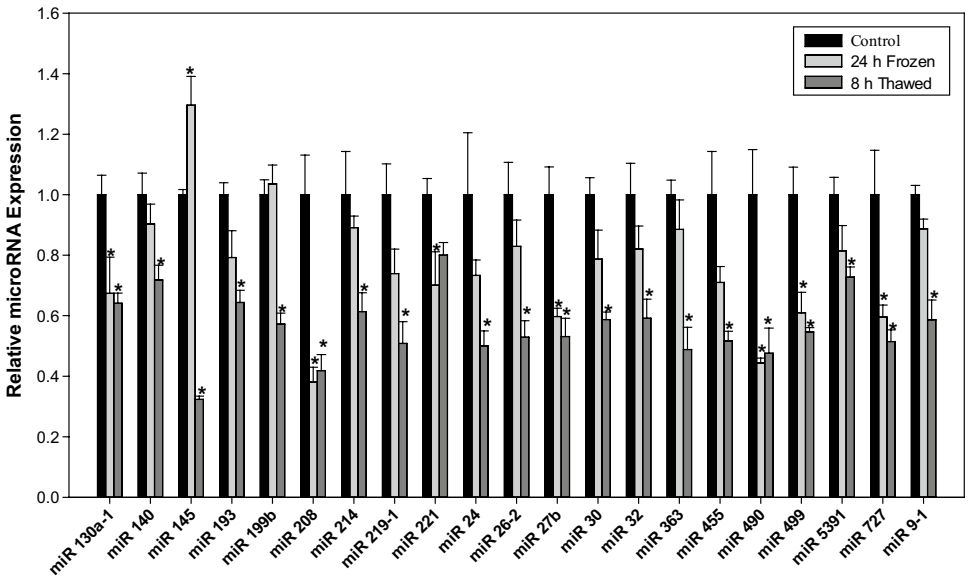

Fig. 1 Relative miRNA expression levels in *R. sylvatica* heart tissue in response to 24 h freezing and 8 h thawing. Data are mean ± SEM, $n = 4$ isolations of RNA from different hearts. *Asterisk* significantly different from the control condition ($p < 0.05$)

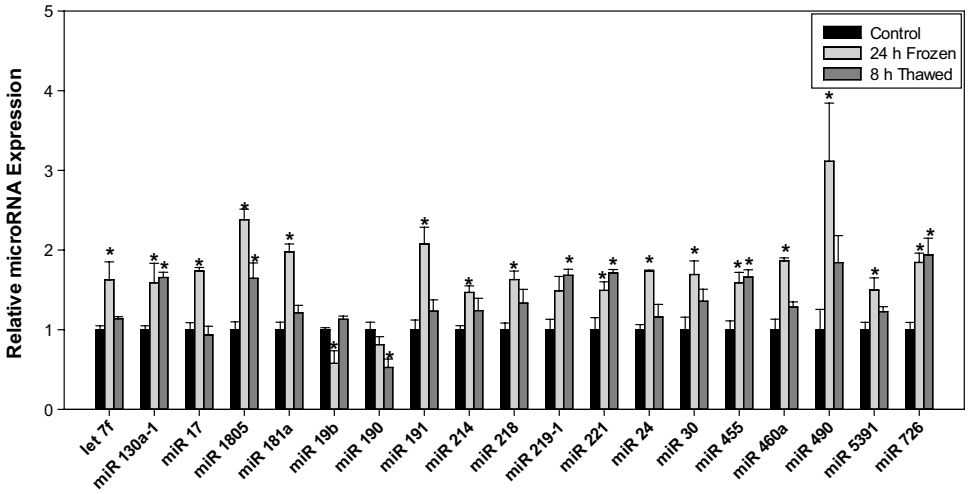

Fig. 2 Relative miRNA expression levels in *R. sylvatica* skeletal muscle tissue in response to 24 h freezing and 8 h thawing. Data are mean ± SEM, $n = 4$ isolations of RNA from different animals. *Asterisk* significantly different from the control condition ($p < 0.05$)

Table 1 MicroRNAs amplified in *R. sylvatica* and recognized by DIANA miRPath v2.0 were used to predict potentially targeted cellular pathways

Tissue	Number of microRNAs	MicroRNAs used for prediction
Heart	18	miR-130a-1, miR-140, miR-145, miR-193, miR-199b, miR-208, miR-214, miR-219, miR-24, miR-26-2, miR-27b, miR-30, miR-32, miR-363, miR-455, miR-490, miR-499, miR-9-1
Skeletal muscle	12	let 7f, miR-130a-1, miR-17, miR-191, miR-181a, miR-214, miR-218, miR-221, miR-24, miR-30, miR-455, miR-490

The microRNAs used for prediction in heart and skeletal muscle were thaw and freeze stress-sensitive, respectively

Table 2 KEGG pathways recognized by DIANA miRPath predicted to be targeted by the differentially expressed *R. sylvatica* microRNAs which were found in study

Tissue	Regulated KEGG pathway	Number of miRNAs involved	Number of genes involved	*p* value
Heart	Actin cytoskeleton	17/18	62	$1.04E^{-15}$
	Arrythmogenic right ventricular cardiomyopathy (ARVC)	13/18	30	$5.21E^{-14}$
	Hypertrophic cardiomyopathy	14/18	29	$5.21E^{-14}$
Skeletal muscle	Actin cytoskeleton	10/12	56	$3.46E^{-15}$
	PI3K-Akt signaling	10/12	73	$1.31E^{-11}$
	MAPK signaling	12/12	59	$1.31E^{-11}$

right ventricular cardiomyopathy (ARVC), and (3) hypertrophic cardiomyopathy (HCM) as KEGG pathways that are potentially regulated by thaw-responsive miRNAs (Table 2). For the actin cytoskeleton pathway, 17 out of the 18 miRNAs were predicted to regulate 62 genes. The ARVC pathway had 30 genes which were potentially targeted by 13 stress-sensitive miRNAs. Lastly, miRPath suggested that 14 miRNAs may be regulating 29 genes in the HCM pathway (Appendix 2).

For skeletal muscle, 12 miRNAs that showed increased expression levels during freezing underwent DIANA miRPath target enrichment analysis (Table 1). The program predicted that the (1) actin cytoskeleton, (2) PI3K-Akt signaling, and (3) MAPK signaling pathways were potentially regulated by miRNAs in response to freezing in skeletal muscle (Table 2). Ten out of the 12 miRNAs were predicted to target 55 genes in the actin cyotskeleton pathway. There were 73 genes in the PI3K-Akt signaling pathway that were predicted to be targeted by 10 miRNAs. Lastly, all 12 identified stress-sensitive skeletal muscle miRNAs were predicted to regulate 59 genes in the MAPK signaling pathway (Appendix 3).

Discussion

The freeze tolerant wood frog, *R. sylvatica,* serves as an excellent animal model to study the biochemical and physiological adaptations necessary for natural freezing survival. The frog uses multiple cryoprotective strategies as well as suppression of metabolic rate to prolong its survival during freezing (Storey and Storey 2013). Changes in gene expression in combination with other modifications help to ensure survival during freezing and its associated stresses of cellular dehydration and anoxia (Storey and Storey 2013). Since their discovery, miRNAs have been implicated in regulatory roles for over 60 % of protein-coding genes in vertebrates (Guo et al. 2010; Esteller 2011). Multiple studies have established their significance in diverse biological functions, including tissue differentiation, cell cycle regulation, apoptosis, metabolism and development (Babar et al. 2008). Dysregulation of miRNAs has been shown to play a role in pathogenic disease states like cancer, diabetes, and muscular dystrophy (Abdellatif 2012). Consequently, they have become important therapeutic targets for many treatments, such as the prevention of tumorigenesis and cardiomyopathy (Rodino-Klapac 2013; Cheng et al. 2014). Not surprisingly, then, the regulation of miRNA expression and discovery of the gene targets of miRNA action has become an active field of study in comparative biochemistry as an aid to understanding the regulation of the significant physiological and biochemical changes that underlie adaptation to severe environmental stress.

The first study of miRNA involvement in wood frog freeze tolerance evaluated only two targets, miR-16 and miR-21, in liver and skeletal muscle of control versus frozen frogs (Biggar et al. 2009). The current study greatly expands our knowledge of miRNA responses in a freeze tolerant species with an analysis of 53 miRNA species in both cardiac and skeletal muscle under control, frozen and thawed conditions. The large number of miRNA species analyzed gives strong insights into the cellular mechanisms that contribute to the preservation of muscle functionality during freeze/thaw. The regulatory actions of miRNAs may mediate freeze/thaw-induced physiological changes in the heart, or minimize damage to skeletal muscle tissues over the wood frog's freeze/thaw cycles. Studies have also begun to reveal that miRNAs have an important role in various animal models of metabolic rate depression (Liu et al. 2010; Biggar and Storey 2012; Biggar et al. 2009). Their role in rapidly suppressing mRNA translation in response to stress, and reversing that effect once the stress is alleviated, i.e., during recovery, might be extremely beneficial in catering to stress-specific needs of heart and skeletal muscle of the frog.

The findings of the present study indicate a tissue specific expression pattern of miRNAs in heart and skeletal muscle of the wood frog in response to freezing and thawing. In heart, of the 53 miRNA species analyzed, 21 miRNAs were responsive to the freeze–thaw cycle. The most robust response in heart was that of rsy-miR-145, the only miRNA to significantly increase during freezing as well as the miRNA that was most strongly reduced after thawing. Given that increased levels of a miRNA implies increased inhibition of its mRNA targets, whereas reduced miRNA levels would facilitate increased translation of specific mRNA transcripts, the response by rsy-miR-145 suggests that reduced translation of its mRNA targets would occur during freezing with a reactivation of their translation during thawing. Overall, these responses may suggest that gene transcripts controlled by rsy-miR-145 code for proteins that are particularly important to freeze/thaw survival. In addition to rsy-miR-145, most miRNAs showed significant decreases during thawing, potentially promoting the translation of their mRNA targets during the recovery period (Fig. 1) and suggesting that a variety of cell systems need attention for the proper recovery of heart function after freezing. For example, rsy-miR-145, rsy-miR-208 and rsy-miR-490 are known to be overexpressed in cardiac diseases like pulmonary arterial hypertension, cardiac fibrosis and arrhythmias (Cooley et al. 2012; Ogorodnikova and Arenz 2015; Huang and Li 2015). The decrease in these miRNAs seen in the heart tissue of thawed frog after freezing could indicate a cardioprotective role for the target genes/proteins controlled by these miRNAs, also supported by the reduced levels of rsy-miR-208 and rsy-miR-490 during freezing. Four other miRNAs, rsy-miR-727, rsy-miR-130a-1, rsy-miR-499 and rsy-miR-27b were also consistently decreased under both frozen and thawed conditions in wood frog heart (Fig. 1). Studies have shown that overexpression of these miRNAs is collectively implicated in cardiac injuries and myocardial infarctions (Cheng et al. 2010; Wang et al. 2012b; McAlinden et al. 2013; Yang et al. 2015). As a result, their decreased abundance through the freeze–thaw cycle might also indicate a putative role in preventing damage to heart tissue during freezing. Lastly, rsy-miR-221 decreased during freezing but showed no change after thawing with respect to control. In vitro studies looking at the role of miR-221 have shown a correlation between its overexpression and hypertrophic cardiomyopathy, characterized by enlargement of heart muscles (Wang et al. 2012a). Although the literature suggests a decrease in this miRNA during freezing may also facilitate a cardioprotective role, it is unlikely that gene expression is actively promoted in a frozen state. Understanding the mechanism of action rsy-miR-221 performs during freezing is of interest, and can be further explored by studying miRNA/RISC interactions, but is beyond the scope of this study. Overall,

21 out of 22 cardiac miRNAs decreased during thawing which suggests a general trend of increased translation of gene transcripts as the frog recovers from severe environmental stress and suggests that despite the presence of cryoprotectants (glucose, urea), a substantial amount metabolic reorganization (perhaps targeted to selected cardiac activities) may be needed for effective recovery of heart function after thawing.

Potential miRNA-affected cardiac functions were suggested by the target enrichment analysis using DIANA miRPath. This analysis suggested that actin cytoskeleton, arrhythmogenic right ventricular cardiomyopathy (ARVC), and hypertrophic cardiomyopathy (HCM) are pathways that are regulated by the set of miRNAs that showed significantly reduced expression in response to 8 h thawing in the heart of the wood frog (Table 2). A total of 18 miRNAs that were differentially expressed in heart (Table 1) were entered into the software program out of which 17 were known to affect a total of 62 genes in the actin cytoskeleton pathway (Table 2; Appendix 2). Actin is an important protein involved in cytoskeletal structure and also has a crucial role in the contractile machinery of muscle (Horowitz et al. 1996; Katz and Lorell 2000; Hopkins 2006). Considering the cell dehydration that accompanies freezing greatly reduces cell volume in the frozen state (even despite the increase in cryoprotective osmolytes), it is perhaps not surprising that the cytoskeleton may be stressed or damaged and that specific signaling pathways may be regulated to maintain or repair the integrity of the actin cytoskeleton and the muscle contractile apparatus during freeze/thaw in order to facilitate the proper resumption of muscle contraction after thawing. These miRNAs likely contribute to this process of muscle cell remodeling over freeze/thaw excursions. The importance of this is emphasized by the fact that resumption of heart beat is not only the first vital sign that is detectable as frogs thaw (Layne and First 1991) but heart pumping is also crucial to the recovery of all other organs after freezing because renewed blood supply is needed in order to restore oxygen and nutrient supplies.

ARVC is a disease where desmosomal proteins of the heart myocardium are impaired. Desmosomes are cell structures specialized in cell–cell adhesion (Romero et al. 2013). In the diseased state, gaps are formed in the myocardium leading to fibrosis and fatty acid deposition. This leads to decreased pumping efficiency and arrhythmias originating in the right ventricle (Romero et al. 2013). Thirteen wood frog miRNAs were involved in the regulation of 30 genes related to this pathway (Table 2). HCM is a cardiac disease characterized by thickening of the cardiac muscles. In other animal models of hypometabolism such as mammalian hibernators, reversible cardiac hypertrophy is observed to accompany cold-induced physiological changes such as a significant decrease in heart rate in the cold torpid state (350–400 to 5–10 bpm) (Luu et al. 2015). A total of 29 genes (Table 2) were regulated by 12 miRNAs in this pathway. Although bioinformatics results highlight cardiac-specific pathways, it is important to note that stress-induced phenotypes (such as hypertrophy) have not been studied in the freeze-tolerant heart. The results of this study suggest that heart miRNAs regulated during thawing may play a cardioprotective role in the freeze-tolerant frog by targeting genes that are present in cardiac-specific pathways.

In skeletal muscle, 19 miRNAs showed stress-responsive changes in expression (Fig. 2). Inhibition of miR-191 has been demonstrated to induce apoptosis and decrease cell proliferation in mouse cancer models (Elyakim et al. 2010). Since a two-fold increase in this miRNA was observed in wood frog muscle during freezing, these results suggest that rsy-miR-191 may contribute to the maintenance of skeletal muscle during freezing by suppressing apoptosis. Similarly, rsy-miR-490, which showed a threefold increase in the frozen state, has been shown to reduce cell proliferation in mammalian cell lines when its expression is increased (Sun et al. 2013). Previous studies have demonstrated that key cell cycle controls are regulated during wood frog freezing, anoxia and dehydration to suppress cell cycle activity under stress (Roufayel et al. 2011; Zhang and Storey 2012). Both apoptosis inhibition and cell cycle suppression would be advantageous for freezing survival to promote cytoprotection and reduce energy demands during freeze/thaw. Rsy-miR-1805 showed a significant increase during freezing that is followed by a partial decrease during recovery—suggesting a role in gene suppression in the frozen state that may also be sustained during thawing. Four other miRNAs (rsy-miR-130a-1, rsy-miR-221, rsy-miR-455, and rsy-miR-726) showed enhanced expression during freezing that remained high after thawing. As some genes certainly have roles at particular periods across the freeze–thaw cycle, and as it is known that the wood frog conserves energy and prolongs its survival in the frozen state by expending energy only on essential transcription/translation (Storey 1987), these results demonstrate that some non-essential gene transcripts may remain suppressed by miRNAs until later stages in the thawing/recovery process. Interestingly, although rsy-miR-130a-1 and rsy-miR-221 are generally suppressed in the heart during freeze/thaw, they are elevated in skeletal muscle. This underscores the versatility of miRNAs and their ability to regulate vastly different pathways. One study demonstrated that miR-130 expression contributed to the cellular hypoxia response by modulating P-bodies to release mRNA transcript for protein translation of HIF-1α (Saito et al. 2011). Another study demonstrated that miR-221 targets MyoD mRNA, ultimately preventing

translation of this gene (Tan et al. 2014). MyoD is a well-studied transcription factor known to promote muscle cell differentiation. Thus, the upregulation of rsy-miR-130 and rsy-miR-221 may benefit skeletal muscle by initiating cellular hypoxia responses while facilitating a state of hypometabolism by targeting MyoD. Finally, this study found that skeletal muscle rsy-miR-190 decreased strongly during thawing (Fig. 2). Suppression of this miRNA was shown to promote insulin resistance and activate the MAPK signaling pathway in mouse skeletal muscle (Chen et al. 2012). Therefore, miRNAs such as rsy-miR-190 may aid in the regulation of metabolic pathways and protein phosphorylation cascades. Ultimately, differential expression of miRNAs in the freeze-tolerant wood frog appear to be making an important contribution to molecular adjustments in skeletal muscle that regulate energetic and cytoprotective processes during freeze/thaw stress exposure.

Target enrichment analysis of miRNAs from skeletal muscle that were freeze responsive predicted that actin cytoskeleton, PI3K-Akt signaling, and MAPK signaling pathways were regulated by 12 miRNAs that showed altered expression in response to freezing (Table 2). Ten out of these 12 miRNAs affected 55 genes involved in actin cytoskeletal regulation in skeletal muscle (Table 2; Appendix 3). Again, given the importance of actin as a structural protein in all types of muscle function, this result may suggest the occurrence of remodeling to facilitate or restore muscle maintenance during freeze/thaw. Similarly, 10 out of the 12 miRNAs used for prediction had implicated roles in regulating 73 genes in the PI3K-Akt pathway (Table 2). The PI3K-Akt signaling pathway is a central regulator of cellular processes that promote cell survival, growth and proliferation. It does this by Akt-mediated phosphorylation of multiple transcription factors and protein kinases, and is particularly involved in regulating protein translation (Fresno Vara et al. 2004). Previous research has shown that the Akt pathway is negatively regulated during freezing in wood frog skeletal muscle, and the effects of elevated microRNA levels on the mRNA transcripts that code for proteins of the Akt pathway may be one of these reasons (Zhang and Storey 2013). Finally, 59 genes involved in MAPK signaling were found to be regulated by all 12 differentially expressed skeletal muscle miRNAs. Members of the mitogen-activated protein kinase family control signaling cascades that rapidly mediate external signals acting at the cell membrane, transducing and amplifying them to achieve coordinated responses by many cell functions to a mitogenic or stress signal (Cowan and Storey 2003). The MAPK pathway was found to be differentially regulated in four different tissues (liver, kidney, heart, brain) of the wood frog during freezing, and reversible protein phosphorylation is now understood to play a pivotal role during the frog's adaptation to freezing and thawing (Greenway and Storey 2000; Storey and Storey 2013). Thus, the present results suggest that miRNAs may facilitate skeletal muscle adaptation to freeze/thaw stress by targeting signaling pathways, some of which have already been shown to be regulated in the freeze-tolerant wood frog.

The present study combines qPCR and bioinformatics to study the role of miRNAs in the wood frog, *R. sylvatica*, as it transitions through periods of freezing and thawing. Relative levels of 53 miRNAs were examined in heart and skeletal muscle of the frogs by employing a modified stem-loop procedure for miRNA analysis. A general trend of decreased miRNA levels was observed in cardiac tissue during thawing. These results suggest that genes are potentially being activated to facilitate the quick restoration of heart function in the thawed frog. Indeed, resumption of heartbeat is the first physiological function that can be seen when frogs thaw, while others such as breathing or voluntary muscle movement occur considerably later. On the contrary, a general increase in miRNA levels was observed in skeletal muscle during freezing, which suggests that mRNA translation is likely being suppressed as the frog transitions into a metabolically suppressed state to conserve energy over the cold winter months. These stress-sensitive frog miRNAs may be controlling pathways by associating with RISC complexes, promoting mRNA degradation, inhibiting protein translation machinery, or localizing transcript to P-bodies and stress granules. Bioinformatics analysis suggested that differentially expressed miRNAs may collectively be involved in regulating cell functions including maintaining cell structure, muscle contraction, and reversible protein phosphorylation. Overall, the results clearly indicate an important regulatory role for differential microRNA expression in wood frog freezing survival and the putative targets of the freeze/thaw responsive miRNAs suggest new areas of cell physiology to explore to fully elucidate the molecular mechanisms of natural freeze tolerance.

Acknowledgments Thanks to J. M. Storey for editorial review of the manuscript. This work was supported by a Discovery grant (#6793) from the Natural Sciences and Engineering Research Council of Canada (NSERC). KBS holds the Canada Research Chair in Molecular Physiology, and BEL holds an NSERC Canada Graduate Scholarship.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Appendix 1

See Table 3.

Table 3 Primers used for analysis of microRNA expression in the heart and skeletal muscle of *R. sylvatica*

Primer	Sequence (5′–3′)
rsy-let-7	ACA CTC CAG CTG GGT GAG GTA GTA GGT TG
rsy-let-7d	ACA CTC CAG CTG GGA GAG GTA GTA GGT TG
rsy-let-7f	ACA CTC CAG CTG GGC TAT ACA ATC TAT TG
rsy-miR-100	ACA CTC CAG CTG GGA ACC CGT AGA TCC GA
rsy-miR-10a	ACA CTC CAG CTG GGT ACC CTG TAG ATC CG
rsy-miR-125	ACA CTC CAG CTG GGT CCC TGA GAC CCT TA
rsy-miR-126	ACA CTC CAG CTG GGC ATT ATT ACT TTT GG
rsy-miR-129a	ACA CTC CAG CTG GGC TTT TTG CGG TCT GG
rsy-miR-130a-1	ACA CTC CAG CTG GGG CTC TTT TCA CAT TG
rsy-miR-133a	ACA CTC CAG CTG GGT TTG GTC CCC TTC AA
rsy-miR-137b	ACA CTC CAG CTG GGA CGG GTA TTC TTG GG
rsy-miR-140	ACA CTC CAG CTG GGC AGT GGT TTT ACC CT
rsy-miR-145	ACA CTC CAG CTG GGG TCC AGT TTT CCC AG
rsy-miR-147	ACA CTC CAG CTG GGG TGT GCG GAA ATG CT
rsy-miR-17	ACA CTC CAG CTG GGC AAA GTG CTT ACA GT
rsy-miR-1805	ACA CTC CAG CTG GGA GTT GTA GTC TTT CA
rsy-miR-181a	ACA CTC CAG CTG GGA ACA TTC AAC GCT GT
rsy-miR-187	ACA CTC CAG CTG GGG CTA CAA CAC AGG AC
rsy-miR-190	ACA CTC CAG CTG GGT GAT ATG TTT GAT AT
rsy-miR-191	ACA CTC CAG CTG GGC AAC GGA ATC CCA AA
rsy-miR-193	ACA CTC CAG CTG GGT GGG TCT TTG CGG GC
rsy-miR-199b	ACA CTC CAG CTG GGC CAG TGT TCA GAC TA
rsy-miR-1a-1	ACA CTC CAG CTG GGA CAT ACT TCT TTA TG
rsy-miR-203	ACA CTC CAG CTG GGA GTG GTT CTT AAC AG
rsy-miR-204a	ACA CTC CAG CTG GGT TCC CTT TGT CAT CC
rsy-miR-205a	ACA CTC CAG CTG GGT CCT TCA TTC CAC CG
rsy-miR-208	ACA CTC CAG CTG GGA AGC TTT TGG CTC GG
rsy-miR-20a	ACA CTC CAG CTG GGT AAA GTG CTT ATA GT
rsy-miR-21	ACA CTC CAG CTG GGT AGC TTA TCA GAC TG
rsy-miR-214	ACA CTC CAG CTG GGT GCC TGT CTA CAC TT
rsy-miR-216a	ACA CTC CAG CTG GGT AAT CTC AGC TGG CA
rsy-miR-218	ACA CTC CAG CTG GGT TGT GCT TGA TCT AA
rsy-miR-219-1	ACA CTC CAG CTG GGA AGA ATT GTG TCT GG
rsy-miR-221	ACA CTC CAG CTG GGA ACC TGG CAT ACA AT
rsy-miR-24	ACA CTC CAG CTG GGT GCC TAC TGA GCT GA
rsy-miR-26-2	ACA CTC CAG CTG GGC CTA TTC TTG ATT AC
rsy-miR-27b	ACA CTC CAG CTG GGA GAG CTT AGC TGA TT
rsy-miR-2970	ACA CTC CAG CTG GGG ACA GTC AGT AGT TG
rsy-miR-30	ACA CTC CAG CTG GGT GTA AAC ATC CTC GA
rsy-miR-32	ACA CTC CAG CTG GGT ATT GCA CAT TAC TA
rsy-miR-363	ACA CTC CAG CTG GGG TGG ATC ACG ATG CA
rsy-miR-375	ACA CTC CAG CTG GGG CGT CGA GCC CCA CG
rsy-miR-455	ACA CTC CAG CTG GGT ATG TGC CCT TGG AC
rsy-miR-458	ACA CTC CAG CTG GGA TAG CTC TTT GAA TG
rsy-miR-460a	ACA CTC CAG CTG GGC CTG CAT TGT ACA CA

Table 3 continued

Primer	Sequence (5′–3′)
rsy-miR-490	ACA CTC CAG CTG GGC CAT GGA TCT CCA GG
rsy-miR-499	ACA CTC CAG CTG GGT TAA GAC TTG CAG TG
rsy-miR-5391	ACA CTC CAG CTG GGT GGA CAG TTA TGA AG
rsy-miR-551	ACA CTC CAG CTG GGG CGA CCC ATA CTT GG
rsy-miR-726	ACA CTC CAG CTG GGT TCA CTA CTA GCA GA
rsy-miR-727	ACA CTC CAG CTG GGT CAG TCT TCA ATT CC
rsy-miR-9-1	ACA CTC CAG CTG GGT CTT TGG TTA TCT AG
rsy-miR-19b	ACA CTC CAG CTG GGT GTG CAA ATC CAT GC
5S rRNA	GGC CTG GTT AGT ACT TGG ATG G
SNORD 96A	ACA CTC CAG CTG GGA TCC TAG TGA TGA CAA G
RT Oligo-dT adapter Primer	CTC ACA GTA CGT TGG TAT CCT TGT GAT GTT CGA TGC CAT ATT GTA CTG TGA GTT TTT TTT TVN
Universal primer	CTC ACA GTA CGT TGG TAT CCT TGT G

Appendix 2

See Table 4.

Table 4 Enriched KEGG pathways from target genes identified by DIANA miRPath v.2.0 based on sequence complementarity to wood frog heart and skeletal muscle microRNAs

Tissue	Regulated KEGG pathway	MicroRNAs involved
Heart	Actin cytoskeleton	miR-130a-1, -140, -145, -193, -199b, -208, -214, -219-1, -24, -26-2, -27b, -30, -32, -363, -490, -499, -9-1
	Arrythmogenic right ventricular cardiomyopathy (ARVC)	miR-130a-1, -145, -193, -199b, -208, -219-1, -26-2, -30, -32, -363, -455, -499, -9-1
	Hypertrophic cardiomyopathy	miR-130a-1, -145, -193, -199b, -208, -214, -219-1, -26-2, -30, -32, -363, -455, -499, -9-1
Skeletal muscle	Actin cytoskeleton	let-7f, miR-130a-1, -17, -181a, -214, -218, -221, -24, -30, -490
	PI3K-Akt signaling	let-7f, miR-130a-1, -17, -181a, -214, -218, -221, -24, -30, -490
	MAPK signaling	let-7f, miR-130a-1, -17, -191, -181a, -214, -218, -221, -24, -30, -455, -490

Appendix 3

See Tables 5 and 6.

Table 5 Gene targets derived from DIANA miRPath v.2.0 based on sequence complementarity to differentially expressed microRNAs in heart tissue of *R. sylvatica* in response to thawing

KEGG pathway	Actin cytoskeleton	ARVC	Hypertrophic cardiomyopathy
1	*Pxn*	*Ctnna1*	*Tgfb2*
2	*Nckap1l*	*Cacng4*	*Prkaa2*
3	*Sos2*	*Dsc2*	*Actc1*
4	*Fgf10*	*Ctnnb1*	*Cacng4*
5	*Pip5k1c*	*Itgb3*	*Ttn*
6	*Pip4k2b*	*Cacnb2*	*Itgb3*
7	*Ppp1r12a*	*Cacnb4*	*Cacnb2*

Table 5 continued

KEGG pathway	Actin cytoskeleton	ARVC	Hypertrophic cardiomyopathy
8	*Fgf14*	*Tcf7*	*Cacnb4*
9	*Pik3r3*	*Lmna*	*Lmna*
10	*Slc9a1*	*Itga5*	*Itga5*
11	*Wasl*	*Actb*	*Actb*
12	*Diap3*	*Cacna1d*	*Cacna1d*
13	*Fgf20*	*Itga1*	*Itga1*
14	*Wasf2*	*Dmd*	*Dmd*
15	*Vav3*	*Dag1*	*Dag1*
16	*Rock1*	*Atp2a2*	*Atp2a2*

Table 5 continued

KEGG pathway	Actin cytoskeleton	ARVC	Hypertrophic cardiomyopathy
17	*Fgf12*	*Cacng2*	*Cacng2*
18	*Itgb3*	*Itga8*	*Itga8*
19	*Myh10*	*Actn1*	*Itgav*
20	*Itga5*	*Cdh2*	*Itgb6*
21	*Cfl2*	*Itgav*	*Itga4*
22	*Actb*	*Itgb6*	*Itgb1*
23	*Itga1*	*Itga4*	*Prkag2*
24	*Arhgef12*	*Itgb1*	*Il6*
25	*Pfn2*	*Sgcb*	*Sgcb*
26	*Fgf13*	*Cacna1c*	*Cacna1c*
27	*Kras*	*Itgb8*	*Itgb8*
28	*Pip5k1b*	*Des*	*Des*
29	*Braf*	*Gja1*	*Itga9*
30	*Nras*	*Itga9*	
31	*Sos1*		
32	*Itga8*		
33	*Mras*		
34	*Rras2*		
35	*Actn1*		
36	*Pik3cb*		
37	*Fgf18*		
38	*Itgav*		
39	*Git1*		
40	*Diap2*		
41	*Itgb6*		
42	*Pik3cg*		
43	*Abi2*		
44	*Myh9*		
45	*Itga4*		
46	*Ssh2*		
47	*Arhgef6*		
48	*Fgf9*		
49	*Itgb1*		
50	*Pdgfra*		
51	*Gna13*		
52	*Pik3cd*		
53	*Pak7*		
54	*Ssh1*		
55	*Itgb8*		
56	*Arpc2*		
57	*Pak2*		
58	*Iqgap2*		
59	*Cyfip2*		
60	*Itga9*		
61	*Pip4k2a*		
62	*Ppp1cc*		

Table 6 Gene targets derived from DIANA miRPath v.2.0 based on sequence complementarity to differentially expressed microRNAs in skeletal muscle tissue of *R. sylvatica* in response to freezing

KEGG pathway	Actin cytoskeleton	PI3K-Akt	MAPK
1	*Pxn*	*Prkaa2*	*Mapk1*
2	*Nckap1l*	*Tsc1*	*Rasa2*
3	*Mapk1*	*Mapk1*	*Fgf14*
4	*Pip4k2b*	*Pik3r1*	*Bdnf*
5	*Ppp1r12a*	*Ywhaz*	*Map4k3*
6	*Pik3r1*	*Ppp2r1b*	*Rps6ka5*
7	*Fgf14*	*Fgf14*	*Map4k4*
8	*Pik3r3*	*Pik3r3*	*Fgf20*
9	*Pak3*	*Bcl2l1*	*Taok3*
10	*Wasl*	*Rps6kb1*	*Map2k1*
11	*Fgf20*	*Ywhag*	*Map3k12*
12	*Vav3*	*Col3a1*	*Taok1*
13	*Map2k1*	*Ddit4*	*Fos*
14	*Rock1*	*Ifnar1*	*Rps6ka1*
15	*Fgf12*	*Fgf20*	*Fgf12*
16	*Itgb3*	*Map2k1*	*Mknk2*
17	*Fgf11*	*Ppp2r2a*	*Ngf*
18	*Tiam2*	*Thbs1*	*Dusp2*
19	*Cfl2*	*Vegfb*	*Fgf11*
20	*Chrm1*	*Insr*	*Rps6ka3*
21	*Arhgef12*	*Reln*	*Cacnb4*
22	*Egfr*	*Gnb1*	*Rapgef2*
23	*Pfn2*	*Fgf12*	*Nlk*
24	*Fgf13*	*Itgb3*	*Cacna1g*
25	*Myh14*	*Ngf*	*Tgfbr1*
26	*Kras*	*Ccne2*	*Il1a*
27	*Pip5k1b*	*Creb1*	*Egfr*
28	*Braf*	*Fgf11*	*Fgf13*
29	*Rdx*	*Lpar1*	*Kras*
30	*Nras*	*Col1a1*	*Braf*
31	*Rock2*	*Col27a1*	*Mapk9*
32	*Sos1*	*Phlpp2*	*Tgfbr2*
33	*Itga8*	*Ifnar2*	*Mecom*
34	*Rras2*	*Chrm1*	*Nras*
35	*Actn1*	*Prlr*	*Sos1*
36	*Tiam1*	*Egfr*	*Cacng2*
37	*Wasf1*	*Ppp2ca*	*Rap1b*
38	*Crk*	*Ghr*	*Rras2*
39	*Diap2*	*Sgk3*	*Rasa1*
40	*Arhgef7*	*Fgf13*	*Map4k2*
41	*Actg1*	*Kras*	*Dusp4*
42	*Ssh2*	*Nras*	*Crk*
43	*Arhgef6*	*Sos1*	*Dusp10*
44	*Araf*	*Foxo3*	*Tnf*

Table 6 continued

KEGG pathway	Actin cytoskeleton	PI3K-Akt	MAPK
45	*Arpc1b*	*Itga8*	*Map3k5*
46	*Itgb1*	*Col5a2*	*Flna*
47	*Pdgfra*	*Jak1*	*Ppp3r1*
48	*Baiap2*	*Col4a6*	*Ppp3ca*
49	*Gna13*	*Osmr*	*Ikbkg*
50	*Pik3cd*	*Col24a1*	*Pdgfra*
51	*Pak7*	*Efna3*	*Akt3*
52	*Ssh1*	*Rbl2*	*Ptprr*
53	*Itgb8*	*Ppp2r5a*	*Atf2*
54	*Itga9*	*Col1a2*	*Map3k11*
55	*Pip4k2a*	*Cdkn1b*	*Dusp1*
56	*Limk2*	*Gng3*	*Mapk8*
57		*Itgb1*	*Zak*
58		*Bcl2l11*	*Dusp8*
59		*Mcl1*	*Rps6ka2*
60		*Ikbkg*	
61		*Pdgfra*	
62		*Il6*	
63		*Rps6kb2*	
64		*Pten*	
65		*Hsp90ab1*	
66		*Pik3cd*	
67		*Itgb8*	
68		*Atf2*	
69		*Ppp2r5c*	
70		*Pkn2*	
71		*Itga9*	
72		*Hsp90b1*	
73		*Prkaa2*	

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