A miR-208–Mef2 Axis Drives the Decompensation of Right Ventricular Function in Pulmonary Hypertension

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Rationale: Right ventricular (RV) failure is a major cause of morbidity and mortality in pulmonary hypertension, but its mechanism remains unknown. Myocyte enhancer factor 2 (Mef2) has been implicated in RV development, regulating metabolic, contractile, and angiogenic genes. Moreover, Mef2 regulates microRNAs that have emerged as important determinants of cardiac development and disease, but for which the role in RV is still unclear.

Objective: We hypothesized a critical role of a Mef2-microRNAs axis in RV failure.

Methods and Results: In a rat pulmonary hypertension model (monocrotaline), we studied RV free wall tissues from rats with normal, compensated, and decompensated RV hypertrophy, carefully defined based on clinically relevant parameters, including RV systolic and end-diastolic pressures, cardiac output, RV size, and morbidity. Mef2c expression was sharply increased in compensating phase of RVH tissues but was lost in decompensation phase of RVH. An unbiased screening of microRNAs in our model resulted to a short microRNA signature of decompensated RV failure, which included the myocardium-specific miR-208, which was progressively downregulated as RV failure progressed, in contrast to what is described in left ventricular failure. With mechanistic in vitro experiments using neonatal and adult RV cardiomyocytes, we showed that miR-208 inhibition, as well as tumor necrosis factor-α, activates the complex mediator of transcription 13/nuclear receptor corepressor 1 axis, which in turn promotes Mef2 inhibition, closing a self-limiting feedback loop, driving the transition from compensating phase of RVH toward decompensation phase of RVH. In our model, serum tumor necrosis factor-α levels progressively increased with time while serum miR-208 levels decreased, mirroring its levels in RV myocardium.

Conclusions: We describe an RV-specific mechanism for heart failure, which could potentially lead to new biomarkers and therapeutic targets. (Circ Res. 2015;116:56-69. DOI: 10.1161/CIRCRESAHA.115.303910.)

Key Words: biomarkers ■ hypertension, pulmonary ■ inflammation ■ metabolism ■ microRNAs

Right ventricular (RV) failure (RVF) is the most important prognostic factor for both morbidity and mortality in pulmonary hypertension (PHT) but remains understudied compared with left ventricular (LV) failure.1,2 Although some forms of pulmonary arterial hypertension (PAH) are relatively rare, overall PHT is a common clinical problem complicating LV dysfunction, chronic parenchymal lung disease, thromboembolic lung disease, as well as the peri- and postoperative care of heart/lung transplant patients.3 Thus, the need for right ventricular (RV)-specific therapies (currently unavailable) is an urgent priority. Mechanisms and therapies for LV failure cannot necessarily be extrapolated to RVF because the 2 heart chambers have critical differences in embryology and myocardial biology.1,2 On the one hand, the RV, which efficiently transitions from the hypertrophied RV of the fetal circulation to the thin-walled RV of the adult circulation, seems to have more plasticity in its ability to switch phenotypes. On the other hand, the RV cannot maintain an adaptive phenotype in conditions of pressure overload. Although in systemic hypertension the hypertrophied LV can maintain an adaptive phenotype and prevent clinical deterioration for decades, in PHT a short-lived adaptive compensating phase of the hypertrophied RV (cRVH) is followed by a catastrophic decompensation phase of RVH (dRVH) leading to accelerated death.3

What drives the switch from cRVH to dRVH is unknown. Some forms of RV hypertrophy (RVH) in humans (e.g., Eisenmenger syndrome)4 or in animal models (pulmonary artery [PA] banding model) can have longer periods of compensation compared with other forms, such as in patients with

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PAH or animal models of PAH (eg, monocrotaline model), where the compensatory phase is dramatically short. Although some patients with PAH can respond to PAH therapies with a decrease in PA pressure, their RVs continue to deteriorate, in contrast to the dogma that RV function is easily reversible when the afterload improves. These data raise the provocative possibility that there may be mechanisms, intrinsic to the RV, that drive RVF independently of the afterload.

We sought to determine what these mechanisms may be and speculated that they may be different than what is described in the LV. We think that there are methodological challenges in preclinical studies of this important problem in the literature. Often authors study RVH without separating between cRVH and dRVH, that is, presuming that RVH is an abnormal, diseased RV; while it is clear that cRVH is an adaptive phenotype because, although briefly, it prevents a decrease in the cardiac output (CO) and clinical symptoms. In other cases, authors may compare a relative compensated RV from one animal model (ie, the PA banding model) to a decompensated RV (dRVH) from another model (ie, monocrotaline or Sugen/hypoxia models). Here is a problem that may arise from studies that do not systematically separate well-defined compensated versus decompensated RVs: It is well-described that the compensatory switch is an adaptive event or a sign of decompensation. We recently showed that when we studied the natural progression of RVF in a rat model of PAH, this metabolic switch was observed only in the cRVH (a state defined by the maintenance of CO) and lost upon entrance to dRVH (where the RV size continued to increase but the CO and survival started decreasing). The glycolytic switch was associated with the activation of angiogenesis, perhaps providing the growing mass of RV myocardium with increased nutrient supply. Loss of this metabolic switch was associated with decreased angiogenesis, contributing to a state of relative ischemia in dRVH and confirming clinical evidence of ischemia in RVF.

This suggests that metabolic switch toward glycolysis is a temporary adaptive state that perhaps should not be treated. We sought to determine the molecular mechanisms that underlie this phenotypic switch from cRVH to dRVH within the same animal model, as PHT progresses.

We focused on what seems to be an RV-specific axis that regulates the development of the RV, not the LV; the Islet1/myocyte enhancer factor 2 (MeF2/Hand2) axis. The master transcription factor MeF2c is required for the alignment of structures and the orientation of the left-right axis during the looping of the embryo. Loss of MeF2c in mice results in embryonic lethality with RV morphogenetic defects, as well as vascular abnormalities. MeF2a, another member of this family, is more expressed in adult cardiac and skeletal muscle. MeF2a knockout mice display increased mortality within the first week of life, with severe spontaneous right ventricular chamber dilation. MeF2 has been implicated in the regulation of metabolic (pyruvate dehydrogenase kinase 4, glucose transporter 4, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha [PGC-1α]) contractile (α- and β-myosin heavy chain [MHC]), and angiogenic genes (vascular endothelium growth factor). Moreover, MeF2 has been implicated in the regulation of microRNAs (miRNAs) that have emerged as important determinants of gene regulation in cardiac development and hypertrophy. We hypothesized that a MeF2-miRNA axis, intrinsic to RV cardiomyocytes, is driving the transition from cRVH to dRVH.

We also speculated that an external, environmental factor, such as inflammation, can be a trigger for the switch from cRVH to dRVH, perhaps explaining why RVs exposed to stronger inflammatory signals seem to fail earlier. Inflammatory cytokines such as tumor necrosis factor-α (TNF-α) are increased in the blood of patients with PAH and patients with PAH that have the highest inflammation burden (eg, in scleroderma or infection with HIV) have the worst prognosis among PAH patients (without having higher pulmonary vascular resistance). Inflammatory cytokines correlate positively with the severity of the disease, suggesting that they may play a direct role on RVF. Thus, inflammatory models of PHT, such as the monocrotaline model, are suitable for the exploration of this hypothesis. We hypothesized that an inflammatory signal may act in synergy with an RV-specific MeF2 axis activation to disturb metabolic and angiogenic signals, triggering the switch from cRVH to dRVH.

### Methods

All experiments were performed with the approval of the University of Alberta Committees on Animal Policy and Welfare. Details are available in the Methods section in the Online Data Supplement.

#### Animal Model of RV Failure

Three hundred to 350 g male Sprague-Dawley rats (n=30) were randomized to injection with monocrotaline 60 mg/kg SC (Sigma...
Aldrich; n=20) versus vehicle (n=10). Animals were euthanized after a comprehensive invasive and noninvasive hemodynamic evaluation, every week between 3 and 6 weeks after monocrotaline. In general, rats in the monocrotaline model appeared to be compensating between third and fourth weeks after monocrotaline injection, while they enter a decompensating phase between the fifth and sixth weeks after monocrotaline with increased morbidity and mortality. However, cRVH (n=10) versus dRVH (n=10) was defined based on strict hemodynamic criteria and clinical signs as discussed in the Results section of this article.

Cardiomyocytes Isolation and Culture

Neonatal RV cardiomyocytes were isolated as previously described, for transfection experiments to manipulate miRNA levels. We also isolated adult cardiomyocytes (aCMs) from compensating RVs and their corresponding LVs, in which we used infections with adenoviruses to manipulate miRNA levels; here, the isolation procedure was adapted from the study by O’Connell et al., originally described for adult mice cardiomyocyte isolation.

Cell Treatments

miR-208 pre-miR mimic and anti-miR sequences, as well as negative and inhibitor-negative controls sequences (pooled in the scramble group), were purchased from Ambion (Life Technology) and were transfected in neonatal cardiomyocytes (nCM) at a final concentration of 20 nmol/L using siPORT NeoFX transfection Agent (Ambion). Adenoviruses were used at a final concentration of 400 multiplicity of infection (10 pfu/25000 cells) to infect aCMs leading to infection rates of 70% to 80% in vitro after 72 hours. TNF-α and phenylephrine were dissolved in dimethyl sulfoxide and used at a final concentration of 100 ng/mL (48 hours, dimethyl sulfoxide <1:10000) and 10 μmol/L (72 hours, dimethyl sulfoxide <1:10000) respectively.

Statistics

Data are presented as mean±SEM. For comparison of 2 means, we used an unpaired t test. For comparison between >2 means, we used a 1-way ANOVA followed by a Tukey post hoc test. For the gene chip microarray analysis statistical analysis, please see the TaqMan analysis section in the in the Online Data Supplement. All analyses were performed using SPSS 21 (IBM Corp). Significance P<0.05 is indicated by *, P<0.01 by **, and P<0.001 by ***.

Results

Modeling the Clinical Stages of RVF in Rat PHT

We first modeled the natural history of RVF in the monocrotaline rat model of PHT. To those studying the monocrotaline model, it is known that rats enter a phase of decompensation (developing signs of heart failure like ascites, decreased appetite and activity, decreased weight, as well as death) in the fifth to sixth week after monocrotaline injection. However, because we were interested in directly comparing tissues from cRVH versus dRVH, it was important to confirm the presence of true hemodynamic and structural signs of compensation versus decompensation. Thus, we prospectively defined criteria for compensation and decompensation, similar to what we used in our patients. Thus, dRVH was defined as a state of ongoing RVH, in which the RV systolic pressure (RVSP) decreases because of contractile failure (while the mean PA pressure remains elevated); the RV end-diastolic pressure increases; the CO decreases; clinical evidence of heart failure (ascites, decreased appetite and activity, >20% weight loss) develops; and death occurs. Our ethics committee protocol does not allow death as an end point. Because these signs of heart failure are absolute predictors of imminent death to our experience, we used this terminal morbidity as a surrogate for mortality. Thus, as soon as the rats developed these signs, we performed echocardiography and catheterization followed by euthanasia, allowing us to study all rats in the dRVH group. To study rats that would presumably have features of hemodynamic compensation, we studied them between the third and fourth week after monocrotaline injection. Here, we confirmed the presence of compensation with invasive hemodynamic assessments and echocardiography, using the parameters and clinical signs discussed above, followed by euthanasia and tissue harvest.

Thus, in our cRVH group, compared with the vehicle-treated (normal) rats, we found increased mean PA pressure, RV systolic pressure, and RV weight (RV/LV+septum or RV weight/body weight), whereas the RV end-diastolic pressure remained unchanged or mildly increased and the CO remained normal or mildly decreased (Figure 1A and 1B). Echocardiography also showed a decrease in the PA acceleration time (a surrogate for increased mean PA pressure) but unchanged tricuspid annular plane systolic excursion (a clinically used surrogate for RV contractility), suggesting preserved RV contractility (Online Figure IA). None of these rats showed any of the signs of terminal morbidity.

However, the dRVH rats, compared with the cRVH rats, exhibited a further increase in RV weight; a decrease in RV systolic pressure and tricuspid annular plane systolic excursion, while the mean PA pressure remained high; a decrease in the CO; a sharp increase in RV end-diastolic pressure; and a dramatic increase in terminal morbidity (with >60% of rats developing these signs; Figure 1A and 1B; Online Figure IA).

We then assessed cardiomyocyte hypertrophy by measuring the cardiomyocyte cross-sectional area in DAPI (4’,6-diamidino-2-phenylindole)-stained sections and tissue remodeling by the degree of fibrosis measured by Masson trichrome staining. We found a 2-fold increase in cross-sectional area in cRVH compared with normal, but no further increase in dRVH, while the degree of fibrosis continued to increase (Figure 1C). Although the cRVH had evidence of fibrosis, there was no apparent disruption in tissue architecture, but in the dRVH there was definite distortion of tissue structure and formation of large fibrous bands. Because severe myocardial fibrosis is associated with an increase in tissue edema, the progressive increase in RV weight in dRVH (without further increase in cardiomyocyte size) is compatible with edema and not ongoing myocardial hypertrophy.

The monocrotaline model of PHT is known to be associated with increased levels of inflammation. We thus measured the level of TNF-α circulating in the serum of the rats in the different stages characterizing our model. We found increasing serum levels of TNF-α as the disease progressed toward RVF (Online Figure IB).

Dynamic Changes of Mef2 in the Progression Toward RVF

Mef2c protein expression was increased in cRVH although it was not increased during the third week (early cRVH) but rather sharply increased in the fourth week (late cRVH); as we recorded the time of our invasive studies, we could separate them between those that had less severe PHT (third week) and
more severe PHT (fourth week after monocrotaline injection; Figure 2A). Intriguingly, the increase in MeF2c was lost again in the dRVH stage. We then studied the subcellular localization of MeF2c using immunofluorescence and found that overall the increased expression of MeF2c in cRVH coincided with its nuclear translocation (Figure 2B), suggesting an increase in its transcriptional activity during cRVH. This was again inhibited in dRVH. This pattern was confirmed by the measurement of the MeF2c target gene for glucose transporter 4, which followed a similar pattern of expression (Figure 2A and 2B). The proportion of glucose transporter 4 translocated to the plasma membrane (stained with a Pan-cadherin antibody) was also increased in cRVH and lost in dRVH (Online Figure IIA). These data show that the fetal factor MeF2c is transiently increased only in cRVH, contributing to the adaptive nature of this stage, perhaps similar to the physiological RVH of the fetal circulation in utero. This transient increase in glucose transporter 4 is in keeping with our recent findings that increased glucose uptake (measured by micro-positron emission tomography-computerized tomographic imaging in vivo) characterizes cRVH, but it is lost during dRVH.10

We then studied the adult isoform of this transcription factor, that is, MeF2a. We found a pattern of expression that was opposite to that of MeF2c during cRVH, that is, MeF2a expression was decreased early on during cRVH (Online Figure II). However, MeF2a failed to increase again to the levels of the normal RV (nRV) and remained downregulated during dRVH. This pattern was confirmed by the levels of the MeF2a target gene for PGC-1α that followed a similar expression pattern (Online Figure II).
miRNA Signature of RV Failure

Although the levels and function of Mef2 are known to be regulated by specific miRNAs in LVH, we first wanted to perform an unbiased screening of miRNAs in our model because miRNA expression has not been previously studied in RVH and RVF progression within the same animal model. Although we were interested mostly in the differences between our well-defined cRVH and dRVH stages, we also studied fetal RVs (fRV) and nRV. This was because the transient expression of Mef2c in cRVH suggested a transient reactivation of a fetal RVH program. We obtained free wall tissues from fRVs (obtained immediately after birth, when the fetal circulation and fetal RVH are present) and RVs from the adult rats in which we performed the hemodynamic studies shown in Figure 1.

We performed an unbiased screening using TaqMan Low-Density Arrays (Applied Biosystems) to determine the expression of the 377 well-characterized miRNAs included in the array. We used several filters and 3 separate standard normalization procedures, as described in Methods section of this article. To assess the variability between samples, we used a hierarchical clustering analysis (Online Figure III), where each column represented 1 sample and each row the profile of expression of 1 miRNA, and a principal component analysis (PCA, as described in Methods in the Online Data Supplement; Online Figure IVA). fRV were clearly separated from all other samples on PCA1, the principal level of variability (fRV PCA1 ≈ −300, nRV/cRVH/dRVH PCA1 ≈ 50), suggesting a specific and strong fRV miRNA program. Although this program was not reactivated in full in the adult RVH stages, we observed several miRNAs that were re-expressed in RVH. Although we observed an overlap between cRVH and dRVH on PCA2, the second level of variability, those 2 groups were separated from nRV in terms of miRNA profiles (nRV PCA2 ≈ 80, cRVH/dRVH PCA2 ≈ 50). To study differences among nRV, cRVH, and dRVH, we performed an adjusted ANOVA with a Benjamini-Hochberg correction on the fold changes among groups (ie, nRV versus cRVH, nRV versus dRVH, and cRVH versus dRVH), using again 3 different normalization procedures, based on U6, median, and quantile corrections. We found 14 miRNAs that were differentially expressed after all 3 normalizations, achieving statistical significance by all
Among them, we were interested in the 6 that showed a different pattern of expression compared with what is known based on published data on LVH or they had not been previously described in LVH models (highlighted in grey in Online Figure IVC): miR-200b, -338-3p, -328, -155, -92a, and -208a. Based on the TargetScan (http://www.targetscan.org/) and DAVID databases (which describe pathways that might be perturbed by changes in the level of specific miRNAs), these 6 miRNAs are strongly implicated in angiogenesis, cytoskeleton, and p53 pathways, in keeping with previously published reports on RVH. The mean data from the arrays for these miRNAs are presented in Online Figure IVD and IVE, showing the progressive decrease in the levels of miR-200b, -208a, -92a, -328, -338-3p, and the progressive increase of 155, as RVF progresses.

We focused on miR-208a for the following reasons:

1. It is a myocardium-specific miRNA, suggesting that if measured in the serum it could potentially be a good biomarker of the molecular state of RVH, allowing one to follow an RV-specific (as the LV is mostly normal in most PHT syndromes) miRNA signature compared with the circulating miRNA changes reflecting the remodeling of pulmonary vessels or peripheral tissues in PHT.

2. It is hosted by the αMHC gene, and cardiac hypertrophy is characterized by a switch from αMHC to β-isotype in cardiomyocytes,33 possibly through Mef2 activation.18

3. Its expression has been shown to be maintained in the progression from normal LV to LVH to LV failure,34 and thus the observation that its levels decreased during RVF progression would suggest a chamber-specific regulatory mechanism.

We confirmed the progressive decrease in miR-208 in cRVH and dRVH compared with nRV by individual quantitative real-time-polymerase chain reaction in the rats studied in Figure 1, this time using a much larger sample size than the n=3 that we used for the screening TaqMan low-density array experiments (ie, n=10/group) (Figure 3A). miR-208 levels were also found to negatively correlate with indexed RV weight (Figure 3A; r=-0.72; n=20; P<0.001). To determine whether miR-208 could be a serum-based biomarker of the molecular state of RVH, we measured miR-208 level in the serum of nRV, cRVH, and dRVH rats (n=3 nRV, 9 cRVH, and 9 dRVH). We detected miR-208 in the late cycles of quantitative real-time-polymerase chain reaction amplification (Ct=37), a fact compatible with its known low levels in the serum, whereas the Ct of our housekeeping gene (U6) was 27, suggesting that there was no significant degradation during our storage and isolation procedures. To ensure that our measurements were within the linear dynamic range of our assay, we also performed a dose-response with a miR-208 mimic and confirmed that our measurements were indeed within the linear range (Material in the Online Data Supplement and Online Fig. VA). We found a decrease in the serum levels of miR-208 during progression toward RVF (Figure 3B; Online Figure VA), suggesting that circulating levels of miR-208 might represent what is happening in RV myocardium. As mentioned above, miR-208 is hosted by the αMHC gene, known to be inhibited in cardiac hypertrophy in favor of the βMHC gene. We confirmed that αMHC is downregulated in cRVH and further downregulated in dRVH and that βMHC expression follows the exact opposite pattern (Figure 3C). This correlation of molecular and physiological features of the 3 stages of RV function presented in Figure 1 further strengthens our model of natural

Figure 3. MicroRNAs (miRNA) signature of right ventricular (RV) failure. A, miR-208 expression measured by individual quantitative real-time (qRT)-polymerase chain reaction (PCR) in RNA extracted from RV free wall tissues (confirming the data from the miRNA arrays) and correlation between miR-208 expression and the progression of the disease assessed by the ratio RV weight/body weight (BW). B, Circulating (serum) miR-208 levels measured by qRT-PCR in RNA extracted from sera in normal, compensated RV hypertrophy (cRVH), and decompensated RV hypertrophy (dRVH) rats. C, α- and β-myosin heavy chain (MHC) mRNA levels measured in RV free wall tissues and (D) correlation between miR-208 levels (from B) and αMHC mRNA levels. For A–D, n=3 normal RV (nRV), 9 cRVH, and 9 dRVH; *P<0.05, **P<0.01, and ***P<0.001.
history of RVF in the rat. Moreover, we found a strong correlation between αMHC and miR-208 levels in the hypertrophied RV myocardium (Figure 3D: r=0.72; n=20; P<0.001), supporting that miR-208 downregulation is subsequent to the inhibition of its host gene (ie, αMHC).

The subunit MED13 of the complex mediator of transcription has been identified as miR-208 direct target gene in the heart.34 Interestingly, another miRNA of our short list, miR-200b, is also able to target MED13 (Online Figure VB) and has a similar pattern of expression with miR-208, that is, it is decreasing while RV weight increases (Online Figure VC and VD; r=-0.64; n=3 nRV, 9 cRVH, and 9 dRVH; P<0.001). Both miR-208 and 200b have binding sites in the MED13 3‘ untranslated region (Online Figure VB) and thus their decrease may lead to an increase in the expression of the MED13/nuclear receptor corepressor 1 (NCoR1) transcription complex. This complex is critical for many metabolic and angiogenic genes, and also the regulation of Mef2 function itself, via negative feedback loops35,36 as we discuss below.

**MED13 Expression Is Upregulated in dRVH**

MED13 is known to act as a functional bridge between nuclear receptors bound to DNA37 and the basal transcription apparatus, including RNA polymerase II.38 It has been identified as a negative regulator of metabolism through repressive effects on nuclear receptor activity and subsequent downregulation of a package of metabolic genes.35 We speculated that changes in MED13 expression could explain why at some point the adaptive metabolic response seen in cRVH is switched off in dRVH, leading to failure. We thus measured the mRNA level of MED13 in our model and found that MED13 mRNA expression is indeed increased in dRVH (Figure 4). To specifically study protein expression in RV cardiomyocytes, we used immunostaining and found a similar pattern with a significant increase in the percentage of cells with nuclear MED13 in dRVH compared with either cRVH or nRV (Figure 4).

Exploring the role of this first description of MED13 activation in RV failure, we studied its transcriptional partner NCoR1.35 NCoR1 N-terminal domains recruit histone deacetylases (including class I [HDAC3] and II [HDAC4]),39,40 resulting in hypoacetylation of histones and a chromatin structure resistant to transcription. NCoR1 knockdown has been associated with a robust increase in muscle mitochondria number and activity, mainly through increased transcription, acetylation and activity of Mef2 family members, and increased transcription

![Figure 4](http://circres.ahajournals.org/)
of metabolically regulated genes including pyruvate dehydrogenase kinase 4, and VEGF. We previously showed that pyruvate dehydrogenase kinase 4 and vascular endothelium growth factor expressions were increased in cRVH and decreased in dRVH, an observation that could potentially be explained by NCoR1 activation. We measured NCoR1 mRNA level in our model and found that NCoR1 expression was slightly decreased in cRVH but significantly increased again in dRVH (Figure 4). We measured NCoR1 protein expression and localization by immunostaining and found a decrease in the percentage of cells with nuclear NCoR1 in cRVH compared with nRV, but then a 4-fold increase in dRVH compared with cRVH (Figure 4). We also evaluated NCoR1 activity using the acetylation status of histones 3 and 4, both known nuclear targets of NCoR1. We found that histones 3 and 4 acetylation is upregulated in late cRVH and downregulated in dRVH, in keeping with NCoR1 expression (Figure 5A and 5B). Although these immunoblot data reflected changes in the whole RV tissue, localizing the signal with acetylated histones 3 immunostaining in RV cardiomyocytes allowed us to confirm the upregulation in cRVH and a more robust inhibition in dRVH myocardium (Figure 5A).

miR-208 and Inflammation Regulates the MED13/NCoR1-Mef2 Axis in the RV but not in the LV

To study whether the miR-208 downregulation is indeed directly responsible for our observed increase in MED13 expression, we performed mechanistic studies using 2 in vitro models: 1 in normal nCM and 1 in aCMs from hypertrophied RVs. We had found that the expression of miR-208 progressively decreased from its levels in nRVs as RVF developed (Figure 3B). We therefore first studied the effect of miR-208 modulation in normal nCM from RVs and corresponding LVs, cultured and transfected with either mimic or anti-miR-208. We found that a similar decrease of miR-208 (80% decrease; Figure 6A) in LV and RV cardiomyocytes was associated with a particularly strong increase in MED13 expression in RV (10-fold increase) compared with LV cardiomyocytes (4-fold increase; Figure 6A). This increase in MED13 expression in RV cardiomyocytes was associated with NCoR1 upregulation and Mef2 downregulation, whereas no changes were measured in LV cardiomyocytes. In contrast, no changes were found after miR-208 upregulation in this model, in either LV or RV cardiomyocytes (Figure 6A). This suggested that perhaps these normal cells from both ventricles express enough miR-208 at baseline to inhibit MED13 and that further increase in its levels has no effect, while the RV is much more sensitive to a decrease in miR-208 than the LV.

Using the UCSC genome browser (online genome browser hosted by the University of California, Santa Cruz at http://genome.ucsc.edu/), we found nuclear factor κB–binding sites (V$NFKAPPAB_01) on the MED13 (in blue) promoter (Online Figure VI), suggesting a possible direct regulation of the MED13/NCoR1 axis by inflammation and TNF-α, a cytokine that is upregulated in the serum of PAH patients and in our rat model (Online Figure I). We thus stimulated normal RV cardiomyocytes (from nRVs) with TNF-α, in the presence or absence of miR-208 mimic or anti-miR-208, and measured MED13 expression (Figure 6B). We found that TNF-α induced a marked increase in MED13 expression, which was significantly reduced by miR-208 (Figure 6B). These results suggest that inflammation, through upregulation of miR-208, may contribute to the increased MED13 expression seen in the RV of patients with PAH.

**Figure 5.** Right ventricular (RV) myocardium histone acetylation during RV hypertrophy (RVH) progression. Levels of acetylated versus total histones 3 (H3; A) and H4 (B) histones measured by immunoblots are shown to assess the nuclear receptor corepressor 1 (NCoR1) deacetylase activity. Mean data correspond to the ratio of acetylated versus total versus actin expression (used as loading control). n=3 rats/group; *P<0.05, **P<0.01, and ***P<0.001. Representative photomicrographs of RV tissues immunostained for the detection of Ac-H3 in red, colocalizing with nuclei stained in blue with DAPI. Myocardial cells were identified using a cardiac myosin heavy chain (MHC) staining (green). A magnified image is shown to the right. The percentages of cells with a positive nuclear staining for Ac-H3 were calculated. n=5 images/rat in 5 rats/group; *P<0.05, **P<0.01, and ***P<0.001. cRVH indicates compensated RV hypertrophy; dRVH, decompensated RV hypertrophy; and nRV, normal RV.
nCM with TNF-α for 48 hours and found a 3-fold increase in MED13 expression in RV but not LV cells (Figure 6B). This suggests a possible RV-specific regulation of MED13 by TNF-α contributing, along with the miR-208 downregulation, to the transition from cRVH to dRVH in a chamber-specific manner.

We then studied the effects of miR-208 modulation in our second model of aCMs from normal and cRVH hearts to determine whether we can recreate the dRVH phenotype (in terms of the MED13-NCoR1-Mef2 axis) in vitro. We first studied the effect of miR-208 downregulation in normal aCMs obtained from the RV and corresponding LV free walls (aCM). Because transfection is not an efficient method for gene delivery in aCMs (in contrast to nCM), we used an adenovirus (10⁷ pfu/mL, 72 hours) coding for an anti-miR-208 sequence (Ad-anti-miR-208). aCM were infected for 72 hours, and the percentage of infected cells was determined using green fluorescent protein (GFP), coexpressed by the virus. We also used a GFP-only adenovirus as control (Ad GFP). After 72 hours, 80% of cells were expressing GFP with a good survival of aCM (Figure 7A). Once again, we found that decreased expression of miR-208 was associated with a much higher increase in MED13 expression in RV compared with LV aCM, despite a similar downregulation of miR-208 (Figure 7B).

We then speculated that in cRVH aCM (where the levels of miR-208 are already decreased compared with nRV), a decrease in miR-208 and an increase in TNF-α may cross a threshold that would trigger the entrance to dRVH. As in neonatal normal cells, TNF-α induced a 2-fold increase in MED13 mRNA level in cRVH aCM, whereas no effect was noticeable on the corresponding LV aCM (Figure 7C). This response was again associated with an increase in NCoR1 mRNA level in cRVH aCM (Figure 7C). Infection with Ad-anti-miR-208 (10⁷ pfu/mL, 72 hours) caused an increase in NCoR1 mRNA levels and a 2-fold decrease in Mef2 mRNA levels, mimicking the transition to dRVH in vivo (Figure 7D). TNF-α in combination with the Ad-anti-miR-208 increased the effects of miR-208 inhibition on NCoR1 mRNA levels, resulting in about a 4-fold increase. We found a 2-fold decrease in vascular endothelium growth factor mRNA levels (another direct target of NCoR1) after infection with Ad-anti-miR-208 and a 3-fold decrease after the combination of Ad-anti-miR-208 and TNF-α (Figure 7D). We then measured the expression level of Mef2, and we found that the inhibition of miR-208 significantly decreased the expression of Mef2. However, in contrast to a potential synergistic effect between miR-208 and TNF-α on NCoR1 and thus on Mef2 function, the addition of TNF-α on
top of anti-miR-208 did not have synergistic effects on Mef2 expression. Interestingly, compared with the empty virus, Ad-anti-miR-208 increased Mef2 expression (Figure 7D).

These data suggested that in contrast to the LV, there is a progressively decreasing level of miR-208 in RVH but perhaps a second trigger (ie, inflammation) is needed for enough MED13/NCoR1 upregulation and Mef2 suppression. This suppression of Mef2 has a potentially dual basis: an inhibition of its activity because of the NCoR1-mediated effects on Mef2 acetylation and an inhibition of its transcription, potentially because of the previously described effects of NcoR1 acetylation on the Mef2 promoter (in addition to the acetylation of Mef2 protein itself). As data in Figure 2A and 5A had shown, the expression of Mef2 in late crVH and its loss in dRVH coincide with the increase in acetylation in late crVH and its suppression again in dRVH, likely mediated by NCoR1.

In the setting of a sustained increase in the afterload, this inhibition of Mef2 activity/expression may push the RV cardiomyocytes away from a normal/compensatory phenotype toward failure. To further study this hypothesis, we studied aCM shape (cross-sectional area and length) under hypertrophic stimuli using a 3D reconstruction with the cellular planes scanned in the z axis on a confocal microscope (Figure 7E),
Discussion

Here, we show for the first time a critical role of a miR-208/Mef2 axis in RVF. Although it has been well-known that Mef2 is critical to the development of the RV specifically, this has not been studied in models of RV failure in PHT. The use of carefully defined stages of the natural history of RVF within the same animal model using clinically relevant criteria of cRVH versus dRVH (CO, RV contractility, RV end-diastolic pressure, clinical signs) allowed us to describe a potential RV-specific molecular trigger for the entrance to dRVH. In contrast to what is known in LVH, the levels of miR-208 (a myocardium-specific miRNA) continuously decrease as RVH progresses, eventually (and particularly in the presence of inflammation) allowing the repression of Mef2 and the exit from a fetal-like compensatory phase (Figure 8). The presence of feedback loops (eg, Mef2 inhibits miR-208 expression by regulating its host αMHC gene, which in turn inhibits Mef2 expression and activity via the MED13/NCoR1 complex) is characteristic of mechanisms that need fine tuning and careful regulation during development, such as the Mef2 axis. Our finding that inflammation (TNF-α) could also serve as a second hit for the entrance to dRVH, in synergy with the decreasing miR-208 (Figure 8), can potentially explain why RVs exposed to strong inflammatory environments are much more prone to decompensation. We showed for the first time that TNF-α, in contrast to miR-208 that inhibits it, activates the MED13/NCoR1 axis, potentially through nuclear factor κB–mediated effects on the MED13 promoter and preferentially on the RV compared with the LV cardiomyocytes (Figure 8).

Another novel aspect of our data is the unbiased discovery of an RV-specific miRNA signature that marks the entrance to the decompensating phase of RVF function. It was through this short list of miRNAs that we chose to study the specific role of miR-208 in RVF. We enhanced the mechanistic aspect of our data by studying in vitro models in parallel to the in vivo models of RV decompensation. Considering the limitations of gene manipulations in cardiac myocytes in vitro, we used a model of neonatal RV cardiomyocytes and a model of aCMs from normal and cRVH RVs to show that decreasing the levels of mir-208 caused an increase in the Med13-NCoR1 axis repressing Mef2. We specifically showed that further decrease of miR-208 in cRVH myocardial cells by anti-miR-208 along with TNF-α stimulation allowed the repression of Mef2, marking the entrance to the dRVH as our in vivo data showed. This is important because, being myocardium-specific and measurable in the serum (Online Figure I; Figure 3C), miR-208 can potentially be a biomarker that along with the levels of TNF-α (or potentially other cytokines that activate nuclear factor κB) could provide serum-based signatures that may correlate with the RV phenotype in vivo and also predict the RVs more likely to fail.

Serum miR-208 has been shown to be a potential biomarker in patients with myocardial infarction, as its levels are increased compared with those from healthy patients.46–48 On the contrary, some authors have failed to detect serum miR-208 in healthy patients, raising concerns whether this will be of practical value.49–50 However, in these studies, relatively small number of patients were studied and not enough details were given to determine whether there was RNA degradation or methodologic differences among studies. Certainly, because miR-208’s only source is one organ (the heart), its serum levels are low, posing challenges in terms of optimal conditions of RNA isolation and prevention of its degradation for future larger studies. However, its specificity in the case of RVF may be particularly high, given
the fact that the left ventricle in these patients is typically normal. miR-208 will not be of value as a biomarker to diagnose RV disease (in other words normal versus RV disease patients) because there are plenty of ways to diagnose RV disease easily and reliably, including physical examination alone. But in the setting of a cohort of patients with PAH, in which baseline levels are measured and miR-208 is detectable, decreasing levels over time (versus sustained levels) may identify early the patients that may be declining clinically later on.

Another important aspect of our work is that the synergy between miR-208 and TNF-α on RV cardiomyocytes did not take place in LV cardiomyocytes. Along with the critical role of HDAC in RV development, our work suggests that there may indeed be an RV-specific mechanism for heart failure. Although its existence has been suspected, we think this may be the first time that a potentially RV-specific mechanism of heart failure is elucidated.

The description of stage-specific clinical and molecular phenotypes in RVH may guide the development of stage-specific therapies. For example, most patients with PHT are diagnosed at late stages because of the absence and nonspecificity of symptoms at early stages of the disease. At best, a patient with PHT is diagnosed during a compensating, that is, cRVH, stage. In addition to the therapies that may aim to reverse the pulmonary vascular disease, what will be important for this patient are therapies that will prevent the entrance to a decompensation phase (ie, dRVH). Our work suggests that therapies that will increase the expression of miR-208 and decrease inflammation will be beneficial. However, it is difficult at this point to pragmatically envision gene therapy trials promoting miR-208 in a chamber-specific manner. However, mimicking the effects of miR-208 upregulation, that is, inhibiting the MED13/NCoR1 axis, may be easier pharmacologically. For example, one of the major ways that the MED13-NCoR1 axis exerts its molecular effects on nuclear factors is through histone acetylation. There is great interest in the use of HDAC inhibitors in the treatment of heart failure. Some work has been published on the role of HDACs in PHT and RVH or RVF syndromes, although at this point most of the HDACs used (such as valproic acid or trichostatin A) have well-characterized pleiotropic effects and regulate multiple mechanisms beyond histone acetylation. Although it is also difficult to separate in vivo the effects of these drugs on the pulmonary vasculature versus the RV myocardium, our work suggests that their efficacy may depend on the exact stage of the disease in which they are given. For example, HDAC inhibitors (which will promote acetylation of histones, antagonizing the enhanced MED13/NCoR1 axis) may prevent the entry to a dRVH stage (where acetylation is decreased) but they will not reverse a cRVH toward an nRV phenotype because acetylation is already increased in cRVH.

Causing a switch toward a nRV phenotype may be an undesirable effect if the afterload (ie, pulmonary vascular remodeling) is not decreased at the same time. Thus, we think our work may help clarify some of the conflicts in the literature on the role of HDAC inhibitors in RV disease in the future.31,52

Two other studies have reported miRNA changes in RVH models, one in the pulmonary artery banding model and another in a study comparing RVs from the PA banding model with RVs from a PHT model (Sugen-hypoxia). Several miRNAs were reported altered in these studies, whether upregulated miR-34 and -21 and downregulated miR-133 in the first study, or downregulated miR-1 and -133 in the second.53 Although in our unbiased screening studies using miRNA microarrays we found a similar expression pattern in those miRNAs during progression toward failure (Online Figure VII), we failed to identify them as markers of a specific stage of the disease, based on our rigorous definition of RV disease stages. The sensitivity of miRNA levels to precise experimental conditions/models and the variety of filters/controls used to expose significant differences may explain the variability of data among reports, a rather frequent (and perhaps unfortunate) observation in the miRNA literature. We think that the precise definition of the different RVH stages within the same RV model and the vigorous control/filter procedures that we followed strengthen our data and may facilitate efforts by others to replicate our data. Although our study used different models and analysis compared with these 2 studies, we cannot help but observe that miR-1 and miR-133 are also known to be muscle-specific (skeletal and cardiac) and both have been shown to be downregulated in the hearts of mice lacking MeF2.54 miR-1 and miR-133 are coded by bicistronic clusters, and several MeF2 enhancer sequences were identified upstream and intragenic of the miR-1 and 133 loci.54,55 We also note that our data (Online Figure II) are in agreement with another study that reported a downregulation of PGC-1α (a product of MeF2) in RVs from rats 4 weeks after monocrotaline injection.56 There is also evidence of MeF2 activation after focal adhesion kinase activation,57 a well-known mechanotransducer activated during pressure overload stages.58 Thus, MeF2 could be implicated in the regulation of miR-1 and miR-133, as well as PGC-1α in addition to miR-208, perhaps emphasizing the importance of the inhibition of MeF2c during dRVH, featured in our work.

Limitations of our work include the lack of causal evidence for the role of the MeF2c/miR-208/MED13/NCoR1 axis in vivo, although previous studies, as discussed, have confirmed a critical role of the MeF2 axis in RV development and function. Although the changes that we described in the RV tissues from our RVH stages are associative, we think that our mechanistic framework experiments support the importance of our proposed mechanism. Specifically, the effects of miR-208 inhibition causing dilatation of hypertrophied RV aCM, while they did not have an effect on the LV (Figure 7F), are supportive of a causal effect of the miR-208 inhibition for dRVH and RVF.

Although the strength of our study was that the definition of the RV function stages was based on objectively measured hemodynamic criteria, a limitation of may be the potential confounding by temporal factors; that is, changes dependent on time after monocrotaline, not necessarily related to myocardial decompensation, that may have affected the morbidity of our rats. In addition, this work would have been strengthened by the description of similar changes in the MeF2c/miR-208/MED13/NCoR1 axis in human RV tissues. However, the supply of tissues from RVs that have been carefully phenotyped (eg, with hemodynamic, clinical, and imaging data) to categorize them as cRVH versus dRVH is particularly challenging. Nevertheless, the potential for the detection of miR-208 and
TNF-α in the blood of such patients is much easier, and we hope that our work will help ignite biomarker discovery research programs in this direction.

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Disclosures

None.

References

What Is Known?

- The prognosis of pulmonary hypertension patients depends mostly on the capacity of the right ventricle (RV) to compensate and maintain the cardiac output under pressure overload.
- Although there is variability among different patients, the compensatory stage is much shorter in the RV than the left ventricle for unknown reasons, leading to rapid RV failure (RVF).
- It is currently not possible to predict the timing of RVF, and no therapy is available specifically treating the failing RV.

What New Information Does This Article Contribute?

- This work uses a single model of RV pressure overload to study the progression toward RVF and describes a RV-specific molecular pathway driving the transition from a compensated RV to a decompensated, failing RV.
- It describes the essential role of a cardiac-specific microRNA and inflammation as triggers for the transition from a compensating RV toward a decompensating RV, both of which could also be used as blood-based biomarkers.
- To the best of our knowledge, this is the first time that a mechanism for heart failure that is specific for the RV, not the left ventricle, is proposed.

RVF is the most important prognostic factor for both morbidity and mortality in pulmonary hypertension. The transition from a compensated stage to a decompensated stage occurs fast in the RV, and the triggers for this typically rapid RV deterioration remain unknown. Our work supports a potential RV-specific mechanism of heart failure, driven by factors intrinsic to the RV myocardium and independent of the afterload. This is critical for the discovery of much-needed biomarkers to predict RVF and the discovery of yet unavailable RVF-specific therapies. We describe a self-limiting feedback loop involving the downregulation of miR-208 and the subsequent activation of complex mediator of transcription 13/nuclear receptor corepressor 1 that limit a molecular and metabolic program, leading to decompensation. We showed that these mechanisms do not seem to be involved in the left ventricles from the same animals. We also found that inflammation (ie, tumor necrosis factor-α) acts in concert with miR-208 to induce complex mediator of transcription 13/nuclear receptor corepressor 1 activation. This may explain the particularity of failure RVs of pulmonary hypertension patients with high inflammatory burden (eg, scleroderma). Because miR-208 is myocardium-specific, it is a potentially attractive biomarker that along with the levels of serum tumor necrosis factor-α could provide an easy-to-use noninvasive composite biomarker/predictor of RVF in patients.
A miR-208–Mef2 Axis Drives the Decompensation of Right Ventricular Function in Pulmonary Hypertension

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**SUPPLEMENTAL MATERIAL**

*Hemodynamic measurements:* Rats were initially anesthetized with 3–4% isoflurane induction and maintained with 2% during procedures. All rats underwent hemodynamic and echocardiographic studies as previously described\(^1\). Pulmonary arterial pressure, RV systolic pressure and RV end-diastolic pressures were measured using a modified Millar catheter with a curved tip (microtip, 1.4F, Millar Instruments Inc) advanced through the jugular vein in close-chest animals. Traces were recorded using a Power Lab device with Chart software 5.4, ADInstruments, as previously described\(^2,3\). Several parameters were assessed by echocardiography using the Vevo770 imaging system with a 716 (70.5MHz for rats) probe. The cardiac output was calculated after determining the left ventricular outflow tract diameter (LVOT), aortic velocity time integral (AoVTI), and heart rate (HR) using the formula: \( CO = 7.85 \times LVOT^2 \times AoVTI \times HR / 1000 \). The pulmonary artery acceleration time PAAT was measured using the pulse-wave Doppler interrogation of the proximal pulmonary artery in the long-axis view. Tricuspid annular plane systolic excursion (TAPSE), was calculated using the M-mode on the apical four chamber view.

*Terminal Morbidity:* We used “terminal morbidity” as an end point, surrogate for mortality, as per our animal ethics committee: 20% body weight loss plus edema/ascites, plus decreased activity/appetite. As soon as all of these signs were identified in a rat, the end point was reached and the rat was studied with echocardiography and catheterization within 24 hours, immediately followed by euthanasia.

*Histology:* Cardiomyocyte cross-sectional area (CSA) was measured using DIC (Differential Interference Contrast) and DAPI nuclear stained sections of RV free wall tissue. Diameters (d) of nucleated cardiomyocytes (n=3 rats/group, 3 random images per rat, n~50 cardiomyocytes/group) were measured and the CSA was determined using the formula: \( \pi (d/2)^2 \).

Tissue fibrotic grade were determined using a Masson Trichrome staining permitting to visualize fibrotic connective tissues in blue. The Ashcroft scale\(^4\) adopted to cardiac fibrosis was used to determine the grades: grade 0: no lesion; grade 1: minimal perivascular fibrosis; grade 3: moderate perivascular and myocardial fibrosis; grade 5: increased fibrosis with definite damage to the muscle structure, formation of fibrous bands or small fibrous masses; grade 7: severe distortion of structure and large fibrous areas (n=5 images/rat in 5 rats/group, *p<0.05  ** p<0.01 and *** p<0.001).
Confocal microscopy and nuclear translocation assay: Imaging was performed using a Zeiss LSM 510 confocal microscope (Carl Zeiss) as previously described\(^2,3,5\). RV tissues (5µm thick slices) were stained with appropriate primary and secondary antibodies. All experiments included secondary antibody-only staining, which in all presented experiments showed no signal, supporting the specificity of the antibodies used. A minimum of 5 random images were taken for each slide, on at least 5 separate slides per animal, in 5 animals per group. The percentage of positive nuclei was determined using a threshold calculated by the mean fluorescence in the control group. Cells showing a nuclear fluorescent intensity superior to the threshold were considered as positive. \(n=20\) cells/field, \(5\) fields/rat in \(5\) rats per group.

TNF\(\alpha\) ELISA: We used the TNF\(\alpha\) Rat ELISA Kit from ABCAM (ab46070). 100uL of serum samples and standards and a biotinylated monoclonal antibody specific for TNF alpha were incubated for 3h in the wells of the provided microtiter strips. After washing, the enzyme Streptavidin-HRP, that binds the biotinylated antibody was added and incubated for 30min. After washing, a Substrate solution acting on the bound enzyme to induce a colored reaction product was added for 20min. The Stop solution was added and absorbance of each well was read on a spectrophotometer using the 450 nm wavelength.

Western Blots: Tissue or cell protein was isolated using NP40 and immunoblotting were performed using standard protocols as previously described\(^2,3,6\). The films were digitized and quantified using Image J software. Expressions were normalized to actin. Antibodies: Mef2c (Abcam, 1/1000), Mef2a (Abcam, 1/1000), Glut-4 (Santa Cruz, 1/200), PGC-1\(\alpha\) (Abcam, 1/1000), Ac-H3 (Millipore, 1/20000), H3 (Cell Signaling, 1/1000), Ac-H4 (Cell Signaling, 1/1000), H4 (Cell Signaling, 1/1000), Actin (Abcam, 1/1000), Ac-Lys (Cell Signaling, 1/1000).

Quantitative Real Time-Polymerase Chain Reaction: qRT-PCRs were performed as previously described\(^2,3\). Total RNAs (miRNAs and mRNAs) were isolated from tissues using the RNeasy fibrous tissue kit, from cardiomyocytes using the RNeasy micro kit, and from serum using the miRNeasy Serum/plasma kit (Qiagen). miRNAs were reverse transcribed using the relevant primers (Applied Biosystems) and then amplified by qRT-PCR. mRNAs were reverse transcribed and amplified using the one step kit TaqMan probes and reagents from Applied Biosytems. qRT-PCR was performed with the ABI Prism 7900 Sequence Detector (Applied Biosystems); \(\beta2M\) and U6 were used as housekeeping
genes for mRNA and miRNA studies respectively (Applied Biosystems). The relative expression level was determined by the $2^{-\Delta \text{Ct}} \times 100$.

For the detection of miRNAs in the serum, as the concentration of isolated RNA and the relative amount of miRNA are usually low and within the qRT-PCR detection limits, the number of qRT-PCR cycles were increased to 50 (instead of 40). All the amplification curves reached the plateau phase of the reaction, permitting the use of completed linear phases to determine Ct. The mean of Ct values was 27 and 37 for U6 and miR-208 respectively, without amplification of the “no template controls” reactions. The linear dynamic range of our miR-208 assay was studied by constructing a standard curve using mimic-208 at an initial concentration of 200 pmol and 9 10-fold consecutive dilutions. Four replicates were run for each dilution point (average standard deviation = 0.15) and 6 no template controls were run in parallel and were not amplified either. The calculated $R^2$ was 0.99. The linear dynamic range is over 7 logs, from Ct=8 to Ct=40, and the limit of detection is around 0.000002 pmol.

**Tagman Low density arrays TLDA:**

We used an Applied Biosystem TaqMan MicroRNA Arrays A permitting the detection from the Sanger miRBase v10 of mature miRNA of 377 highly characterized, expressed and with high biological importance miRNAs.

Total RNA was isolated from free wall RV using the mirVana miRNA isolation Kit (Ambion, Austin, TX, USA). RNA concentrations were determined using a Nanodrop 8000 spectrometer (Thermo scientific, Wilmington, DE, USA) and quality control was assessed on Agilent Bioanalyser (Agilent Technology, Mississauga, ON, CANADA). We used 12 Rodent MicroRNA pool A Arrays v2.0 to determine the expression of 377miRNAs [including mice miRNAs (mmu), and unique rat miRNAs (rno)]. Amplification data were reduced using several filters (threshold 0.2, detection before cycle 35 and miRNAs altered by at least 2 fold) leading to a working list of 173miRNAs. Raw CTs were then normalized using 3 different normalization procedures: one relative to U6 small nuclear RNA, one relative to the median CT and one relative to the quantile. Hierarchical Clustering Analysis (HCA) was performed using the multiple experiment viewer Software (MeV) using expression values obtained after the normalization relative to the median CT. The HCA shown in Supplementary Fig. 3 represents the Pearson un-centered cluster of miRNAs that are differentially expressed between fRVs and nRVs (non parametric Wilcoxon test, $p<0.05$). We also performed a Principal Component Analysis (PCA) using orthogonal projections to convert observed data into co-ordinates. Co-ordinates that had the largest variance (the largest variability observed between data) were plotted on the Principal component (X axis, called PCA1), and each succeeding component (PCA2=Y axis) separated co-
ordinates with the highest variance possible but uncorrelated to the previous component (that are not separated based on the previous component). We performed an adjusted ANOVA on the fold changes obtained between nRV, cRVH and dRVH (i.e. nRV vs cRVH, nRV vs dRVH and cRVH vs dRVH). We used a Benjamini-Hochberg correction to adjust the individual p-value of each miRNA. The Benjamini-Hochberg correction provides a good balance between discovery of statistically significant miRNAs and false positive occurrences.

**Neonatal cardiomyocytes isolation and culture:** Hearts from neonatal Sprague-Dawley rat pups were isolated and placed in ice-cold phosphate-buffered saline (PBS) solution. Atria were removed, right and left ventricles were separated and minced with scissors. The minced tissue was washed three times in ice-cold PBS and then underwent 3 subsequent cycles of digestion:
1) Digestion in a T-25-cm² tissue culture flask containing 19.5 ml of ice-cold PBS, 0.025% DNase, 0.10% collagenase, and 0.05% trypsin on rotary shaker at 37°C for 20min.
2) Reaction stopped in 20mL of DF20 media, 20% fetal bovine serum FBS
3) Centrifugation at 114g for 1min. The supernatant containing cells were conserved; the pellets were digested further as in step 1.

Supernatants were pooled (except the first supernatant that mainly contains fibroblasts and red blood cells), and centrifuged at 300g for 7 min at 4 °C. Cardiomyocytes were then separated from fibroblasts by differential plating: the resulting pellet was re-suspended in 10ml of DMEM/F12 media (Gibco) supplemented with 5% fetal bovine serum (Sigma), 10% horse serum (Gibco), 50µg/ml gentamicin (Sigma) and incubated at 37°C in a T75 culture flask for 1h. After 60 min the supernatant was removed and placed in another T75 tissue culture flask for an additional hour. After serial plating, cells were centrifuged at 200g for 2min and the resulting pellet was re-suspended in plating media. The cells were plated at a density of 2.0*10⁶ cells/mL in DMEM/F12 media (Gibco) supplemented with 5% fetal bovine serum (Sigma), 50µg/ml gentamicin (Sigma) and 10µmol/L cytosine arabinoside AraC (Sigma) to inhibit fibroblast growth. Immunocytochemistry for myosin heavy chain confirmed that the studied cultured cells were cardiomyocytes.

**Adult rat cardiomyocytes isolation and culture:** Adult Sprague Dawley (100-150g) rats were injected intra-peritoneally with heparin (2U/g) 15min prior to anesthesia; then anesthetized with Euthanyl (Sodium Pentobarbitol 65mg/mL). The chest was opened, the heart exposed and isolated by cutting the trachea and isolating the aorta. The heart was placed in a 60mm dish containing a perfusion buffer (120.4mM NaCl, 14.7mM KCl, 0.6mM KH₂PO₄, 0.6mM Na₂HPO₄, 1.2mM MgSO₄-7H₂O,
10 mM Na-HEPES, 4.6 mM NaHCO₃, 30 mM Taurine, 10 mM BDM and 5.5 mM Glucose, pH=7.0). Extraneous tissues were removed and the heart was cannulated by the aorta and attached on a Langerdorf apparatus. Once cannulated, the heart was perfused with perfusion buffer (37°C and oxygenated) for 4 min at 6 mL/min to flush blood from the vasculature and remove extracellular calcium to stop contractions. After 4 min the solution was switched to the myocyte digestion buffer (100 mL of perfusion buffer containing 2.4 mg/mL of Collagenase Type II). The heart was perfused for 3 min and 45 µL of CaCl₂ 100 mM was added to the Myocyte Digestion Buffer to reintroduce calcium during the remaining digestion time. By perfusing with 100 mL of digestion buffer, the total perfusion time was 15 min or less. Once the digestion was complete, the heart was removed from the cannula, ventricles were separated using fine scissors and placed in 2 different sterile 35 mm dish containing 2.5 mL of myocyte digestion buffer and teased into small pieces. Ventricular pieces were then transferred into 2 T25 flasks containing 5 mL of myocyte digestion buffer each (7.5 mL total) and incubated on rotary shaker at 37°C for 10 min. Ventricular pieces looked at this step very flaccid, requiring little force to dissociate. Under a laminar flow culture hood and using sterile technique, by pipetting several times with a sterile 3 mL transfer pipette (2 mm opening), 2 cell suspensions were obtained and transferred into 15 mL falcons containing 7.5 mL of stopping buffer (perfusion buffer with 10% calf serum and 100 mM CaCl₂). Cell suspensions were then centrifuged 3 min at 20 g. Supernatant containing non-myocytes and dead myocytes were removed and pellets containing viable cardiomyocytes were re-suspended in 10 mL of stopping buffer supplemented with 2 mM of ATP. After centrifugation (3 min at 20 g) pellets were re-suspended in stopping buffer containing 100 µM of CaCl₂. After 2 min in this solution, this last step was repeated with stopping buffer containing 400 µM and 900 µM of CaCl₂. Final pellets were then re-suspended in plating media (M199 media with 10% calf serum, 1% Anti-Anti, 10 mM BDM, 2 mM glutamine, 2 mM ATP, 10 mM Insulin Transferin Selenium). Cardiomyocyte number and shape were assessed using a hemacytometer. Rod-shaped cardiomyocytes were numbered and plated at a concentration of 25000/mL on Laminin (Roche) coated plates. After 1 h, the plating media was removed and replaced by culture media (M199 media with 0.1% BSA, 1% Anti-Anti, 10 mM BDM, 2 mM glutamine, 10 mM Insulin Transferin Selenium).

**Cardiomyocyte 3D reconstruction and shape measurement:** To investigate the shape changes of cardiomyocytes we performed 3D reconstruction of cardiomyocytes using the cellular planes scanned in the Z axis. Cardiomyocytes in culture assume a cross-sectional area (CSA) that resembles a flattened ellipse. Twenty cardiomyocytes per experimental condition were examined to obtain cell length (L) and width (W) in the Y plane and to analyze the CSA diameter (D) by 3D reconstruction and
rotation of the entire reconstructed cell around its central axis. Assuming an elliptical cross section, the area was calculated as CSA=$\pi (W/2)(D/2)$, and cell volume was calculated as $V=CSA*L^2$.

**Cardiomyocyte treatments:** miR-208 pre-miR mimic and anti-miR sequences, as well as negative and inhibitor-negative controls sequences (pooled in the “scramble” group) were purchased from Ambion (Life Technology) and were transfected in neonatal cardiomyocytes at a final concentration of 20nmol/L using siPORT NeoFX tranfection Agent (Ambion). Adenoviruses carrying GFP plus anti-miR-208 or antago-miR-208 were used at a final concentration of 400 MOI ($10^7$pfu/25000 cells) to infect adult cardiomyocytes leading to infection rates of 70-80% in vitro after 72h. We used empty adenoviruses carrying only GFP as a control. Ideally, the control virus should include scrambled-miR in addition to GFP, but these constructs are not commercially available. However, we used transfections with plasmids carrying scramble-miR extensively throughout this paper and did not detect any significant biologic effects of the scramble-miR. Thus while the GFP alone or scramble alone do not have any significant effects, it is unlikely that their combination would.

TNF-α and Phenylephrine (PHE) were dissolved in DMSO and used at a final concentration of 100ng/mL (48h, DMSO<1:10,000) and 10uM (72h, DMSO<1:10,000) respectively.

**Statistics:** Data are presented as mean±SEM. For comparison of 2 means, we used an unpaired t-test. For comparison between more than two means we used a one-way ANOVA followed by a Tukey post hoc-test. For the gene chip miRNA analysis statistical analysis please see the TaqMan analysis section in the supplement. All analyses were performed using SPSS 21 (IBM Corp.). Significance $p<0.05$ is indicated by *, $p<0.01$ by ** and $p<0.001$ by ***.
Online Figure I: A. Representative images and mean data of Pulmonary Artery Acceleration Time (PAAT) measured using the pulse-wave Doppler interrogation of the proximal pulmonary artery in the long-axis view, and Tricuspid Annular Plane Systolic Excursion (TAPSE) measured using the M-mode on an apical four chamber view. B. Serum levels of TNFα measured by ELISA in nRV, cRVH and dRVH animals. TNFα levels are expressed in pg.mL^-1. n=5 rats/group, *p<0.05

Online Figure II: A. Representative confocal images RV tissues immunostained for the detection of GLUT4 in green, co-localizing with Pan-cadherin stained in red (plasma membrane marker). B. Protein levels of Mef2a and PGC1α (a Mef2a target gene) were studied with immunoblots in RV free wall tissues from nRV, early (3d week post MCT) and late (4 weeks post MCT) cRVH and dRVH. Mean data are expressed as the ratio over actin. n=3 rats/group. *p<0.05 ** p<0.01

Online Figure III: Hierarchical clustering analysis showing the profile of expression in all the groups of miRNA differentially expressed between fetal RV (fRV), nRV, cRVH and dRVH. n=3 /group. Colors from green to red (through black) express increased level of expression.

Online Figure IV: A. Principal component analysis assessing the variability of data obtained in the miRNA Taqman low-density arrays, shown in Supplemental Figure 3. The first degree of variability is plotted on the X axis (PCA1) and the second level on the Y axis (PCA2). B. Venn diagram representing the number of miRNAs with significant differential expression between nRV, cRVH and dRVH after an adjusted ANOVA applied on fold-change obtained after U6, median, and quantile standard normalization procedures. 14 miRNAs are found in the overlap in the 3 normalizations. C. List of these 14 miRNAs and their associated p-value (in the normalization by U6). Although we show the p values from one analysis (U6), we achieved significance in all 3 analysis approaches; i.e. we achieved significance in the quantile and the median strategies as well. In gray, we highlight 6 miRNAs with a pattern of expression different to the one described in LVH or not previously described in LVH. D. Levels of the 6 miRNAs of interest measured by miRNA arrays: miR-200b, -338-3p, -328, -155, -92a and -208a. n=3 rats/group; the p-value indicated corresponds to the ANOVA with Benjamini-Hochberg correction under U6 normalization. E. Cluster representing these 6 miRNAs of interest and their relative expression. Colors from green to red (through black) express increased level of expression.
Online Figure V: A. Linear Dynamic Range of our miR-208 detection Taqman assay: Amplification plot showing consecutive 10-fold dilutions of mimic-miR-208 starting at a dose of 200 pmol. Out of the 9 serial dilutions the last one did not amplify. Four replicates were run for each dilution point (average standard deviation = 0.15) and 6 no template controls were run in parallel and were not amplified either. The calculated $R^2$ is 0.99. The linear dynamic range is over 7 logs, from Ct=8 to Ct=40, and the limit of detection is around 0.000002 pmol. B. Human MED13 3’UTR and conserved sites for miR-208 and miR-200b binding as predicted and presented by TargetScan. C-D. miR-200b expression measured by individual qRT-PCR in RNA extracted from RV tissues (C) and correlation between miR-200b expression and the progression of RVH assessed by the RV weight/body weight (D). n=20 rats (n=3 nRV, 8 cRVH and 9 dRVH), *p<0.05.

Online Figure VI: Selection of UCSC genome Browser predicted conserved MED13 (in blue) transcription factors binding sites. Of interest is the presence of NFκB (V$NFKAPPAB_01) binding site.

Online Figure VII: Hierarchical clustering of the expression of miR-1, -133a, 133b and miR-21, -34a, 34b-3p, 34c in nRV, cRVH and dRVH. Colors from green to red (through black) express increased level of expression.

References


Serum levels of TNF-α (pg mL⁻¹)

B

Online Figure I

A

PAAT (ms)

TAPSE (mm)

4 chamber view

RV, LV, RA, LA

Online Figure I

B
Online Figure IV

miRNA | p-value  
--- | ---  
mmu-miR-208b-4395401 | 0.002  
mmu-miR-132-4373143 | 0.003  
mmu-miR-31-4373331 | 0.005  
mmu-miR-208-4373091 | 0.005  
mmu-miR-150-4373127 | 0.005  
mmu-miR-338-3p-4395363 | 0.007  
mmu-miR-200b-4395362 | 0.011  
mmu-miR-21-4373090 | 0.021  
mmu-miR-486-4378096 | 0.038  
mmu-miR-221-4373077 | 0.049  
mmu-miR-222-4395387 | 0.052  
mmu-miR-155-4395701 | 0.068  
mmu-miR-92a-4373013 | 0.088  
mmu-miR-328-4373049 | 0.099  
mmu-miR-200b-4395362 | 0.011  
mmu-miR-208-4373091 | 0.005  
mmu-miR-328-4373049 | 0.007  
mmu-miR-92a-4373013 | 0.099  
mmu-miR-155-4395701 | 0.068  

D

p-value=0.011
miR-200b 2^ΔCt*100 to U6
p-value=0.088
miR-92a 2^ΔCt*100 to U6
p-value=0.099
miR-338-3p 2^ΔCt*100 to U6
p-value=0.007
miR-208 2^ΔCt*100 to U6

E

nRV | cRVH | dRVH
--- | --- | ---  
mmu-miR-338-3p-4395363  | mmu-miR-200b-4395362  | mmu-miR-208-4373091  | mmu-miR-208-4373091  | mmu-miR-328-4373049  | mmu-miR-92a-4373013  | mmu-miR-155-4395701  |
A Amplification Plot

B miR-208 Assay Ct

Human MED13 3' UTR

Conserved sites for miR RNA families broadly conserved among vertebrates

C miR-200b ΔCt *100 to U6

D miR-200b ΔCt *100 to U6
NFκB binding site
Online Figure VII

mmu-miR-21-4373036
mmu-miR-34a-4395168
mmu-miR-34b-3p-4395748
mmu-miR-34c-4373090

mmu-miR-1-4395333
mmu-miR-133b-4395358
mmu-miR-33a-4395375