A Novel circRNA Highlights a Path to Cardiac Hypertrophy in Spontaneously Hypertensive Rats.

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Abstract

Background

Cardiac hypertrophy can be considered a maladaptive response which in many cases results in heart failure and sudden death. Genetic predisposition to cardiac hypertrophy is ill-defined, but current research has given credence to a role of non-protein-coding RNAs in the development of cardiac hypertrophy.

Results

We used microarrays, RNA-Seq and quantitative genetics with the spontaneously hypertensive, SHR (SHR/OlaIpcv) rat strain, the normotensive Brown Norway (BN-Lx/Cub) rat strain, and a recombinant inbred (RI) panel of rats (HXB/BXH) derived from these strains to examine the areas of the genome associated with cardiac hypertrophy. We identified circular (circ) RNAs coded within these areas. Of the 122 differentially expressed circRNAs in left ventricle of SHR and BN-Lx rats, three were transcribed from areas corresponding to the QTLs we identified for cardiac hypertrophy using the HXB/BXH rat panel. We then identified microRNAs which are “sponged” by the circ RNAs and the mRNAs which are destabilized by the microRNAs. 265 miRNAs could be identified as targets for the three circRNAs. We focused on the four miRNAs that were also differentially expressed between SHR and BN-Lx rats. One of the miRNAs (Mir-210-5p) was a target of the differentially expressed circ H2afy and circ H2afy was located within the QTL on Chr 17 (genome wide p-value = 0.011). Mir-210-5p has a binding site on the 3’ UTR of DR6, which is differentially expressed and in turn controls the expression level of NFκB. NFκB is an important component of the oxidative stress response leading to hypertrophy.

Conclusions

Our work identified a novel circRNA that may be the mediator of a cascade of events that influence a cardiac hypertrophy phenotype. The circRNA and miRNA which we identified may become useful as markers of the risk for cardiac hypertrophy.

Introduction

Approximately 6.2 million adults in the United States suffer from heart failure, and, in 2018, more than 379,000 deaths were attributed to heart failure(1). An important risk factor contributing to heart failure is hypertension, and left ventricular hypertrophy (LV hypertrophy) is a morphologic concomitant to prolonged hypertension. In humans, the prevalence of LV hypertrophy increases with the severity of the hypertensive disease and up to 50% of hypertensive subjects demonstrate LV hypertrophy(2-4). In turn, LV hypertrophy leads to myocardial infarction, arrhythmias and heart failure resulting in sudden death.

Numerous factors have been shown to contribute to the development of hypertension and LV hypertrophy including genetically determined components(5-8). However, the correlation between blood pressure and
LV mass in humans is at best modest and thus the environmental and genetic factors contributing to hypertension and LV hypertrophy must not be identical.

The use of animal models to disengage components of environmental and genetic contributors to a phenotype is of benefit when studying complex traits such as hypertension and LV hypertrophy. The ability to raise animals of different genetic backgrounds in similar (if not identical) environments allows for isolation and magnification of the genetic components of the phenotype. Genetic selection and inbreeding have produced several strains of rats that exhibit hypertensive and LV hypertrophy traits that are significantly influenced by their genome. Of these, the spontaneously hypertensive rat (SHR) has gained the greatest popularity for studying cardiovascular disease. Several colonies of SHR rats are available for study, but the choice of comparator animals, as well as the source of the SHR stock, is an important component of the acquired results. In our work, we chose an SHR strain (SHR/OlaLpcv) that was one of the progenitors of a recombinant inbred (RI) panel of rats of which the other progenitor strain was the BN-Lx (BN-Lx/Cub). The HXB/BXH RI panel allows for use of quantitative genetic analysis (quantitative trait locus [QTL] analysis) of phenotypic traits to identify the genomic regions that can influence the magnitude of those traits. This panel has been used extensively to study a number of cardiovascular traits.

In using quantitative genetic techniques, much attention has been paid to the protein coding genes that reside in a QTL region as possible contributors to the trait being studied. While DNA variants that affect the function of protein-coding genes can affect the trait of interest, current knowledge has exposed the fact that a multitude of non-coding RNAs (ncRNA) can be transcribed from DNA. These non-protein coding elements have major effects on transcriptional, splicing, and translational processes, as well as protein-coding RNA stability. Inclusion of non-coding RNAs in the interpretation of a QTL analysis has not yet become popular, however, we have previously demonstrated that the element responsible for a QTL may be a long ncRNA. One could posit that differentially expressed RNA, including ncRNA, measured in two strains of isogenic animals that differ significantly in a trait of interest, would be more likely to contribute to that trait if this RNA were expressed from a genetic region defined by a QTL for the trait being studied.

A novel species of RNA has recently come to the attention of the research community. CircRNA (circular RNA) is generated by post transcriptional processing that produces back splicing of a pre-mRNA, where the 5’-terminus of a pre-mRNA upstream exon is spliced with the 3’-terminus of a downstream exon to form a covalently closed continuous loop which is resistant to hydrolysis by exonucleases. These circRNAs can contain both introns and exons, and can play a number of regulatory roles in vivo. One of the most studied roles is that of circRNAs acting as “sponges” to sequester miRNAs, thus reducing miRNA availability for transcription/translation activities.

The role of circRNAs in cardiac hypertrophy and heart failure has started to gain attention and recent studies have suggested a role for two circRNAs expressed in heart tissue in mouse models of
hypertrophy. HRCR and circSLC8A1-1 have been found to act as sponges for miR-223-5p and miR-133, respectively, and thus regulate hypertrophy(15). Because of the relative stability of circRNA when released into the circulation, measurement of circRNA in blood has also been proposed for use as a biomarker for diagnosing and measuring the severity of various forms of heart disease in humans, and three circRNAs have been reported as potential biomarkers of hypertrophic cardiomyopathy(17).

To understand whether circRNA represents a genetic factor that contributes to cardiac hypertrophy and potentially heart failure, we performed studies using the SHR and BN-Lx rats and the HXB/BXH RI panel. In these studies, we combined identification and quantification of circRNAs in the left ventricle of the rat and mapping of the DNA locations giving rise to the circRNAs. The overlap in location of the QTL for LV hypertrophy and the coding sites of differentially expressed circRNAs provided a reason to consider a functional relationship between the circRNA and LV hypertrophy. We then used informatics techniques to generate information on the relationships of the circRNA, the miRNA controlled by the circRNA, and the mRNA that can be influenced by the cognate miRNA.

Results

QTL Marker Set

In total, 20,283 SNPs were originally included in the STAR genetic marker set (18). Of these, 19,391 SNPs corresponded to probes that uniquely and perfectly aligned to the rn6 version of the rat genome. Removing SNPs that did not differ between the parental strains (BN-Lx and SHR) resulted in 13,103 remaining SNPs. Removing SNPs containing a heterozygous genotype call in either or both parental strains, and SNPs for which more than 5% of the RI strains had a missing or heterozygous call, resulted in 10,582 SNPs. Finally, SNPs with large genetic distances compared to physical distances, as well as double recombinant SNPs, were removed, resulting in 10,486 retained SNPs. From these SNPs, 1,478 unique strain distribution patterns (SDPs) for the HXB/BXH RI panel of 30 strains were identified.

Left Ventricle Weight QTL

Left ventricle weight ranged from 136.3 to 212.9 mg per 100 g of body weight (Fig. 1). The left ventricle weight QTL analysis resulted in 1 significant peak (genome-wide p-value = 0.011) located on chromosome 17 with a 1.5 LOD support interval from 5.4 to 15.3 Mb, and 1 suggestive peak (genome-wide p-value = 0.140) located on chromosome 8 with a 1.5 LOD support interval from 113.6 to 129.5 Mb (Fig. 2A). Pravenec, et al. (19) previously identified the QTL on chromosome 17, but the chromosome 8 peak is a new finding. As mentioned in Pravenec, et al. (19), the strains with higher left ventricle weight have the BN-Lx genotype at the chromosome 17 QTL, while for the chromosome 8 QTL the strains with higher left ventricle weight have the SHR genotype (Additional File 1, Supplementary Fig. 1).

To determine if these QTLs were unique for left ventricle weight, rather than overlapping the QTLs for systolic blood pressure (SBP) and/or diastolic blood pressure (DBP), QTLs for SBP and DBP were also
mapped in the same population of rats. SBP and DBP data were available for individual rats, which enabled the calculation of a broad sense heritability (DBP = 35.8% and SBP = 56.1%). In general, for both SBP and DBP, the parental strains (BN-Lx and SHR) represented the extremes of the distribution of strain means (Additional File 1, Supplementary Fig. 2). The two blood pressure phenotypes are significantly correlated across strains (correlation coefficient = 0.68; p-value = 2×10^−5), but neither measure of blood pressure was correlated with relative left ventricle weight (Additional File 1, Supplementary Table 1). The QTLs for SBP and DBP replicated some previously identified QTLs for these traits (19), but the significant/suggestive QTLs for SBP and DBP did not overlap either of the QTLs for left ventricle weight (Additional File 1, Supplementary Fig. 3). SBP had 2 genome-wide significant and 2 suggestive peaks, while DBP had 2 suggestive peaks.

Circular Rna Candidates

Of the 12,134 circRNAs tested for differential expression between BN-Lx and SHR strains, 122 were differentially expressed (FDR < 0.05). Of these, 67 were up-regulated in SHR and 55 were down-regulated in SHR by comparison to BN-Lx (Fig. 3). The physical locations of three differentially expressed circRNAs fell within the 1.5 LOD support interval of the suggestive or significant QTLs for relative left ventricle weight. Two differentially expressed circRNAs were within the 1.5 LOD support interval for the QTL on chromosome 17 and 1 was within the 1.5 LOD support interval of the QTL on chromosome 8 (Table 1 and Fig. 2B).

Small Rna Sequencing

The small RNA-Seq dataset (ribosomal RNA-depleted total RNA fragments which passed a 200 base pair size filtration step), contained over 299 million read fragments across the 8 left ventricle samples. These reads were aligned to 404 miRNA features (determined by MirDeep) including 258 which were previously annotated. Over 73 million reads aligned to precursor miRNA sequences and/or to mature miRNA sequences. Of the 404 miRNAs features considered, 204 were detected above background in left ventricle.

Microrna Candidates

There were 265 unique miRNA targets for the 3 candidate circRNAs. All target miRNAs expressed above background and their corresponding candidate circRNAs are shown in Additional File 1, Supplementary Table 2. In our miRNA expression dataset, 30 miRNA targets were detected above background. Of these 30, 4 (Mir-139-5p, Mir-146b-5p, Mir-196c-5p, and Mir-210-5p) were differentially expressed (p-value < 0.05) between BN-Lx and SHR strains (Table 2).

Three of these are targets for the same circRNA, rno_circRNA_015936 (probe ID ASCRPR013891), aka circZcwpw2, which is down-regulated in SHR and physically located in the Chr 8 QTL for relative left
ventricle weight (Table 2). Two of these miRNAs (Mir-146b-5p and Mir-196c-5p) are up-regulated in SHR, which would follow the expectation based on our current working hypothesis, i.e., when expression of the circRNA is decreased, the level of the miRNA is increased. These miRNAs are considered “tier 1” candidates. The other miRNA (Mir-139-5p) is up-regulated in BN-Lx and is considered a “tier 2” candidate, since it does not follow the direction of our hypothesized relationship. The fourth differentially expressed miRNA (Mir-210-5p) is a target for mro_circRNA_006400 (probe ID ASCPRR002506), aka circH2afy, which is located in the QTL on Chr 17. The differential expression of this miRNA is in the opposite direction from the differential expression of the circRNA, and therefore this miRNA is considered a “tier 1” candidate. The statistical information reported on the 4 candidate miRNAs is shown in Table 2, and Additional File 1, Supplementary Fig. 4 illustrates circH2afy with binding sites for the target miRNA.

**Total Rna Sequencing**

For the total RNA-Seq data, over 1.5 billion read fragments (approximately 790 million paired-end reads) derived from ribosomal RNA-depleted total RNA were generated across the 8 left ventricle samples. There were 21,260 Ensembl genes that were detected above background in left ventricle.

**Messenger Rna Candidates**

MultiMiR identified 48 unique mRNA targets for the 4 candidate miRNAs described above. Of these 48 mRNAs, 10 were differentially expressed between BN-Lx and SHR (FDR < 0.05). Eight of these were targets for Mir-139-5p, which was the “tier 2” candidate miRNA. One mRNA was a target for Mir-196c-5p, and the final mRNA was a target for Mir-210-5p. All 10 mRNA targets and their respective differential expression results are reported in Additional File 1, Supplementary Table 3. Only 1 mRNA (Tnfrsf21) was finally considered a “tier 1” mRNA candidate (Additional File 1, Supplementary Fig. 5). This designation was based on the differential expression of circH2afy, which targets Mir-210-5p (down-regulated in SHR), which in turns targets Tnfrsf21, which is up-regulated in SHR (Fig. 4).

**Discussion**

Heart failure continues to be one of the major causes of death in the U.S. and countries around the world (1). A number of pathophysiologic pathways are associated with and culminate in heart failure, including cardiac hypertrophy (particularly LV hypertrophy), inflammation, regeneration, and angiogenesis (20, 21). During the last decade, a number of RNAs which do not code functional proteins (i.e., non-coding(nc) RNAs) have been implicated in the etiology of cardiac hypertrophy (22). These ncRNAs include miRNAs (23) and long ncRNAs, and the long ncRNA category includes circular (circ)RNAs (15, 17). A canonical
regulatory pathway has been proposed, where circRNA “sponges” and diminishes the concentration of miRNA, and in turn produces an increase in the mRNA which is a demonstrated target of the miRNA (24).

The pathophysiological role of circRNA in cardiac hypertrophy is currently being recognized and the primary mechanism for the circRNA effects has been proposed to be via the canonical regulatory pathway (15, 17, 22, 25). Numerous instances of miRNA influenced cardiac hypertrophy have been noted (22), and the identified miRNA has led investigators to focus on the protein coding genes (through mRNA levels) which would be the entities mediating the terminal events of the pathologic pathway to cardiac hypertrophy.

While these studies are important, they are generally based on identification of differences in expression of miRNAs in models of cardiac hypertrophy (26). Such changes could contribute to development of cardiac hypertrophy, or could represent a response to this pathological state. Our goal was to investigate genetically-based pathways by which non-coding RNAs can be associated with cardiac hypertrophy.

The HXB/BXH recombinant inbred panel which was derived from the initial mating of BN-Lx and SHR strains (11) has been used extensively to study cardiovascular traits. We performed QTL analyses of blood pressure and relative left ventricle weight using a newly established set of genomic markers. The outcome of the blood pressure QTL analysis demonstrated that the QTLs for systolic-blood pressure and diastolic blood pressure were located on different segments of the rat genome, although there was a significant correlation between the phenotypes of systolic and diastolic blood pressure across the 30 RI strains tested.

As mentioned earlier, prior work using the HXB/BXH RI panel generated QTL results for systolic blood pressure similar to those that were generated in the current work, but in both past and present studies, QTL analysis for diastolic blood pressure did not generate any significant QTLs. The reason for this finding may well be the modest number of strains and few loci with a substantial impact on the variance for diastolic blood pressure. On the other hand, if one ascribes to Cheverud's conjecture (27) on the relationship between phenotypic and genetic variance, one might assume that the high phenotypic correlation between systolic and diastolic blood pressure would indicate that there is a genetic relationship between these two traits.

There was, however, no correlation between the phenotypes of LV weight with either systolic or diastolic blood pressure. This is reminiscent of data obtained with human subjects where only a modest correlation between systolic blood pressure and cardiac hypertrophy has been reported across populations (2). However, a Mendelian analysis, using a population with extreme genetic loading based on markers previously associated with systolic blood pressure, indicated that the genetic loading for systolic blood pressure also significantly exacerbates LV hypertrophy (28). These analyses were performed by utilizing 300 individuals representing the extremes of a genetic risk score based on 107 previously established markers for systolic blood pressure (150 individuals with the highest and 150 with the lowest scores). The larger impact of using genetic markers rather than measured blood pressure on
the phenotype of LV hypertrophy may be due to the effect size generated by the combined effect of 107 predisposing or protective genotypes.

In our study, the QTLs for LV hypertrophy did not correspond to the QTLs that we and others have calculated for systolic or diastolic blood pressure using the HXB/BXH RI panel. The lack of a phenotypic correlation between blood pressure and LV weight, as well as different QTL locations for these traits, indicate that different genetic factors may be contributing to these events. Hypertension can be considered as a causative factor for LV hypertrophy via increased myocardial stress (3), and thus can be construed as an “environmental” cause (trigger) of hypertrophy, even though the genetic factors predisposing hypertension and LV hypertrophy may differ. The dissociation of the genetic contributors to blood pressure and LV weight, however, could also be a consequence of the age of the rats at which we obtained the data for QTL analysis (i.e., approximately 12 weeks of age). A detailed study of progression of hypertension and LV hypertrophy in SHR rats (29) noted that hypertension was evident by 6–8 weeks of age while LV hypertrophy became significant at 12–20 weeks of age when compared to normotensive WKY rats (9, 29). We also found that the LV weight to body weight ratio increased between 12 and 18 weeks of age in the SHR rats used for our studies (mg LV weight/100g body weight: 12 weeks: 185.6 ± 11.8; 18 weeks, 201.5 ± 15.1, mean ± SD, n = 8, P < 0.05, t-test). Thus, blood pressure changes occur earlier than LV hypertrophy in the SHR rats, again suggesting that hypertension may be a genetically independent, but etiologically connected factor contributing to LV hypertrophy.

To continue the exploration of genetic factors contributing to LV hypertrophy, and particularly quantitative differences in circRNAs, we identified three circRNAs that were differentially expressed in the left ventricle between SHR and BN-Lx strains and were located within QTLs for LV hypertrophy. These circRNAs were derived from the gene loci for Zcwpw2 on Chr 8 and for the H2afy gene and Agtpbp1 gene on Chr 17. The human homologues of these genes are located on chromosome 3, chromosome 5, and chromosome 9, respectively. These circRNAs had binding sites for 46 miRNAs, but only 4 miRNAs of the 46 were present at significantly different levels (FDR < 0.05) in left ventricular tissue of the SHR versus the BN-Lx rats. Three of these miRNAs (Mir-139-5p, Mir-146b-5p, Mir-196c-5p) were associated with a single circRNA (circZcwpw2) which was produced from the locus of the QTL on Chr 8. The fact that this QTL did not reach genome-wide significance led us to consider circZcwpw2 a provisional candidate for contributing to LV hypertrophy. In an earlier study with the HXB/BXH RI rats on QTLs for LV hypertrophy (19), the Chr 8 locus was not evident, and we postulate that our use of a more extensive panel of markers allowed for the emergence of this QTL. We will, however, postpone a further discussion of the miRNAs and mRNAs influenced by circZcwpw2 until more evidence is available to support the existence of the QTL on Chr 8.

CircH2afy, the coding region for which is located within the QTL on Chr 17, has not been previously well annotated, but can be found in the human, as well as the rat, transcriptome. The quantity of circH2afy was higher in the SHR strain, but the marker associated with the SHR genotype at the Chr 17 QTL peak was associated with a phenotype of lower LV weight/body weight ratio. An interpretation of these results would be that the QTL region on Chr 17 produces a protective (lower LV weight) effect. circH2afy contains 2 binding sites for Mir-210-5p. If circH2afy acts as a sponge sequestering Mir-210-5p, the higher
expression of the circRNA in the SHR left ventricle, compared to the BN-Lx, would be expected to result in lower levels of Mir-210-5p in the SHR left ventricle, which is what was observed. It is of interest that Mir-210-5p is one of many microRNAs found to be up-regulated in a model of cardiac hypertrophy in engineered heart tissue (26). In this study, lowering the level of upregulated miRNAs reduced the pathological hypertrophy. The lower level of Mir-210-5p in SHR left ventricle, compared to the normotensive BN-Lx strain, could reflect a protective effect in the SHR strain.

When we examined which mRNAs expressed in the LV had binding sites for Mir-210-5p, we found that Tnfrs21 (Death Receptor 6 (DR6)) had such a binding site in its 3’ UTR (Additional File 1, Supplementary Fig. 5) and was differentially expressed in the left ventricle between SHR and BN-Lx rats (higher levels in the SHR rats). Prior work had also demonstrated that Mir-210-5p could control the levels of Tnfrs21 (30). Mir-210-5p is induced by hypoxia, via hypoxia-inducing factor, and is also induced by NF-κB (31, 32). Higher levels of Tnfrs21 can also lead to activation of NF-κB, generating a further interaction between Mir-210-5p and Tnfrs21 (30, 33). In addition, the activation of NF-κB by Tnfrs21 can potentially affect pathways modulating apoptosis (30). Activation of the NF-κB pathway can produce both pro-apoptotic and anti-apoptotic effects, depending on the stimulus and cellular environment (34). Interestingly, activation of the NF-κB signaling pathway is sufficient to suppress apoptosis in ventricular myocytes under hypoxic conditions (35–38).

The apoptotic pathway can play an important role in development of pathological LV hypertrophy (34). It has become clear that, although the mechanical stress of pressure overload is one of the factors contributing significantly to ventricular hypertrophy, a plethora of other factors contribute to the development of this condition (3). These factors include both neurohumoral and genetic components. The influence of genetic factors on development of cardiac hypertrophy was well described as early as 1991 (39). The initial stages of hypertrophy can reflect an adaptive increase in cardiomyocyte growth to help maintain ejection fraction, but continued hemodynamic overload induces what is referred to as cardiac remodeling. The remodeling process involves lengthening of myocytes, the rearrangement of myocytes, cell death and infiltration of fibrous tissue into the ventricular wall (40). The remodeling process depends on apoptotic and non-apoptotic cardiomyocyte death and can progress to heart failure (40). Apoptosis can be initiated by factors such as cytokines acting through their receptors and recruiting Fas-Associated Death Domain-containing proteins (e.g., DR6), and by genetic factors which can be intrinsically activated through transcriptional, post-transcriptional, and post-translational control mechanisms (40). These mechanisms may involve miRNAs and circRNAs.

It needs to be noted that another strong candidate for regulation of LV hypertrophy has been proposed to reside in the genomic interval defining the QTL on Chr 17. Petretto et al. (41) proposed osteoglycin (Ogn) RNA and protein expression to be associated with LV hypertrophy in rats and humans. Their conclusions were in large part based on RNA expression values derived by use of Affymetrix RAE230 v2.0 hybridization arrays. They speculated on the presence of two isoforms of Ogn RNA differing in the sequence of the 3’UTR, with only one isoform being correlated with measures of LV hypertrophy. Our sequencing data substantiates the presence of two 3’UTR variants of Ogn. Our results (Additional File 1,
Supplementary Fig. 6) indicate that the short 3'UTR isoform is expressed at higher levels in the BN-Lx rat ventricle, compared to SHR, and the long form of the Ogn 3'UTR is contained in the transcript expressed at higher levels in the SHR left ventricle. At the time of publication of the work of Petretto et al. (41), the importance of the 3'UTR for miRNA binding and mRNA translation was not fully appreciated, but since then Ogn protein expression has been shown to be related to Mir-1305 binding to Ogn RNA and to control of Mir-1305 levels by a circRNA hsa_circ_0076906 (42).

In all, our study generated the identification of a novel genetically-mediated pathway initiated by circH2afy and leading to modulation of NF-κB activity. Suppression of apoptosis by the NF-κB pathway may well be one factor associated with protection of myocytes from cell death under hypoxic conditions induced by ventricular overload in the SHR animals, which develop hypertension early in their lifespan. However, our findings on the association of circH2afy with LV hypertrophy do not exclude influence from other genes which are located within the Chr 17 QTL interval.

In addition to tracing the etiology of hypertrophy to a control element (i.e., circRNA) that would be expected to have pleiotropic effects, we also considered that a circRNA could be monitored as a circulating marker for identifying predisposition, severity, or prognosis of at least one element leading to heart failure. Several miRNAs have already been explored as markers for extent of left ventricular function in subjects who have experienced an acute myocardial infarction (17) and Mir-210-5p may provide diagnostic information for ascertaining the presence of cardiovascular remodeling. The question of whether the novel circRNA identified in our studies can also function as a diagnostic marker would require some substantial research being initiated by investigations of whether it can be measured in blood or plasma of rats or humans.

**Conclusions**

Using a quantitative genetic approach, we identified a genetically-mediated pathway that implicates a circular RNA, its microRNA target, and the mRNA target of the microRNA, in the processes contributing to left ventricle hypertrophy. The function of the identified non-coding RNAs and protein coding mRNA influence the NF-κB pathway and apoptotic processing involved in cardiac hypertrophy that can lead to heart failure. The non-coding RNAs (miRNA and circRNA) may be candidate biomarkers for the risk of cardiac failure.

**Methods**

**Overview**
In addition to the description below, a detailed flowchart of the methods used is available as Additional File 1, Supplementary Fig. 7. Our first goal was to examine relative left ventricle weight and blood pressure to assess genetic regulation of these traits via quantitative trait loci (QTL) analysis in the HXB/BXH RI panel. We then measured circRNA expression levels in left ventricle and identified circRNA which 1) are differentially expressed between BN-Lx and SHR strains (progenitors of the HXB/BXH RI panel) and 2) arise from the DNA regions located within the QTLs for the traits of interest. As circRNAs are commonly described as miRNA sponges, we identified miRNAs potentially targeted by the candidate circRNAs and determined which of these miRNAs are differentially expressed in left ventricle between SHR and BN-Lx strains and also follow the expected expression pattern, i.e., if the circRNA is expressed at higher levels in one strain, we would expect less miRNA to be available in that strain. Subsequently, we identified putative mRNA targets of the differentially expressed miRNAs that were also differentially expressed in left ventricle and followed the expected direction, i.e., if there is a higher level of miRNA in a specific strain, we would expect a lower amount of target mRNA in that same strain.

**Relative Left Ventricle Weight And Blood Pressure**

Data on relative left ventricle weight (mg per 100 g of body weight) in 12 week old male rats from the HXB/BXH RI panel was retrieved from GeneNetwork (http://genenetwork.org;/ HRP_10022). Additional experimental details about this phenotype are available through (19). Information on the recombinant inbred strains was available through GeneNetwork as strain means, and individual rat values from the BN-Lx and SHR (progenitor strains of the HXB/BXH panel) were provided by Dr. Michal Pravenec.

Telemetric daytime blood pressure (both systolic and diastolic blood pressure, SBP and DBP) was measured every day for 1 week on 226 rats in total (approximately 7 rats/strain). Data were obtained from the HXB/BXH RI strains as well as the BN-Lx and SHR strains. These data have been previously published in Pravenec et al. (19). For our study, data were screened for extreme outliers (+/- 3 SD from both rat and strain means). Each individual rat was summarized by its mean blood pressure over the 7 days, and for further analyses each strain was summarized by taking the means of the individual rat summaries.

30 HXB/BXH strains had data available for all three phenotypes: relative left ventricle weight, systolic blood pressure and diastolic blood pressure. Strain means and a Pearson correlation coefficient were used to measure genetic correlations among these three traits.

**Dna Sequence Variants**

The genetic marker set used for QTL analyses was previously calculated and reported by the STAR Consortium (18). Many of the adjacent markers (SNPs) display the same genotype pattern among all the RI strains (i.e., no recombination events for any strain between SNPs), which would result in the same level of statistical significance (p-value) for all of these SNPs when performing QTL analysis. Therefore,
we reduced the number of association tests, without losing information, by identifying unique strain distribution patterns (SDPs, i.e., the genotypes for all strains at a particular SNP) for the 30 relevant strains in the HXB/BXH RI panel.

Qtl Analyses

We identified quantitative trait loci (QTL) for relative left ventricle weight and blood pressure (both systolic and diastolic) using the marker set described above. All QTLs were calculated using strain means and marker regression. Genome-wide p-values were determined using 1,000 permutations, and a 1.5 LOD support interval was estimated for significant (genome-wide p-value < 0.05) and suggestive (genome-wide p-value < 0.63 (43)) QTLs. All QTL analyses including permutations and support intervals were calculated using the \textit{R/qtl} package (44) (v1.46-2) in \textit{R} (45) (v4.0.2).

Rna Expression Levels

Circular RNA Arrays (circRNA data source)

Total RNA was isolated from samples of the left ventricle of the heart of 4 male SHR and 4 male BN-Lx 12-week-old rats using QIAzol (Qiagen, Valencia, CA, USA). The RNAeasy Plus Universal Midi Kit (Qiagen) was used to separate long (> 200 nt) and short (< 200 nt, miRNA-enriched) fractions. The long RNA fraction was purified using the RNeasy Mini Kit (Qiagen). The long RNA fraction underwent RNAse R treatment by Arraystar, Inc (Rockville, MD) to enrich for circRNAs, which were amplified and transcribed into fluorescent cRNA utilizing a random priming method (Arraystar Super RNA Labeling Kit; Arraystar). The labeled cRNAs were hybridized onto the Arraystar Rat circRNA Array (8x15K, Arraystar) and scanned by the Agilent Scanner G2505C. Agilent Feature Extraction software (version 11.0.1.1) was used to analyze acquired array images. Quantile normalization and subsequent data processing were performed using the \textit{limma} package (46) in \textit{R}.

Small RNA Sequencing (miRNA data source)

Illumina 1x50bp raw reads from the short RNA fractions purified as described above from the left ventricles of the BN-Lx and SHR strains were trimmed for adapter sequence and read quality with CutAdapt (47)(v.1.9.1), removing reads with any lengths shorter than 18 nucleotides. Reads that aligned to rRNA using Bowtie2 (48)(v2.3) were removed. Next, reads were aligned to a custom precursor miRNA database generated for the PhenoGen website using STAR (49)(v2.7.3a). Only reads that aligned to precursor miRNAs were subsequently aligned with STAR (49) to do a more precise alignment to a similar custom mature miRNA database generated for PhenoGen. The median nucleotide read depth in a sample was used to represent the expression level of mature miRNA in statistical analyses. A filter for detection above background was applied, where at least 50% of the heart samples were required to have a median
read depth of 1 or more for a particular miRNA in order for the mature miRNA to be retained in the dataset for analysis.

The custom precursor and custom mature miRNA databases for PhenoGen were generated using miRNA predictions from MirDeep (50), that were based on small RNA expression data across three different tissues (heart, brain, and liver) and annotated miRNAs (Ensembl and RefSeq). The custom PhenoGen miRNA databases contain strain-specific miRNA sequences for the SHR and BN-Lx strains and are available through the PhenoGen website (http://phenogen.org). Detailed methods on the development of these custom databases are available in Additional File 2.

**Total RNA Sequencing (mRNA data source)**

Sequencing libraries for the long RNA fraction were constructed using the Illumina TruSeq Stranded Total RNA Sample Preparation Kit with Ribo-Zero ribosomal RNA reduction chemistry (Illumina, San Diego, CA) in accordance with the manufacturer's instructions. Samples were sequenced (2X100 paired end reads) on an Illumina HiSeq2000 with 2 samples per lane.

 Reads were quantified on the gene level using the RNA-Seq Expectation Maximization (RSEM v1.2.31) algorithm (51) and the Ensembl v96 rat transcriptome (consisting of 32,377 genes) as the reference for quantitation. All subsequent analyses were performed on the RSEM expected counts. A filter for detection above background was applied that required at least 2/3 of the samples to have 1 count or more for a gene to be retained in the analysis.

**Differential Expression**

**Circular RNA Arrays**

The MAS5 algorithm (52) was implemented in the GeneSpring software to evaluate whether expression level measurements for circRNA were above background (present, absent or marginal). circRNA were included for statistical analysis if at least 50% of the samples were classified as either present or marginal. For determining differential expression, a t-test was performed between the BN-Lx and SHR groups at each probe. A correction for multiple testing was applied using a False Discovery Rate (FDR) (53). Differential expression was considered to be statistically significant when FDR < 0.05. The location of the DNA sequences coding for the circRNA transcripts was investigated to ascertain whether the DNA sequence was located within the trait-related QTL regions.

**Small and Total RNA Sequencing**

A negative binomial generalized linear model from the DESeq2 package (v 1.30.0; (54)) in R was used to determine differential expression between BN-Lx and SHR strains for both the mature miRNA and the
protein-coding transcripts. Although all mature miRNAs and all protein-coding transcripts (mRNA) were included in the differential expression analysis to stabilize the dispersion estimates, only mature miRNAs that were targets of differentially expressed circRNAs, and protein-coding transcripts that had binding sites for these miRNAs, were considered as candidates contributing to LV hypertrophy. Therefore, multiple testing was not performed and an unadjusted p-value < 0.05 was considered statistically significant for differential expression.

Identification Of Mirnas Targeted By Circrnas

Three approaches were used to identify miRNAs targeted by the circRNAs which were differentially expressed and located within the trait QTL regions. First, Arraystar applied an in-house software to predict miRNA targets based on prior versions of miRANDA (55, 56) and TargetScan toolsets (57, 58). Second, we utilized mirDB (59, 60) on full length circRNA sequences provided by Arraystar to predict miRNA candidates with a score of 50 or higher. Finally, we used the TargetScan 7.0 perl script to predict additional miRNAs that are potential targets of the circRNAs of interest. We modified full length circRNA sequences by substituting U’s for T’s and created a database of miRNA seed sequences from the full miRBase fasta file using a custom python script. The identified miRNAs were then required to be differentially expressed between the SHR and BN-Lx strains, in order to be considered candidate miRNAs.

Identification Of Mrna Targets

Target mRNAs of the candidate miRNAs were identified using the multiMiR package in R (v 1.6.0, (61)) utilizing database version 2.3.0). In brief, this software searches multiple databases to identify mRNA targets for miRNAs provided to the program. We used default search parameters and required an mRNA target to be identified in 2 or more databases. To be considered as “candidates”, the identified mRNAs had to be differentially expressed between the SHR and BN-Lx strains.

Declarations

Ethics Approval:

All animal experiments were in agreement with the Animal Protection Law of the Czech Republic and were approved by the Ethics Committee of the Institute of Physiology of the Czech Academy of Sciences, Prague.

Consent for Publication:

Yes

Availability of Data and Materials
BN-Lx and SHR data including blood pressure strain means, normalized circRNA array data, miRNA RNASEq read counts, mRNA read counts, custom precursor and custom mature miRNA databases, and the genetic marker data set are available for download at https://phenogen.org/publications.

**Competing Interests:**

The authors declare that they have no competing interests.

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**Author's contributions:**

B.T. Conceived and designed research; analyzed data and interpreted results; edited and revised manuscript.

J.M. Collected and analyzed data; prepared figures and tables; drafted manuscript.

L.A.V. Analyzed data, prepared tables and figures

L.M.S. Analyzed data; edited tables and figures; edited manuscript

P.L.H. Analyzed data and interpreted results; edited manuscript.

M.P. Provided data; edited and approved manuscript.

S.M. Analyzed data; drafted manuscript and prepared figures and tables.

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### Tables

#### Table 1: Differentially expressed circRNAs physically located within a relative left ventricle weight QTL.

| probeID (circRNA ID) | circRNA     | p-value | FDR  | Log2 Fold Difference (SHR - BN-Lx) | SHR Regulation | Chr  | strand | Start Location (bp) | End Location (bp) | CircRNA Type       | Gene Symbol |
|-----------------------|-------------|---------|------|------------------------------------|----------------|------|--------|-------------------|-------------------|------------------|-------------|
| ASCRPR002506 (mo_circRNA_006400) | circH2afy   | 1.10e-04 | 0.028 | 1.25                               | up             | chr17| +      | 9,313,518          | 9,314,164         | Exonic           | H2afy       |
| ASCRPR007585 (mo_circRNA_006858) | circAgtbp1  | 1.01e-05 | 0.011 | -1.54                              | down           | chr17| +      | 5,513,193          | 5,604,275         | Exonic           | Agtbp1      |
| ASCRPR013891 (mo_circRNA_015936) | circ2cw0w2  | 2.53e-04 | 0.042 | -1.62                              | down           | chr8  | -      | 126,333,477        | 126,390,804       | sense overlapping | Zcw0w2      |

#### Table 2: Differential expression results for the 4 candidate miRNAs which are targets for the candidate circRNAs.

| miRNA | Physical Location: Chr (Mb) | Log2 Difference (SHR - BN-Lx) | p-value | FDR  | miRNA DE Direction | circRNA(s) | circRNA DE Direction | Phenotype | Candidate Tier |
|-------|-----------------------------|-------------------------------|---------|------|---------------------|-------------|----------------------|-----------|-----------------|
| MIR-139-5p | Chr1: 166.6 Mb | -0.56 | 3.58E-03 | 0.032 | BN-Lx up-regulated | circ2cw0w2 | BN-Lx up-regulated | LV weight (chr17 peak, opposite direction) | #2, Not Correct DE Direction |
| MIR-146b-5p | Chr1: 266.1 Mb | 0.95 | 6.06E-05 | 0.0023 | BN-Lx up-regulated | circ2cw0w2 | BN-Lx up-regulated | LV weight (chr8 peak) | #1, DE Correct Direction |
| MIR-196c-5p | Chr10: 84.1 Mb | 3.69 | 2.18E-04 | 0.0038 | BN-Lx up-regulated | circ2cw0w2 | BN-Lx up-regulated | LV weight (chr8 peak) | #1, DE Correct Direction |
| MIR-210-5p | Chr1: 214.2 Mb | -0.39 | 3.67E-03 | 0.032 | BN-Lx up-regulated | circH2afy | SHR up-regulated | LV weight (chr17 peak, opposite direction) | #1, DE Correct Direction |

### Figures
Figure 1

Relative Left Ventricle Weight in the HXB/BXH Recombinant Inbred Panel. Strain means are plotted as individual bars. The progenitor strains (BN-Lx/Cub and SHR/OlaIpcv) are represented by blue bars and the RI strains are represented by black bars. Strains have been sorted based on relative left ventricle weight (milligrams / 100 grams of body weight). * next to a strain name on the x-axis indicates that genotype data is not currently available for that strain and therefore, that strain was not included in quantitative trait loci analyses.
Figure 2

Quantitative Trait Loci (QTL) Analysis of Relative Left Ventricle Weight in the HXB/BXH Recombinant Inbred Panel. Relative left ventricle weight (mg/100g of body weight) was downloaded for the HXB/BXH panel from GeneNetwork (http://genenetwork.org; HRP_10022). Strain means were mapped to genetic markers in the RN6 version of the rat genome. A. LOD Scores for the association of relative left ventricle weight and genetic markers across the genome. The red dotted line represents a significant threshold (genome-wide p-value < 0.05) and the blue dotted line represents a suggestive threshold (genome-wide p-value < 0.63) based on 1000 permutations. B. Statistically significant or suggestive QTL peaks for relative left ventricle weight and number of differentially expressed circRNAs physically located within the QTL. Each physiological QTL peak is depicted as a row. The chromosome (chr) and genomic location (position) reported are for the most significant marker in that region. The +/- 1.5 LOD support interval is reported along with the maximum LOD score for each peak. The final column indicates the number of circRNAs that physically reside in the QTL 1.5 LOD support interval and that are differentially expressed in brain between the SHR/OlaIpcv and BN-Lx/Cub strains.
Differential Expression of Circular RNA in Brains for BN-Lx/Cub and SHR/Olaipcv Rats. Differential expression results for the circRNA array probes with the log2 fold difference of SHR to BN-Lx on the x-axis and the $-\log_{10}$ p-value on the y-axis. Each dot represents a probe and those in blue are differentially expressed between BN-Lx and SHR (FDR < 0.05), highlighted in orange are the 3 circRNAs that are differentially expressed and physically located with a relative left ventricle weight QTL. A gray line is drawn at the FDR = 0.05 threshold.
Differential expression evidence for modulation of Tnfrsf21 through differences in expression of circH2afy and miR-210-5p. Candidate trios of circRNA, miRNA, and mRNA were identified based on a series of filtered steps designed to identify circRNA/miRNA/mRNA that may work together to produce differences in relative left ventricle weight. The top row of graphics indicates differences in expression of circH2afy, miR-210-5p, and Tnfrsf21 (left to right). circH2afy is physically located in a QTL for relative left ventricle weight and is over-expressed in the spontaneously hypertensive SHR/OlaIpcv strain compared to the normo-tensive BN-Lx/Cub strain. miR-210-5p fits the biological hypothesis that circRNA act as miRNA sponges and therefore, decrease nascent miRNA expression because it has a binding site on circH2afy and is under-expressed in SHR compared to BN-Lx. Likewise, based on the role of microRNAs in promoting mRNA degradation, under-expression of miR-210-5p may lead to over-expression of Tnfrsf21 in the SHR strain, since Tnfrsf21 has a binding site in its untranslated region for miR-210-5p. Each circle represents a separate biological replicate. The short horizontal line represents the strain mean. All expression values have been normalized and log transformed.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

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