Canadian Journal of Physiology and Pharmacology

Endoplasmic reticulum and the microRNA environment in the cardiovascular system.

Journal:	<i>Canadian Journal of Physiology and Pharmacology</i>
Manuscript ID	cjpp-2018-0720.R1
Manuscript Type:	Review
Date Submitted by the Author:	31-Jan-2019
Complete List of Authors:	Groenendyk, Jody; University of Alberta Fan, Xiao; University of Alberta Peng, Zhenling; University of Alberta Kurgan, Lukasz; Virginia Commonwealth University, Department of Computer Science Michalak, Marek; University of Alberta
Is the invited manuscript for consideration in a Special Issue:	2018 IACS Cuba
Keyword:	ER stress, micro RNA, unfolded protein response, endoplasmic reticulum, heart

SCHOLARONE™
Manuscripts

Endoplasmic reticulum and the microRNA environment in the cardiovascular system.

Jody Groenendyk¹, Xiao Fan², Zhenling Peng³, Lukasz Kurgan⁴ and Marek Michalak¹

¹Department of Biochemistry, University of Alberta, Edmonton, Alberta Canada T6G 2S7, ²Irving Medical Center, Columbia University, New York, NY, U.S.A. 10032, ³Center for Applied Mathematics, Tianjin University, Tianjin, China 300072, ⁴Department of Electrical and Computer Engineering, University of Alberta, Edmonton, Alberta, Canada T6G 2V4 and ³Department of Computer Science, Virginia Commonwealth University, Richmond, 23284 USA.

Address correspondence to:

Dr. M. Michalak

Department of Biochemistry,

University of Alberta,

Edmonton, Alberta,

Canada T6G 2H7

Tel: 780-492-2256

Fax: 780-492-0886

E-mail: marek.michalak@ualberta.ca

and

Dr. Jody Groenendyk

E-mail: jody.groenendyk@ualberta.ca

ABSTRACT

Stress responses are important to human physiology and pathology and the inability to adapt to cellular stress leads to cell death. To mitigate cellular stress and re-establish homeostasis, cells, including those in the cardiovascular system, activate stress coping response mechanisms. The endoplasmic reticulum, a component of the cellular reticular network in cardiac cells, mobilizes so-called endoplasmic reticulum stress coping responses, such as the unfolded protein response (UPR). MicroRNAs play an important part in the maintenance of cellular and tissue homeostasis, perform a central role in the biology of the cardiac myocyte, and are involved in pathological cardiac function and remodeling. In this report we review a link between endoplasmic reticulum homeostasis and microRNA with an emphasis on the impact on stress responses in the cardiovascular system.

Key words: ER stress, micro RNA, unfolded protein response, endoplasmic reticulum, heart

INTRODUCTION

Stress responses are central to human physiology and pathology and failure to adapt to cellular stress leads to cell death. To mitigate cellular stress and re-establish homeostasis cardiac cells must activate stress coping response mechanisms (Cao and Kaufman 2014; Dicks et al. 2015; Groenendyk et al. 2013). In eukaryotic cells, including muscle cells, the endoplasmic reticulum (ER) plays a central role in cellular stress responses. The ER mobilizes so-called ER stress coping responses, such as the unfolded protein response (UPR) (Groenendyk et al. 2013). The UPR involves three ER associated transmembrane signaling proteins, the inositol-requiring 1 (IRE1), an ER kinase, dsRNA-activated protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6) (Groenendyk et al. 2013; Hetz and Papa 2018; Wang and Kaufman 2016). Activation of UPR signaling pathways leads to translational attenuation, transcriptional activation of genes encoding proteins involved in protein folding, and the ER-associated degradation (ERAD) pathway (Groenendyk et al. 2013; Hetz and Papa 2018; Wang and Kaufman 2016). Under normal physiological conditions IRE1, PERK and ATF6 are silenced by binding to BiP, an ER chaperone. Upon stress, BiP dissociates from these proteins, resulting in activation of UPR signaling pathways (Groenendyk et al. 2013; Hetz and Papa 2018). Activation of UPR pathways has been implicated in many cardiovascular diseases including hypoxia, ischemia/reperfusion, hypertrophy, pressure overload, and drug-induced insults (Agellon and Michalak 2018; Groenendyk et al. 2013; Marambaio et al. 2010). For example, inhibition of IRE1 α signaling protects the heart from cardiac fibrosis (Groenendyk et al. 2016) and atherosclerosis (Tufanli et al. 2017). In recent years, numerous studies have reported an extra level of regulation of stress responses by microRNAs. As of March 2018 (miRBase Release 22) there has been over 38,500 microRNAs identified (<http://www.mirbase.org/>) and the list is likely to grow. Many of these microRNAs have been implicated in cardiac development, physiology and pathophysiology (Olson 2014; Romaine et al. 2015).

SARCO-ENDOPLASMIC RETICULUM.

All eukaryotic cells, including cells of cardiovascular system, contain network of linked intracellular organelles that form a cellular reticular network (Agellon and Michalak 2018). These include the ER, Golgi apparatus, lysosomes, membranous components of the endocytic pathway, peroxisomes, and nuclear envelope (Agellon and Michalak 2018). The ER is responsible for many cellular functions including the synthesis, folding and posttranslational modification of proteins; the synthesis of lipids and steroids; stress signaling, and signaling to the nucleus, cytoplasm, mitochondria, and plasma membrane (Braakman and Hebert 2013; Krebs et al. 2015; Schwarz and Blower 2016). The ER is also responsible for storage, release and uptake of Ca^{2+} (Krebs et al. 2015). The ER is a major Ca^{2+} storage compartment with a number of Ca^{2+} buffering proteins involved in maintaining this concentration at approximately 500 μM , in conjunction with the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) that continuously pumps Ca^{2+} back into the ER (Krebs et al. 2015). This Ca^{2+} is used for signaling events that activate the inositol 1,4,5-triphosphate receptor (InsP_3R), an ER membrane InsP_3 -activated Ca^{2+} channel that releases Ca^{2+} into the cytoplasm. As cytoplasmic Ca^{2+} concentrations are very low, in the nM range, the increase in cytoplasmic Ca^{2+} levels can regulate a variety of cellular processes, including cell proliferation, metabolism and apoptosis. The depletion of Ca^{2+} from the ER triggers a unique process termed store-operated Ca^{2+} entry (SOCE), which sustains the increase in cytoplasmic Ca^{2+} concentrations via Ca^{2+} influx across the plasma membrane, thereby allowing the refilling of ER Ca^{2+} stores through the action of SERCA. Stromal interaction molecule 1 (STIM1), an ER membrane type I transmembrane protein, acts as an ER Ca^{2+} sensor (Liou et al. 2005; Roos et al. 2005; Zhang et al. 2005). Upon ER Ca^{2+} depletion, STIM1 extends its conformation, inducing cluster formation and movement towards the plasma membrane where it binds to the cytoplasmic domain of ORAI1, a plasma membrane Ca^{2+} channel, to form membrane contact sites (MCS) between the ER and the plasma membrane (Baba et al. 2006; Bhardwaj et al. 2016; Feske et al. 2006; Luik et al. 2008; Luik et al. 2006; Muik et al. 2008; Nwokonko et al. 2017; Park et al. 2009; Peinelt et al. 2006; Phillips and Voeltz 2016; Stathopoulos et al. 2006; Vig et al. 2006; Yang and Rana 2012). The interaction between STIM1 and ORAI1 at the MCS causes influx of

Ca^{2+} via the ORAI1 Ca^{2+} channel (Hou et al. 2012; Mignen et al. 2008; Penna et al. 2008; Prakriya et al. 2006; Vig et al. 2006). Any disturbance in ER Ca^{2+} homeostasis results in significant ER stress. For example, treatment with thapsigargin, an irreversible inhibitor of SERCA, results in a disruption in ER Ca^{2+} homeostasis, depletion of ER Ca^{2+} stores, and activation of SOCE triggering ER stress. Therefore, cells must actively maintain homeostatic cellular Ca^{2+} concentrations to prevent ER stress. Here we review a link between ER homeostasis and levels microRNA with a major emphasis on the cardiovascular system (Fig. 1).

MICRORNAS IN THE CARDIOVASCULAR SYSTEM

MicroRNAs play an important role during cardiovascular differentiation, physiology and pathology (Olson 2014; Small et al. 2010). For example, embryonic stem (ES) cells downregulate expression of miR-302 via BMP4 (Kang et al. 2012) to control differentiation into cardiac precursor cells, while increased expression of miR-302 maintains ES cells in a pluripotent and naïve state (Card et al. 2008). miR-1 and miR-133 have been used to reprogram human fibroblasts into myocardial cells (Nam et al. 2013). The muscle specific transcription factor MEF2c binds and turns on expression of these two microRNAs via an intragenic muscle specific enhancer that is active during embryogenesis (Liu et al. 2007). miR-499 is also strongly associated with cardiac differentiation of human ES cells (Fu et al. 2011; Wilson et al. 2010). One of the earliest families of microRNAs that is increased in abundance during cardiomyocyte differentiation is the miR-322/503 cluster, located on the X chromosome (Shen et al. 2016). This family of microRNAs targets Celf1 (Le Tonqueze et al. 2016), reducing expression of a protein that is involved in the differentiation of neuroectoderm and inhibition of cardiac differentiation. Additionally, cardiac specific deletion of DICER, the endoribonuclease enzyme involved in the generation of all microRNAs, leads to dilated cardiomyopathy and postnatal lethality within 4 days (Chen et al. 2008). During development, the microRNA, miR-128 is upregulated during the switch from proliferation to differentiation. Conditional deletion of miR-128 in adult heart reactivates cardiomyocyte proliferation and heart regeneration, while overexpression prevents proliferation and results in reduced

cardiac function (Huang et al. 2018). Therefore, maintenance of optimal levels of microRNAs during heart function is essential and could be used as a regenerative therapy.

MicroRNAs play an important part in the maintenance of cellular and tissue homeostasis, perform a central role in the biology of the cardiac myocyte, and are involved in cardiac pathology and tissue remodeling (Divakaran and Mann 2008; Small et al. 2010). MicroRNAs are also upregulated during reactivation of the fetal gene program and modulate cardiac hypertrophic growth (Thum et al. 2007). When microRNAs were analyzed in fetal hearts and failing hearts, there was a high degree of similarity between the two transcriptomes, indicating a convergent relationship between the two gene expression programs (Thum et al. 2007). Interestingly, similar to a number of other microRNA deficient mouse models, the miR-208a-deficient mouse had no discernable phenotype unless stressed with cardiac overload (van Rooij et al. 2007), while targeted silencing of miR-208a in hypertension-induced heart failure in rats improved heart function and survival (Montgomery et al. 2011). The cardiac specific miR-208a is located in an intron of the α MHC gene (van Rooij et al. 2007), and overexpression of miR-208a leads to cardiomyocyte hypertrophy (Callis et al. 2009). Abundance of miR-25 in the heart is increased during heart failure (Dirkx et al. 2013), while miR-214 protects the heart from ischemic injury by targeting members of the Ca^{2+} signaling pathway (Aurora et al. 2012). More recently, microRNAs have been used to profile cardiac biopsies and predict allograft rejection (Di Francesco et al. 2018). Delivery of microRNA mimics or inhibitors is achieved by viral infection and shows clinical and therapeutic promise. MicroRNAs are also importantly therapeutically during cancer, type 2 diabetes, and hepatitis C infections (Baumann and Winkler 2014; Mellis and Caporali 2018).

A LINK BETWEEN MICRORNA AND STRESS RESPONSES

How does ER stress regulate the expression of microRNAs? One possible explanation may be that microRNA expression is directly regulated by members of the ER stress pathway. ER stress-dependent activation of ATF6 down regulates miR-455, thereby increasing the expression of the downstream genes, providing a level of protein folding relief by the expression of molecular chaperones (Belmont et al.

2012b). This suggests that ER stress may control gene expression through ATF6 mediated alterations in microRNA abundance, and in this manner contributing to the protective effects of ATF6 in the heart (Belmont et al. 2012b). Similarly, the transcription factor CHOP regulates miR-708, with the conserved region encoding miR-708 found in the 3' UTR region of a CHOP regulated gene (Behrman et al. 2011). Furthermore, the XBP1 transcription factor regulates microRNAs, including upregulation of miR-346, silencing expression of the antigen peptide transporter TAP1, and thereby affecting immune regulatory genes (Bartoszewski et al. 2011). In turn, miR-30* and miR-214 regulate XBP1 abundance during cardiac hypertrophy and heart failure (Duan et al. 2015). ATF4 targets expression of several microRNAs, which may contribute to the attenuation of protein translation or protein synthesis (Cortesio et al. 2008). Furthermore, ER stress directly modifies lipid metabolism and triggers increased abundance of miR-33 and CHOP in atherosclerotic plaques and macrophages (Sun et al. 2018). As well, ischemia that occurs during myocardial infarction, activates the ATF6 branch of the ER stress response, which in turn regulates downstream microRNA abundance (Belmont et al. 2012a; Doroudgar et al. 2009). During the remodeling after a myocardial infarction, miR-199b is up-regulated, affecting the fibrotic pathway and contributing to cardiac remodeling (Duygu et al. 2017). Finally, oxidative stress, similar to that seen during ischemia/reperfusion, results in reduced abundance of the miR-30 family. One of the targets for this family is a gene encoding HSPA5, indicating a possible protective feedback effect with upregulation in expression of the molecular chaperone (Chen et al. 2014).

Interestingly, IRE1 α can directly cleave a number of microRNAs, leading to degradation of the microRNAs and subsequently the activation of apoptosis (Upton et al. 2012). ER stress may cause a decrease in stability of mRNAs or microRNAs, thereby indirectly regulating the protein expression of target genes. Much evidence points toward an overlap between microRNA deregulation and increased ER stress during cardiovascular pathologies (Read et al. 2014).

The disruption in Ca²⁺ homeostasis triggered by the buildup of misfolded proteins also controls the transcriptional regulation of microRNAs. A deficiency in ORAI1 in mouse fibroblast leads to a

significant increase in the expression of miR-322 (Groenendyk et al. 2014c) with degradation of miR-322 by IRE1 α dependent on intracellular Ca²⁺ levels. The release of Ca²⁺ observed during ER stress with the resultant increase in cytoplasmic Ca²⁺ triggers activation of Ca²⁺ dependent enzymes and signaling pathways that control transcription. For example, intracellular Ca²⁺ oscillations regulate NF κ B transcriptional activity, phosphorylation of I κ B α , and Ca²⁺-dependent gene expression (Zhu et al. 2011). ATF4 and NF κ B are co-activators under conditions of anoxia, inducing expression of a key gene SKIP3, with temporal stimulation of the NF κ B pathway under anoxia protecting cells from the undesirable consequences of oxygen stress (Rzymiski et al. 2008). NF κ B is linked to the regulation of two microRNAs that are located very close to one another, miR-199a2 and miR-214, both with multiple NF κ B binding sites (Duan et al. 2012). Furthermore, the tumor suppressor p53 is activated by DNA damage, upregulates transcription of miR-34, and processing of miR-16, miR-103, miR-143, miR-145, miR-16a and miR-206 to promote cell growth arrest and apoptosis (Leung and Sharp 2010).

The UPR can be stimulated by various conditions, including protein deficiency, energy deficit, or drug treatment. Fibroblasts lacking ER associated chaperones, including calnexin and PDIA3 (ERp57), have an increase in the UPR as monitored using the XBP1 splicing reporter assay (Coe et al. 2008; Coe et al. 2009). BiP deficient or silenced cells also demonstrate a significant increase in the UPR (Eletto et al. 2012; Groenendyk et al. 2014c). Importantly, a small change in ER protein abundance can trigger a massive ER stress response. For example, only 30% silencing of BiP using siRNA commands in an 800% increase in ER stress under basal conditions (Groenendyk et al. 2014c). We used several different conditions to induce the UPR in NIH-3T3 and HEK-293 cells; including thapsigargin, an inhibitor of the ER Ca²⁺-ATPase (SERCA); calreticulin deficiency, to reduced ER Ca²⁺ capacity; cyclosporine A, an inhibitor of peptidyl prolyl isomerases; and DTT, a reducing agent that induces protein mis-folding and ER stress (Fig. 2).

To identify what role microRNAs may play in the ER stress coping response, we carried out microRNA deep sequencing analysis using mouse NIH-3T3 fibroblasts treated with 0.5 μ M thapsigargin for 24 hours

as well as deep sequencing analysis of wild-type and calreticulin-deficient mouse ES cells. We identified a number of microRNAs that were either up- or down-regulated upon thapsigargin treatment or in calreticulin-deficient ES cells that have reduced ER luminal Ca^{2+} capacity (Table 1).

INGENUITY PATHWAY ANALYSIS (IPA) ANALYSIS OF MICRORNA AND STRESS RESPONSE

A number of the microRNAs found in the thapsigargin treated fibroblasts analyzed by deep sequencing were similar to that seen in a screen carried out by Behrman *et al.* (Behrman et al. 2011). These included miR-322, miR-708*, miR-671-5p, and miR-503 (Behrman et al. 2011) (Table 1). We used multiple target prediction programs in tandem, including TargetScan (Friedman et al. 2009; Lewis et al. 2005), DIANAmicroT (Maragkakis et al. 2009), and RepTar (Elefant et al. 2011), to generate a list of candidate target transcripts for microRNAs. Using the IPA platform, mmu-miR-322 gene targets were separated into several canonical pathways, including seven genes involved in tight junction signaling (4.3%), five genes involved in TGF β signaling (5.6%), and six genes involved in WNT/ β -catenin signaling (3.5%) (Table 2). As miR-322 is one of the earliest microRNA families expressed during cardiac differentiation, it may play a role in the regulation of the WNT pathway (Shen et al. 2016). The WNT signaling pathway performs a central role in cardiac myocyte differentiation during the early stages of development and is tightly controlled during differentiation (Tian et al. 2010). The mmu-miR-503 gene target canonical pathways include TGF β signaling (12.4%), PTEN signaling (7.3%), and BMP signaling (8.8%). Finally, analysis of all gene targets for mmu-miR-322, 503, 101b, 708*, and 671-5p showed that the top canonical pathways included PTEN signaling (35.5% coverage), WNT/ β -catenin signaling (33.3% coverage), and TGF β signaling (36% coverage) (Table 2). Sustained ER stress is linked to an increase in TGF β expression in the murine lung (Roberson et al. 2011), as well as in mouse heart, along with expression of fibrotic genes (Groenendyk et al. 2016). Interestingly, TGF β inhibits NF κ B, which plays an important role during the ER stress coping response and may be involved in the regulation of numerous microRNAs, including miR-322 and miR-503 (Davis and Hata 2009; Duan et al. 2012).

Calreticulin-deficient ES cells have impaired cardiogenesis (Groenendyk and Michalak 2014). The deep sequencing analysis of calreticulin-deficient ES cells revealed significant changes in expression of several genes involved in cellular growth, proliferation, development, and movement, including the WNT signaling pathway (50%) and the cell cycle pathway (56.1%) (Table 3). This is in agreement with an earlier report on the impact of calreticulin deficiency on the WNT signaling pathway in ES cells (Li et al. 2002; Mery et al. 2005). Two additional signaling pathways affected by the microRNAs in calreticulin-deficient ES cells were the cardiac hypertrophy signaling pathway (46.1%) and TGF β signaling pathway (60.5%) (Table 3). Both of these pathways have been validated in calreticulin-deficient cells (Groenendyk and Michalak 2014; Mesaeli et al. 1999). These findings indicated that microRNAs also contribute to the cardiac pathology associated with calreticulin deficiency (Mesaeli et al. 1999).

MIR-322 EXPRESSION DURING THE STRESS RESPONSE

Initial validation of the role of miR-322 during ER stress in cells treated with thapsigargin revealed a decrease in the abundance of miR-322 (Groenendyk et al. 2014c) (Fig. 3A). Furthermore, under conditions that stimulate ER stress, such as cyclosporine, tunicamycin, or brefeldin A, there was also a decrease in miR-322 abundance (Fig. 3A). miR-322 is located on the reverse strand of the X-chromosome at site 50,407,432 - 50,407,526 (Pinheiro et al. 2011), in close proximity to miR-503, suggesting that both microRNAs may be regulated in a similar manner. Both miR-322 and miR-503 are associated with cardiac development (Shen et al. 2016), and deep sequencing analysis indicated that miR-503 was downregulated to a similar extent as miR-322 (Table 1). Some of the putative targets of miR-322 that we have identified include Smad3 and PPI γ (Fig. 3B), and PDIA6 (Groenendyk et al. 2014c). When thapsigargin stimulated mouse fibroblasts were treated with a miR-322 mimic, Smad3 and PPI γ mRNA were both down-regulated (Fig. 2B), supporting a role of miR-322 in regulation of Smad3 and PPI γ during ER stress. Smad3 may be negatively regulated by miR-322 under basal conditions to prevent induction of the TGF β pathway involved in cellular response to inflammation. PPI γ is a nuclear localized peptidylprolyl isomerase involved in catalyzing the isomerization of proline peptide bonds and the

regulation of pre-mRNA splicing (Dubourg et al. 2004), a necessary part of the ER stress response (Upton et al. 2012). Cyclosporine binds to PPI γ (Davis et al. 2010), and this may play a role in the cardiac pathology observed during cyclosporine treatment (Owunwanne et al. 1993). PDIA6 is involved in the regulation of IRE1 α activity under normal conditions with silencing of PDIA6 leading to a significant decrease in IRE1 α activity (Groenendyk et al. 2014c). PDIA6 is an ATF6-dependent ER stress gene that prevents cardiac myocyte death during ischemia/reperfusion (Vekich et al. 2012). Thapsigargin dependent down-regulation of miR-322/503 may be responsible for an optimal ER stress response.

A LINK BETWEEN IRE1 α AND miR-322

Recent evidence points to the endoribonuclease function of IRE1 α in the degradation of microRNAs (Upton et al. 2012). IRE1 α can cleave a select group of microRNAs, all containing a similar consensus nucleotide sequence as that seen in XBP1 mRNA. Importantly, IRE1 α cleaves microRNAs to elevate expression of a putative target protein, such as caspase 2, in response to ER stress. Analysis of the nucleotide sequence of miR-322 determined that it has a putative IRE1 α cleavage site (Fig. 4A), similar to that seen previously (Upton et al. 2012). This cleavage may lead to premature degradation of this microRNA, correlating with activation of IRE1 α , and a significant decrease in miR-322 upon thapsigargin treatment that activates IRE1 α . The miR-322/503 family is upregulated during early stages of cardiogenesis (Shen et al. 2016), while activation of IRE1 α is linked to human heart failure and ischemia/reperfusion damage (Szegezdi et al. 2006; Thuerlauf et al. 2006). Silencing of IRE1 α in mouse fibroblasts resulted in significant increase in abundance of miR-322, indicating a role for IRE1 α in the degradation of miR-322 (Fig. 4B). Therefore, under conditions of disrupted ER Ca²⁺ homeostasis, such as in ORAI1-deficient ES cells (Groenendyk et al. 2014c) (Fig. 4C), IRE1 α activity modified miR-322 expression and this may be ORAI1/Ca²⁺ dependent (Fig. 4C).

THAPSIGARGIN DEPENDENT UP-REGULATION OF miR-101b AND EFFECT ON POTENTIAL TARGETS

Thapsigargin treatment increased the abundance of miR-101b (Fig. 5A). IPA analysis of miR-101b targets include; Gene Expression, Cellular Development, and Post-translational Modification, but what

was striking was that the Top Toxicity List for miR-101b included Cardiac Hypertrophy and Cardiac Fibrosis (Table 4). Under conditions of thapsigargin dependent ER stress, miR-101b abundance was significantly up-regulated (Fig. 5A). Recent work has demonstrated that increased abundance of miR-101b resulted in improvement in cardiac function in rats with chronic myocardial infarction by suppressing cardiac fibroblast proliferation and collagen production (Pan et al. 2012). We determined that miR-101b has a number of gene targets that participate in many aspects of cellular function, including regulation of expression of COX-2 and GSK3 β genes. Addition of miR-101b mimic had a direct effect on both mRNA and protein levels of COX-2, supporting the notion that miR-101b targets the COX-2 mRNA (Fig. 5B). Furthermore, miR-101b mimic resulted in significant decreases to the XBP1 splicing reporter activity during thapsigargin treatment (Fig. 5C), implying that the targets of miR-101b are involved in the ER stress response. Silencing of COX-2 resulted in significant decreases in XBP1 splicing, implying a direct role for COX-2 in the ER stress response, similar to what we have seen previously (Fig. 5D) (Groenendyk et al. 2018). COX-2 is a novel regulator of ER stress (Groenendyk et al. 2018) that is down-regulated during ER stress conditions by miR-101b. COX-2 is involved in the conversion of arachidonic acid to prostaglandin endoperoxide H₂. Prostaglandins play an important role in the inflammatory process in the heart (Ricciotti and FitzGerald 2011). COX-2 up-regulation is commonly seen in inflammatory microenvironments, with prostaglandins possessing anti-inflammatory properties and able to inhibit NF κ B and I κ K as well as activate PPAR γ (Plummer et al. 1999; Yu et al. 2002). COX-2 inhibitors increase the risk of heart disease in patients that have been using coxibs for a long period of time (Streicher and Wang 2008). COX-2 is linked to ER stress responses and may be responsible for the UPR seen during conditions that induce oxidative stress (Choo-Wing et al. 2013; Groenendyk et al. 2018). GSK3 β is also a target of miR-101b (Tian et al. 2016). Treatment of thapsigargin stimulated fibroblasts with miR-101b mimic results in decreased abundance of GSK3 β mRNA (Fig. 5E), a kinase central to a number of signaling pathways, including WNT signaling. GSK3 β mRNA expression was increased during thapsigargin treatment (Fig. 5E) (Song et al. 2002). GSK3 β up-regulation is directly linked to activation of caspase 3 and stimulation of apoptosis (Song et al. 2002). miR-101b is currently under

investigation as a therapeutic target during myocardial infarction as deletion of GSK3 β prevented ventricular remodeling and improved cardiac function after myocardial infarction (Zhou et al. 2016). Therefore, up-regulation of miR-101b by thapsigargin treatment may play a role in control of the abundance of GSK3 β and consequently maintain ER stress at sufficient level, allowing the cell to efficiently deal with the stressor.

CALRETICULIN-DEFICIENT ES CELLS AND THE miR-302 CLUSTER

Recent work from our laboratory (Groenendyk and Michalak 2014) has identified a critical microRNA cluster, miR-302, that is significantly down-regulated in calreticulin deficient ES cells (Table 1; Fig. 6A). This may help explain, at least in part, why calreticulin-deficient ES cells have impaired cardiogenesis (Groenendyk and Michalak 2014). The miR-302 cluster, containing miR-302a,b,c,d, plays an essential role during cardiac differentiation, proliferation, and regeneration (Tian et al. 2015). The miR-302 cluster is vital for the maintenance of pluripotency and can be used to reprogram epithelial cells to return to the stem cell state, a technique that generates cells termed iPS cells (induced pluripotent stem cells) (Anokye-Danso et al. 2011; Barroso-del Jesus et al. 2009; Kelley and Lin 2012; Liao et al. 2011; Lin et al. 2008; Lin et al. 2011). Downstream targets of miR-302 play a central role in the cell cycle, specifically by targeting the cell cycle inhibitor, cyclin D1 (Card et al. 2008; Lin et al. 2010). Expression of miR-302 degrades the cell cycle inhibitor, allowing the cell cycle to progress. WNT signaling has been suggested to keep levels of the ES cell specific microRNAs, including the miR-302 cluster high, thereby maintaining ES cells in a naïve ES cell pluripotent state (Xia 2008; Zhou et al. 2012). The miR-302 cluster also degrades inhibitors of the WNT signaling pathway, leading to hyper-activation of WNT signaling (Anton et al. 2011). IPA analysis of miR-302c demonstrated that the top canonical pathways affected were S-phase entry and G1/S checkpoint regulation as well as WNT/ β -catenin signaling (Table 5). This provides evidence for the inherent need for microRNAs in the essential process of ES pluripotency and cardiac differentiation via the WNT signaling pathway. A deficiency in calreticulin disrupts WNT signaling, as well as preventing cell cycle progression (Groenendyk and Michalak 2014).

These functions may be partially dependent on the Ca^{2+} buffering capacity of calreticulin, which regulates ER Ca^{2+} release and activation of Ca^{2+} -dependent kinase and phosphatase cascades. Validation of putative targets of miR-302 showed that mRNA for Ca^{2+} dependent genes calmodulin-dependent kinase II (CaMKII) and calcineurin were significantly increased in calreticulin-deficient ES cells (Fig. 6B), possibly due to the substantial decrease in miR-302 abundance (Fig. 6A). A deficiency in calreticulin disrupts the Ca^{2+} signaling capacity of the ER (Mesaeli et al. 1999; Nakamura et al. 2001), leading to down-regulation in expression of miR-302. Similar to miR-322 (Groenendyk et al. 2014c), miR-302 is regulated by a disruption in Ca^{2+} signaling.

IDENTIFICATION OF A NOVEL MIRNA THAT IS UP-REGULATED WITH A DEFICIENCY IN CALRETICULIN

As part of our deep sequencing analysis, we identified a novel miRNA that was up-regulated when calreticulin was deficient in mouse ES cells (Fig. 7A). We termed this microRNA, miR-0034*. We performed a targeting analysis using three targeting algorithms to identify putative targets. We chose two possible targets for further validation, Trinucleotide Repeat Containing Gene 9 (TOX3) and Lysine Deficient Protein Kinase 1 (WNK1). The novel microRNA miR-0034* that targets TOX3 was significantly increased (3.5 fold) in calreticulin-deficient ES cells (Fig. 6A), resulting in a decreased abundance of both TOX3 mRNA (Fig. 7B) and protein (Fig. 7C). In addition, WNK1 mRNA abundance was also reduced (Fig. 7B). TOX3 is involved in Ca^{2+} -dependent transcription in neurons and functions to modify chromatin structure and is involved in CREB binding (Dittmer et al. 2011). TOX3 overexpression plays a protective role against cell death caused by ER stress in neuronal cells (Dittmer et al. 2011). Therefore, the down regulation of TOX3 by miR-0034* may be involved in the apoptotic pathway stimulated by ER stress. WNK1 is a serine/threonine kinase involved in electrolyte homeostasis, including sodium and chloride transport (Park et al. 2012). WNK1 has also been shown to regulate embryonic cardiac development (Xie et al. 2013), with WNK1 deficient mice embryonic lethal at day 10.5 due to cardiovascular developmental defects (Xie et al. 2009). WNK1 involvement in ER stress may be linked to the disruption in Ca^{2+} homeostasis, with a trickle-down effect on the transport of sodium and

chloride. WNK kinases are also positive regulators of canonical WNT signaling (Serysheva et al. 2013), and therefore may be connected to the regulation of WNT signaling observed in the calreticulin-deficient ES cells as a downstream mechanism (Groenendyk and Michalak 2014). Therefore, miR-0034* may play a regulatory role in the ‘side effects’ of ER stress, modulating the cellular response to damage.

CONCLUSIONS

MicroRNA have been implicated in many cardiovascular disorders including cardiac hypertrophy, cardiac remodelling, arrhythmias, atherosclerosis and heart failure. MicroRNA has also been involved in cardiovascular development including cell proliferation, differentiation, apoptosis, angiogenesis, fibrosis, cardiac regeneration and inflammation. Some of the many microRNAs that have been implicated in cardiovascular physiology and disease will continue to emerge a potential biomarkers and drug targets.

Methods

Cell Culture: NIH-3T3 mouse fibroblasts and HEK-293 human kidney cells were maintained under standard tissue culture conditions, including 5% CO₂ with high humidity. Tissue culture media included 10% fetal bovine serum in DMEM (both from Sigma). *ORAI1*^{-/-} cells were a generous gift from Dr. Gwack. Mouse wild type and calreticulin deficient embryonic stem cells were maintained according to previously published protocols (Groenendyk and Michalak 2014).

Plasmid: The pRL-IXFL XBP1 splicing reporter was a generous gift from Dr. R. Kaufman and contains an internal Renilla control and the nucleotide sequence encoding XBP1 followed by firefly luciferase separated by an internal ribosomal entry site (IRES) initiation region (Back et al. 2006a). This reporter will only generate firefly luciferase if XBP1 is spliced, allowing the firefly luciferase to be in frame. Transfection, cell viability and luciferase Assays were described previously (Groenendyk et al. 2014c).

Reverse transcriptase-PCR (RT-PCR) and Quantitative-PCR (QPCR) for mRNA and miRNA: To monitor the levels of mRNA, cells were harvested at day three after siRNA transfection using Trizol (Invitrogen). For drug test conditions, cells were harvested 24 hours later (day four). Drug conditions include: 0.5 μ M thapsigargin, 20 μ M cyclosporine, 1 mM DTT, 5 μ g/ml BFA, 5 μ g/ml tunicamycin. RNA was isolated using the RNeasy kit (Qiagen) according to manufacturer's protocol and concentration was measured spectrophotometrically. Total RNA (200 ng) was subsequently used in RT-PCR for mRNA to generate cDNA for each sample. For monitoring of the mRNA levels, the cDNA was diluted 10-fold, with 2 μ l of cDNA used in subsequent PCR reactions with primers targeting controls, selected genes, ER stress markers and was performed in duplicate on a minimum of three separate occasions. Quantitative PCR was performed using a LightCycler rapid thermal cycler system (Corbett Research) or RotorGene 3000 (Qiagen) according to the manufacturer's instructions. Reactions were performed in a 20 μ l volume with 0.5 μ M primers. Other reagents including nucleotides, Taq DNA polymerase and buffer were used as provided in the SYBR Green Master Mix (Q uanta). The amplification protocol included: 10 min 95°C denaturation; forty cycles with 95°C denaturation for 15 s, 58°C annealing for 10 s and 72°C extension for 15 s. Detection of the fluorescent product occurred at the end of the 72°C extension periods. Specificity of the amplification product from each primer pair was confirmed by a melting curve analysis of the PCR product. Quantification was performed by online monitoring for identification of the exact time-point at which the logarithmic linear phase could be distinguished from the background (crossing point) by setting a threshold. The threshold was expressed as a cycle number (Ct) and was normalized to housekeeping control (HKC) gene GAPDH. The analysis was performed by the equation: $1/2^{(Ct(\text{gene}) - Ct(\text{HKC}))}$ and normalized internally for the untreated negative siRNA control.

The following nucleotide primers were used for RT-PCR and QPCR analyses:

Smad3

Forward 5'-TGACTACAGCCATTCCATTCC-3'; Reverse 5'-GGTCACTGGTTTCTCCATCTT-3'

PPI γ

Forward 5'-CACCTGCTGTGTGACAAATAAA-3'; Reverse 5'-GTCAGGGAGAGCAAATGAGTAA-3'

GSK3 β

Forward 5'-TTGGAGCCACTGATTACACGTCCA-3'; Reverse 5'-AATGTGCACAAGCTTCCAGTGGTG-3'

Calcineurin

Forward 5'-GTCCCCTTTCCTCCAACCCGACGGCTAAC-3'; Reverse 5'-TGATCCCCCAACTTCAAACAACCTCA-3'

CAMKII

Forward 5'-CATCCTCACCCTATGCTG-3'; Reverse 5'-ATCGATGAAAGTCCAGGCCC-3'

Tox3

Forward 5'-TGATGCACTGCTACCCCTTTC-3'; Reverse 5'-TCCACCAGATTCCTTGAGATTG-3'

Wnk1

Forward 5'-TGCTGGAAGACGGTTTATAGTG-3'; Reverse 5'-GGGATGAAGCAGAGTGAGATAAG-3'

COX-2

Forward: 5'-ACTGGGCCATGGAGTGGACTTAAA-3'; Reverse: 5'-AACTGCAGGTTCTCAGGGATGTGA-3'

GAPDH

Forward: 5'-TTCACCACCATGGAGAAGGC-3'; Reverse: GGCATGGACTGTGGTCATGA-3'.

Deep sequencing analysis, annotation and selection of miRNA: Deep sequencing was performed by Plant BioSys (University of Lethbridge deep sequencing facility). Analysis performed as described in (Groenendyk et al. 2014c).

miRNA expression and treatment with miRNA mimic: The sequence specific mimics were purchased from Qiagen and were used at 100 nM. Primers for mmu-miR-322, hsa-miR-101b, mmu-miR-302c and

mmu-miR-0034* were purchased from Exiqon or from Qiagen. Transfection conditions for mimics were similar to that described earlier (Groenendyk et al. 2014c). Experiments were performed in duplicate on three separate occasions. To measure the amount of miRNA present in samples, the samples were isolated with Trizol (Invitrogen) and subjected to RNA purification using the RNeasy kit (Qiagen). To monitor the miRNA, a specific microRNA RT-PCR to generate cDNA was performed using the miRCURY LNA Universal RT miRNA PCR kit (Exiqon). Briefly, the short length miRNA does not contain a poly-A tail, and therefore regular RT-PCR using the oligo dT cannot be performed to generate cDNA. RT-PCR generates cDNA that corresponds to miRNA and contains a specific motif that can be recognized by specific primers for miRNA analysis (Exiqon). The cDNA was diluted 10-fold and miRNA specific primers were used in the subsequent qPCR. The qPCR was prepared as follows; 4 µl of cDNA, 1 µl of PCR primer mix (prepared by diluting forward and reverse primers 1:1) and 5 µl of SYBR Green Master Mix (Quanta). The amplification protocol included: 10 min 95°C denaturation; forty cycles with 95°C denaturation for 15 s, 58°C annealing for 10 s and 72°C extension for 15 s. Detection of the fluorescent product occurred at the end of the 72°C extension periods. Specificity of the amplification product from each primer pair was confirmed by a melting curve analysis of the PCR product. Quantification was performed as described above. The threshold was expressed as a cycle number (Ct) and the analysis was performed by the equation: $1/2^{(Ct(\text{gene}) - Ct(\text{HKC}))}$ and was normalized to a housekeeping control 18S ribosomal RNA (HKC) (Exiqon Cat# 203906).

The mmu-miR-322 PCR Primer Set (Cat #:205182 Exiqon) and mmu-miR-322 mimic (Cat #:MSY0000548 Qiagen) based on 5'-CAGCAGCAAUUAUGUUUUGGA-3'

Mmu-miR-302c PCR Primer Set (Cat #: 205064 Exiqon) based on 5'-AAGUGCUUCCAUGUUUCAGUGG-3'

Hsa-miR-101b PCR Primer Sets (Cat#: 205072 Exiqon) and hsa-miR-101b mimic (Cat#: MSY0000099 Qiagen) based on 5' - UACAGUACUGUGAUAGCUGAA-3'

Mmu-miRNA PCR primers (Exiqon) were ordered based on the novel sequence for miR-0034*. 5'-UGUAGUGUUUCCUACUUUAUGGA-3'

siRNA treatment: As described in (Groenendyk et al. 2014c).

Immunoblot Analysis: Samples prepared using RIPA buffer (Groenendyk et al. 2014c) and run on SDS-PAGE followed by immunoblot analysis using COX-2 antibody (Abcam Cat# ab15191) and GAPDH antibody (Abcam Cat# ab9485).

REFERENCES

- Agellon, L.B., and Michalak, M. 2018. Stress coping strategies in the heart: an integrated view. *Front. Cardiovasc. Med* **21**: 168.
- Anokye-Danso, F., Trivedi, C.M., Juhr, D., Gupta, M., Cui, Z., Tian, Y., Zhang, Y., Yang, W., Gruber, P.J., Epstein, J.A., and Morrissey, E.E. 2011. Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency. *Cell Stem Cell* **8**(4): 376-388. doi: 10.1016/j.stem.2011.03.001.
- Anton, R., Chatterjee, S.S., Simundza, J., Cowin, P., and Dasgupta, R. 2011. A systematic screen for micro-RNAs regulating the canonical Wnt pathway. *PLoS One* **6**(10): e26257. doi: 10.1371/journal.pone.0026257.
- Aurora, A.B., Mahmoud, A.I., Luo, X., Johnson, B.A., van Rooij, E., Matsuzaki, S., Humphries, K.M., Hill, J.A., Bassel-Duby, R., Sadek, H.A., and Olson, E.N. 2012. MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca²⁺ overload and cell death. *J Clin Invest* **122**(4): 1222-1232. doi: 10.1172/JCI59327.
- Baba, Y., Hayashi, K., Fujii, Y., Mizushima, A., Watarai, H., Wakamori, M., Numaga, T., Mori, Y., Iino, M., Hikida, M., and Kurosaki, T. 2006. Coupling of STIM1 to store-operated Ca²⁺ entry through its

- constitutive and inducible movement in the endoplasmic reticulum. *Proc Natl Acad Sci U S A* **103**(45): 16704-16709. doi: 0608358103 [pii]10.1073/pnas.0608358103.
- Back, S.H., Lee, K., Vink, E., and Kaufman, R.J. 2006a. Cytoplasmic IRE1 {alpha}-mediated XBP1 mRNA Splicing in the Absence of Nuclear Processing and Endoplasmic Reticulum Stress. *J Biol Chem* **281**(27): 18691-18706.
- Back, S.H., Lee, K., Vink, E., and Kaufman, R.J. 2006b. Cytoplasmic IRE1alpha-mediated XBP1 mRNA splicing in the absence of nuclear processing and endoplasmic reticulum stress. *J Biol Chem* **281**(27): 18691-18706. doi: M602030200 [pii]10.1074/jbc.M602030200.
- Barroso-del Jesus, A., Lucena-Aguilar, G., and Menendez, P. 2009. The miR-302-367 cluster as a potential stemness regulator in ESCs. *Cell Cycle* **8**(3): 394-398.
- Bartoszewski, R., Brewer, J.W., Rab, A., Crossman, D.K., Bartoszewska, S., Kapoor, N., Fuller, C., Collawn, J.F., and Bebek, Z. 2011. The unfolded protein response (UPR)-activated transcription factor X-box-binding protein 1 (XBP1) induces microRNA-346 expression that targets the human antigen peptide transporter 1 (TAP1) mRNA and governs immune regulatory genes. *J Biol Chem* **286**(48): 41862-41870. doi: 10.1074/jbc.M111.304956.
- Baumann, V., and Winkler, J. 2014. miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents. *Future Med Chem* **6**(17): 1967-1984. doi: 10.4155/fmc.14.116.
- Behrman, S., Acosta-Alvear, D., and Walter, P. 2011. A CHOP-regulated microRNA controls rhodopsin expression. *The Journal of cell biology* **192**(6): 919-927. doi: 10.1083/jcb.201010055.
- Belmont, P.J., Chen, W.J., Thuerlauf, D.J., and Glembotski, C.C. 2012a. Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. *J Mol Cell Cardiol* **52**(5): 1176-1182. doi: 10.1016/j.yjmcc.2012.01.017.
- Belmont, P.J., Chen, W.J., Thuerlauf, D.J., and Glembotski, C.C. 2012b. Regulation of microRNA expression in the heart by the ATF6 branch of the ER stress response. *J Mol Cell Cardiol*. doi: 10.1016/j.yjmcc.2012.01.017.

- Bhardwaj, R., Hediger, M.A., and Demaurex, N. 2016. Redox modulation of STIM-ORAI signaling. *Cell Calcium* **60**(2): 142-152. doi: 10.1016/j.ceca.2016.03.006.
- Braakman, I., and Hebert, D.N. 2013. Protein folding in the endoplasmic reticulum. *Cold Spring Harb Perspect Biol* **5**(5): a013201. doi: 10.1101/cshperspect.a013201.
- Callis, T.E., Pandya, K., Seok, H.Y., Tang, R.H., Tatsuguchi, M., Huang, Z.P., Chen, J.F., Deng, Z., Gunn, B., Shumate, J., Willis, M.S., Selzman, C.H., and Wang, D.Z. 2009. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. *J Clin Invest* **119**(9): 2772-2786. doi: 10.1172/JCI36154.
- Cao, S.S., and Kaufman, R.J. 2014. Endoplasmic reticulum stress and oxidative stress in cell fate decision and human disease. *Antioxid Redox Signal* **21**(3): 396-413. doi: 10.1089/ars.2014.5851.
- Card, D.A., Hebbar, P.B., Li, L., Trotter, K.W., Komatsu, Y., Mishina, Y., and Archer, T.K. 2008. Oct4/Sox2-regulated miR-302 targets cyclin D1 in human embryonic stem cells. *Mol Cell Biol* **28**(20): 6426-6438. doi: 10.1128/MCB.00359-08.
- Chen, J.F., Murchison, E.P., Tang, R., Callis, T.E., Tatsuguchi, M., Deng, Z., Rojas, M., Hammond, S.M., Schneider, M.D., Selzman, C.H., Meissner, G., Patterson, C., Hannon, G.J., and Wang, D.Z. 2008. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. *Proc Natl Acad Sci U S A* **105**(6): 2111-2116. doi: 10.1073/pnas.0710228105.
- Chen, M., Ma, G., Yue, Y., Wei, Y., Li, Q., Tong, Z., Zhang, L., Miao, G., and Zhang, J. 2014. Downregulation of the miR-30 family microRNAs contributes to endoplasmic reticulum stress in cardiac muscle and vascular smooth muscle cells. *Int J Cardiol* **173**(1): 65-73. doi: 10.1016/j.ijcard.2014.02.007.
- Choo-Wing, R., Syed, M.A., Harijith, A., Bowen, B., Pryhuber, G., Janer, C., Andersson, S., Homer, R.J., and Bhandari, V. 2013. Hyperoxia and interferon-gamma-induced injury in developing lungs occur via cyclooxygenase-2 and the endoplasmic reticulum stress-dependent pathway. *Am J Respir Cell Mol Biol* **48**(6): 749-757. doi: 10.1165/rcmb.2012-0381OC.

- Coe, H., Bedard, K., Groenendyk, J., Jung, J., and Michalak, M. 2008. Endoplasmic reticulum stress in the absence of calnexin. *Cell Stress Chaperones* **13**(4): 497-507. doi: 10.1007/s12192-008-0049-x.
- Coe, H., Jung, J., Groenendyk, J., Prins, D., and Michalak, M. 2009. ERp57 modulates STAT3 signaling from the lumen of the endoplasmic reticulum. *J Biol Chem* **285**(9): 6725-6738. doi: M109.054015 [pii]10.1074/jbc.M109.054015.
- Cortesio, C., Chan, K., Perrin, B., Burton, N., Zhang, S., Zhang, Z., and Huttenlocher, A. 2008. Calpain 2 and PTP1B function in a novel pathway with Src to regulate invadopodia dynamics and breast cancer cell invasion. *The Journal of cell biology* **180**(5): 957-971.
- Davis, B.N., and Hata, A. 2009. Regulation of MicroRNA Biogenesis: A miRiad of mechanisms. *Cell Commun Signal* **7**: 18. doi: 10.1186/1478-811X-7-18.
- Davis, T.L., Walker, J.R., Campagna-Slater, V., Finerty, P.J., Paramanathan, R., Bernstein, G., MacKenzie, F., Tempel, W., Ouyang, H., Lee, W.H., Eisenmesser, E.Z., and Dhe-Paganon, S. 2010. Structural and biochemical characterization of the human cyclophilin family of peptidyl-prolyl isomerases. *PLoS Biol* **8**(7): e1000439. doi: 10.1371/journal.pbio.1000439.
- Di Francesco, A., Fedrigo, M., Santovito, D., Ntarelli, L., Castellani, C., De Pascale, F., Toscano, G., Fraiese, A., Feltrin, G., Benazzi, E., Nocco, A., Thiene, G., Valente, M., Valle, G., Schober, A., Gerosa, G., and Angelini, A. 2018. MicroRNA signatures in cardiac biopsies and detection of allograft rejection. *J Heart Lung Transplant*. doi: 10.1016/j.healun.2018.06.010.
- Dicks, N., Gutierrez, K., Michalak, M., Bordignon, V., and Agellon, L.B. 2015. Endoplasmic reticulum stress, genome damage, and cancer. *Front Oncol* **5**: 11. doi: 10.3389/fonc.2015.00011.
- Dirkx, E., Gladka, M.M., Philippen, L.E., Armand, A.S., Kinet, V., Leptidis, S., El Azzouzi, H., Salic, K., Bourajjaj, M., da Silva, G.J., Olieslagers, S., van der Nagel, R., de Weger, R., Bitsch, N., Kisters, N., Seyen, S., Morikawa, Y., Chanoine, C., Heymans, S., Volders, P.G., Thum, T., Dimmeler, S., Cserjesi, P., Eschenhagen, T., da Costa Martins, P.A., and De Windt, L.J. 2013. Nfat and miR-25 cooperate to reactivate the transcription factor Hand2 in heart failure. *Nat Cell Biol* **15**(11): 1282-1293. doi: 10.1038/ncb2866.

- Dittmer, S., Kovacs, Z., Yuan, S.H., Siszler, G., Kogl, M., Summer, H., Geerts, A., Golz, S., Shioda, T., and Methner, A. 2011. TOX3 is a neuronal survival factor that induces transcription depending on the presence of CITED1 or phosphorylated CREB in the transcriptionally active complex. *Journal of cell science* **124**(Pt 2): 252-260. doi: 10.1242/jcs.068759.
- Divakaran, V., and Mann, D.L. 2008. The emerging role of microRNAs in cardiac remodeling and heart failure. *Circ Res* **103**(10): 1072-1083. doi: 10.1161/CIRCRESAHA.108.183087.
- Doroudgar, S., Thuerauf, D.J., Marcinko, M.C., Belmont, P.J., and Glembotski, C.C. 2009. Ischemia activates the ATF6 branch of the endoplasmic reticulum stress response. *J Biol Chem* **284**(43): 29735-29745. doi: 10.1074/jbc.M109.018036.
- Duan, Q., Chen, C., Yang, L., Li, N., Gong, W., Li, S., and Wang, D.W. 2015. MicroRNA regulation of unfolded protein response transcription factor XBP1 in the progression of cardiac hypertrophy and heart failure in vivo. *J Transl Med* **13**: 363. doi: 10.1186/s12967-015-0725-4.
- Duan, Q., Wang, X., Gong, W., Ni, L., Chen, C., He, X., Chen, F., Yang, L., Wang, P., and Wang, D.W. 2012. ER stress negatively modulates the expression of the miR-199a/214 cluster to regulates tumor survival and progression in human hepatocellular cancer. *PLoS One* **7**(2): e31518. doi: 10.1371/journal.pone.0031518.
- Dubourg, B., Kamphausen, T., Weiwad, M., Jahreis, G., Feunteun, J., Fischer, G., and Modjtahedi, N. 2004. The human nuclear SRcyp is a cell cycle-regulated cyclophilin. *J Biol Chem* **279**(21): 22322-22330. doi: 10.1074/jbc.M400736200.
- Duygu, B., Poels, E.M., Juni, R., Bitsch, N., Ottaviani, L., Olieslagers, S., de Windt, L.J., and da Costa Martins, P.A. 2017. miR-199b-5p is a regulator of left ventricular remodeling following myocardial infarction. *Noncoding RNA Res* **2**(1): 18-26. doi: 10.1016/j.ncrna.2016.12.002.
- Elefant, N., Berger, A., Shein, H., Hofree, M., Margalit, H., and Altuvia, Y. 2011. RepTar: a database of predicted cellular targets of host and viral miRNAs. *Nucleic Acids Res* **39**(Database issue): D188-194. doi: 10.1093/nar/gkq1233.

- Eletto, D., Maganty, A., Dersh, D., Makarewich, C., Biswas, C., Paton, J.C., Paton, A.W., Doroudgar, S., Glembotski, C.C., and Argon, Y. 2012. Limitation of individual folding resources in the ER leads to outcomes distinct from the unfolded protein response. *Journal of cell science*. doi: 10.1242/jcs.108928.
- Feske, S., Gwack, Y., Prakriya, M., Srikanth, S., Puppel, S.H., Tanasa, B., Hogan, P.G., Lewis, R.S., Daly, M., and Rao, A. 2006. A mutation in *Orai1* causes immune deficiency by abrogating CRAC channel function. *Nature* **441**(7090): 179-185.
- Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. 2009. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**(1): 92-105. doi: 10.1101/gr.082701.108.
- Fu, J.D., Rushing, S.N., Lieu, D.K., Chan, C.W., Kong, C.W., Geng, L., Wilson, K.D., Chiamvimonvat, N., Boheler, K.R., Wu, J.C., Keller, G., Hajjar, R.J., and Li, R.A. 2011. Distinct roles of microRNA-1 and -499 in ventricular specification and functional maturation of human embryonic stem cell-derived cardiomyocytes. *PLoS One* **6**(11): e27417. doi: 10.1371/journal.pone.0027417.
- Groenendyk, J., Agellon, L.B., and Michalak, M. 2013. Coping with endoplasmic reticulum stress in the cardiovascular system. *Annu Rev Physiol* **75**: 49-67. doi: 10.1146/annurev-physiol-030212-183707.
- Groenendyk, J., Lee, D., Jung, J., Dyck, J.R., Lopaschuk, G.D., Agellon, L.B., and Michalak, M. 2016. Inhibition of the Unfolded Protein Response Mechanism Prevents Cardiac Fibrosis. *PLoS One* **11**(7): e0159682. doi: 10.1371/journal.pone.0159682.
- Groenendyk, J., and Michalak, M. 2014. Disrupted WNT signaling in mouse embryonic stem cells in the absence of calreticulin. *Stem Cell Rev* **10**(2): 191-206. doi: 10.1007/s12015-013-9488-6.
- Groenendyk, J., Paskevicius, T., Urrea, H., Viricel, C., Wang, K., Barakat, K., Hetz, C., Kurgan, L., Agellon, L.B., and Michalak, M. 2018. Cyclosporine A binding to COX-2 reveals a novel signaling pathway that activates the IRE1 α unfolded protein response sensor. *Sci Rep* **8**(1): 16678. doi: 10.1038/s41598-018-34891-w.
- Groenendyk, J., Peng, Z., Dudek, E., Fan, X., Mizianty, M.J., Dufey, E., Urrea, H., Sepulveda, D., Rojas-Rivera, D., Lim, Y., Kim, D.H., Baretta, K., Srikanth, S., Gwack, Y., Ahnn, J., Kaufman, R.J., Lee, S.-K., Hetz, C., Kurgan, L., and Michalak, M. 2014a. Interplay Between the Oxidoreductase PDIA6 and

- microRNA-322 Controls the Response to Disrupted Endoplasmic Reticulum Calcium Homeostasis. *Science Signaling* **7**(329): ra54-ra54. doi: 10.1126/scisignal.2004983.
- Groenendyk, J., Peng, Z., Dudek, E., Fan, X., Mizianty, M.J., Dufey, E., Urrea, H., Sepulveda, D., Rojas-Rivera, D., Lim, Y., Kim, D.H., Baretta, K., Srikanth, S., Gwack, Y., Ahnn, J., Kaufman, R.J., Lee, S.K., Hetz, C., Kurgan, L., and Michalak, M. 2014b. Interplay between the oxidoreductase PDIA6 and microRNA-322 controls the response to disrupted endoplasmic reticulum calcium homeostasis. *Sci Signal* **7**(329): ra54. doi: 10.1126/scisignal.2004983.
- Groenendyk, J., Peng, Z., Dudek, E., Fan, X., Mizianty, M.J., Dufey, E., Urrea, H., Sepulveda, D., Rojas-Rivera, D., Lim, Y., Kim do, H., Baretta, K., Srikanth, S., Gwack, Y., Ahnn, J., Kaufman, R.J., Lee, S.K., Hetz, C., Kurgan, L., and Michalak, M. 2014c. Interplay between the oxidoreductase PDIA6 and microRNA-322 controls the response to disrupted endoplasmic reticulum calcium homeostasis. *Sci Signal* **7**(329): ra54. doi: 10.1126/scisignal.2004983.
- Hetz, C., and Papa, F.R. 2018. The Unfolded Protein Response and Cell Fate Control. *Mol Cell* **69**(2): 169-181. doi: 10.1016/j.molcel.2017.06.017.
- Hou, X., Pedi, L., Diver, M.M., and Long, S.B. 2012. Crystal structure of the calcium release-activated calcium channel Orai. *Science* **338**(6112): 1308-1313. doi: 10.1126/science.1228757.
- Huang, W., Feng, Y., Liang, J., Yu, H., Wang, C., Wang, B., Wang, M., Jiang, L., Meng, W., Cai, W., Medvedovic, M., Chen, J., Paul, C., Davidson, W.S., Sadayappan, S., Stambrook, P.J., Yu, X.Y., and Wang, Y. 2018. Loss of microRNA-128 promotes cardiomyocyte proliferation and heart regeneration. *Nat Commun* **9**(1): 700. doi: 10.1038/s41467-018-03019-z.
- Kang, H., Louie, J., Weisman, A., Sheu-Gruttadauria, J., Davis-Dusenbery, B.N., Lagna, G., and Hata, A. 2012. Inhibition of microRNA-302 (miR-302) by bone morphogenetic protein 4 (BMP4) facilitates the BMP signaling pathway. *J Biol Chem* **287**(46): 38656-38664. doi: 10.1074/jbc.M112.390898.
- Kelley, K., and Lin, S.L. 2012. Induction of somatic cell reprogramming using the microRNA miR-302. *Progress in molecular biology and translational science* **111**: 83-107. doi: 10.1016/B978-0-12-398459-3.00004-6.

- Krebs, J., Agellon, L.B., and Michalak, M. 2015. Ca^{2+} homeostasis and endoplasmic reticulum (ER) stress: An integrated view of calcium signaling. *Biochem Biophys Res Commun* **460**(1): 114-121. doi: 10.1016/j.bbrc.2015.02.004.
- Le Tonqueze, O., Gschloessl, B., Legagneux, V., Paillard, L., and Audic, Y. 2016. Identification of CELF1 RNA targets by CLIP-seq in human HeLa cells. *Genom Data* **8**: 97-103. doi: 10.1016/j.gdata.2016.04.009.
- Leung, A.K., and Sharp, P.A. 2010. MicroRNA functions in stress responses. *Mol Cell* **40**(2): 205-215. doi: 10.1016/j.molcel.2010.09.027.
- Lewis, B.P., Burge, C.B., and Bartel, D.P. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**(1): 15-20. doi: 10.1016/j.cell.2004.12.035.
- Li, J., Pucat, M., Perez-Terzic, C., Mery, A., Nakamura, K., Michalak, M., Krause, K.-H., and Jaconi, M.E. 2002. Calreticulin reveals a critical Ca^{2+} checkpoint in cardiac myofibrillogenesis. *The Journal of Cell Biology* **158**(103-113.).
- Liao, B., Bao, X., Liu, L., Feng, S., Zovoilis, A., Liu, W., Xue, Y., Cai, J., Guo, X., Qin, B., Zhang, R., Wu, J., Lai, L., Teng, M., Niu, L., Zhang, B., Esteban, M.A., and Pei, D. 2011. MicroRNA cluster 302-367 enhances somatic cell reprogramming by accelerating a mesenchymal-to-epithelial transition. *J Biol Chem* **286**(19): 17359-17364. doi: 10.1074/jbc.C111.235960.
- Lin, S.L., Chang, D.C., Chang-Lin, S., Lin, C.H., Wu, D.T., Chen, D.T., and Ying, S.Y. 2008. Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA* **14**(10): 2115-2124. doi: 10.1261/rna.1162708.
- Lin, S.L., Chang, D.C., Lin, C.H., Ying, S.Y., Leu, D., and Wu, D.T. 2011. Regulation of somatic cell reprogramming through inducible mir-302 expression. *Nucleic Acids Res* **39**(3): 1054-1065. doi: 10.1093/nar/gkq850.

- Lin, S.L., Chang, D.C., Ying, S.Y., Leu, D., and Wu, D.T. 2010. MicroRNA miR-302 inhibits the tumorigenicity of human pluripotent stem cells by coordinate suppression of the CDK2 and CDK4/6 cell cycle pathways. *Cancer Res* **70**(22): 9473-9482. doi: 10.1158/0008-5472.CAN-10-2746.
- Liou, J., Kim, M.L., Heo, W.D., Jones, J.T., Myers, J.W., Ferrell, J.E., Jr., and Meyer, T. 2005. STIM is a Ca^{2+} sensor essential for Ca^{2+} -store-depletion-triggered Ca^{2+} influx. *Curr. Biol.* **15**(13): 1235-1241.
- Liu, N., Williams, A.H., Kim, Y., McAnally, J., Bezprozvannaya, S., Sutherland, L.B., Richardson, J.A., Bassel-Duby, R., and Olson, E.N. 2007. An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. *Proc Natl Acad Sci U S A* **104**(52): 20844-20849. doi: 10.1073/pnas.0710558105.
- Luik, R.M., Wang, B., Prakriya, M., Wu, M.M., and Lewis, R.S. 2008. Oligomerization of STIM1 couples ER calcium depletion to CRAC channel activation. *Nature* **454**(7203): 538-542. doi: nature07065 [pii]10.1038/nature07065.
- Luik, R.M., Wu, M.M., Buchanan, J., and Lewis, R.S. 2006. The elementary unit of store-operated Ca^{2+} entry: local activation of CRAC channels by STIM1 at ER-plasma membrane junctions. *The Journal of cell biology* **174**(6): 815-825. doi: jcb.200604015 [pii]10.1083/jcb.200604015.
- Maragkakis, M., Reczko, M., Simossis, V.A., Alexiou, P., Papadopoulos, G.L., Dalamagas, T., Giannopoulos, G., Goumas, G., Koukis, E., Kourtis, K., Vergoulis, T., Koziris, N., Sellis, T., Tsanakas, P., and Hatzigeorgiou, A.G. 2009. DIANA-microT web server: elucidating microRNA functions through target prediction. *Nucleic Acids Res* **37**(Web Server issue): W273-276. doi: 10.1093/nar/gkp292.
- Marambio, P., Toro, B., Sanhueza, C., Troncoso, R., Parra, V., Verdejo, H., Garcia, L., Quiroga, C., Munafo, D., Diaz-Elizondo, J., Bravo, R., Gonzalez, M.J., Diaz-Araya, G., Pedrozo, Z., Chiong, M., Colombo, M.I., and Lavandero, S. 2010. Glucose deprivation causes oxidative stress and stimulates aggresome formation and autophagy in cultured cardiac myocytes. *Biochim Biophys Acta* **1802**(6): 509-518. doi: 10.1016/j.bbadis.2010.02.002.

- Mellis, D., and Caporali, A. 2018. MicroRNA-based therapeutics in cardiovascular disease: screening and delivery to the target. *Biochem Soc Trans* **46**(1): 11-21. doi: 10.1042/BST20170037.
- Mery, A., Aimond, F., Menard, C., Mikoshiba, K., Michalak, M., and Puceat, M. 2005. Initiation of embryonic cardiac pacemaker activity by inositol 1,4,5-trisphosphate-dependent calcium signaling. *Mol Biol Cell* **16**(5): 2414-2423.
- Mesaeli, N., Nakamura, K., Zvaritch, E., Dickie, P., Dziak, E., Krause, K.H., Opas, M., MacLennan, D.H., and Michalak, M. 1999. Calreticulin is essential for cardiac development. *J Cell Biol* **144**(5): 857-868.
- Mignen, O., Thompson, J.L., and Shuttleworth, T.J. 2008. Orai1 subunit stoichiometry of the mammalian CRAC channel pore. *J Physiol* **586**(2): 419-425. doi: jphysiol.2007.147249 [pii]10.1113/jphysiol.2007.147249.
- Montgomery, R.L., Hullinger, T.G., Semus, H.M., Dickinson, B.A., Seto, A.G., Lynch, J.M., Stack, C., Latimer, P.A., Olson, E.N., and van Rooij, E. 2011. Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure. *Circulation* **124**(14): 1537-1547. doi: 10.1161/CIRCULATIONAHA.111.030932.
- Muik, M., Frischauf, I., Derler, I., Fahrner, M., Bergsmann, J., Eder, P., Schindl, R., Hesch, C., Polzinger, B., Fritsch, R., Kahr, H., Madl, J., Gruber, H., Groschner, K., and Romanin, C. 2008. Dynamic coupling of the putative coiled-coil domain of ORAI1 with STIM1 mediates ORAI1 channel activation. *J Biol Chem* **283**(12): 8014-8022. doi: M708898200 [pii]10.1074/jbc.M708898200.
- Nakamura, K., Zuppini, A., Arnaudeau, S., Lynch, J., Ahsan, I., Krause, R., Papp, S., De Smedt, H., Parys, J.B., Muller-Esterl, W., Lew, D.P., Krause, K.H., Demaurex, N., Opas, M., and Michalak, M. 2001. Functional specialization of calreticulin domains. *J Cell Biol* **154**(5): 961-972. doi: 10.1083/jcb.200102073.
- Nam, Y.J., Song, K., Luo, X., Daniel, E., Lambeth, K., West, K., Hill, J.A., DiMaio, J.M., Baker, L.A., Bassel-Duby, R., and Olson, E.N. 2013. Reprogramming of human fibroblasts toward a cardiac fate. *Proc Natl Acad Sci U S A* **110**(14): 5588-5593. doi: 10.1073/pnas.1301019110.

- Nwokonko, R.M., Cai, X., Loktionova, N.A., Wang, Y., Zhou, Y., and Gill, D.L. 2017. The STIM-Orai Pathway: Conformational Coupling Between STIM and Orai in the Activation of Store-Operated Ca^{2+} Entry. *Adv Exp Med Biol* **993**: 83-98. doi: 10.1007/978-3-319-57732-6_5.
- Olson, E.N. 2014. MicroRNAs as therapeutic targets and biomarkers of cardiovascular disease. *Sci Transl Med* **6**(239): 239ps233. doi: 10.1126/scitranslmed.3009008.
- Owunwanne, A., Shihab-Eldeen, A., Sadek, S., Junaid, T., Yacoub, T., and Abdel-Dayem, H.M. 1993. Is cyclosporine toxic to the heart? *J Heart Lung Transplant* **12**(2): 199-204.
- Pan, Z., Sun, X., Shan, H., Wang, N., Wang, J., Ren, J., Feng, S., Xie, L., Lu, C., Yuan, Y., Zhang, Y., Wang, Y., Lu, Y., and Yang, B. 2012. MicroRNA-101 inhibited postinfarct cardiac fibrosis and improved left ventricular compliance via the FBJ osteosarcoma oncogene/transforming growth factor-beta1 pathway. *Circulation* **126**(7): 840-850. doi: 10.1161/CIRCULATIONAHA.112.094524.
- Park, K.S., Poburko, D., Wollheim, C.B., and Demarex, N. 2009. Amiloride derivatives induce apoptosis by depleting ER Ca^{2+} stores in vascular endothelial cells. *Br J Pharmacol* **156**(8): 1296-1304. doi: BPH133 [pii] 10.1111/j.1476-5381.2009.00133.x.
- Park, S., Hong, J.H., Ohana, E., and Muallem, S. 2012. The WNK/SPAK and IRBIT/PP1 pathways in epithelial fluid and electrolyte transport. *Physiology (Bethesda)* **27**(5): 291-299. doi: 10.1152/physiol.00028.2012.
- Peinelt, C., Vig, M., Koomoa, D.L., Beck, A., Nadler, M.J., Koblan-Huberson, M., Lis, A., Fleig, A., Penner, R., and Kinet, J.P. 2006. Amplification of CRAC current by STIM1 and CRACM1 (Orai1). *Nat Cell Biol*.
- Penna, A., Demuro, A., Yeromin, A.V., Zhang, S.L., Safrina, O., Parker, I., and Cahalan, M.D. 2008. The CRAC channel consists of a tetramer formed by Stim-induced dimerization of Orai dimers. *Nature* **456**(7218): 116-120. doi: nature07338 [pii] 10.1038/nature07338.
- Phillips, M.J., and Voeltz, G.K. 2016. Structure and function of ER membrane contact sites with other organelles. *Nat Rev Mol Cell Biol* **17**(2): 69-82. doi: 10.1038/nrm.2015.8.

- Pinheiro, I., Dejager, L., and Libert, C. 2011. X-chromosome-located microRNAs in immunity: might they explain male/female differences? The X chromosome-genomic context may affect X-located miRNAs and downstream signaling, thereby contributing to the enhanced immune response of females. *Bioessays* **33**(11): 791-802. doi: 10.1002/bies.201100047.
- Plummer, S.M., Holloway, K.A., Manson, M.M., Munks, R.J., Kaptein, A., Farrow, S., and Howells, L. 1999. Inhibition of cyclo-oxygenase 2 expression in colon cells by the chemopreventive agent curcumin involves inhibition of NF-kappaB activation via the NIK/IKK signalling complex. *Oncogene* **18**(44): 6013-6020. doi: 10.1038/sj.onc.1202980.
- Prakriya, M., Feske, S., Gwack, Y., Srikanth, S., Rao, A., and Hogan, P.G. 2006. Orai1 is an essential pore subunit of the CRAC channel. *Nature* **443**(7108): 230-233.
- Read, D.E., Gupta, A., Ladilov, Y., Samali, A., and Gupta, S. 2014. miRNA signature of unfolded protein response in H9c2 rat cardiomyoblasts. *Cell Biosci* **4**(1): 56. doi: 10.1186/2045-3701-4-56.
- Ricciotti, E., and FitzGerald, G.A. 2011. Prostaglandins and inflammation. *Arterioscler Thromb Vasc Biol* **31**(5): 986-1000. doi: 10.1161/ATVBAHA.110.207449.
- Roberson, E.C., Tully, J.E., Guala, A.S., Reiss, J.N., Godburn, K.E., Pociask, D.A., Alcorn, J.F., Riches, D.W., Dienz, O., Janssen-Heininger, Y.M., and Anathy, V. 2011. Influenza Induces ER Stress, Caspase-12- Dependent Apoptosis and JNK Mediated TGF-beta Release in Lung Epithelial Cells. *Am J Respir Cell Mol Biol*. doi: 10.1165/rcmb.2010-0460OC.
- Romaine, S.P., Tomaszewski, M., Condorelli, G., and Samani, N.J. 2015. MicroRNAs in cardiovascular disease: an introduction for clinicians. *Heart* **101**(12): 921-928. doi: 10.1136/heartjnl-2013-305402.
- Roos, J., DiGregorio, P.J., Yeromin, A.V., Ohlsen, K., Lioudyno, M., Zhang, S., Safrina, O., Kozak, J.A., Wagner, S.L., Cahalan, M.D., Velicelebi, G., and Stauderman, K.A. 2005. STIM1, an essential and conserved component of store-operated Ca²⁺ channel function. *J Cell Biol* **169**(3): 435-445.
- Rzymiski, T., Paantjens, A., Bod, J., and Harris, A.L. 2008. Multiple pathways are involved in the anoxia response of SKIP3 including HuR-regulated RNA stability, NF-kappaB and ATF4. *Oncogene* **27**(33): 4532-4543. doi: 10.1038/onc.2008.100.

- Schwarz, D.S., and Blower, M.D. 2016. The endoplasmic reticulum: structure, function and response to cellular signaling. *Cell Mol Life Sci* **73**(1): 79-94. doi: 10.1007/s00018-015-2052-6.
- Serysheva, E., Berhane, H., Grumolato, L., Demir, K., Balmer, S., Bodak, M., Boutros, M., Aaronson, S., Mlodzik, M., and Jenny, A. 2013. Wnk kinases are positive regulators of canonical Wnt/beta-catenin signalling. *EMBO Rep*. doi: 10.1038/embor.2013.88.
- Shen, X., Soibam, B., Benham, A., Xu, X., Chopra, M., Peng, X., Yu, W., Bao, W., Liang, R., Azares, A., Liu, P., Gunaratne, P.H., Mercola, M., Cooney, A.J., Schwartz, R.J., and Liu, Y. 2016. miR-322/-503 cluster is expressed in the earliest cardiac progenitor cells and drives cardiomyocyte specification. *Proc Natl Acad Sci U S A* **113**(34): 9551-9556. doi: 10.1073/pnas.1608256113.
- Small, E.M., Frost, R.J., and Olson, E.N. 2010. MicroRNAs add a new dimension to cardiovascular disease. *Circulation* **121**(8): 1022-1032. doi: 10.1161/CIRCULATIONAHA.109.889048.
- Song, L., De Sarno, P., and Joep, R.S. 2002. Central role of glycogen synthase kinase-3beta in endoplasmic reticulum stress-induced caspase-3 activation. *J Biol Chem* **277**(47): 44701-44708. doi: 10.1074/jbc.M206047200.
- Stathopulos, P.B., Li, G.Y., Plevin, M.J., Ames, J.B., and Ikura, M. 2006. Stored Ca²⁺ depletion-induced oligomerization of stromal interaction molecule 1 (STIM1) via the EF-SAM region: An initiation mechanism for capacitive Ca²⁺ entry. *J Biol Chem* **281**(47): 35855-35862.
- Streicher, J.M., and Wang, Y. 2008. The role of COX-2 in heart pathology. *Cardiovasc Hematol Agents Med Chem* **6**(1): 69-79.
- Sun, Y., Zhang, D., Liu, X., Li, X., Liu, F., Yu, Y., Jia, S., Zhou, Y., and Zhao, Y. 2018. Endoplasmic Reticulum Stress Affects Lipid Metabolism in Atherosclerosis Via CHOP Activation and Over-Expression of miR-33. *Cell Physiol Biochem* **48**(5): 1995-2010. doi: 10.1159/000492522.
- Szegezdi, E., Duffy, A., O'Mahoney, M.E., Logue, S.E., Mylotte, L.A., O'Brien, T., and Samali, A. 2006. ER stress contributes to ischemia-induced cardiomyocyte apoptosis. *Biochem Biophys Res Commun* **349**(4): 1406-1411. doi: 10.1016/j.bbrc.2006.09.009.

- Thuerauf, D.J., Marcinko, M., Gude, N., Rubio, M., Sussman, M.A., and Glembotski, C.C. 2006. Activation of the unfolded protein response in infarcted mouse heart and hypoxic cultured cardiac myocytes. *Circ Res* **99**(3): 275-282. doi: 10.1161/01.RES.0000233317.70421.03.
- Thum, T., Galuppo, P., Wolf, C., Fiedler, J., Kneitz, S., van Laake, L.W., Doevendans, P.A., Mummery, C.L., Borlak, J., Haverich, A., Gross, C., Engelhardt, S., Ertl, G., and Bauersachs, J. 2007. MicroRNAs in the human heart: a clue to fetal gene reprogramming in heart failure. *Circulation* **116**(3): 258-267. doi: 10.1161/CIRCULATIONAHA.107.687947.
- Tian, T., Mingyi, M., Qiu, X., and Qiu, Y. 2016. MicroRNA-101 reverses temozolomide resistance by inhibition of GSK3beta in glioblastoma. *Oncotarget* **7**(48): 79584-79595. doi: 10.18632/oncotarget.12861.
- Tian, Y., Cohen, E.D., and Morrissey, E.E. 2010. The importance of Wnt signaling in cardiovascular development. *Pediatr Cardiol* **31**(3): 342-348. doi: 10.1007/s00246-009-9606-z.
- Tian, Y., Liu, Y., Wang, T., Zhou, N., Kong, J., Chen, L., Snitow, M., Morley, M., Li, D., Petrenko, N., Zhou, S., Lu, M., Gao, E., Koch, W.J., Stewart, K.M., and Morrissey, E.E. 2015. A microRNA-Hippo pathway that promotes cardiomyocyte proliferation and cardiac regeneration in mice. *Sci Transl Med* **7**(279): 279ra238. doi: 10.1126/scitranslmed.3010841.
- Tufanli, O., Telkoparan Akillilar, P., Acosta-Alvear, D., Kocaturk, B., Onat, U.I., Hamid, S.M., Cimen, I., Walter, P., Weber, C., and Erbay, E. 2017. Targeting IRE1 with small molecules counteracts progression of atherosclerosis. *Proc Natl Acad Sci U S A* **114**(8): E1395-E1404. doi: 10.1073/pnas.1621188114.
- Upton, J.P., Wang, L., Han, D., Wang, E.S., Huskey, N.E., Lim, L., Truitt, M., McManus, M.T., Ruggero, D., Goga, A., Papa, F.R., and Oakes, S.A. 2012. IRE1alpha cleaves select microRNAs during ER stress to derepress translation of proapoptotic Caspase-2. *Science* **338**(6108): 818-822. doi: 10.1126/science.1226191.

- van Rooij, E., Sutherland, L.B., Qi, X., Richardson, J.A., Hill, J., and Olson, E.N. 2007. Control of stress-dependent cardiac growth and gene expression by a microRNA. *Science* **316**(5824): 575-579. doi: 10.1126/science.1139089.
- Vekich, J.A., Belmont, P.J., Thuerauf, D.J., and Glembotski, C.C. 2012. Protein disulfide isomerase-associated 6 is an ATF6-inducible ER stress response protein that protects cardiac myocytes from ischemia/reperfusion-mediated cell death. *J Mol Cell Cardiol* **53**(2): 259-267. doi: 10.1016/j.yjmcc.2012.05.005.
- Vig, M., Beck, A., Billingsley, J.M., Lis, A., Parvez, S., Peinelt, C., Koomoa, D.L., Soboloff, J., Gill, D.L., Fleig, A., Kinet, J.P., and Penner, R. 2006. CRACM1 multimers form the ion-selective pore of the CRAC channel. *Curr Biol* **16**(20): 2073-2079. doi: S0960-9822(06)02153-1 [pii]10.1016/j.cub.2006.08.085.
- Wang, M., and Kaufman, R.J. 2016. Protein misfolding in the endoplasmic reticulum as a conduit to human disease. *Nature* **529**(7586): 326-335. doi: 10.1038/nature17041.
- Wilson, K.D., Hu, S., Venkatasubrahmanyam, S., Fu, J.D., Sun, N., Abilez, O.J., Baugh, J.J., Jia, F., Ghosh, Z., Li, R.A., Butte, A.J., and Wu, J.C. 2010. Dynamic microRNA expression programs during cardiac differentiation of human embryonic stem cells: role for miR-499. *Circ Cardiovasc Genet* **3**(5): 426-435. doi: 10.1161/CIRCGENETICS.109.934281.
- Xia, H.P. 2008. Great Potential of MicroRNA in Cancer Stem Cell. *J. Cancer Mol.* **4**(3): **79-89**, **2008**(4(3)): 4(3): 79-89.
- Xie, J., Wu, T., Xu, K., Huang, I.K., Cleaver, O., and Huang, C.L. 2009. Endothelial-specific expression of WNK1 kinase is essential for angiogenesis and heart development in mice. *Am J Pathol* **175**(3): 1315-1327. doi: 10.2353/ajpath.2009.090094.
- Xie, J., Yoon, J., Yang, S.S., Lin, S.H., and Huang, C.L. 2013. WNK1 protein kinase regulates embryonic cardiovascular development through the OSR1 signaling cascade. *J Biol Chem* **288**(12): 8566-8574. doi: 10.1074/jbc.M113.451575.

- Yang, C.S., and Rana, T.M. 2012. Learning the molecular mechanisms of the reprogramming factors: let's start from microRNAs. *Molecular bioSystems*. doi: 10.1039/c2mb25088h.
- Yu, Y., Correll, P.H., and Vanden Heuvel, J.P. 2002. Conjugated linoleic acid decreases production of pro-inflammatory products in macrophages: evidence for a PPAR gamma-dependent mechanism. *Biochim Biophys Acta* **1581**(3): 89-99.
- Zhang, S.L., Yu, Y., Roos, J., Kozak, J.A., Deerinck, T.J., Ellisman, M.H., Stauderman, K.A., and Cahalan, M.D. 2005. STIM1 is a Ca^{2+} sensor that activates CRAC channels and migrates from the Ca^{2+} store to the plasma membrane. *Nature* **437**(7060): 902-905.
- Zhou, A.D., Diao, L.T., Xu, H., Xiao, Z.D., Li, J.H., Zhou, H., and Qu, L.H. 2012. beta-Catenin/LEF1 transactivates the microRNA-371-373 cluster that modulates the Wnt/beta-catenin-signaling pathway. *Oncogene* **31**(24): 2968-2978. doi: 10.1038/onc.2011.461.
- Zhou, J., Ahmad, F., Parikh, S., Hoffman, N.E., Rajan, S., Verma, V.K., Song, J., Yuan, A., Shanmughapriya, S., Guo, Y., Gao, E., Koch, W., Woodgett, J.R., Madesh, M., Kishore, R., Lal, H., and Force, T. 2016. Loss of Adult Cardiac Myocyte GSK-3 Leads to Mitotic Catastrophe Resulting in Fatal Dilated Cardiomyopathy. *Circ Res* **118**(8): 1208-1222. doi: 10.1161/CIRCRESAHA.116.308544.
- Zhu, L., Song, S., Pi, Y., Yu, Y., She, W., Ye, H., Su, Y., and Hu, Q. 2011. Cumulated Ca^{2+} spike duration underlies Ca^{2+} oscillation frequency-regulated NFkappaB transcriptional activity. *Journal of cell science* **124**(Pt 15): 2591-2601. doi: 10.1242/jcs.082727.

FIGURE LEGENDS

Figure 1. Schematic representation of a link between ER stress, microRNA and cardiac physiology and pathology. PDIA6, protein disulfide isomerase A6; PERK, dsRNA-activated protein kinase-like ER kinase; IRE, inositol-requiring kinase; ATF6, activating transcription factor 6; COX-2, cyclooxygenase-2; Casq, calsequestrin.; BiP, immunoglobulin binding protein.

Figure 2. Induction of the UPR. NIH-3T3 mouse fibroblasts (**A**) and HEK-293 human kidney cells (**B**) were maintained under standard tissue culture conditions. Mouse embryonic stem cells wild type or calreticulin deficient (*Calr*^{-/-}) (**C**) were maintained according to previously published protocols (Groenendyk and Michalak 2014). A.U., absorbance units. Drug conditions were performed for 24 hours and included: 0.5 μ M thapsigargin (*Thap*), 20 μ M cyclosporine (*CSA*), or 1 mM DTT. RT-PCR analysis was carried out as described previously (Groenendyk et al. 2014b).

Figure 3. Abundance of miR-322 under ER stress conditions. NIH-3T3 mouse fibroblasts were treated with: 0.5 μ M thapsigargin (*Thap*), 20 μ M cyclosporine (*CSA*), 1 mM DTT, 5 μ g/ml tunicamycin (*Tun*), or 5 μ g/ml brefeldin A (*BFA*), and microRNA was isolated using the miRNeasy kit according to manufacturer's protocol. (**A**) The mmu-miR-322 abundance (A.U.=absorbance units) was determined using qPCR. (**B**) Primers used in quantitative PCR to measure mRNA levels included: m-Smad3, m-PPI γ and were normalized to m-GAPDH. The mmu-miR-322 mimic treatment was at 100 nM for 24 hours, cells were harvested with RNeasy followed by qPCR analysis (Groenendyk et al. 2014b).

Figure 4. miR-322. (**A**) Identification of a putative IRE1 α cleavage site within mmu-miR-322. (**B**) NIH-3T3 cells (*Neg siRNA*) and IRE1 α silenced NIH-3T3 cells (*IRE1 α siRNA*) were treated with 0.5 μ M thapsigargin (*Thap*) for 24 hours. MicroRNA was harvested and qPCR performed to monitor mmu-miR-322 abundance (Groenendyk et al. 2014b). (**C**) NIH-3T3 cells (*control*) and ORAI1-deficient MEFs in NIH3T3 cells (*ORAI*^{-/-}) were treated with 0.5 μ M thapsigargin (*Thap*) for 24 hours. MicroRNA was harvested and qPCR performed to monitor mmu-miR-322 abundance (Groenendyk et al. 2014b).

Figure 5. A role of miR-101b. HEK-293 cells were treated with 0.5 μ M thapsigargin (*Thap*) for 24 hours or not (*control*) followed by RNA isolation (Groenendyk et al. 2014b). (A) The hsa-miR-101b abundance was determined using qPCR. (B) HEK-293 cells were treated with 0.5 μ M thapsigargin with or without (*control*) 24-hour treatment of 100 nM hsa-miR-101b mimic. (C) Activity of XBP1 reporter. The pRL-IXFL XBP1 contains an internal Renilla control and the nucleotide sequence encoding XBP1 followed by firefly luciferase separated by an internal ribosomal entry site (IRES) initiation region (Back et al. 2006b; Groenendyk et al. 2014a). (D) HEK-293 cells were transfected with siRNA specific for COX-2 (Groenendyk et al. 2018), treated for 24 hours with 0.5 μ M thapsigargin (Groenendyk et al. 2014b). (E) HEK-293 cells were transfected with hsa-miR-101b mimic, treated for 24 hours with 0.5 μ M thapsigargin and mRNA harvested.

Figure 6. miR-302c. Wild type and calreticulin deficient mouse (*Calr*^{-/-}) embryonic stem cells (ES) were maintained under standard tissue culture conditions. (A) MicroRNA was isolated using the miRNeasy kit (Qiagen) and qPCR was performed to monitor mmu-miR-302c expression and was based on the sequence 5'-AAGUGCUUCCAUGUUUCAGUGG-3'. (B) mRNA was isolated using the RNeasy kit (Qiagen) and quantitative PCR carried out to determine mRNA abundance of m-calcineurin A.U. = absorbance units.

Figure 7. Novel microRNA miR-0034*. (A) MicroRNA was isolated from wild type and calreticulin deficient (*Calr*^{-/-}) ES cells and mmu-miR-0034* abundance was monitored using qPCR. (B) Quantitative PCR was carried out to determine mRNA abundance of m-TOX3, m-WNK1 and normalized to m-GAPDH. (C) Protein was isolated from wild-type and calreticulin-deficient ES cells and immunoblotting carried out using an antibody specific for TOX3. A.U. = absorbance units.

Table 1. MicroRNA in thapsigargin (*Thap*) treated fibroblasts and in calreticulin-deficient (*Calr*^{-/-}) ES cells.

Deep sequencing analysis was performed by the Plant BioSys (University of Lethbridge). Additional statistical analysis was carried out as described previously (Groenendyk et al. 2014c). *Thap*, thapsigargin; T/C, thapsigargin/control; KO/WT, *Calr*^{-/-}/wild-type

miRNA <i>Thap</i> treated	T/C	miRNA <i>Calr</i> ^{-/-}	KO/WT
mmu-miR-671-5p	0.068966	mmu-miR-302d*	0
new_miRNA_star0666	0.071429	new_miRNA_star0377	0.037037
mmu-miR-669f-3p	0.117647	mmu-miR-302b	0.061224
mmu-miR-503	0.137931	new_miRNA0374	0.061983
mmu-miR-143*	0.263514	mmu-miR-2137	0.074074
mmu-miR-322	0.312573	mmu-miR-302d	0.080346
new_miRNA0980	1.917352	new_miRNA0377	0.080666
mmu-miR-708*	2.496441	mmu-miR-302a*	0.081967
new_miRNA0795	2.634454	new_miRNA_star0376	0.083333
mmu-miR-101b	2.779874	new_miRNA0375	0.090909
mmu-miR-92a-1*	3.769231	mmu-miR-302c*	0.130435
mmu-miR-1937a	4.060738	mmu-miR-302c	0.132075
new_miRNA_star0242	4.272727	mmu-miR-653	0.145833
new_miRNA0055	8.333333	new_miRNA0376	0.159851
mmu-miR-216b	8.666667	mmu-miR-302a	0.164234
new_miRNA0056	18.33333	new_miRNA0466	0.248299
mmu-miR-216a*	19	mmu-miR-211	0.251656
		mmu-miR-351	0.314572
		mmu-miR-342-3p	0.334783
		mmu-miR-150	0.384913
		mmu-miR-335-3p	0.478696
		mmu-miR-17	0.478849
		mmu-miR-92a	0.553597
		new_miRNA0510	0.589958
		mmu-miR-191	0.592348
		mmu-miR-183	0.706784
		mmu-miR-127	1.913413
		mmu-miR-300	2.429967
		mmu-miR-134	2.745946
		mmu-miR-142-5p	2.860963
		mmu-miR-142-3p	3.517241
		new_miRNA_star0034	3.565217

Table 2. Ingenuity Pathway Analysis of mouse fibroblast microRNAs

Ingenuity Pathway Analysis carried out on targets of mmu-miR-322 and on microRNA affected by 24-hour thapsigargin (0.5 μ M) treatment.

Targets of mmu-miR-322	
Molecular and Cellular Functions	# of Molecules
Cellular Assembly and Organization	29
Cell Morphology	28
Molecular Transport	29
Carbohydrate Metabolism	9
Top Canonical Pathways	Ratio
Tight Junction Signaling	0.04
TGF β Signaling	0.06
WNT/ β -catenin Signaling	0.03
Targets of Thapsigargin Affected microRNAs	
Molecular and Cellular Functions	# of Molecules
Cellular Growth and Proliferation	958
Post-translational Modifications	429
Cell Cycle	525
Top Canonical Pathways	Ratio
PTEN Signaling	0.35
WNT/ β -catenin Signaling	0.33
TGF β Signaling	0.36

Table 3. Ingenuity Pathway Analysis of calreticulin-deficient ES cells.

Ingenuity Pathway Analysis carried out on microRNA affected by a deficiency in calreticulin in mouse ES cells.

Targets of microRNA affected by a Deficiency in Calreticulin	
Molecular and Cellular Functions	# of Molecules
Cellular Growth and Proliferation	883
Cellular Development	763
Cellular Movement	584
Top Canonical Pathways	Ratio
WNT/ β -catenin Signaling	0.50
TGF β Signaling	0.60
Cardiac Hypertrophy Signaling	0.41
Cell Cycle	0.56

Draft

Table 4. Ingenuity Pathway Analysis of gene targets of hsa-miR-101b.

Targets of hsa-miR-101b	
Molecular and Cellular Functions	# of Molecules
Gene Expression	94
Cellular Development	103
Post-translational Modification	33
Top Toxicity List	Ratio
Cardiac Hypertrophy	0.04
Cardiac Fibrosis	0.06
Vasopressin-induced Genes	0.25
Renal Necrosis/Cell Death	0.04

Draft

Table 5. Ingenuity Pathway Analysis of gene targets for mmu-miR-302c.

Targets of mmu-miR-302c	
Molecular and Cellular Functions	# of Molecules
Gene Expression	51
Cell Morphology	47
Cellular Development	48
Top Canonical Pathways	Ratio
Estrogen-mediated S-phase Entry	0.11
Cell Cycle: G1/S Checkpoint Regulation	0.09
WNT/β-catenin Signaling	0.03
Molecular Mechanisms of Cancer	0.02

Draft

Figure 2

Figure 3

miR-322

Figure 4

Figure 5

Figure 6

Figure 7