SEX DIFFERENCES IN CARDIAC STRUCTURE AND FUNCTION OF ZUCKER DIABETIC FATTY MALE AND FEMALE RATS

A Thesis presented to the Faculty of the Graduate School

at the University of Missouri – Columbia

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

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by

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MAY 2016

The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

SEX DIFFERENCES IN CARDIAC STRUCTURE AND FUNCTION OF ZUCKER DIABETIC FATTY MALE AND FEMALE RATS

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a candidate for the degree of Master of Science,

and hereby certify that, in their opinion, it is worthy of acceptance.

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ACKNOWLEDGEMENTS

I would first like to thank my advisor, Lakshmi Pulakat, PhD, who has provided guidance and support in developing this project as well as in my academic endeavor. I especially thank her for her patience and mentorship. I additionally thank and appreciate my committee members, Craig Emter, PhD, and Gerald Meininger, PhD, for their insightful comments, suggestions, and careful reviews in completion of my thesis.

I also would like to thank members of the Pulakat laboratory that I have worked with throughout the past two years. To Anna Ślusarz, PhD, Purushotham Koppula, PhD, Maininder Malik, PhD, Christian Luck, Abuzar Mahmood, Jamal Bajwa, Ahmad Raja, Fanding Gao and Nicholas Arnold: thanks!

I would be remiss if I did not thank Lisa Watkinson and Terry Carmack, who completed animal care and monitoring throughout this study. These two individuals have been a valuable resource of information and a great help in any and all matters related to animal studies.

Resources and support at the Harry S. Truman Memorial Veterans Hospital made this work possible. Research reported in this thesis was supported by the National Institutes of Health and the Missouri Foundation for Veterans' Medical Research.

I appreciate Javad Habibi, PhD, who taught me immunohistochemistry and who has answered many of my seemingly silly questions regarding immunostaining.

I thank Misha Kovalenko, MSc, and Dmitriy Shin, PhD, WSI Analytics Lab at the University of Missouri for digital slide scanning of histological samples.

I would like to acknowledge the Histology section at IDEXX Laboratories, Inc. (Columbia, MO), especially Jill Hansen, Janet Adair and Bonita Cowan, for their assistance in tissue embedding, sectioning and staining.

Last and certainly not least, I would like to thank my friends and family who have provided emotional support, encouragement, and shoulders to cry on.

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LIST OF ABBREVIATIONS

CVD – cardiovascular disease

DM – diabetes mellitus

hCAVSMC – human coronary artery smooth muscle cell

LV – left ventricle

ZDF-F – Zucker diabetic fatty female

ZDF-M – Zucker diabetic fatty male

ZL-F – Zucker lean female

ZL – M – Zucker lean male

ABSTRACT

Cardiovascular disease (CVD) is the leading cause of death for American adults, and diabetes mellitus (DM) is an independent risk factor for CVD. Diabetic women, both pre- and postmenopausal, have a greater risk for CVD compared to age-matched diabetic men. However, the factors that dictate the increased risk of heart disease in diabetic females remains unclear. We have observed that in young, male and female Zucker diabetic fatty (ZDF-M and ZDF-F) rats display hyperglycemia, mild diastolic dysfunction and increased body fat at 5-months of age. However, only ZDF-F showed regions of gross cardiac damage. In an effort to understand why diabetic females have a greater risk for CVD, we examined differences in cardiac gene expression of two families of microRNAs (miR-29 and miR-208) and genes they target by qRT-PCR. We hypothesized that sex differences exist in the expression of both miRNA families which may contribute to not only disease progression, but increased risk of myocardial damage in diabetic females. Notably, we observed a sex bias in healthy rats: ZL-F had greater *Agtr2*, *Med13* and miR-208 expression, while ZL-M had increased cardiac *Gata4*, *Gdf11*, *Nppb* and miR-29b. In both ZDF-F and ZDF-M, the miR-29 family of miRNAs were upregulated whereas only ZDF-F showed a suppression in *Agtr2*. We additionally show that *in vitro*, transfection of primary human coronary artery smooth muscle cells (hCAVSMCs) with miR-29b increases the number of TUNEL- and PI-positive cells, but a 4 day exposure to 25mM glucose failed to induce an upregulation of miR-29. This suggests that short-term hyperglycemia is not enough to upregulate miR-29 expression in hCAVSMCs. We conclude that sex differences in *Agtr2* and miR-29b expression may underlie higher risk for CVD in young ZDF-F.

INTRODUCTION

For nearly a century, cardiovascular disease (CVD) has been the leading cause of death for American adults (Jones, 2012). Due to its prevalence, heart disease remains a problem, in spite of continuing advances in healthcare. While CVD risk factors such as smoking, obesity and lack of physical activity equally apply to both men and women, there are some risk factors that uniquely affect women, including polycystic ovary syndrome and menopause (Tan, 2010). Incidents of CVD generally occur about 10 years later in women than in men (NHLBI, 2006), and previous studies have alluded to estrogens accounting for this cardioprotective effect (Barrett-Connor, 1991, Mendelsohn, 1994, Farhat, 1996). Importantly, diabetes mellitus (DM) has been shown to be an independent risk factor for CVD in both men and women in population and epidemiological studies such as the Framingham study (Kannel, 1979), the Multiple Risk Factor Intervention Trial (MRFIT) (Stamler, 1993), the Strong Heart Study (Howard, 1996) and INTERHEART (Yusuf, 2004). A recent meta-analysis utilizing data from 64 cohorts found that diabetic women have a 44% greater risk for coronary heart disease compared to diabetic men, even after adjusting for age (Peters, 2014). Accordingly, DM negates the protective effect, if any, provided by estrogens. Thus, important sex differences regarding diabetes-associated risk of CVD have been established.

Sex differences in heart disease risk, symptoms and outcomes

Understanding gender-based differences in healthy and diabetic individuals provides insight to disease susceptibility and progression. It has been established that sex differences exist regarding the clinical presentation of CVD. Common symptoms for

acute coronary syndrome include chest pain, shortness of breath and sweating; women, however, are less likely to report chest pain (Canto, 2007), and more likely to report atypical symptoms such as indigestion, palpitations, fatigue and numbness in the hands (DeVon, 2008). For both genders, elevated low-density lipoprotein (LDL) can be a predictor of CVD (Howard, 2000), though low high-density lipoprotein (HDL) may pose a greater risk (Gordon, 1977, Jenkins, 1978). Additionally, compared to men, women have been found to have more multiple risk factors for CVD (Manson, 1990). In the population-based Worcester Heart Attack Study, women diagnosed with acute myocardial infarction had additional comorbidities such as systemic hypertension, diabetes mellitus and heart failure, compared to age-matched men (Milner, 2004), which may play a role in sex differences of clinical presentations. Furthermore, while improvements in technology greatly improve patient outcomes, diabetic women have increased incidences of CVD-related mortality (Garcia, 1974, Gu, 1999, Franco, 2007). Indeed, sex differences exist in the risk, clinical presentation and outcome of diabetic individuals with CVD. However, the mechanism(s) underlying sex differences in diabetes-related risk of CVD remain to be identified.

Animal models of diabetes

There are several animal models utilized in diabetes research. The $Lepr^{db/db}$ mouse contains an autosomal recessive mutation in the leptin receptor (OB-R) (Chen, 1996). These mice, commonly referred to as db/db mice, are usually inbred on a C57BLKS/J background and are resultantly and spontaneously hyperphagic, obese, hyperglycemic and insulin resistant. These characteristics are similar to what is observed in diabetic humans, making this a popular murine model for diabetes research. Studies examining

cardiac function by measuring hemodynamics (Belke, 2000, Li, 2010, Mori, 2014) or by echocardiography (Semeniuk, 2002) show that db/db hearts display diastolic dysfunction. Li *et. al* found that in using speckle tracking echocardiography, compared to 16-week old wild-type mice, db/db mice showed reductions in circumferential and radial strain (2014). While these studies provide valuable insight to how the diabetic milieu can lead to impairments in cardiac function, it is important to note that they utilize male mice only. One study included both male and female db/db mice and found impaired calcium handling at the cellular and organ level and systolic dysfunction (Pereira, 2006), but did not specifically delineate sex differences. However, cardiac function has been examined in female db/db mice (Aasum, 2003), though there are few studies aimed at addressing sex-specific differences (Shimoni, 2004, Bowden, 2015).

The Zucker diabetic fatty (ZDF) rat is a popular, inbred rodent model of type 2 DM (T2DM). It is characterized by a leptin receptor (*Lepr*) missense mutation (fa/fa) and pancreatic ß-cell dysfunction. Hyperphagia results in development of obesity, with concomitant hyperglycemia and hyperinsulinemia at a relatively young age (as reviewed in Srinivasan, 2007). Additionally, reduction in pancreatic ß-cell function, due to an unmatched need of ß-cell expansion from a high insulin demand and ß-cell death, leads to development of insulin resistance (Pick, 1998, Finegood, 2001). ZDF males (ZDF-M) readily develop diabetes around 6-10 weeks of age as a result of insulin resistance and consequential hyperglycemia (Christopher, 2010). Interestingly, ZDF females (ZDF-F), exhibit obesity and insulin resistance, but do not become diabetic (Christopher, 2010), even when maintained on a similar diet as ZDF-M (i.e. Purina 5008). However, when ZDF-F rats are fed high-fat diets, they can develop diabetes (Corsetti, 2000). Notably,

ZDF-M has been reported to be suitable for modeling human disease, namely CVD, based on molecular marker similarities by urinary proteome analysis (Siwy, 2012). Cardiac structure-function studies by the Pulakat group and others have characterized the cardiac pathology in male ZDF rats, including left ventricle (LV) chamber dilation in 36-week old rats without increases in LV mass (Baynes, 2009), and mild diastolic dysfunction (characterized by reduced $-dP/dt_{max}$, increased τ, isovolumetric relaxation time and LV end diastolic pressure) (van den Brom, 2010, Pulakat, 2011, Daniels, 2012). To date, there are no reports of cardiac structure-function analyses in hyperglycemic ZDF female (ZDF-F) rats, and importantly, no studies comparing cardiac function between ZDF-F and ZDF-M. Mice and rats serve as useful preclinical models for studying disease progression and pharmacological interventions, however, their limitations involve small size and distinct differences between rodent and human physiology, especially cardiovascular physiology. Nonetheless, in examining CVD, rats are advantageous because they are easy to handle, and their larger size allows surgical procedures to be more readily performed.

Sex differences in ZDF rats

The overarching hypothesis for this project was that there are key sex differences in young, age-matched, hyperglycemic ZDF-F and ZDF-M, and these differences may contribute to increased risk of CVD in ZDF-F. The Pulakat laboratory has characterized metabolic and cardiac phenotypic sex differences in ZDF rats (Lum-Naihe *et al.* 2016, manuscript in preparation). Briefly, we observed that the progression of diabetes, and the development of diabetes-associated cardiac pathology, is different in 5-month old male and female ZDF rats. ZDF-M exhibited “classic” diabetes progression, characterized by

an initial onset of hyperglycemia, followed by hyperinsulinemia and insulinopenia, whereas ZDF-F became hyperglycemic at a later time point but remained hyperinsulinemic. Both groups of ZDF rats were insulin resistant, as assessed by homeostatic model assessment (HOMA-IR). Interestingly, both ZDF-F and ZDF-M showed mild diastolic dysfunction and cardiac ultrastructural disorganization. However, only ZDF-F displayed gross structural damage to the heart. These findings are summarized in Fig. 1.

In order to understand which molecular players may be involved in the development of DM, we investigated whether sex differences exist in the cardiac expression of two families of microRNAs (miRNAs): miR-29 and miR-208. The Pulakat laboratory has previously found via microarray that both families of miRNAs are upregulated in cardiac tissues of male ZDF rats at 13-weeks of age (unpublished data).

The miR-29 family of miRNAs, and a putative target, Agtr2

Many classes of small, non-coding RNAs have been discovered, with various roles in cellular functions. In particular, miRNAs (or miRs), which are highly conserved, endogenous nucleic acids about 18-24 nucleotides in length, have been found to regulate gene expression (Carthew, 2009). miRNAs can interact with messenger RNA (mRNA) molecules by complementary binding, targeting it for degradation or translational repression (Gu, 2010). The miR-29 family is composed of three members, miR-29a, b, and c, who share an identical sequence and have common targets. However, there are some differences in their association with disease. For example, miR-29a has been implicated as a biomarker of hypertrophy and fibrosis in humans with hypertrophic

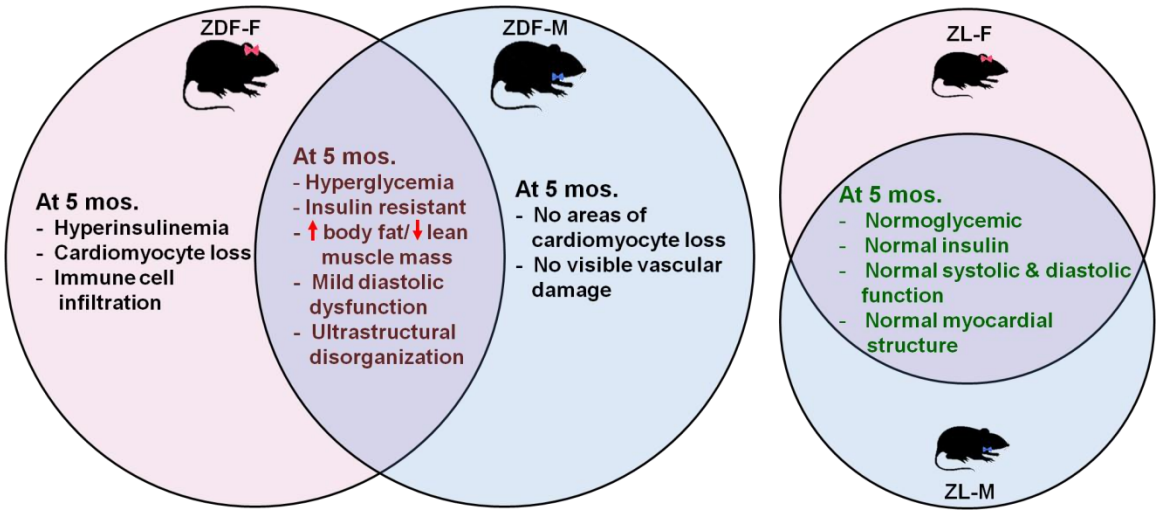


FIGURE 1. Diagram shows observed sex differences in young (5-month old) ZL and ZDF rats regarding metabolic phenotype and cardiac structure and function.

cardiomyopathy (Roncarati, 2014); miR-29b has been found to be involved in early development of aortic aneurysm in Marfan syndrome (Merk, 2012); and miR-29c was reported to be a key molecule, upregulated in hyperglycemic conditions *in vitro* and *in vivo* in db/db glomeruli (Long, 2011). Consistent with our unpublished microarray data, the Pulakat laboratory has previously reported that diabetes progression in young (15-week), male ZDF rats was associated with an increase in cardiac miR-29 expression, and that increased miR-29 expression corresponded with cardiomyocyte disarray (Arnold, 2014). miR-29, then, serves as a potential link between diabetes and CVD. Since diabetic females are more susceptible to CVD, we hypothesized that sex differences may exist in cardiac miR-29 expression, which may play a role in why diabetic females have a greater risk of heart disease.

The angiotensin II type 2 receptor (AT2R) is a member of vasodilative, cardioprotective branch of the renin-angiotensin system. Activation and/or upregulation of AT2R is reported to be involved in cardiac (Altarche-Xifró, 2009, Ludwig, 2012, Qi, 2012) and vascular (Okumura, 2005) repair, following cardiac injury. *Agtr2*, the gene coding for AT2R, is X-linked, and it has been previously reported to have increased expression in the female vasculature of rodents (Armando, 2002, Sampson, 2008). An intriguing observation about *Agtr2* is that *in silico* analysis reveals it to be a putative target by all miR-29 members. We postulated that sex differences may exist in the cardiac expression of *Agtr2* in ZDF-F and ZDF-M.

The miR-208 family of miRNAs and some of their predicted targets

Muscle-specific miRNAs, or myomiRs, have roles in muscle development. Their differential expression after resistance training or endurance exercise, and in skeletal muscle of diabetics implicate their role in healthy and diseased states (McCarthy, 2007, Nielsen, 2010, Drummond, 2011). Of note, the cardiac-specific miR-208 has roles in cardiomyocyte development. The miR-208 family includes miR-208a and miR-208b. miR-208a is encoded in an intron of the *Myh6* gene, which codes for the fast, alpha-myosin heavy chain (α-MHC), while miR-208b is encoded in an intron of the *Myh7* gene, coding for the slow, ß-MHC (van Rooij, 2007, van Rooij, 2009). Callis *et al.* found that cardiac-specific overexpression of miR-208a induced hypertrophy, while deletion of miR-208a disrupted cardiac conduction (2009). Recent reports suggest miR-208 may be a potential biomarker of drug-induced cardiotoxicity in rats (Nishimura, 2015) and cardiac injury in humans (Corsten, 2010, Bostjancic, 2010). Thus, the miR-208 family of miRNAs plays an important role in normal cardiac physiology. It is unknown whether sex differences exist in miR-208 expression. We hypothesized that cardiac miR-208 expression is different in male and female ZDF rats.

A known target of miR-208 is mediator complex 13 (MED13). Grueter *et al.* reported that in C57BL6 mice on a high-fat diet, cardiac-specific overexpression of MED13 or inhibition of miR-208a improves systemic insulin sensitivity and glucose tolerance (2012). It is unknown whether sex differences occur in the expression of cardiac *Med13*, or if *Med13* plays a role in cardiac pathology associated with hyperglycemia. To test this, we investigated the cardiac expression of *Med13* in male and female Zucker lean (ZL) and ZDF rats.

In response to metabolic stress, the adult heart can revert back to the so-called fetal gene program as an adaptive mechanism to improve cell survival (Rajabi, 2007). Arguably, diabetics exhibit a metabolically stressed environment. Key occurrences with the return of this program include a change from the preferential oxidation of fatty acids to the utilization of carbohydrates for energy metabolism; upregulation of atrial and brain natriuretic peptides (ANP and BNP, respectively); and a switch in isoform of contractile proteins (myosin heavy chains) and other enzymes involved in metabolism (Taegtmeyer, 2010). In regards to the myosin heavy chains, α- MHC and ß-MHC, it has been observed that their relative expression can change in a number of pathological conditions, such as heart failure (Nakao, 1997, Reiser, 2001). In addition, an upregulation of ß-MHC, at both the transcript and protein levels, has been reported to occur in response to hypertrophic stimuli (Izumo, 1987, Waspe, 1990), implicating ß-MHC as a marker of hypertrophy. ß-MHC is characterized by a slow adenosine triphosphatase (ATPase) activity and Krenz & Robbins reported cardiac overexpression of ß-MHC induces detrimental effects following coronary ligation or an isoproterenol challenge (2004). Previous publications showed that sex differences in the mRNA expression of the myosin heavy chains in rats (Rosenkranz-Weiss, 1994) and humans (Reiser, 2014) exist. Importantly, in male rats, streptozotocin-induced diabetes was accompanied by MHC isoform switch (Schaffer, 1989, Depre, 2000). Interestingly, two members of the fetal gene program, *Myh6* and *Myh7*, are predicted targets of miR-208a. Therefore, we sought to investigate whether sex differences exist in the expression of the fetal program genes, *Nppb, Myh6* and *Myh7*, which encode for BNP, α-MHC and ß-MHC, respectively.

The transcription factor GATA-4 is another protein whose expression is known to be regulated by miR-208 (Callis, 2009). It has been shown to have an important role in cardiac development, especially in the embryo (Kuo, 1997, Molkentin, 1997). GATA-4 is a zinc finger member of the GATA family of transcription factors and its role in regulating genes involved in cardiac differentiation and growth is further evidenced by temporal and spatial expression in endocardial and myocardial tissues in murine embryos (Heikinheimo, 1994). Interestingly, genes regulated by GATA-4 included α-MHC (Molkentin, 1994), ß-MHC, ANP (Charron, 1999) and BNP (Thuerauf, 1994). Missense mutations in *Gata4* that affect transcriptional activity have been identified in humans with congenital heart defects (Garg, 2003, Tomita-Mitchell, 2007). Oka *et al.* examined GATA4's role in adult mouse hearts and found that cardiac-specific deletion of *Gata4* reduced the hypertrophic response following transverse aortic constriction (2006). Thus, GATA4 is critical during fetal development and additionally plays a role in the postnatal myocardium. It is unknown whether sex differences exist in the expression of *Gata4* in the setting of diabetes; we therefore determined cardiac *Gata*4 expression in male and female ZL and ZDF rats.

Growth differentiation factor 11 (GDF-11) is a member of the transforming growth factor ß (TGF-ß) superfamily of proteins. It is a circulating protein, related to myostatin, and recently reported to be implicated as a negative regulatory of cardiac hypertrophy in the hearts of aged mice (Loffredo, 2013). GDF11 is also referred to as bone morphogenetic protein 11 (BMP-11) and is involved in a number of biological processes during development in a number of organs (Hogan, 1996). A recent short publication showed that in humans without diabetes, plasma GDF-11 decreases with age,

whereas plasma GDF-11 was increased in those with type 2 diabetes and CVD (Fadini, 2015). Furthermore, *Gdf11* is a predicted target of miR-208a. We examined whether there are sex differences in the cardiac expression of *Gdf11* in ZL and ZDF rats.

Previous studies (Barrett-Connor, 1991, Mendelsohn, 1994, Farhat, 1996) have alluded to estrogens accounting for the so-called "female advantage," based on the observation that healthy women have fewer incidences of cardiovascular events than age-matched men. Others have demonstrated that 17-ß-estradiol can induce endothelium-dependent vasodilation in ovariectomized rabbit femoral arteries (Gisclard, 1988), rat aorta (Wassman, 2001) and rat coronary arteries (Santos, 2004). In a 2003 study, ovariectomy in estrogen receptor alpha (ERα) knockout mice reduced acetylcholine-dependent relaxation in coronary arteries, though supplementation with 17-ß-estradiol restored the vasodilative response (Muller-Delp). Moreover, estrogen, signaling through ERα, has been shown to modulate vasodilation by both upregulation and increased activation of endothelial nitric oxide synthase (eNOS) (MacRitchie, 1997, Chambliss, 2002). These studies, coupled with the fact that ERα and ERß are expressed in endothelial cells, vascular smooth muscle cells (Karas, 1994, Hodges, 2000) and cardiac myocytes (Grohé, 1997), highlight estrogen's role in the cardiovascular system. We sought to investigate whether a sex difference in *Esr1*, the gene encoding for ERα, exists in healthy and diabetic male and female rats. Of note, *Esr1* is a hypothetical target of miR-208a.

MATERIALS & METHODS

Animal care

All animal procedures were approved prior to the start of these studies by the Harry S. Truman Memorial Veterans' Hospital (HSTMVH) Subcommittee for Animal Safety and the University of Missouri Institutional Animal Care & Use Committee. Six-week old male and female ZL and ZDF rats were purchased from Charles River Laboratories. Animals were individually housed in the HSTMVH animal housing facility and maintained at ~22˚C with reverse light/dark cycle (dark cycle from 0400 to 1600; light cycle from 1600 to 0400). Animals had *ad libitum* access to food and water, unless otherwise specified. All groups were fed Purina #5008 (16.97% calories from fat; Lab Diet, St. Louis, MO), except ZDF females, who were maintained on Research Diets #D12468 (47.7% calories from fat; Research Diets, Inc., New Brunswick, NJ). At 19-weeks (~5-months), animals were euthanized with isofluorane. The heart was excised and a coronal section through the ventricles was cut for histological analysis and fixed in 10% neutral buffered formalin. The remaining parts of the heart were snap frozen in liquid nitrogen.

Histology

Formalin-fixed, paraffin-embedded heart sections were sectioned at 5µm and were dewaxed in CitriSolv (Fisher Scientific), rehydrated in an ethanol series and HEPES wash buffer, followed by a heat-mediated antigen retrieval step in sodium citrate buffer. To block non-specific binding sites, sections were incubated with blocking buffer (5% donkey serum, 5% BSA) for 4h at room temperature, followed by incubation with *Helix*

pomatia agglutinin (HPA) conjugated to Alexa Fluor 647 (Life Technologies; 1:400, 2.5µg/mL) and *Griffonia simplicifolia* isolectin B4 (IB4) conjugated to Alexa Fluor 594 (Life Technologies; 1:200, 5µg/mL) for 4h at room temperature. The former was used to stain cardiomyocyte membranes, while the latter was used to identify endothelial cells. Sections were thoroughly washed and slides were mounted using Mowiol (Sigma). Imaging was performed using a Leica DMI4000B inverted confocal microscope at 40X and 63X. Cross-sectional area (μm^2) of cardiomyocytes were measured using ImageJ (NIH, Bethesda, MD). Only circuluar or nearly circular cardiac myocytes were selected for analysis. To assess capillary density, the ratio of capillaries to cardiomyocytes was calculated.

RNA isolation and real-time PCR

Both mRNA and miRNA were isolated from frozen heart tissues using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol. RNA isolates were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Complementary DNA (cDNA) synthesis for miRNA was performed using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems), and for mRNA, the Omniscript RT Kit (Qiagen) was used, following manufacturer instructions. Quantitative real-time PCR (qRT-PCR) reactions were performed in triplicate using either TaqMan Faster Universal PCR Master Mix (2X) (Applied Biosystems) or SYBRSelect Master Mix (2X) (Life Technologies), using the 7500 Fast Real-Time PCR System platform (Applied Biosystems). TaqMan MicroRNA Assays (Life Technologies) primers for miR-29a, b, c, miR-208a, b and U6 snRNA were used for miRNA targets; and Esr1, Gdf11, Med13 and 18S rRNA for mRNA targets. Primer sequences of 18S, forward:

CTGAGAAACGGCTACCACATC, reverse: TTGGATGGTTTAGTGAGGCC; Agtr2, forward: ATGAAGGACAACTTCAGTTTTGCTGCCACCAGC, reverse: TTAAGACACAAAGGTGTCCATTTCTCTAAGAG; Gata4, forward: ATGTACCAAAGCCTGGCTATGGCCGCCAAC, reverse: TTACGCGGTGATTATGTCCCCATGACTGTC; Myh6, forward: GACACCAGCGCCCACCTG, reverse: ATAGCAACAGCGAGGCTCTTTCTG; Myh7, forward: GGAGCTCACCTACCAGACAGA, reverse: CTCAGGGCTTCACAGGCATCC; and Nppb, forward: TTCCGGATCCAGGAGAGACTT, reverse: CCTAAAACAACCTCAGCCCGT. For all reactions, relative quantification (Rq) values were obtained by using the formula $2^{(-\Delta\Delta Ct)}$), by determining ΔCt values, followed by ΔΔCt.

Cell culture

Primary human coronary artery smooth muscle cells (hCAVSMC) of female donor origin were purchased from ATCC (Manassas, VA), and were cultured according to manufacturer instructions at 37˚C and 5% CO_2. These cells were certified and analyzed by ATCC, including positive smooth muscle α-actin staining. Cells were used from passages 7 to 10 and maintained in Medium 231 (Life Technologies) supplemented with Smooth Muscle Growth Supplement (SMGS, Life Technologies) and 10µg/mL gentamicin and 0.25µg/mL amphotericin B, unless otherwise indicated. To assess cell death, two experiments were performed: propidium iodide (PI) exclusion and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. For PI exclusion, primary hCAVSMCs were seeded ($3.0x10^4$) per well in a 24-well plate, allowed to grow and attach for ~36h, and then transfected with AllStars Negative Control siRNA (100nM;

Qiagen, Valencia, CA) or miR-29 mimics (100nM; mirVana miRNA mimics, Life Technologies) using siPORT Amine (Applied Biosystems) according to manufacturer instructions. After 24h, cells were incubated with PI (400nM; Life Technologies) and Hoechst 33342 (5μM; Life Technologies) for 30min at 37˚C, followed by live-cell imaging at 10X. For TUNEL assay, hCAVSMCs were seeded ($3.0x10^4$) on glass coverslips, and transfection with miRNAs was performed as described as above. TUNEL assay was performed using Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection (Life Technologies) according to manufacturer's protocol, and samples were mounted using Fluoroshield with DAPI (Sigma). PI- and TUNEL-positive cells were observed using a Leica DMI 4000B inverted confocal microscope using Leica Application Suite software. Experiments were performed at least with four replicates. For hyperglycemic conditions, hCAVSMCs were seeded in T150 flasks ($2.5x10^4/cm^2$) and allowed to grow until approximately 80% confluency. Cells were then switched to low serum conditions [Medium 231 supplemented with Smooth Muscle Differentiation Supplement (Life Technololologies)] and either 5mM or 25mM glucose (Sigma) for four days with daily medium changes. Note that basal Medium 231 contains 4.6mM glucose. We maintained cells in low serum medium to inhibit proliferation; previous preliminary experiments have verified that such conditions stop hCAVSMC growth and induce morphological changes, consistent with the manufacturer's instructions. Cells were washed with ice-cold phosphate buffered saline (PBS, Life Technologies), collected using a cell scraper, centrifuged for 5 min at 3,000rpm and snap frozen in liquid nitrogen. RNA was isolated as described above.

Atomic force microscopy

To investigate whether hyperglycemic conditions alter cell stiffness, hCAVSMCs were cultured under normo- and hyperglycemic conditions, identical to that stated above, and cell stiffness was assessed by atomic force microscopy (AFM). Cells were seeded in 60mm dishes ($5.0x10^4$), allowed to reach ~50-60% confluency and then switched to either 5mM or 25mM glucose in low serum medium. Evaluation of cell stiffness and calculation of Young's elastic modulus (E-modulus), using a Hertz model, were performed as described previously (Hong, 2015). Three replicates were performed for either glucose condition and cell stiffness was measured on 10 cells per replicate. An average of 30 force curves were collected per cell. AFM is a useful technique which allows for examination of cell surface measurements with high resolution (i.e. nanometer). Briefly, a probe/tip attached to a flexible cantilever is mounted to a piezoelectric scanner for fine control in the x, y and z directions. A sample is placed below the piezo and as the tip approaches the sample surface and begins to make contact, the cantilever spring bends. A laser diode, positioned on the back of the tip, deflects the laser beam upon cantilever bending, which is detected by a sensitive photodetector. On the basis of cantilever deflection, one can procure measurements such as topography, adhesion forces and/or cell stiffness. A representative force curve is shown in Fig. 2.

Statistics

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA). For multiple comparisons, one- or two-way ANOVA, followed by uncorrected Fisher's LSD post-hoc test, was performed as appropriate, and unpaired

two-tailed *t*-test was performed for pairwise comparisons. A *p*-value <0.05 was deemed significant.

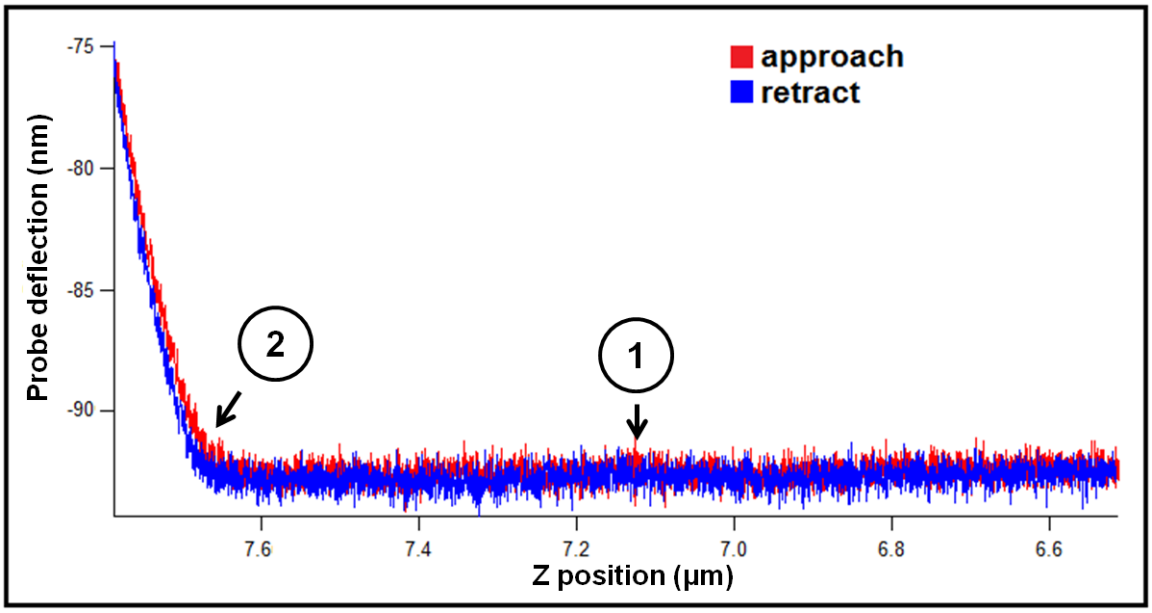


FIGURE 2. An example of a force curve measurement from atomic force microscopy is shown. At point (1), cantilever deflection is unchanged as the tip of the probe is not in contact with the sample surface. At point (2), contact is made by the tip, due to repulsive forces (namely van der Waals interactions), leading to cantilever deflection.

RESULTS

Cardiac hypertrophy was observed only in diabetic female rats, however, both diabetic females and males exhibit reduced capillary density

At 5-months, ZDF-F exhibited a significant increase in heart size (normalized to tibia length) relative to ZL-F, indicative of cardiac hypertrophy (Fig. 3A). In contrast, ZDF-M did not show any significant disease-dependent changes in heart size when compared to ZL-M (Fig. 3A). To evaluate cardiomyocyte hypertrophy, heart sections were stained with HPA conjugated to Alexa Fluor 647 and costained with IB4 to determine capillary density. ZDF-F exhibited the highest cardiomyocyte cross-sectional area among the four groups (Fig. 3B and 3D), consistent with our finding of hypertrophy at the organ level. Both ZDF-F and ZDF-M displayed a significant reduction in cardiac capillary density (Fig. 3C and D).

Cardiac miR-29 family of miRNAs expression is upregulated in diabetic rats

Cardiac expression of all miR-29 family members increased in both ZDF-F and ZDF-M when compared to ZL-F and ZL-M (Fig. 4A-C). In pairwise comparisons, miR-29b expression was the highest in ZDF-F (compared to ZL-F), whereas miR-29c expression was greatest in ZDF-M (compared to ZL-M). In comparing cardiac miR-29 expression in healthy animals, miR-29a and c were not significantly different between ZL-F and ZL-M, while miR-29b expression was ~3-fold higher in ZL-M ($p>0.05$), as compared to ZL-F (Fig. 4A-C).

Agtr2 *expression is suppressed in diabetic female, but not male, hearts*

Fig. 5 shows cardiac *Agtr2* expression, determined by qRT-PCR, was higher in ZL-F rats compared to ZL-M rats. Cardiac *Agtr2* was ~2-fold higher in ZL-F, consistent with previous reports of increased expression of AT2R in the vasculature of female rodents (Armando, 2002, Sampson, 2008). However, in the setting of diabetes, there was about 60% suppression of *Agtr2* in ZDF-F compared to ZL-F (diabetes main effect). There was no significant difference in cardiac *Agtr2* expression between ZL-M and ZDF-M.

miR-208a expression is greater in ZL-F than ZL-M, and is increased in ZDF hearts

Expression of the miR-208 family of miRNAs is shown in Fig. 6. Both miR-208a (Fig. 6A) and miR-208b (Fig. 6B) were significantly decreased (~90% each) in ZL-M than in ZL-F hearts. Cardiac miR-208a was significantly increased in ZDF-F and was ~3-fold higher in ZDF-M (Fig. 6A) as compared to ZL-F and ZL-M, respectively. Interestingly, miR-208b, the second member of the miR-208 family, had a different expression pattern than that of miR-208a: in ZDF-F, cardiac miR-208b was largely unchanged, compared to ZL-F, whereas miR-208b was significantly upregulated in ZDF-M hearts, compared to ZL-M (Fig. 6B).

Cardiac Med13 *expression is decreased in ZDF rats*

Cardiac MED13 plays an important role in maintaining insulin sensitivity. Since we observed differences in serum insulin levels between both ZDF groups (unpublished data, Pulakat laboratory), we examined whether this would affect *Med13* expression. As shown in Fig. 7, cardiac expression of *Med13* was 50% lower in ZL-M compared to ZL-F, indicating a sex bias in *Med13* expression (significant sex main effect). In both ZDF-F

and ZDF-M, *Med13* was suppressed compared to their respective lean counterparts (diabetes main effect).

Genes involved in the fetal gene program are largely unchanged in ZDF rat hearts

Cardiac expression of *Myh6*, encoding for α-MHC, is shown in Fig. 8A. *Myh6* was not significantly difference between lean males and females, between lean and diabetic females, nor between lean and diabetic males. In comparing ZL-F and ZL-M, *Myh7* expression, encoding for ß-MHC, was similarly not significant (Fig. 8B). In ZDF-F, *Myh7* trended to increase but was not statistically significant. In contrast, cardiac *Myh7* was significantly increased in ZDF-M compared to ZL-M. In ZL-M hearts, *Nppb*, encoding for BNP, was significantly upregulated (~6.8-fold) compared to ZL-F (Fig. 8C). In diabetic animals, there were opposing trends: there was a slight increase in *Nppb* expression in ZDF-F (~2-fold, $p>0.05$) and a decrease in *Nppb* expression in ZDF-M (40% suppression, $p>0.05$).

Expression of the transcription factor Gata4 *exhibits sex bias in healthy rats*

Cardiac expression of *Gata4* is significantly upregulated in ZL-M hearts as compared to ZL-F hearts (Fig. 9). ZDF-F hearts exhibit a slight, but insignificant increase in *Gata4* expression, while in ZDF-M hearts it was suppressed ($p<0.05$).

Cardiac Gdf11 *expression is upregulated in ZL-M, but is not significantly different in diabetic rats*

Expression of cardiac *Gdf11* in ZL and ZDF rats at 5-months of age is shown in Fig. 10. *Gdf11* expression was significantly increased (~3.5-fold) in hearts of ZL-M

compared to ZL-F. In the diabetic groups, there was no significant difference in *Gdf11* expression between ZDF-F and ZL-F or ZDF-M and ZL-M hearts.

Esr1 *expression is similar in male and female ZL and ZDF rat hearts*

Cardiac *Esr1* expression was not statistically different for any of the groups at 5-months of age (Fig. 11).

miR-29b induces cell death in human coronary artery vascular smooth muscle cells

We observed some interesting characteristics that were only present in ZDF-F hearts, and that included (1) miR-29b expression was greatest in ZDF-F (Fig. 4B) and (2) only this group of animals displayed areas of gross structural damage (summarized in Fig. 1). We therefore hypothesized that miR-29b may exert detrimental effects to the heart. In order to investigate the effects of miR-29 *in vitro*, we individually transfected primary cultures of human coronary artery smooth muscle cells (hCAVSMCs) using miR-29 mimics (100nM), or AllStars Negative Control siRNA (100nM). We examined membrane integrity by using propidium iodide (PI) exclusion and fluorescent microscopy, and cell death by TUNEL assay 24 hours post-transfection. As shown in Fig. 12A, miR-29b resulted in the highest number of TUNEL-positive cells and PI-stained cells (Fig. 12B). Compared to AllStars, transfection with miR-29a or c did not significantly change the number of TUNEL- or PI-positive cells.

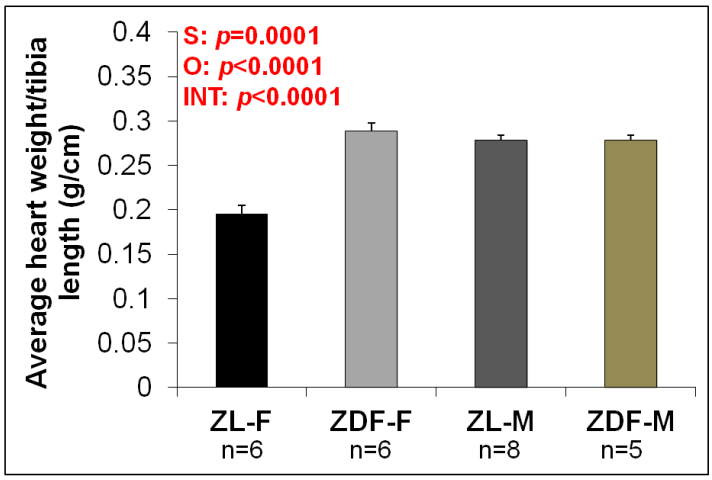
Hyperglycemic conditions and miR-29 expression in vitro

The miR-29 families of miRNAs are negative regulators of many genes, including extracellular matrix components (reviewed by Kriegel, 2012). While miR-29c has been previously reported to be a marker of hyperglycemia (Long, 2011), it is unknown

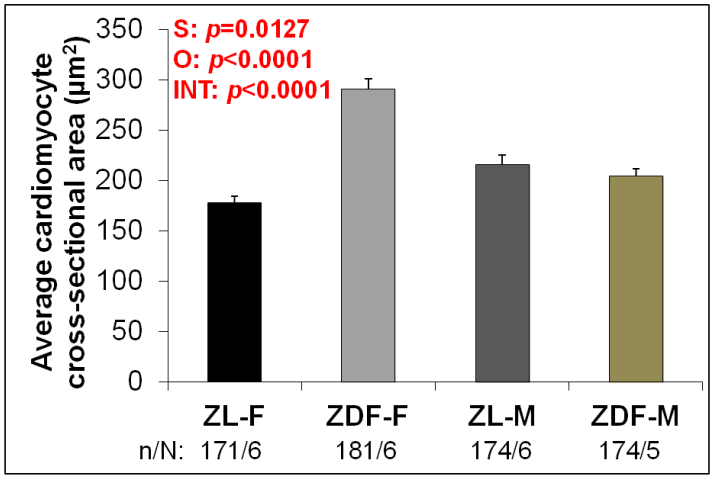
whether hyperglycemic conditions similarly upregulate miR-29a and b. To gain insight to the mechanism by which cardiac miR-29 expression increases, hCAVSMCs were treated under physiological normo- (5mM) and hyperglycemic (25mM) conditions for 96h. Expression of miR-29 was evaluated by qRT-PCR and data is shown in Fig. 13A. At this time point and under these conditions, there was no significant change in miR-29 expression.

Previous studies have shown that hyperglycemia is associated with an increase in arterial wall stiffness in humans (Rubin, 2012) and an increase in stiffness in primary neonatal rat cardiac myocytes (Michaelson, 2014). We hypothesized that hyperglycemia would similarly induce an increase in hCAVSMC stiffness. Under normoglycemic (5mM) or hyperglycemic (25mM) conditions, cell stiffness, assessed by AFM, was not significantly different between either group (Fig. 13B).

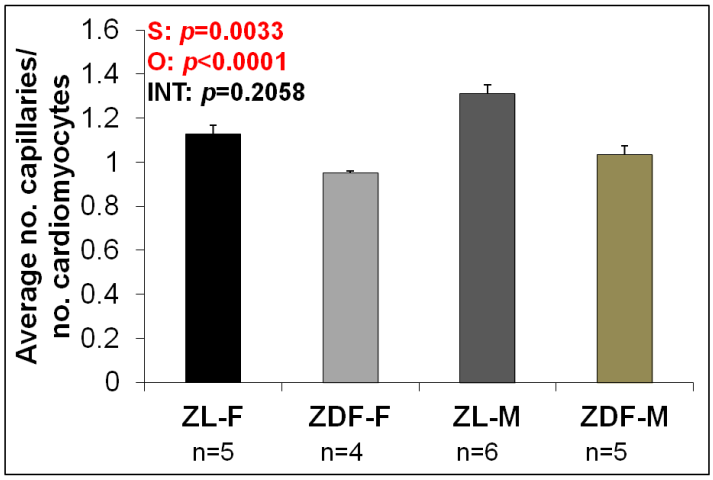
A


Average heart weight/tibia length (g/cm)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
S: p=0.0001
O: p<0.0001
INT: p<0.0001
ZL-F
n=6
ZDF-F
n=6
ZL-M
n=8
ZDF-M
n=5

B


Average cardiomyocyte cross-sectional area (µm²)
350
300
250
200
150
100
50
0
S: p=0.0127
O: p<0.0001
INT: p<0.0001
ZL-F
n/N: 171/6
ZDF-F
181/6
ZL-M
174/6
ZDF-M
174/5

C


Average no. capillaries/ no. cardiomyocytes
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
S: p=0.0033
O: p<0.0001
INT: p=0.2058
ZL-F
n=5
ZDF-F
n=4
ZL-M
n=6
ZDF-M
n=5

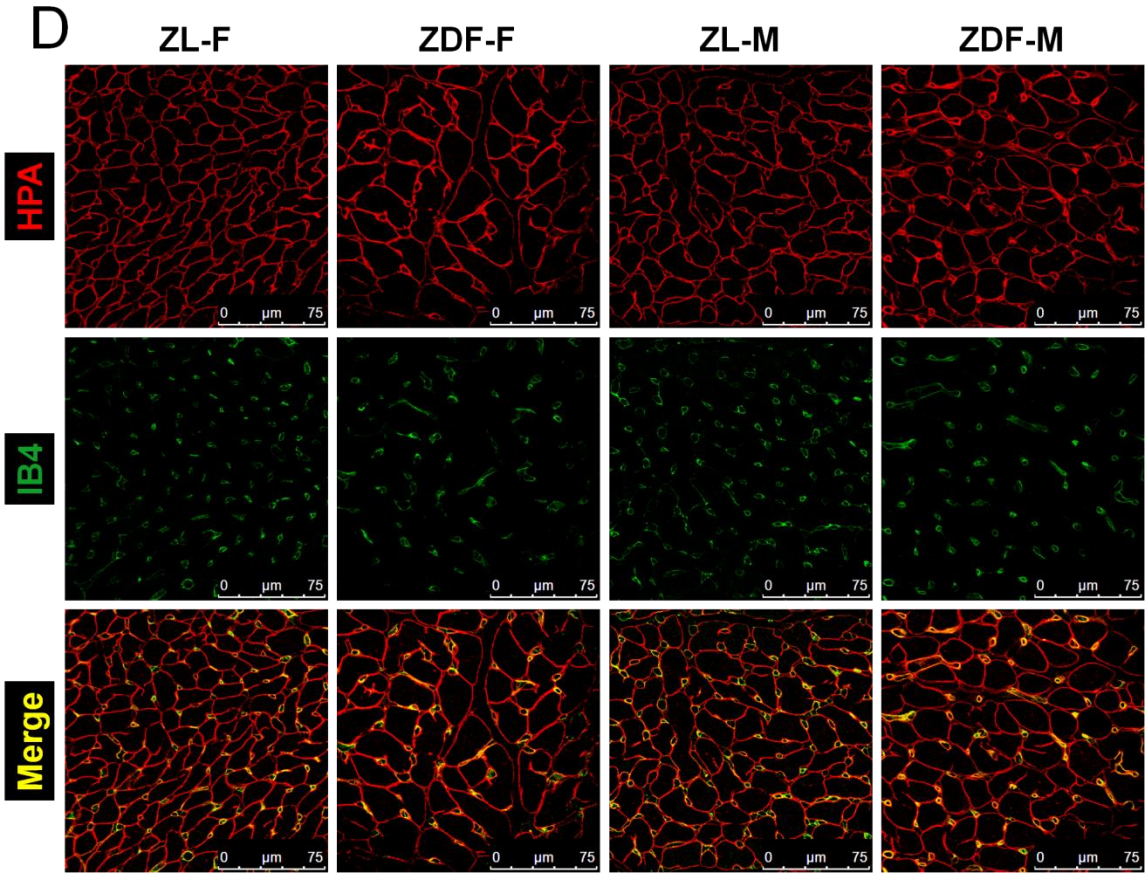
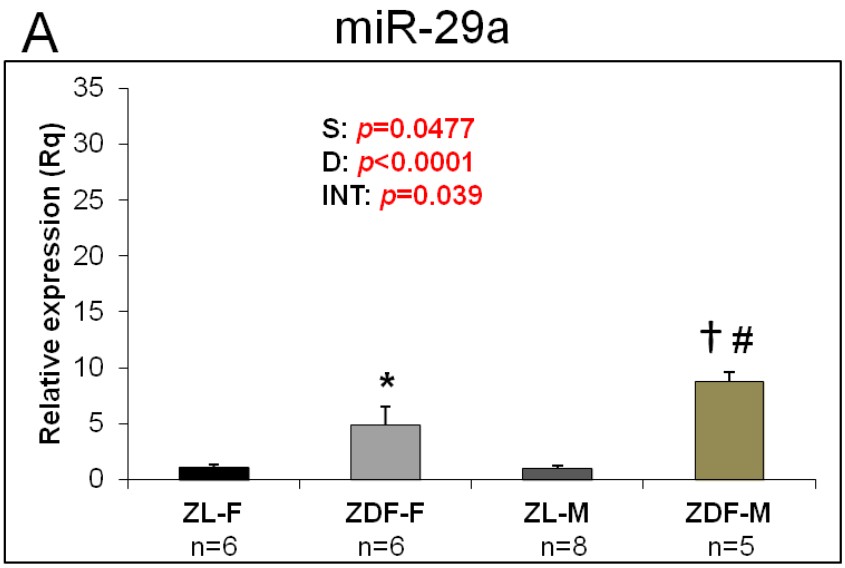
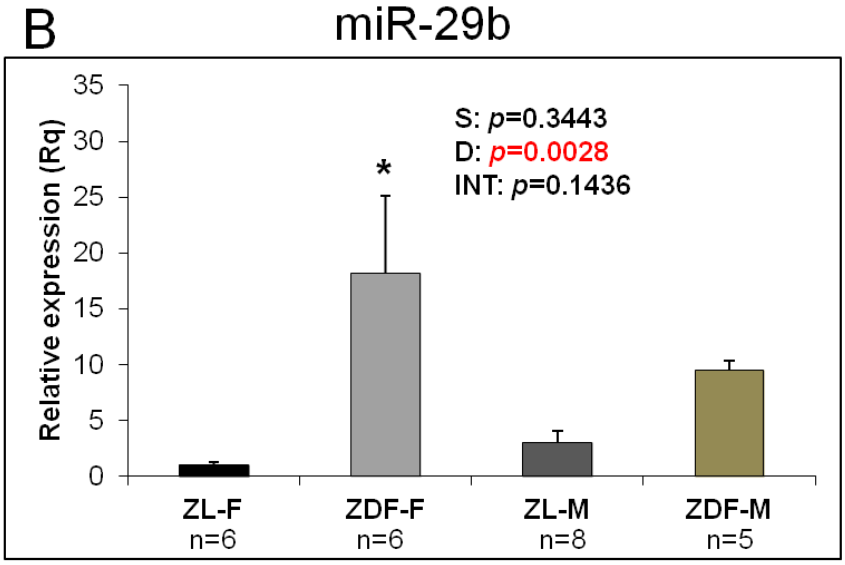


FIGURE 3. (A) Graph shows heart weight adjusted to tibia length. (B) Quantification of cardiomyocyte cross-sectional area in (D), where n = number of cardiomyocyte cross-sections and N = number of animals in each group. (C) Quantification of capillary density in (D). (D) Representative images are of heart sections stained with HPA conjugated to Alexa Fluor 647 (scale bars = 78µm) to visualize cardiomyocyte membrane and IB4 conjugated to Alexa Fluor 594 to visualized capillaries. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction.


A
miR-29a
Relative expression (Rq)
35
30
25
20
15
10
5
0
S: p=0.0477
D: p<0.0001
INT: p=0.039
*
† #
ZL-F
n=6
ZDF-F
n=6
ZL-M
n=8
ZDF-M
n=5


B
miR-29b
Relative expression (Rq)
35
30
25
20
15
10
5
0
S: p=0.3443
D: p=0.0028
INT: p=0.1436
*
ZL-F
n=6
ZDF-F
n=6
ZL-M
n=8
ZDF-M
n=5

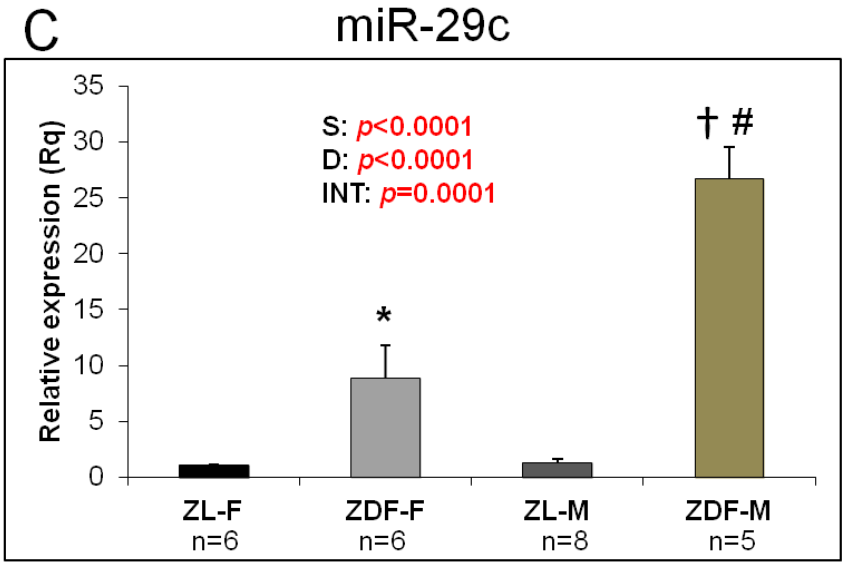

C
miR-29c
Relative expression (Rq)
35
30
25
20
15
10
5
0
S: p<0.0001
D: p<0.0001
INT: p=0.0001
*
† #
ZL-F
n=6
ZDF-F
n=6
ZL-M
n=8
ZDF-M
n=5

FIGURE 4. Graphs showing qRT-PCR data on cardiac expression of the miR-29 family of miRNAs in 5-month old lean and diabetic male and female rats. (A-C) Expression of miR-29 miRNAs were increased in both ZDF-F and ZDF-M, however, their expression pattern was different. Both miR-29a and miR-29c were highest in ZDF-M. miR-29b levels were higher in ZDF-F compared to ZDF-M. Data was normalized to U6 snRNA and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. **p*<0.05 vs. ZL-F; †*p*<0.05 vs. ZL-M; #*p*<0.05 vs. ZDF-F.

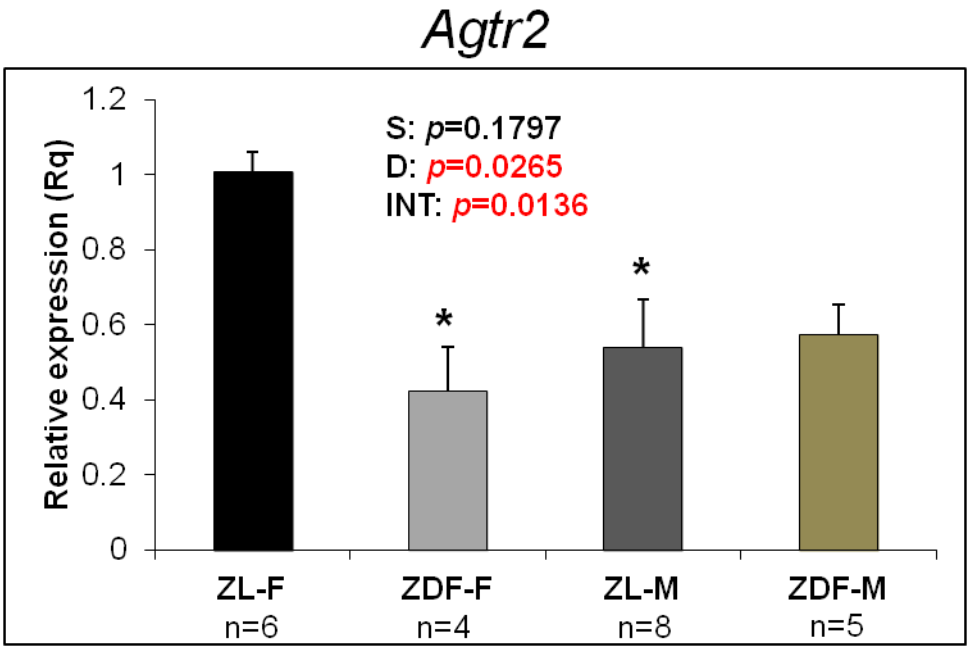


FIGURE 5. Graph shows cardiac *Agtr2* mRNA expression in lean and diabetic male and female rats at 5-months of age. Compared to ZL-M, *Agtr2* expression was higher in healthy ZL-F rats (~2-fold). There was a nearly 60% suppression in ZDF-F compared to ZL-F, however, there was no significant difference in *Agtr2* expression in ZL-M and ZDF-M rats. Data was normalized to 18S and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. **p*<0.05 vs. ZL-F; †*p*<0.05 vs. ZL-M; #*p*<0.05 vs. ZDF-F.

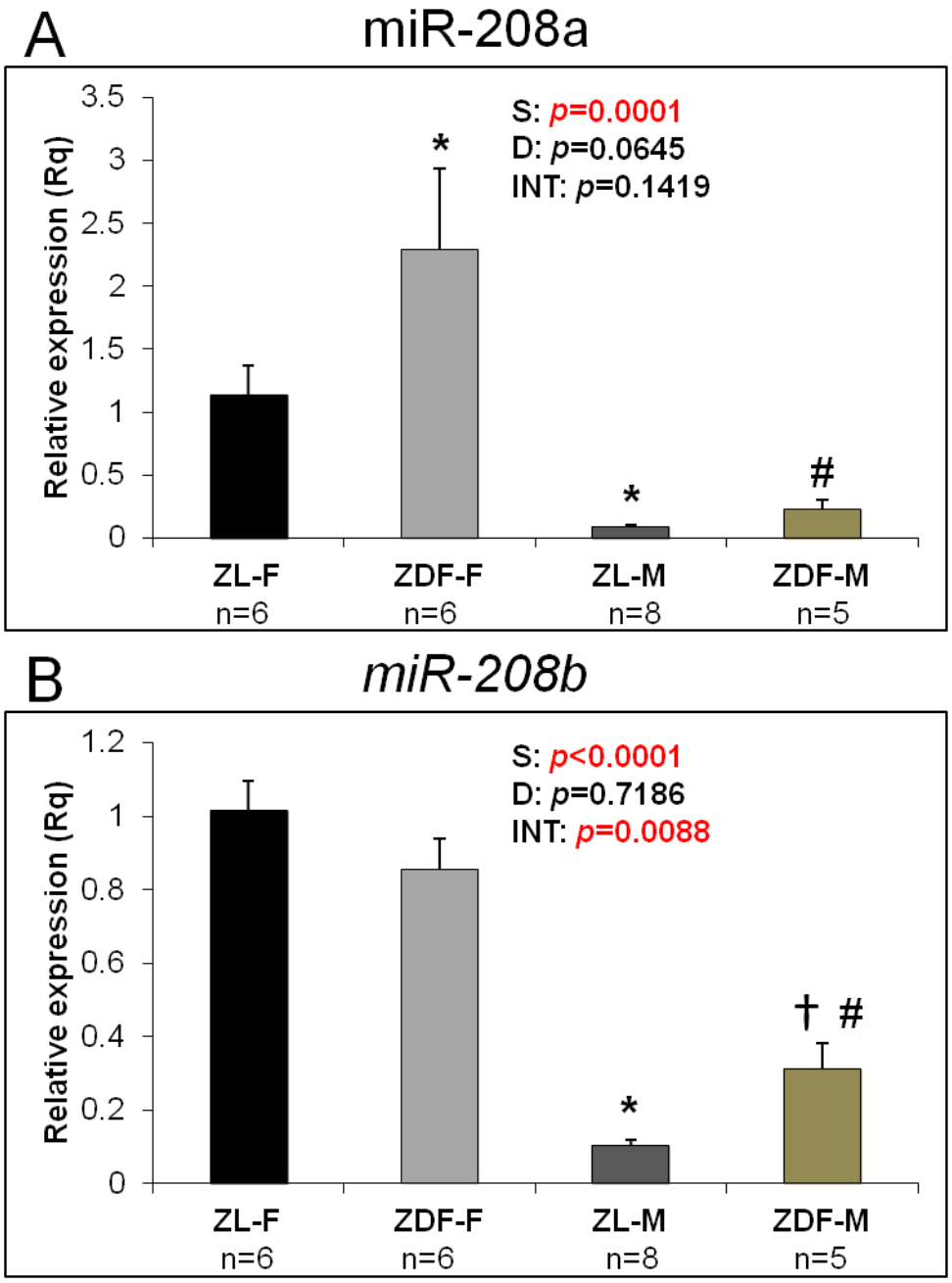


FIGURE 6. Cardiac expression of the miR-208 family of miRNAs in ZL and ZDF rats at 5-months. (A and B) The miR-208 family, composed of miR-208a and miR-208b, was significantly higher in ZL-F hearts compared to ZL-M. miR-208a expression trended to increase, while miR-208b expression was largely unchanged, in ZDF-F. In ZDF-M, cardiac miR-208a and b were significantly upregulated, compared to ZL-M. Data was normalized to U6 snRNA and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. *$p<0.05$ vs. ZL-F; †$p<0.05$ vs. ZL-M; #$p<0.05$ vs. ZDF-F.

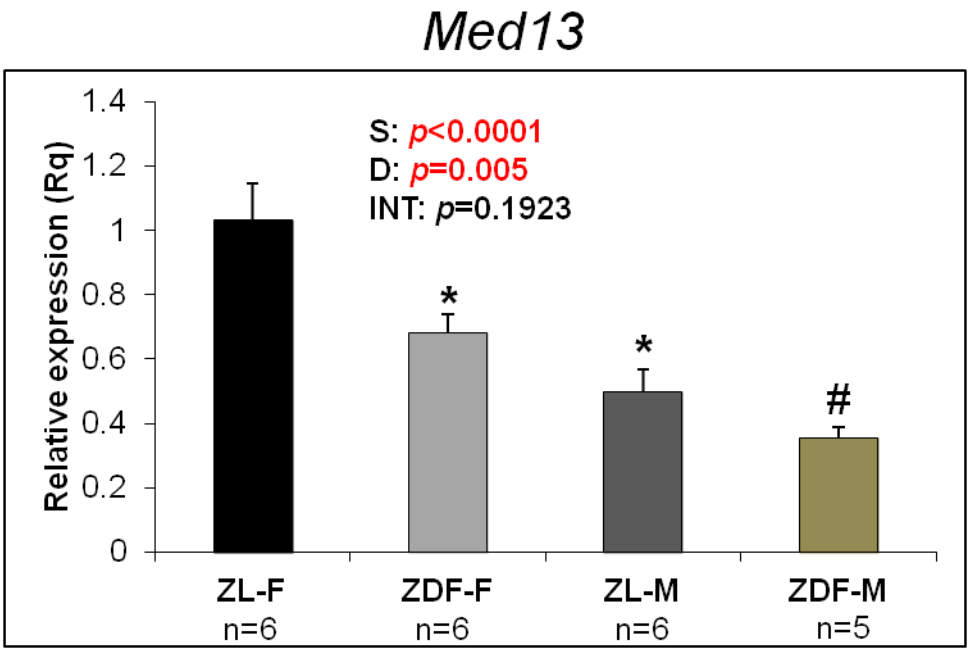


FIGURE 7. Cardiac expression of *Med13* in male and female ZL and ZDF rats at 5-months. ZL-M hearts had about 50% less *Med13* expression than that of ZL-F. *Med13* was slightly but significantly suppressed in ZDF-F hearts, while there was a trend towards *Med13* suppression in ZDF-M. Data was normalized to 18S and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. *$p<0.05$ vs. ZL-F; †$p<0.05$ vs. ZL-M; #$p<0.05$ vs. ZDF-F.

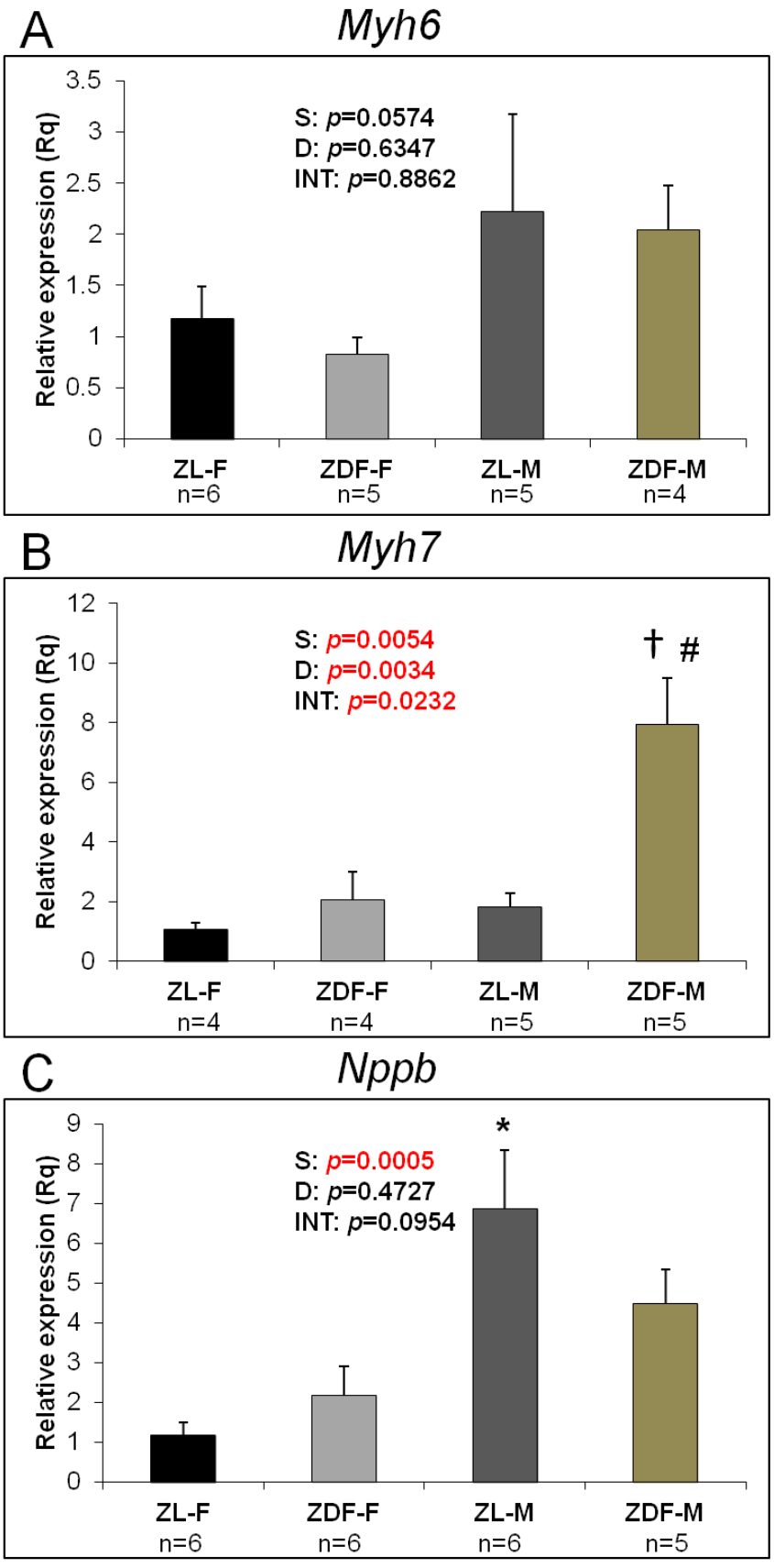

A
Myh6
Relative expression (Rq)
3.5
3
2.5
2
1.5
1
0.5
0
S: p=0.0574
D: p=0.6347
INT: p=0.8862
ZL-F
n=6
ZDF-F
n=5
ZL-M
n=5
ZDF-M
n=4
B
Myh7
Relative expression (Rq)
12
10
8
6
4
2
0
S: p=0.0054
D: p=0.0034
INT: p=0.0232
† #
ZL-F
n=4
ZDF-F
n=4
ZL-M
n=5
ZDF-M
n=5
C
Nppb
Relative expression (Rq)
9
8
7
6
5
4
3
2
1
0
S: p=0.0005
D: p=0.4727
INT: p=0.0954
*
ZL-F
n=6
ZDF-F
n=6
ZL-M
n=6
ZDF-M
n=5

FIGURE 8. Expression of genes part of the fetal gene program are mostly unchanged in male and female ZL and ZDF rats. (A) Cardiac *Myh6* expression, which codes for the α-MHC protein, is not significantly different in healthy, lean rats, or between lean and diabetic female or male rats. (B) Graph show qRT-PCR data for expression of cardiac *Myh7* mRNA, which codes for ß-MHC. Only ZDF-M exhibited an increase in *Myh7* expression, compared to ZL-M (F). (C) Cardiac *Nppb* expression, which codes for BNP, is significantly higher in ZL-M than in ZL-F (~7-fold). In comparing lean and diabetic groups by sex, there were no significant differences in *Nppb* expression. Data was normalized to 18S and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. **p*<0.05 vs. ZL-F; †*p*<0.05 vs. ZL-M; #*p*<0.05 vs. ZDF-F.

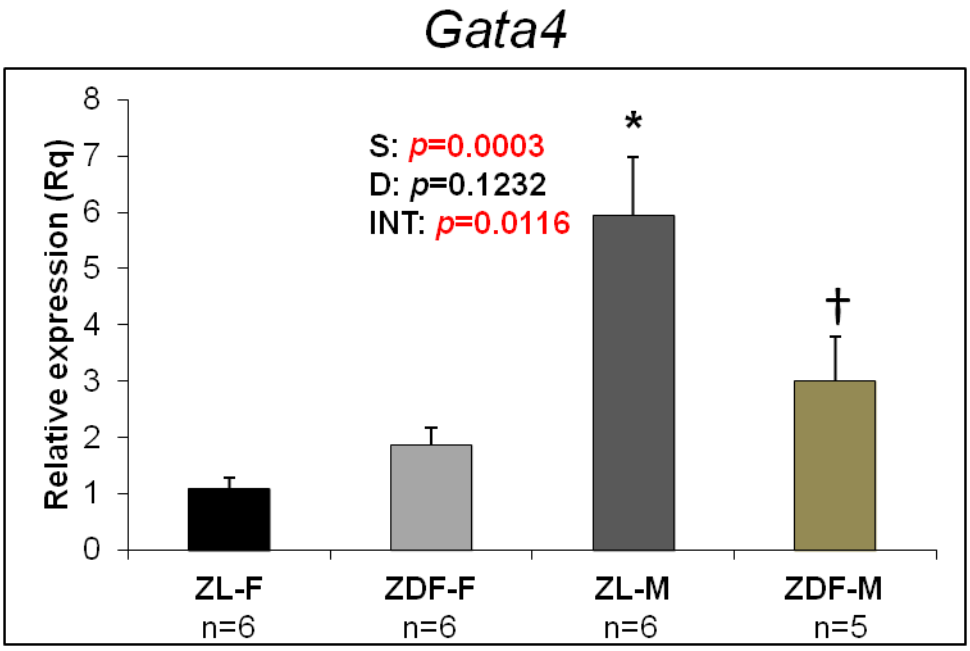


FIGURE 9. Expression of the transcription factor *Gata4* is markedly higher (~6-fold) in ZL-M hearts than in ZL-F. ZDF-F hearts have a slight but insignificant upregulation of *Gata4* compared to ZL-F, while *Gata4* expression in ZDF-M trended to decrease when compared to ZL-M. Data was normalized to 18S and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. **p*<0.05 vs. ZL-F; †*p*<0.05 vs. ZL-M; #*p*<0.05 vs. ZDF-F.

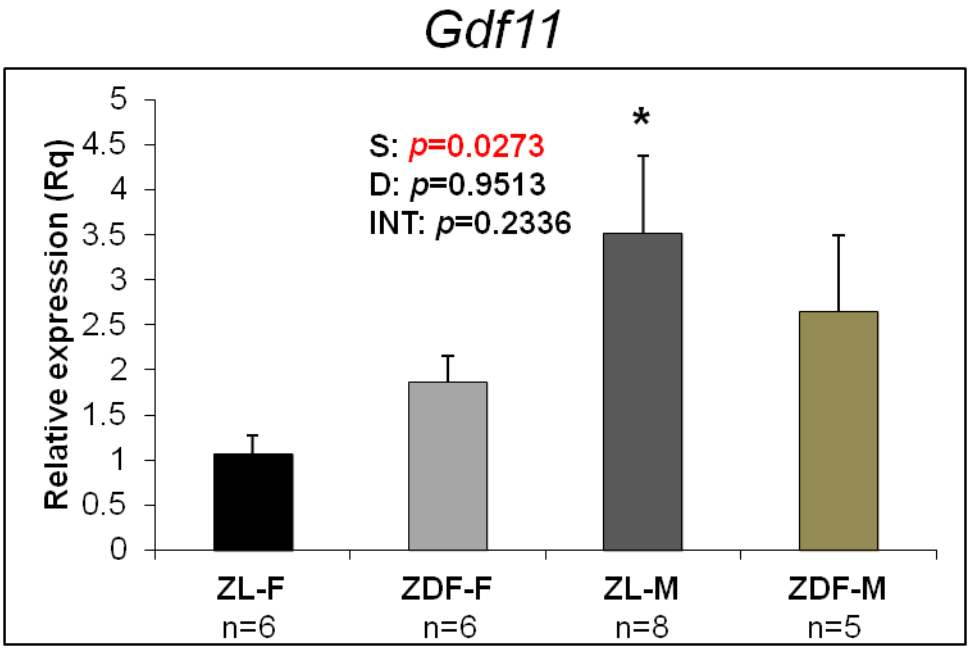


FIGURE 10. Cardiac expression of *Gdf11* is significantly higher (~3.5-fold) in ZL-M hearts than in ZL-F. ZDF-F hearts have a slight upregulation of *Gdf11* compared to ZL-F, while there was a slight decrease in *Gdf11* expression in ZDF-M when compared to ZL-M, but these were not significant. Data was normalized to 18S and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. *$p<0.05$ vs. ZL-F; †$p<0.05$ vs. ZL-M; #$p<0.05$ vs. ZDF-F.

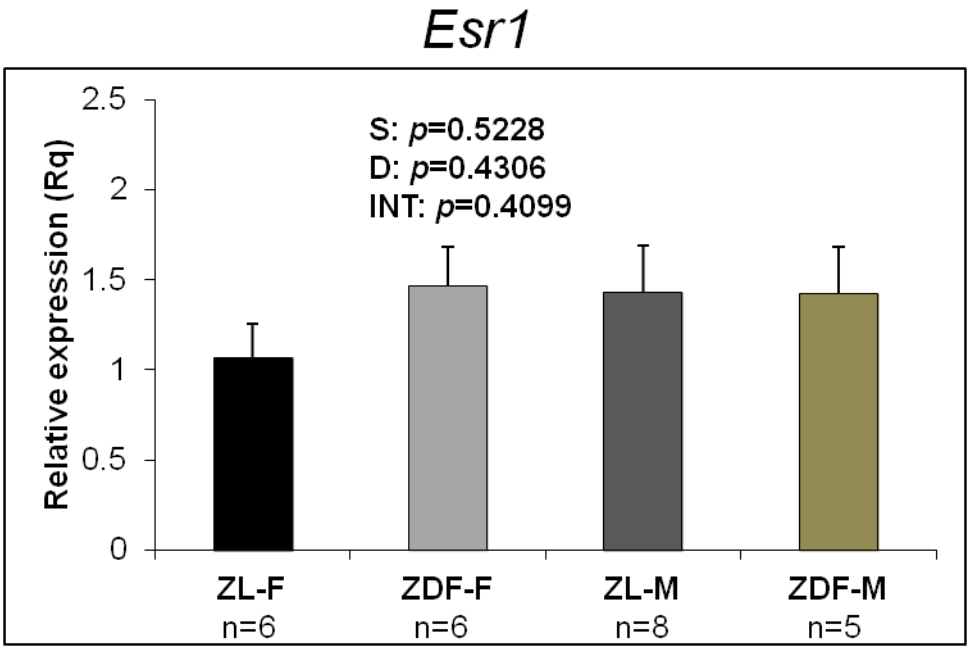


FIGURE 11. Expression of cardiac *Esr1* is not significantly different between male and female ZL and ZDF rats. Data was normalized to 18S and expression is relative to ZL-F. Values are means ± SEM. *p*-values are noted and were determined by two-way ANOVA. *S*, sex; *D*, diabetes; *INT*, interaction. **p*<0.05 vs. ZL-F; †*p*<0.05 vs. ZL-M; #*p*<0.05 vs. ZDF-F.

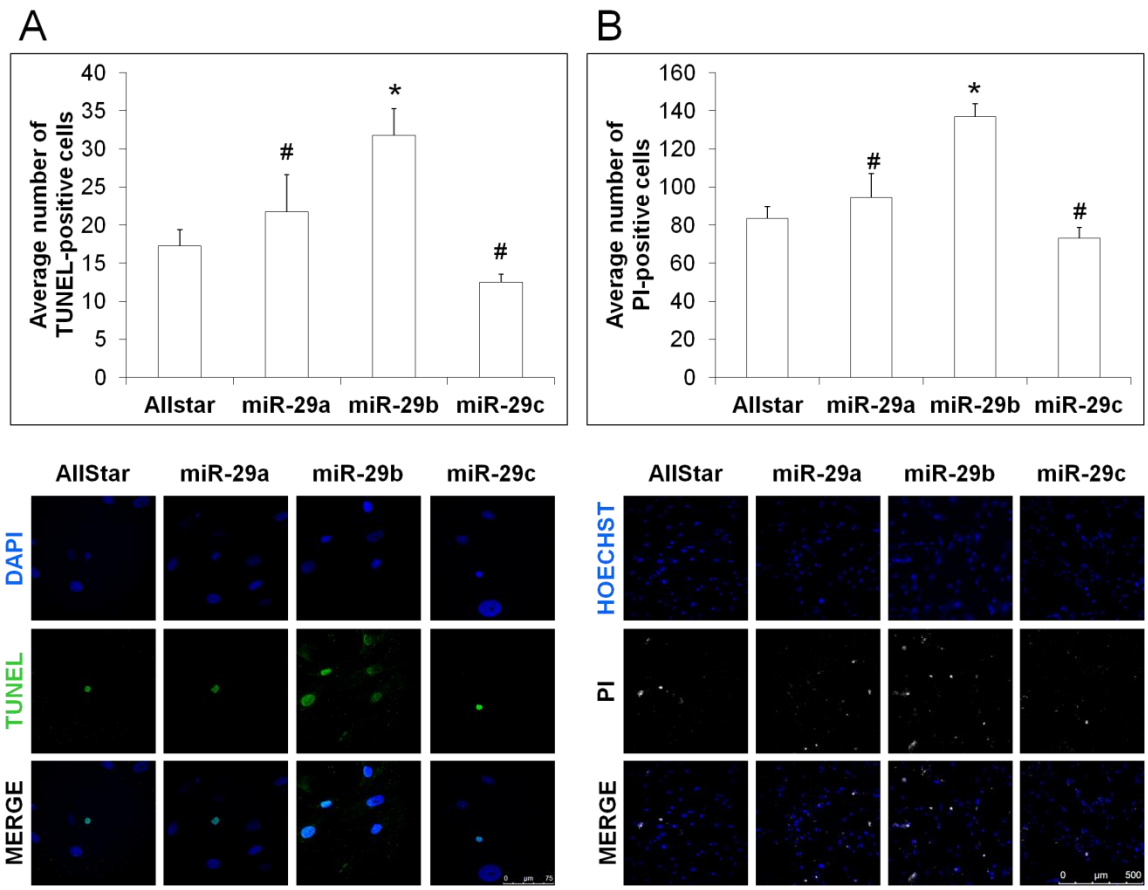


FIGURE 12. Transfection of primary human coronary artery vascular smooth muscle cells (hCAVSMCs) with AllStars Negative Control siRNA (100nM) or miR-29a, b and c mimics (100nM). Extent of cell death was examined 24 hours post-transfection. (A) Quantification of TUNEL-positive cells for indicated treatments and representative images below. Scale bars = 75µm. (B) Quantification of PI-positive cells for each corresponding treatment and representative images below. Scale bars = 500µm. Live cell imaging was assessed by fluorescent microscopy after cell staining with PI (400nM) and Hoechst 33342 (5µM). DAPI, 4’,6-diamidino-2-phenylindole; PI, propidium iodide. *p<0.05 vs. AllStar, #p<0.05 vs. miR-29b by one-way ANOVA.

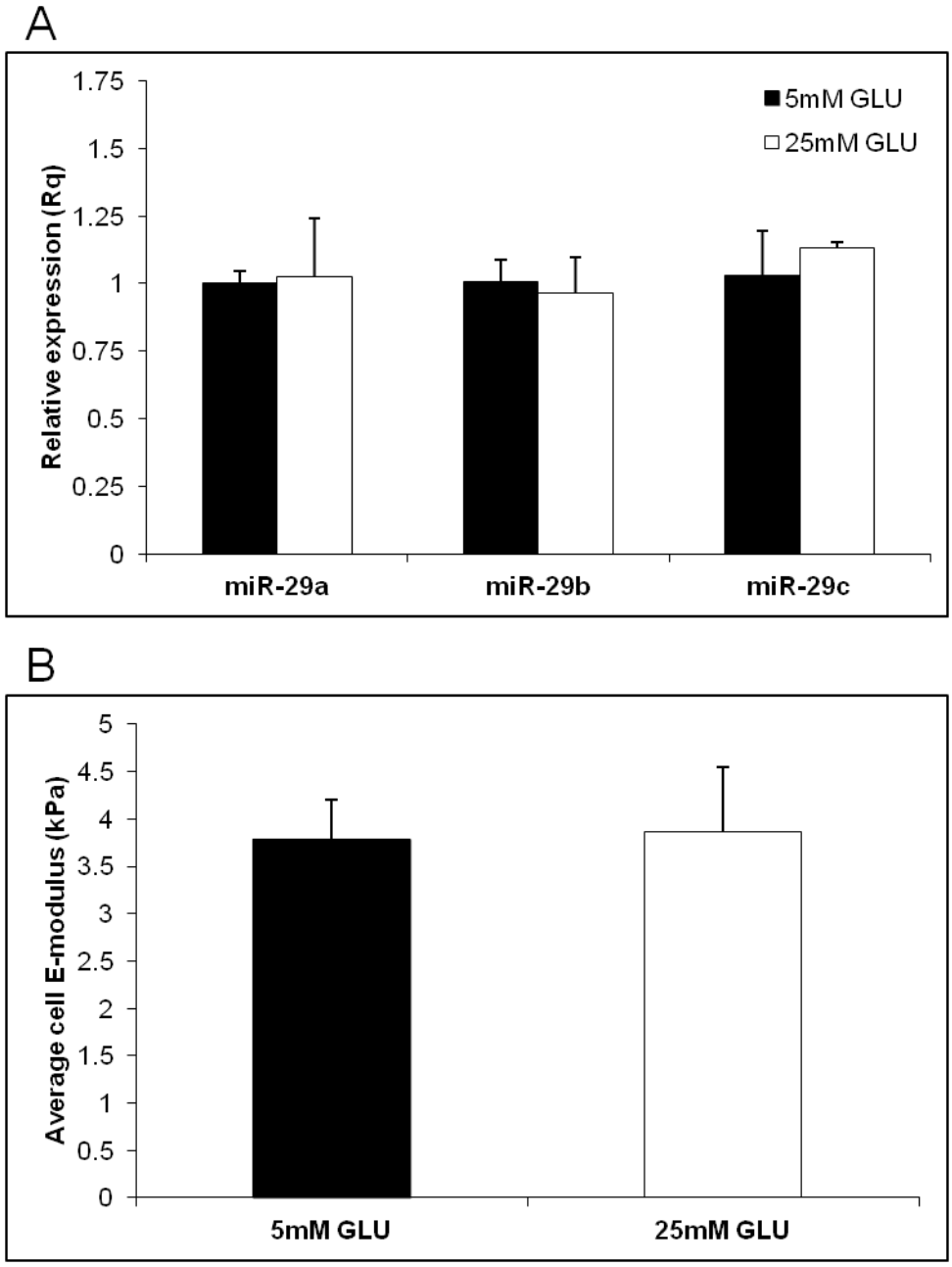


FIGURE 13. Treatment of primary hCAVSMCs in normo- (5mM) or hyperglycemic (25mM) conditions for 96h and assessment of (A) miR-29 family of miRNA expression, and (B) Young's elastic modulus. (A) Expression of the miR-29 family members was not different under the indicated treatments with glucose. (B) Average E-modulus for three groups of hCAVSMCs per condition shows no difference in either group, indicating no significant change in cell stiffness. Values are means ± SEM.

DISCUSSION

The results from this study highlight that there are sex-associated differences in young, diabetic male and female ZDF rats. First, only ZDF-F exhibited cardiac hypertrophy at both the organ and cardiomyocyte levels (Fig. 3), and gross structural damage to the heart (unpublished data, Pulakat laboratory). Such pathological changes were not observed in any of the ZDF-M rats. Second, there are sex differences in the cardiac expression of several genes (Figs. 4-11). Importantly, we observed a co-upregulation of both the miR-29 family of miRNAs and miR-208a in both ZDF groups. Because both miR-29 and miR-208 families target genes involved in normal cardiac function and repair, we propose that dysregulation of one or both families in ZDF rat hearts contributes to development of diabetes-associated cardiac pathology.

Cardiac hypertrophy occurs in response to changes in pressure, volume and workload of the heart. There are distinct stimuli that lead to the development of pathological or physiological cardiac hypertrophy (as reviewed in McMullen, 2007). Human based studies have demonstrated that diabetic individuals have an associated increase in LV mass (Galderisi, 1991, Eguchi, 2008). We observed cardiac hypertrophy, at both the organ and cellular level, only in hyperglycemic ZDF-F rats (Fig. 3), as compared to ZL-F. While heart mass (normalized to tibia length) was larger in ZDF-F, compared to ZL-F, they were similar in size compared to both groups of male rats. Additionally, body weights of ZL-M, ZDF-F and ZDF-M were comparable at the end of the study (unpublished data, Pulakat laboratory). This suggests that myocardial growth might simply be a reflection of the animal size (i.e. a biological scaling) and not necessarily pathological in nature.

The miR-29 family of miRNAs are involved in a number of cellular processes and alterations in miR-29 expression has been associated with various disease states (reviewed in Kriegel, 2012, Ślusarz, 2015). Importantly, expression of miR-29 members have been shown to be upregulated in skeletal muscle (He, 2007) and cardiac tissues (Arnold, 2014) of diabetic rats, and in liver of db/db mice (Pandey, 2011). In human studies, miR-29s have been detected in urine (Peng, 2013) and serum (Kong, 2011) of patients with type 2 diabetes. The Pulakat laboratory has previously published that cardiac miR-29 expression is increased in young, ZDF-M rats, and that such increases in miR-29 were associated with cardiomyocyte disarray (Arnold, 2014). These observations indicate that miR-29 may serve as a link between diabetes and CVD.

Previous studies in the Pulakat laboratory have demonstrated that, *in vitro*, insulin suppresses miR-29a, b, and c (Arnold, 2014). We observed that ZDF females were severely hyperinsulinemic throughout the study (as summarized in Fig. 1), and that the high insulin levels did not lead to a suppression of miR-29 family expression at 5-months (Fig. 4). Interestingly, all miR-29 members were upregulated in response to diabetes in both sexes, though the expression pattern was different. In comparing ZL-F and ZL-M, cardiac miR-29a and miR-29c expression were similar in both groups, however, ZL-M displayed ~3-fold increase in miR-29b (Fig. 4). Cardiac miR-29b showed the most differential expression between ZDF-F and ZL-F, whereas miR-29c expression showed the greatest expression between ZDF-M and ZL-M. Elevation of miR-29a was observed in both diabetic groups, relative to their respective lean controls. van Rooij *et al.* have reported that miR-29a is a negative regulator of hypertrophy, in part by targeting genes involved in cardiac extracellular matrix formation (2008). It should be noted that

increases in miR-29b has an important role in early abdominal aortic aneurysm (Merk, 2012). It is conceivable that the increased expression of miR-29b in the ZDF-F heart can confer a detrimental effect to the cardiac vasculature, but this is speculative and warrants further investigation. miR-29c has been shown to be a miRNA upregulated in hyperglycemic db/db mice (Long, 2011). Consistent with this finding, we observed increased miR-29c expression in both ZDF-F and ZDF-M hearts, relative to ZL rats.

Our observation that cardiac *Agtr2* expression was about 2-fold higher in healthy, ZL-F rats than in ZL-M was not surprising, and is consistent with previous reports of upregulated AT2R expression in the female rodent vasculature (Armando, 2002, Sampson, 2008). Our data shows that diabetic female rats display a loss of this cardioprotective molecule while diabetic male rats do not. Recent reports highlight the importance of AT2R in mediating cardiac repair. For example, in rats, a population of $cKit^+$ $AT2R^+$ progenitor cells increases following acute myocardial infarction (Altarche-Xifró, 2009), and that AT2R activation exerts protective effects (Xu, 2013). These are two studies of an emerging body of evidence that illustrates the role of AT2R in response to a cardiac insult. Due to its cardioprotective effects, we posit that loss of cardiac *Agtr2* expression may be an important contributor to the increased risk of myocardial damage in ZDF-F rats. Further investigations aimed at examining AT2R activation in ZDF-F, in spite of suppressed *Agtr2* mRNA, are needed to confirm whether this attenuates structural damage to the myocardium. Of note, *Agtr2* is a putative target of all miR-29 family members. While we observed upregulation of the miR-29 family in both ZDF groups, we found *Agtr2* to be downregulated in ZDF-F only. This suggests that miR-29-mediated suppression of *Agtr2* may be sex specific.

The miR-208 family of miRNAs includes miR-208a and miR-208b (Callis, 2009), and are considered to be "myomiRs" since their expression is muscle-specific, but more so because they are located within myosin genes (van Rooij, 2009). Previous reports have examined the functional role of miR-208a, including its role in cardiac conduction and hypertrophic growth in mice (Callis, 2009), and cardiac remodeling and function in Dahl hypertensive rats (Montgomery, 2011). We found that both miR-208a and miR-208b were ~90% suppressed in ZL-M compared to ZL-F (Fig. 6) In ZDF-F, miR-208a expression only trended to increase, while miR-208b was not significantly different, compared to ZL-F. Interestingly, in ZDF-M, both miR-208a and miR-208b expression was significantly upregulated, compared to ZL-M.

Mediator complex subunit 13 (MED13) is also known as thyroid hormone receptor-associated protein 1 (THRAP1) because of its role as a transcriptional coactivator for many different genes regulated by thyroid hormone receptor and other nuclear receptors. Cardiac overexpression of *Med13* or inhibition of miR-208a, a negative regulator of MED13, was found to reduce diet-induced obesity and improve glucose tolerance and insulin sensitivity in mice (Grueter, 2012). We observed that there was ~50% less cardiac *Med13* expression in ZL-M than in ZL-F (Fig. 7). Although ZL-F had considerably lower body weights than ZL-M rats, their body compositions were largely similar (unpublished data, Pulakat laboratory). This suggests that male and female ZL rats have sex differences in cardiac MED13 contribution to overall energy metabolism. While some sex differences have been reported in regards to metabolism (reviewed in Wu, 2011), further studies are required to elucidate the functional role of upregulated *Med13* mRNA in healthy female hearts. In both groups of ZDF rats, *Med13*

was suppressed in comparison to ZL rats. This indicates that loss of cardiac *Med13* is a diabetes-associated phenomenon, independent of sex. Consistent with this observation, we found miR-208a to be increased ~2.3-fold in ZDF-F ($p<0.05$) and ~3-fold in ZDF-M ($p>0.05$), relative to respective lean controls. This indicates a functional miR-208a-Med13 axis in diabetic animals. It is tempting to speculate that under nondiabetic conditions, cardiac *Med13* is not entirely regulated by miR-208, especially in light of our observation that ZL-F hearts had greater *Med13* and miR-208 member expression than ZL-M, however further studies are required to evaluate this idea.

Regarding the role of cardiac MED13 improving insulin sensitivity, as reported previously by Grueter, *et al.* (2012), we found a negative correlation between *Med13* expression and HOMA-IR, an index of insulin resistance, in ZL rats as well as female rats at 5-months of age (Fig. 14). Our data shows that increases in *Med13* expression is strongly negatively correlated (r=-0.64, $p<0.05$ in ZL; r=-0.59, $p<0.05$ in females) with increases in HOMA-IR. Such a correlation was not observed in male rats nor in ZDF rats.

In response to pathophysiological conditions, e.g. diabetes, the adult heart is capable of activating pathways that lead to hypertrophy. This cardiac remodeling process parallels cardiac growth during fetal development (as reviewed in Rajabi, 2007, Dirkx, 2013). The reversion back to the fetal gene program includes expression of ANP, BNP, α-MHC and ß-MHC, which are coded by the respective genes *Nppa*, *Nppb*, *Myh6* and *My7*. In mice, expression of the contractile myosin proteins are temporally distinct: slow-acting ß-MHC is higher in embryonic hearts while fast-acting α-MHC is the major isoform in adults (Sassoon, 1988). In humans, ß-MHC expression is present in adulthood, and was found to account for the majority of MHC content in non-failing left ventricles

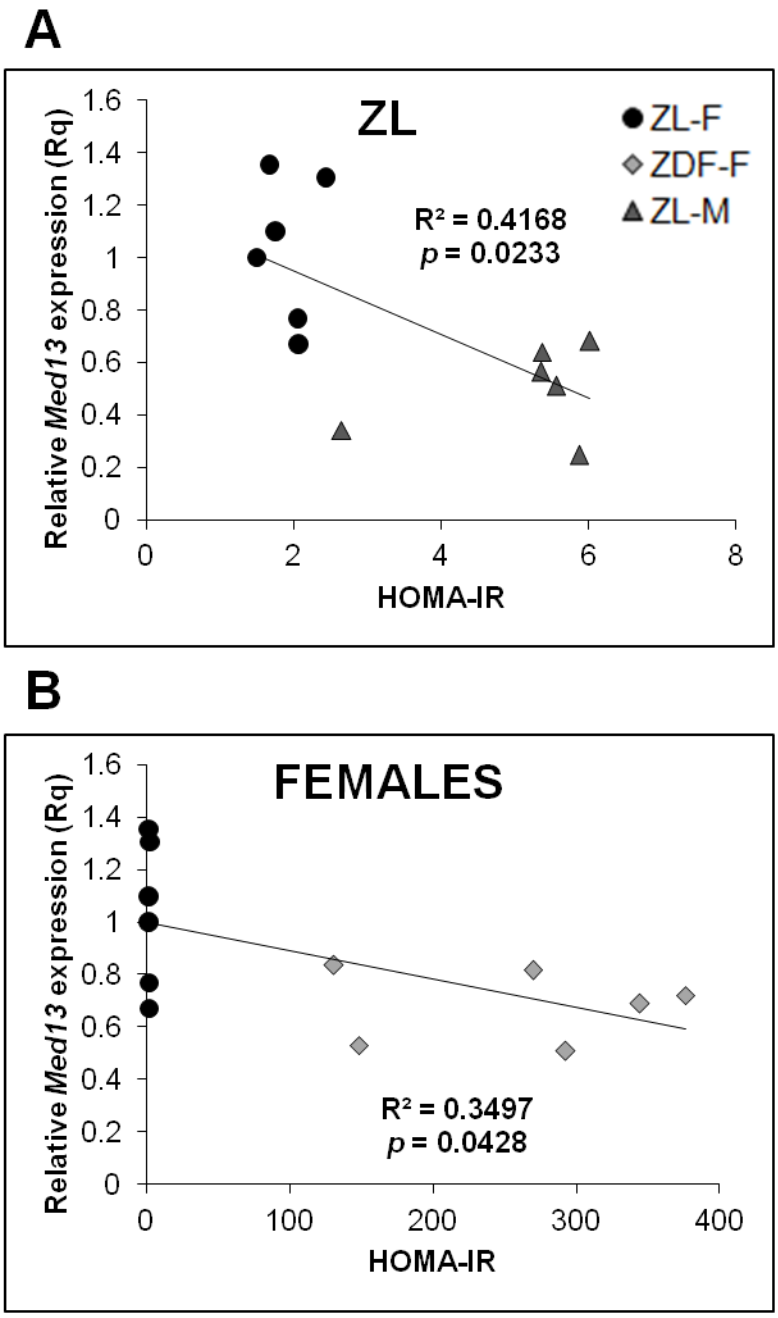


FIGURE 14. Graph depict a strong negative correlation between *Med13* expression and HOMA-IR in 5-month old rats. (A) ZL includes ZL-F and ZL-M. (B) Females includes ZL-F and ZDF-F. The squared Pearson's correlation coefficient and *p*-value indicated are for both groups of rats. Each dot represents measurements from one animal.

(Reiser, 2001). Marked upregulation of ß-MHC, at both the mRNA and protein levels, has been reported to occur in cardiac hypertrophy (Izumo, 1987, Waspe, 1990), congestive heart failure (Huang, 2001) and is considered to be an early marker of hypertrophy. In ZL rats, there was no significant difference between the sexes in the expression of either myosin gene (Fig. 8). In ZDF-F, there was a small but insignificant increase in *Myh7* expression, while in ZDF-M, *Myh7* was significantly upregulated, relative to respective ZL groups. While fetal gene program activation is reported to increase in conditions of pathological hypertrophy (as reviewed in McMullen, 2007), and because we only observed cardiac hypertrophy in ZDF-F, the lack of *Myh7* upregulation further supports the notion that cardiac remodeling in ZDF-F could simply be an adaptive mechanism. In support of this, it has been reported that in renin transgenic mice, a model of cardiac hypertrophy, reexpression of ß-MHC occurs in some myocytes but not all, and that hypertrophic cells are not always accompanied by increased ß-MHC expression (Pandya, 2006). Consistent with this report, we did not observe cardiac hypertrophy in ZDF-M (compared to ZL-M) in spite of upregulated *Myh7*.

The natriuretic peptides, ANP and BNP, are produced respectively by the atrial and ventricular myocytes, and secreted in response to various stimuli such as sympathetic activation and cardiac distension (London, 2006). They exert their actions through natriuretic peptide receptors to regulate blood volume, blood pressure and renal salt and water balance (Silberbach, 2001). Horio *et al*. found that in neonatal rat ventricular myocytes, ANP may act as a negative regulator of hypertrophy (2000). In canine cardiac fibroblasts, BNP was found to decrease production of collagen and increase metalloproteinase activity (Tsuruda, 2002), suggesting a role of BNP as a modulator of

cardiac growth. In addition, elevated plasma BNP has been reported in patients with hypertrophic cardiomyopathy (Hasegawa, 1993) and left ventricular hypertrophy (Kohno, 1995). Of course, plasma N-terminal proBNP (NT-proBNP) is used clinically as a marker of heart failure (Hammerer-Lercher, 2008). Here, we found that cardiac *Nppb* was significantly increased in ZL-M hearts (~7-fold) than in ZL-F. In ZDF-F, there was no insignificant changes in *Nppb* expression, while in ZDF-M, *Nppb* trended to decrease, relative to corresponding ZL groups. In examining cardiac proBNP expression by immunohistochemistry (unpublished data, Pulakat laboratory), both ZDF-F and ZDF-M had increased proBNP, compared to corresponding lean controls. It is conceivable to conclude that increased levels of proBNP are indicative of an adaptive mechanism that occurs in diabetes.

The miR-208 family of miRNAs are located within introns of myosin genes: miR-208a is encoded in intron 29 of *Myh6*, and miR-208b is encoded in intron 31 of *Myh7*; expression of each miR-208 member has been reported to be in conjunction with its myosin host gene (Callis, 2009). Surprisingly, our data is not in agreement with that report. In healthy rats, miR-208a and b were significantly higher in ZL-F than in ZL-M (Fig. 6), although there were no significant differences in *Myh6* or *Myh7* expression (Fig. 8). ZDF-F rats had a small increase in cardiac miR-208a but no significant difference in *Myh6* expression, compared to ZL-F. On the other hand, miR-208b expression was not different from ZL-F, yet there was a trend towards upregulated *Myh7*. In ZDF-M, both miR-208a and b were significantly increased, though only *Myh7* was upregulated. Because adult rodents primarily express the α-MHC isoform (Sassoon, 1988, Huang, 2001), it is likely that there were no observable differences at the transcript level.

Additionally, our observed differences in miR-208 and myosin gene expression may be attributed to transcript stability and/or post-transcriptional modifications. van Rooij *et al.* reported that miR-208a, by regulating expression of *Thrap1/Med13*, leads to an upregulation of ß-MHC (2007). It is possible that because ZDF-M exhibited upregulation of both miR-208 members and increased *Myh7*, and that miR-208a and b share a similar seed sequence, that a certain threshold of miR-208 expression is necessary for *Myh7* upregulation. However, further investigations are needed to evaluate whether miR-208b controls *Myh7* expression, perhaps in a feed forward mechanism.

GATA-4 is a transcription factor expressed in the heart that regulates genes involved in embryonic cardiac development (Kuo, 1997, Molkentin, 1997). Genes regulated by GATA-4 include *Myh6*, *Myh7*, *Nppa* and *Nppb* (Dirkx, 2013). In this study, cardiac *Gata4* was significantly higher (~6-fold) in ZL-M than in ZL-F (Fig. 7), suggesting a sex bias in its expression. In the diabetic groups, *Gata4* was slightly upregulated in ZDF-F, but significantly decreased in ZDF-M, compared to ZL-F and ZL-M, respectively. This data suggests there is a diabetes-associated difference in *Gata4* expression, which is dependent on sex. Furthermore, *Gata4* is a predicted target of the miR-208 family of miRNAs. Our observation of transcriptional suppression of *Gata4* in ZDF-M only could similarly be due to a combined upregulation of miR-208a and miR-208b, similarly observed only in ZDF-M, additionally suggesting that miR-208 regulation of *Gata4* is sex dependent.

The circulating protein GDF-11, also referred to as BMP-11, is a member of the TGF-ß superfamily of proteins that plays an important role in embryonic development (Gamer, 1999). It was recently reported that GDF-11 prevents cardiac hypertrophy in

aged mice (Loffredo, 2013). A recent publication from the same group illustrated that daily injections of GDF-11, over a period of 9 days, similarly led to reduced heart mass and cardiomyocyte size in both young (2-month) and old (22-months) mice (Poggioli, 2016). Smith *et al.* reported that treatment of 24-month old mice with recombinant GDF-11 failed to reduce cardiac hypertrophy (2015), which may be attributed to differences in experimental design (McNally, 2016). Interestingly, a recent publication demonstrated that plasma GDF-11 decreases with age in non-diabetic individuals, while patients with type 2 diabetes and macroangiopathy had significantly elevated plasma GDF-11 levels (Fadini, 2015). In our study, ZL-M rats had greater cardiac *Gdf11* expression than ZL-F (~3.5-fold) (Fig. 7). We observed a trend of increased *Gdf11* in ZDF-F, relative to ZL-F, and no significant difference between either male group. While *Gdf11* was slightly increased in ZDF-F rats, we still observed cardiac hypertrophy. This is inconsistent with the idea that GDF-11 mediates antihypertrophic effects in both young and old animals, and may reflect that *Gdf11* expression at the transcript level does not necessarily correlate with protein and/or circulating plasma levels. GDF-11 shares a high degree of homology with another TGF-ß member, myostatin, which is known to inhibit skeletal muscle growth (Breitbart, 2011). We observed that ZDF-F rats had the highest percent of body fat and lowest percent of lean mass among the four groups (as summarized in Fig. 1), and it is tempting to suggest that this was associated with the slight (but not significant) increase in *Gdf11*. This, however, may not be causal especially since ZDF-M rats, which similarly had more fat than ZL-M rats, did not display the same trend. Based on our results and the fact that *Gdf11* is a putative target of miR-208a, it is possible that miR-208 does not contribute to regulation of *Gdf11* in ZDF rats.

Based on our observation that cardiac miR-29b expression was highest in ZDF-F, that only ZDF-F hearts exhibited areas of structural damage (unpublished data, Pulakat laboratory), and miR-29b plays a role in abdominal aortic aneurysm (Merk, 2012), we were interested in examining the effects of miR-29 *in vitro*. We transfected primary cultures of hCAVSMCs with miR-29 mimics and assessed cell death 24 hours post-transfection. We hypothesized that miR-29b might affect survival of hCAVSMCs .We observed that miR-29b increased the number of TUNEL-positive cells, relative to the negative control, and increased membrane permeability, assessed by PI exclusion (Fig. 12). Transfection with miR-29a or miR-29c did not significantly change the number of TUNEL- or PI-positive cells, compared to the negative control. Here we only used two simple methods to assess cell death. Arguably, additional experiments should be performed to elucidate the cell death pathway, if any, mediated by increasing miR-29b expression. Merk, *et al.* previously published that miR-29b participates in early aneurysm formation in Marfan mice which was associated with increased apoptosis and decreased medial elastin deposition in the ascending aorta (2012). Unsurprisingly, the miR-29 family negatively regulates genes involved in forming the extracellular matrix (reviewed by Kriegel, 2012). Regarding our cell culture data, it is tempting to make an argument that the role of miR-29b in aneurysm formation, or more generally, blood vessel wall weakness, could conceivably occur in the vasculature of the heart, i.e. coronary arteries. This might provide an explanation to the structural damage we observed in ZDF-F (unpublished data, Pulakat laboratory). However, it is purely speculative and requires a thorough investigation in diabetic animals. For example, pharmacological inhibition of miR-29b with an anti-miR in ZDF-F rats may attenuate such damage.

In order to determine whether hyperglycemia can upregulate miR-29 family miRNA expression, we subjected hCAVSMCs to normoglycemic (5mM) or hyperglycemic (25mM) conditions for 96h and observed no significant difference in miR-29 member expression between the two groups (Fig. 13). As reported previously, miR-29c is a signature miRNA upregulated in renal microvascular endothelial cells and podocytes stimulated under hyperglycemic conditions (Long, 2011). While glucose concentrations between our study and that reported in Long *et al*. are identical, our observation of no signification change in miR-29c expression may largely reflect differences in cell type. Additionally, the cells used by Long *et al.* were immortalized and of murine origin, while our study utilized a primary human culture. We further hypothesized that long-term hyperglycemia (96h in culture) would alter cell stiffness in hCAVSMCs. Vascular smooth muscle cell stiffness, determined as Young's modulus, is a key mechanical property that plays a role in overall vascular stiffness (Sehgel, 2013). Importantly, diabetic conditions (namely hyperglycemia or hyperlipidemia) have been shown to increase stiffness of neonatal cardiac myocytes (Michaelson, 2014). Contrary to our expectations, high glucose failed to affect cell stiffness as compared to cells treated in normoglycemic conditions (Fig. 13).Our data suggests that in hCAVSMCs, hyperglycemia does not elicit an upregulation of miR-29 family of miRNA expression, nor did it contribute to alterations in cell stiffness. It is conceivable to think that some other component in the diabetic milieu, e.g. inflammatory cytokines, which have been found to be associated with development of type 2 diabetes (Spranger, 2003), regulates miR-29 expression. It should be noted that it has been observed that cultured VSMCs isolated from diabetic rats show phenotypical morphometric changes, including a loss of

so-called “hill and valley” morphology (Pandolfi, 2003). While we did not carefully examine morphological changes in our cell culture studies, it is possible that hyperglycemia affects other cellular properties in hCAVSMCs.

In conclusion, we observed that the progression of diabetes, and the development of diabetes-associated cardiac pathology, is different in young male and female ZDF rats. Only ZDF-F displayed cardiac hypertrophy, at both the cellular and organ level compared to ZL-F. In healthy ZL rat hearts, we observed that a number of molecules exhibit a sex bias: ZL-F had more *Agtr2*, *Med13* and miR-208 family of miRNAs, while ZL-M displayed increased *Gata4*, *Gdf11*, *Nppb* and miR-29b. Importantly, we observed that cardiac expression of the miR-29 family of miRNAs and miR-208a were upregulated in both groups of ZDF rats compared to ZL rats. A summary figure outlining differences in gene expression is provided (Fig. 15). Moreover, we observed that increased miR-29b occurs in the setting of diabetes, although in *in vitro* studies of hCAVSMCs, upregulation of miR-29 does not appear to be induced by short-term (4 days) hyperglycemia. This suggests that either chronic hyperglycemia and/or other component(s) within the diabetic milieu mediate increases in miR-29. Notably, the loss of cardiac *Agtr2*, which codes for cardioprotective AT2R, and an increase in miR-29b, which promoted increased hCAVSMC TUNEL- and PI-staining, may underlie the increased susceptibility of ZDF-F to cardiac damage. We propose that co-upregulation of one or both miR-29 and miR-208 families of miRNAs may contribute to development of cardiac pathology in diabetic rats.

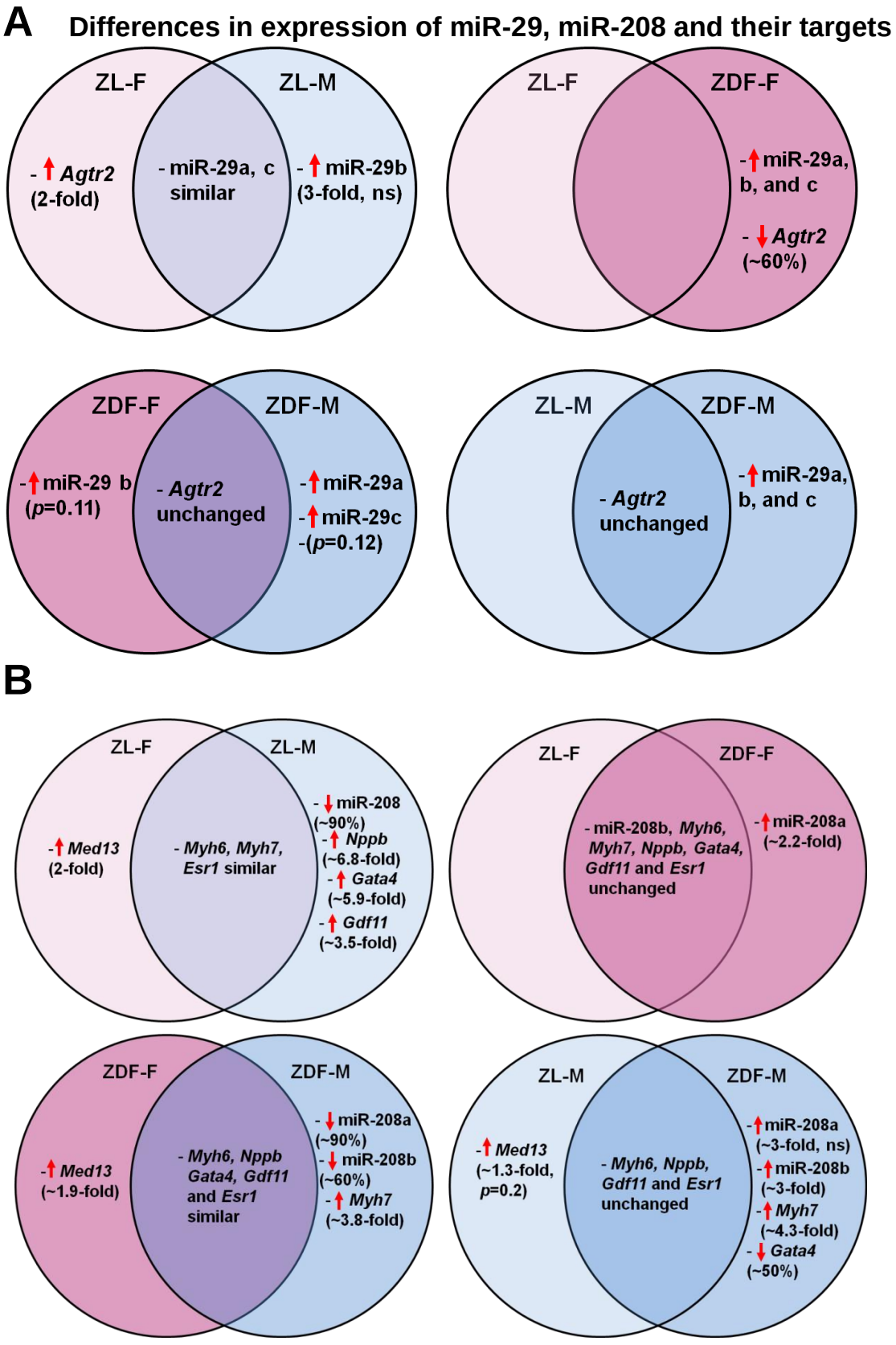

A
Differences in expression of miR-29, miR-208 and their targets
ZL-F
ZL-M
- ↑ Agtr2 (2-fold)
- miR-29a, c similar
- ↑ miR-29b (3-fold, ns)
ZL-F
ZDF-F
- ↑ miR-29a, b, and c
- ↓ Agtr2 (~60%)
ZDF-F
ZDF-M
- ↑ miR-29 b (p=0.11)
- Agtr2 unchanged
- ↑ miR-29a
- ↑ miR-29c -(p=0.12)
ZL-M
ZDF-M
- Agtr2 unchanged
- ↑ miR-29a, b, and c
B
ZL-F
ZL-M
- ↑ Med13 (2-fold)
- Myh6, Myh7, Esr1 similar
- ↓ miR-208 (~90%)
- ↑ Nppb (~6.8-fold)
- ↑ Gata4 (~5.9-fold)
- ↑ Gdf11 (~3.5-fold)
ZL-F
ZDF-F
- miR-208b, Myh6, Myh7, Nppb, Gata4, Gdf11 and Esr1 unchanged
- ↑ miR-208a (~2.2-fold)
ZDF-F
ZDF-M
- ↑ Med13 (~1.9-fold)
- Myh6, Nppb Gata4, Gdf11 and Esr1 similar
- ↓ miR-208a (~90%)
- ↓ miR-208b (~60%)
- ↑ Myh7 (~3.8-fold)
ZL-M
ZDF-M
- ↑ Med13 (~1.3-fold, p=0.2)
- Myh6, Nppb, Gdf11 and Esr1 unchanged
- ↑ miR-208a (~3-fold, ns)
- ↑ miR-208b (~3-fold)
- ↑ Myh7 (~4.3-fold)
- ↓ Gata4 (~50%)

FIGURE 15. Diagrams outlining key findings from this study, outlining differences in cardiac expression of (A) the miR-29 family of miRNAs and one of its putative targets, *Agtr2*; and (B) the miR-208 family of miRNAs and some of their known and predicted target genes. Each set of Venn diagrams represents comparative expression between two groups of rats.

LIMITATIONS AND FUTURE DIRECTIONS

An important consideration of the animal study was the fact that two different diets were utilized in order to produce ZDF rats that were hyperglycemic. The ZL male and female rats and ZDF-M were maintained on Purina #5008 and it has been demonstrated that ZDF-M spontaneously and progressively develop hyperglycemia (Shiota, 2012). In contrast, ZDF-F females maintained on the same diet do not develop hyperglycemia, and were instead fed Research Diets #D12468. While both diets provide a large amount of calories from carbohydrates (56.6% for Purina #5008 and 42.4% for RD #12468), the latter has nearly three times as much calories provided by fat (47.7% vs. 16.9% in Purina #5008). Notably, the source of fat in the latter is due to lard. The analyses provided within this thesis do not include diet as a factor, though the effects of diet cannot be ignored. In a population-based study, individuals with physician-diagnosed heart failure showed poor dietary patterns (Lemon, 2010), implicating an important role for dietary considerations. Carbone *et al.* recently reported that a Western diet (high sugar and saturated fat content) leads to systolic and diastolic dysfunction in CD-1 mice (2015). One of the goals of this study was, in part, to develop and characterize a hyperglycemic ZDF female rat whose metabolic and cardiac phenotype could be compared to that of age-matched ZDF males. Consequently, we have ***tried*** to draw the most conservative conclusions, though an additional group of ZDF males maintained on RD #D12468 and ZDF females on Purina #5008 are necessary to adequately make conclusive statements about this study.

A second limitation of this study was that most of the data evaluating expression was completed by performing qRT-PCR, examining mRNA rather than protein. While

this technique is valuable in providing information about changes at the transcript level, we often rely on mRNA expression as an index for predicting protein expression. For non-coding RNAs, i.e. microRNAs, qRT-PCR is suitable, since they exert their actions as nucleic acids. For genes involved in functional aspects of the cell, i.e. myosin genes, which encode for motor proteins that are part of the contractile apparatus in myocytes, examination of protein content would have been a better metric of expression, or at least would have supported the qRT-PCR data. Given the small number of animals per group (6-8) and the limited amount of tissue available for analysis, we opted for examining mRNA expression. Of course, kits and techniques have greatly enhanced the level of protein able to be detected, and such analyses would only have contributed to the data reported.

Regarding the cell culture data, it should be noted that for culture conditions of simulated normo- and hyperglycemia, we did not adjust for changes in osmolarity. Based on particle permeability, differences is osmolarity can indeed affect cell volume. Glucose uptake and metabolism are important for overall vascular smooth muscle function (Hardin and Paul, 1995). While our results indicate no significant impact of hyperglycemia (25mM) on mediating increases in miR-29 or in alterations in cell stiffness, the osmotic conditions were not identical, which may have influenced our results. Importantly, alterations in osmolarity can lead to NFAT5 (the transcription factor, nuclear factor of activated T-cells 5) translocation to the nucleus (Scherer, 2014) where it presumably affects gene expression involved in maintaining cell volume (Miyakawa, 1999).

Finally, another limitation of this study involves the cell culture experiments and the decision to use vascular smooth muscle cells as the *in vitro* model for examining effects of miR-29 and hyperglycemia. Because the data obtained in the animal study focused on cardiac function at the organ level and structure at the cellular and organ level, it appears to be a completely random transition. We attempted and subsequently failed to culture isolated rat ventricular myocytes for more than 24 hours. In addition, the experiment we designed included liposome transfection of miR-29 mimics. It has been reported that such a technique in isolated adult cardiomyocytes has low transfection rates (reviewed in Louch, 2011). In terms of cell number, cardiac fibroblasts are the most abundant cell type within the adult myocardium (Gerdes, 2012). Indeed, cardiac fibroblasts can be isolated from both neonatal and adult hearts, or cell lines are available for purchase. Although immortalized cell lines, such as the murine atrial HL-1 and rat myoblast H9C2, are suitable for transfection experiments, we decided to use a primary cell culture that might more closely resemble characteristics observed *in vivo*.

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