Title: microRNA Network Changes in the Brainstem Underlie the Development of Hypertension

Authors: Danielle DeCicco, Haisun Zhu, Anthony Brureau, James S. Schwaber, Rajanikanth Vadigepalli

Affiliation: Daniel Baugh Institute for Functional Genomics/Computational Biology, Department of Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, USA

Running: brainstem microRNA regulatory networks characterize hypertension

Address for Correspondence:
Rajanikanth Vadigepalli
314 C Jefferson Alumni Hall
Thomas Jefferson University
1020 Locust Street
Philadelphia, PA 19107

Email: rajanikanth.vadigepalli@jefferson.edu
Phone: 215-955-0576
Abstract: Hypertension is a major chronic disease whose molecular mechanisms remain poorly understood. We compared neuroanatomical patterns of microRNAs in the brainstem of the spontaneous hypertensive rat (SHR) to the Wistar Kyoto rat (WKY; control). We quantified 419 well-annotated microRNAs in the nucleus of the solitary tract (NTS) and rostral ventrolateral medulla (RVLM), from SHR and WKY rats, during three main stages of hypertension development. Changes in microRNA expression were stage- and region-dependent, with a majority of SHR vs. WKY differential expression occurring at the hypertension onset stage in NTS versus at the prehypertension stage in RVLM. Our analysis identified 24 microRNAs showing time-dependent differential expression in SHR compared to WKY in at least one brain region. We predicted potential gene regulatory targets corresponding to catecholaminergic processes, neuroinflammation and neuromodulation using the miRWALK and RNA22 databases, and we tested those bioinformatics predictions using high-throughput qPCR to evaluate correlations of differential expression between the microRNAs and their predicted gene targets. We found a novel regulatory network motif consisting of microRNAs likely down-regulating a negative regulator of pro-hypertensive processes such as angiotensin II (Ang II) signaling and leukotriene-based inflammation. Our results provide new evidence on the dynamics of microRNA expression in the development of hypertension and predictions of microRNA-mediated regulatory networks playing a region-dependent role in potentially altering brainstem cardiovascular control circuit function leading to the development of hypertension.

Key Words: Hypertension, microRNA, Neuroinflammation, Angiotensin II, Brainstem
Introduction:

Hypertension is a major chronic disease worldwide, affecting about 50 million adults in the United States alone. It is a multi-organ disease and a major cause of heart failure, coronary heart disease, atrial fibrillation, stroke, and end-stage renal disease, and it has an enormous economic impact. About 90-95% of cases are “essential” with no known medical cause (11). Moreover, only about a third of hypertensive patients have their blood pressure fully controlled (11). A significant and increasing population (~25%) of hypertensive patients is drug resistant. There remains an unmet need for antihypertensive therapy.

In the context of the recent discovery of novel lines of therapeutic interventions involving approaches to modulate microRNA we decided to elucidate changes in microRNA levels during the development of SHR hypertension. The recent explosion in microRNA research has produced new computational and experimental approaches for studying microRNA functions in cell culture and in vivo, which we take advantage of here. MicroRNAs are an abundant class of small (approximately 22 nt) endogenous noncoding RNAs that direct post-transcriptional regulation of gene expression. There is ample evidence that dysregulation of microRNAs is associated with the pathogenesis of human diseases, including cardiovascular diseases as markers and therapeutic targets (26) specifically in hypertensive patients (26, 29) and show great promise in application to understanding SHR hypertension (5, 44, 57). Disease-associated microRNAs represent a new class of targets for the development of microRNA-based therapeutic modalities, which may yield patient benefits unobtainable by other therapeutic approaches (7, 16, 36). However, there has been no characterization performed on microRNA expression in the brainstem during hypertension development.
Due to the distinct roles of the NTS and the RVLM in the physiological regulation of blood pressure, it is likely that microRNAs are involved and their expression profiles differ between these two regions. Our goal is to characterize microRNAs that mediate hypertension in the brainstem, and to understand their physiological relevance. There are two main types of signaling in the NTS and RVLM that contribute to the development of neurogenic hypertension, including angiotensin II (Ang II) signaling and specific neuroinflammatory pathways involving leukotriene B4 (LTB4) and interleukin 1 (IL-1) signaling (4, 32, 42, 43, 55, 58).

With many signaling pathway changes occurring during the development and onset of hypertension, we wondered whether microRNAs could be involved in such mechanisms and potentially act as master regulators of these physiologically relevant signaling pathways. Given the associations between microRNAs and brain pathologies and their mechanistic potential to contribute to multigenic phenotypes by regulating networks of gene expression, we also wondered if different microRNA expression patterns exist in brain regions associated with blood pressure regulation in hypertension compared to control. Here, we present an extensive characterization of microRNA expression patterns and correlated gene targets in two key structures responsible for regulating cardiovascular function, the NTS and the RVLM. We hypothesized a putative signature exists in each anatomical region of microRNAs and respective targets characterizing the hypertensive state. We aimed to collect data that offers insight into the potential role of microRNAs in an animal model relevant to hypertension susceptibility, and that enables further examination into the complexity underlying this disease. Our use of micro punches and probe hybridization and qPCR technologies enabled us to obtain precise neuroanatomical resolution of microRNA and putative gene target expression. Our results reveal different brain regions show distinct microRNA changes with corresponding target-correlation signatures to different molecular signaling pathway targets implicated in hypertension.
Materials and Methods:

Animal model: Male, Wistar Kyoto (WKY/NHsd) rats and spontaneous hypertensive rats (SHR/NHsd) obtained from Harlan Laboratories were housed 1 per cage in the Thomas Jefferson animal facility to avoid animal to animal stress from dominance that could affect blood pressure. Each condition had an n=3 or 4 animals. Facilities were maintained at constant temperature and humidity with 12/12 hour light cycles (lights on at Zeitgeber time [ZT] 0). All protocols were approved by the TJU Institutional Animal Care and Use Committee.

Tissue sample punches: There were three time points of interest to this experiment, referred to as prehypertension, hypertension onset, and chronic hypertension. These time points correspond to the age of the rat at the time of sacrifice and mean arterial blood pressure measurements at that time. The prehypertensive stage is 6 to 7 weeks old, hypertension onset is at 10 to 12 weeks, and chronic hypertension occurs around 16 weeks old. At the assigned time of sacrifice, rat was sacrificed via rapid decapitation and brainstems were excised, placed into ice-cold artificial cerebral spinal fluid (ACSF: 10mM HEPES, pH 7.4; 140mM NaCl; 5mM KCl; 1mM MgCl2; 1mM CaCl2; 24 mM d-glucose) and secured with agarose for sectioning (4% UltraPure™ low melting point agarose [Invitrogen] in ACSF). 275mm transverse sections were made with a McElwain Tissue Chopper (Gamshall, England) DVC microdissection with size-matched micropunches (1.25mm; Stoelting, Wood Dale, IL), as previously reported (27). Bilateral region punches from one animal were treated as a single sample.

RNA extraction and processing: Total RNA was extracted with the miRNeasy extraction kit (Qiagen, Valencia, CA), which captures all RNA greater than 18 nucleotides in length, DNase treated (DNA-Free RNA kit, Zymo Research, Orange, CA), and stored at -80°C. Concentration and integrity were assessed with an ND-1000 (NanoDrop, Philadelphia, PA). 100 ng of RNA per experimental condition was processed in the nanoString microRNA assay following
manufacturer's protocol. Expression levels of 419 microRNAs were measured from two sections of the brainstem: the NTS and RVLM.

**MicroRNA expression data analysis:** nanoString data set has endogenous controls and RNA spike-ins, i.e., additional negative controls from other species purposefully added to the assay to assure that reads were not from non-specific binding, to assure quality. Total count normalization was performed per the manufacturer's protocol for normalizing nanoString data.

The normalized expression level for $i^{th}$ microRNA in $j^{th}$ sample was calculated as:

$$
exp_{ij} = \frac{Count_{ij}}{TotalCount_j} * 100000
$$

Where $Count_{ij}$ is the raw count, $TotalCount_j$ is the sum of counts for all microRNAs, positive and negative controls in $j^{th}$ sample, and 100000 is a normalization factor. In essence, this calculation accounts for different amounts of total number of counted molecules across samples (45). Data was log normalized (base 2), and all microRNAs with maximum normalized expression < 6.0 were removed from subsequent analysis. The threshold of 6.0 corresponded to the maximum value of the negative controls including spike-ins. This analysis yielded 197 microRNAs with expression above background detection limits. The data for each anatomical region (NTS and RVLM) was considered separately in statistical analysis. Data was statistically assessed via a two-way ANOVA using stage of hypertension development and rat strain as independent and interacting factors, p=0.05, we also confirmed results with Tukey Honest Significant Difference post-hoc testing and corrected using an FDR cut-off of 0.05. For visualization purposes, we followed established approaches for normalizing the expression of each microRNA by subtracting the median expression value of that microRNA across all samples from the same neuroanatomical region.
MicroRNA target prediction: Targets of key microRNAs identified from the nanoString profiling were determined using the miRWalk 1.0 database that provides predicted as well as validated microRNA binding sites for human, mouse and rat (18). This database utilizes 8 established programs for predicting microRNA target genes and combines these results across three genomes, to improve the sensitivity and specificity. Database settings were altered from default to include both the 5'UTR and CDS for microRNA target matching. Target predictions were filtered based on Gene Ontology and known functional role of in neuronal function. We also used RNA22 to find non-canonical predicted microRNA interactions, and combined these lists to compile all possible microRNA-target putative interactions (35).

High-throughput PCR: Intron-spanning PCR primers and probes for gene target assays, were designed using Roche’s Universal Probe Library Assay Design Center (www.universalprobelibrary.com). The standard BioMark™ protocol was used to pre-amplify cDNA samples for 12 cycles using TaqMan® PreAmp Master Mix per the manufacturer’s protocol (Applied Biosystems, Foster City, CA). qPCR reactions were performed using 3 – 48.48 BioMark™ Dynamic Arrays (Fluidigm, South San Francisco, CA) enabling quantitative measurement of multiple genes and samples under identical reaction conditions. Runs were 30 cycles (15s at 95°C, 5s at 70°C, 60s at 60°C). The primers are listed in Table S6.

Gene expression data analysis: Targets assayed were chosen based on their role in neuromodulation, inflammation and catecholaminergic regulation from literature and previous unpublished data from this lab. Ct values were calculated by the Real-Time PCR Analysis Software (Fluidigm) and software-designated failed reactions were discarded from analysis. Data was median-centered per anatomical region of the brainstem (NTS, RVLM). An independent statistical analysis was conducted using a 2-way ANOVA with stage of hypertension development and rat strain as independent and interacting factors, p=0.05. Pearson correlations between mRNA and microRNA expression were calculated. The
microRNA:mRNA pairs with correlation values less than -0.4 or above 0.4 were considered in the downstream network analysis. Hierarchal clustering based on Pearson correlation was performed for each data set using MultiExperiment Viewer part of the TM4 software tool suite (48), and organized graphically with Cytoscape software (12, 49, 50).

**Results:**

*Global microRNA Expression Patterns Vary by Strain and Brain Region*

Using the SHR and WKY animals, we measured and compared the expression of 419 microRNAs in NTS and RVLM at three stages: prehypertension, hypertension onset, and chronic hypertension (Fig. 1). In the NTS, 21 microRNAs were differentially expressed between SHR and WKY in a stage-independent manner (Fig. 2A VI). A set 53 microRNAs were differentially expressed across stage, with a similar temporal pattern between SHR and WKY (Fig. 2A II-V). Seven microRNAs were differentially expressed across stage in distinct ways between SHR and WKY (Fig. 3A I). In the RVLM, 12 microRNAs demonstrated differential expression in SHR compared to WKY (Fig. 3B IV-V), and 20 microRNAs demonstrated differential expression in a stage-dependent manner similarly across animal strains (Fig. 3B II-III). Sixteen microRNAs demonstrated stage-dependent differential expression in SHR relative to WKY (Fig. 3B I) in RVLM.

Our results revealed that majority of differential expression occurred at distinct stages in the two brainstem regions studied. In the NTS, the microRNA expression of SHR was higher at the hypertension onset stage compared to controls (Fig. 2B). In the RVLM, the microRNA expression of SHR was lower at the prehypertension stage compared to controls (Fig. 3A). Additionally, 12 microRNAs demonstrated differential expression by stage that was consistent and overlapping between the two brain regions. Only one microRNA, miR-674-5p, was common
between the RVLM and NTS with regard differential expression in SHR relative to WKY. Finally, two microRNAs, miR34a and miR-19b, were differentially expressed between SHR and WKY in a stage-dependent manner in both the NTS and the RVLM. All of the dynamically-expressed, strain-dependent, differentially expressed microRNAs showed strong correlation at the hypertension onset stage and the chronic hypertension state, whereas they showed an inverse correlation between regions at the prehypertension stage, meaning that when a microRNA had high expression levels, its corresponding putative target has lower expression levels (Fig. 4).

Relating key microRNAs to potential gene regulatory targets with known functional impact on hypertension

We prioritized a set of microRNAs that showed distinct temporal patterns between SHR and WKY for further analysis to relate these changes to target gene expression (Fig. 2A-I, B-I and Fig 3A-I; B-I). We focused on potential target genes that are involved processes with known functional impact on development and maintenance of hypertension. We developed a list of 144 potential target transcripts that are associated with AT1R signaling through Pkc, Camkl1, Mapk, and Pi3k pathways, immediate early genes (e.g., Fos, Egr1, Egr2, Egr3, Jun, Junb), and ion channels and transporters, guided by our previous studies (27, 34, 39, 40, 42, 51, 53, 54), as well as genes relevant to inflammatory pathways shown to affect blood pressure regulation, including interleukins, chemokines, and leukotrienes (19, 52, 60). Several of these pathways are enriched in astrocytes (e.g., inflammatory processes) compared to neurons (e.g., ion channels) compared to endothelial cells (e.g. junctional proteins), whereas the transcriptional regulators and signaling pathways may be common to all cell types (38).
We pursued a combination of approaches for microRNA:mRNA target prediction, including use of miRWALK (18) database, RNA22 (35) tool for pattern matching, and literature-derived known functional interactions (19, 51, 56, 59, 61). Our results revealed that several of the hypertension-relevant functional processes could be targeted by the key differentially expressed microRNAs, yielding a putative microRNA:mRNA regulatory network (Table S1). A total of 65 transcripts were predicted to be targets of miR-135a including key transcripts implicated in blood pressure regulation such as angiotensin II receptor antagonist protein (Agtrap), interleukin 1 receptor antagonist (Il1rn), and prostaglandin reductase 1 (Ptgr1). 45 transcripts from the potential target transcript list were predicted to be targeted by miR-376a including Agtrap, Orexin Receptors 1 and 2 (Hcrtr1, Hcrtr2), and Chemokine Receptor 5 (Ccr5).

High-Throughput mRNA Target Expression Level Patterns Vary by Strain and Brain Region

We evaluated the expression levels of the 144 prioritized mRNA targets for differential levels that may be consistent with the observed dynamics of microRNA regulation. We employed a high-throughput real-time PCR approach to assay expression of 144 transcripts in 45 samples using the Fluidigm BioMark platform. In the NTS, 24 transcripts were differentially expressed in SHR compared to WKY, and 21 transcripts showed a stage-dependent response. Two transcripts, Tnf and Ccr5, demonstrated stage-dependent differential expression in SHR relative to WKY (Fig. 5). In the RVLM, seven transcripts showed a strain-dependent differential expression, and 41 transcripts were differentially expressed in a stage-dependent manner. Seven transcripts were significantly differentially expressed over time in SHR versus WKY (Slc1a4, Tlr4, Rgs1, Gabrd, Ace, Nox4, and Gad1) (Fig. 6).

In general, the gene expression patterns were distinct in NTS versus RVLM, consistent with the observations in the microRNA expression profiles. In NTS, the gene expression levels were
lower in SHR at the hypertension onset stage compared to controls (Fig. 5). In contrast, the RVLM gene expression level profiles did not show an obvious grouping based on stage-specific response (Fig. 6).

Key Differentially Expressed microRNAs and Putative Transcriptional Targets Comprise Regulatory Networks Contributing to Hypertension

Of the stage and strain-dependent microRNAs (NTS: Fig. 2A-1, RVLM: Fig.3B-I), two were common between these regions. Interestingly, these two microRNAs were shown to be enriched in different cell types: miR-135a in astrocytes and miR-376a in neurons (25). We focused the subsequent analysis on this pair of microRNAs and their putative targets. We observed that there were correlational relationships between microRNA and predicted transcript targets when we examined the expression of microRNA:putative target pairs (Fig. 7). Therefore, we assessed all of the microRNA-putative target transcript correlations, and we filtered the microRNA:mRNA network (Table S1) to focus on the microRNAs with strain-specific and stage-dependent differential response and their predicted targets (Fig. 8A). We mapped the differential expression data as well as the linear correlation between microRNA expression and target transcript levels on to the postulated regulatory network. We filtered the results to include microRNA:mRNA interactions corresponding to absolute correlation values larger than 0.4. Our analysis revealed a similar extent of positive versus negative correlation between the microRNA and predicted target transcript patterns. Correlations across SHR animals differed significantly compared to those of WKY animals. Ultimately, we discovered a pair of microRNAs whose regulatory network showed differences in correlations in hypertension compared to control (Fig. 8). Of note are the inflammation transcripts in NTS for microRNA-135a. There appears to be a much stronger inversely correlative relationship between miR-135a and these transcripts in SHR compared to WKY (Fig. 8B).
Based on this analysis in both NTS and RVLM, we constructed a model to show how microRNAs affect hypertension. We focused on a pair of microRNAs, miR-135a and miR-376a, which were (1) differentially expressed between SHR and WKY in both NTS and RVLM, (2) predicted to target transcripts that play a key role in the development of hypertension and (3) inversely correlated in expression with those key putative targets (Fig. 9). Our model includes miR-135a mediated putative down-regulation of Ptgr1 transcript. The corresponding protein, PTGR1 degrades LTB4 and hence, serves as a negative regulator of leukotriene-mediated inflammation. Similarly, our model includes miR-135a mediated putative down-regulation of Il1rn transcript. The corresponding protein IL-1RA serves as a negative regulator of IL-1 mediated inflammation. In addition, our model incorporates putative down-regulation of Agtrap transcript by miR-135a and miR-376a, likely leading to disinhibition of AT1R signaling.

**Discussion:**

To our knowledge this is the first study that has examined microRNAs and target gene expression in a high-throughput manner from the NTS and RVLM over the course of development of hypertension. We used the SHR animal model that closely mimics several features of the development and chronic state of human hypertension. Through an unbiased global microRNA expression analysis, we found extensive differential expression of microRNAs in these two key brainstem regions during the development of hypertension. We correlated these changes to their putative gene expression targets with a focus on how the changes evolve over time (dynamics). We developed a regulatory network model that connects changes in microRNA expression to modulation of key transcript levels participating in inflammation and Ang II signaling to drive hypertension development. The network contains a novel double-negative regulatory motif in which up-regulated microRNAs likely down-regulate inhibitors of inflammation and Ang II signaling processes.
The recent literature offers abundant evidence for “neurogenic hypertension” in which the central neuronal mechanisms of blood pressure regulation play a key role, in particular autonomic structures in the brainstem, the nucleus of the solitary tract (NTS) and the rostral ventrolateral medulla (RVLM). Fortunately, current evidence indicates that hypertensive strains of rats and susceptible humans have much in common, both phenotypically and genotypically with respect to neurogenic contributions to hypertension (5, 28, 31, 51), and the SHR is by far the most widely studied animal model of hypertension (44). The SHR has the advantage of hypertension developing from a young age over several weeks, which permits study of transcriptional changes in the pre-hypertensive age, thereby separating effects of chronic high blood pressure from transcriptional processes that may lead to hypertension during the prehypertension stage. Thus, temporal profiling addresses the cause and effect regarding the association between transcript changes and blood pressure. The animal model supports the time-series study of developmental vs. chronic gene expression changes, which is beneficial for clarifying an optimal therapeutic intervention time point.

Our results highlight the stage-dependent dysregulation of molecular networks in the development of hypertension. These findings are consistent with observations indicating a stage-dependent role for Ang II signaling processes in driving hypertension. Blocking Ang II mediated AT1R signaling at a young age (4-8 weeks of age) in SHR model, corresponding to intervention at the prehypertension stage, prevented development of hypertension even up to 48 weeks later (6). These results, together with our findings, implicate the prehypertensive stage as a key period in the development of hypertension. Our results uncovered a microRNA-mediated regulation as potentially underlying this stage-dependent sensitivity. The microRNAs identified in our study could serve as novel targets for manipulation to influence the Ang II signaling and interfere with the development of hypertension.
Our results suggest that microRNA regulation and downstream effects on putative target transcript levels vary by brainstem region during development of hypertension. RVLM showed differential microRNA expression prior to the onset of hypertension, at an earlier stage than was observed in the NTS, which showed microRNA expression changes during the hypertension onset stage. The functional consequences of these distinct stage-dependent responses need to be interpreted through the location and role of these brainstem nuclei in the blood pressure control circuit. The NTS is known to be the primary site of cardiorespiratory regulatory integration (1, 8, 9, 13, 33, 46, 47). The RVLM receives inhibitory projections from the caudal ventrolateral medulla. Disruption of these inhibitory projections leads to the development of hypertension (3, 9, 10, 15, 20, 22, 24). The RVLM is crucial in tonic and reflexive regulation of arterial pressure, and it has been shown to contribute to elevated sympathetic nerve activity and mean arterial pressure in obese Zucker hypertensive rats (24). Studies have shown that RVLM-enhanced angiotensinergic activation and reduced GABAergic inhibition contributes to hypertension in these rats, and the low levels of microRNAs observed in our results are consistent with putative microRNA-mediated regulation of these processes (9, 24, 37). Our results suggest that by the time increases in mean arterial pressure are seen in the hypertension onset stage, it is likely that the aberration in the molecular networks have already occurred in the RVLM. It is interesting to speculate whether the processes that are disrupted earlier in RVLM lead to changes in the microRNA expression and corresponding dysregulation of transcript levels in the NTS at a later stage. In that regard, a question arises as to whether the NTS microRNA network expression changes at hypertension onset are compensatory or are further advancing the pathology leading to aberrant wiring across the baroreflex control circuit.

We interpret the microRNA and putative target gene expression correlations observed in our results as signatures of the underlying mechanistic relationships that are candidates for further experimental testing. Our results show that the microRNA and putative target gene expression
correlations differ significantly between the SHR and WKY animals, indicating differences in the underlying regulatory networks between the two genotypes. We prioritized a subset of these relationships based on inverse correlation between microRNAs and putative targets for constructing a regulatory network. The positive correlations observed in our results could arise due to multiple possibilities. For instance, microRNAs have been shown to up-regulate certain targets by stabilizing the mRNAs (41). Additionally, the microRNAs could be affecting transcript levels in a positive manner indirectly by down-regulating a negative regulator of the transcript.

In order to examine the effects of microRNA changes on the molecular pathways implicated in hypertension, such as angiotensin II signaling and inflammation, we employed bioinformatics analyses to predict regulatory targets corresponding to these pathways. From the cohort of differentially expressed microRNAs, we examined two cell type specific microRNAs in additional detail: miR-135a, which is enriched in astrocytes, and miR-376a, which is enriched in neurons (25). Given the expected cell-type enrichment, it is important to localize the correlations between microRNAs and putative targets to specific cell types, as these may be masked or altered when considering tissue-scale samples that we employed in the present study. Literature evidence points to likely cell-type specificity of these pathways, for example, AT1R signaling in neurons and leukotriene metabolism in astrocytes (38, 56). Both microRNAs showed significant differences in expression in NTS and RVLM, and are predicted to target transcripts and networks associated with high blood pressure. We developed a regulatory network model containing these two microRNAs and putative target transcripts (Fig. 6).

miR-135a is particularly enriched in astrocytes relative to neurons, oligodendrocytes, and microglia (25). Our target prediction and expression correlation analysis revealed that miR-135a is likely to act by down regulating Ptgr1 transcript, which has been shown to be down-regulated in adult SHR rats compared to WKY rats (56), which is consistent with the higher expression of
miR-135a observed in our data. Thus, we have hypothesize that miR-135a down regulates the $Ptgr1$ expression to increase the levels of a key pro-inflammatory leukotriene LTB4, likely leading to the development of hypertension. High levels of LTB4 in the NTS have been shown to be prohypertensive, and blocking LTB4 receptor resulted in lowered arterial pressure in SHR (56). In RVLM, proinflammatory cytokines have been shown to be elevated (2), and microinjecting pentoxifylline, an anti-inflammatory drug whose mechanism is partly mediated through leukotriene inhibiton has been shown to lower blood pressure in LPS-induced hypertensive rats (59). Our analyses also pointed to additional routes of influence via miR-135a down-regulation of $Il1rm$, a key anti-inflammatory regulatory player. IL-1RN has been shown to exhibit an anti-inflammatory effect via IL-1 signal attenuation (21). Furthermore, the levels of IL-1, a proinflammatory molecule, are higher in the brainstems of 22-week-old SHR compared to age-matched WKY (51). Additional human studies have also found that $Il1rm$ has single nucleotide polymorphisms that contribute to acute coronary syndrome, which further implicates this gene in cardiovascular disease (19). In our results, $Il1rm$ is expressed at higher levels in WKY than in SHR. We predict that the down-regulation of $Il1rm$ transcript via miR-135a contributes to the development of hypertension by disinhibiting an inflammatory signal mediated through IL-1. Another hypothesized route of miR-135a influence predicted by our computational analysis is through modulation of Ang II signaling. NTS contains the highest amount of Ang II receptors in the medulla, and this is evolutionarily conserved (4, 14, 23, 30). RVLM also contains a high amount of Ang II receptors compared to the rest of the medulla (22, 23). miR-135a putative mediation of Ang II signaling occurs via down-regulation of $Agtrap$, angiotensin II receptor associated mRNA. AGTRAP protein is a key down-regulator of the angiotensin II receptor type 1 (17). Based on this target prediction and expression correlation, we postulate that miR-135a may be down-regulating a negative regulator AT1R, therefore increasing Ang II signal transduction, leading to blood pressure elevation.
In contrast to miR-135a, miR-376a is highly expressed in neurons relative to astrocytes, microglia and oligodendrocytes (25). miR-376a was one of the highly expressed microRNAs in our results with whole tissue samples. Based on the target prediction analysis and transcript and microRNA expression correlation results, we hypothesize that the neuron-enriched miR-376a could act via targeting Agtrap to disinhibit Ang II signaling in NTS. In the rat transcript of Agtrap, there appears to be only one miR-376a predicted binding site in the 3'UTR beginning at the 5' end nucleotide 1646 based on RNA22 predictions; however, in the human there is one in the 3' UTR beginning at 936 from 5' end of the transcript, and an additional predicted binding site in the 5' UTR beginning at nucleotide 53 from the 5' end on the transcript (Fig. S4). miR-135a has more predicted binding sites on the Agtrap transcript than does miR-376a. miR-135a has two predicted target sites on Agtrap, both in the 3' UTR beginning at nucleotide 1125 and 1509 from 5' end. In contrast, the human Agtrap transcript has five predicted target sites for miR-135a-1, all of which are located in the 3' UTR at locations: 714, 749, 839, 906, and 961 from 5' transcript end. Notably, there is no competition between these two microRNAs to bind Agtrap as none of the predicted binding sites overlap.

Our results also highlight several additional microRNAs differentially expressed between the SHR and WKY rats and correlated with their downstream transcript targets, providing a cadre of putative microRNA regulated pathways underlying the development of hypertension. Our correlation analysis pointed to several putative influences of microRNA changes aside from the canonical direct targeting via seed region base pairing in the 3' UTR. For instance, miR-135a expression was inversely correlated with that of Ace, which was not predicted to be a direct target of miR-135a in our analysis. This suggests a regulatory network interaction involving multiple intermediate steps, or alternatively, potential novel direct targeting of Ace by miR-135a.
Additional experiments involving manipulation of microRNAs in disease models are required to further develop the putative target transcript correlations into a mechanistic causative role for microRNAs in the development of hypertension. Our results identify several microRNAs, including those with a cell type specific role, for prioritization in such follow-on experimental studies. We would propose miR-135a and 376a as an initial point for future functional studies as we predict they are positioned to act as “hubs” or “master regulators” for several relevant transcripts that may drive development of cardiovascular disease in SHRs. Additional opportunities for investigation include analysis of other neuroanatomical regions participating in blood pressure regulation, such as the caudal ventrolateral medulla and the paraventricular nucleus of the hypothalamus. Understanding the dynamics of microRNA mediated changes across the nuclei in the blood pressure control circuit would yield new targets for testing for effects on prevention as well as potential rescue from the hypertensive condition.

Acknowledgements: D.D. acknowledges Warren A. Anderson for guidance with comparative network analysis and Sirisha Achanta for assistance in conducting initial high-throughput qPCR experiments.

Grants: The work presented here was funded through NIH NIGMS R01 GM083108 and NIH NHLBI R01HL111621.

Disclosures: No conflicts of interest, financial or otherwise, are declared by the authors.
References:


46. Rogers RF, Paton JF, Schwaber JS. NTS neuronal responses to arterial pressure and pressure changes in the rat NTS neuronal responses to arterial and pressure changes in the rat. *Am J Physiol Regul Integr Comp Physiol*.


Figure Captions:

Figure 1. Comparison of blood pressure in SHR versus WKY over the time course of hypertension development. Prehypertension stage corresponds to 6-7 weeks of age, hypertension onset stage corresponds to 10-12 weeks of age, and chronic hypertension corresponds to 16 weeks of age.

Figure 2. Dynamics of microRNA expression in NTS during the development of hypertension. A) Clustered groups of microRNAs showing differential expression: (I) across strain (SHR v. WKY) in a stage-dependent manner; (II-V) in a stage-dependent and strain-independent manner which shows a gradient of high to low expression from (II) –(V); (VI) between strains (SHR v. WKY) without dynamic regulation over time. B) Dynamic profiles of all microRNAs in group I and a select subset from other groups. *p-value <0.05

Figure 3. Dynamics of microRNA expression in RVLM during the development of hypertension. A) Dynamic profiles of all microRNAs differentially expressed across strain (SHR v. WKY) in a stage-dependent manner. B) Clustered groups of microRNAs showing differential expression (I) across strain (SHR v. WKY) in a stage-dependent manner; (II and III) in a stage-dependent and strain-independent manner; (IV and V) between strains (SHR v. WKY) without dynamic regulation over time. * p-value <0.05

Figure 4. Comparison of differential microRNA expression between NTS and RVLM through multiple stages of hypertension development. A) Prehypertension stage shows an inverse, inverse correlative relationship B) Onset of Hypertension shows a correlative relationship and C) Chronic Hypertension shows no relationship.
Figure 5. NTS differentially expressed transcripts show strain-dependent and stage-dependent differential expression. A) Differentially expressed transcripts show (I) stage-dependent differential expression independent of strain; (II) strain-dependent differential expression independent of stage, which cluster into two groups based on Pearson correlation; (III) strain-dependent dynamic regulation. p<0.05. B) Summary of transcripts differentially expressed in strain, stage as well as strain-dependent stage effects.

Figure 6. RVLM differentially expressed transcripts show strain-dependent and stage-dependent differential expression. A) Differentially expressed transcripts show (I) stage-dependent differential expression independent of strain; (II) strain-dependent differential expression independent of stage, which cluster into two groups based on Pearson correlation; (III) strain-dependent dynamic regulation. p<0.05. B) Summary of transcripts differentially expressed in strain, stage as well as strain-dependent stage effects.

Figure 7. Comparison of microRNA expression patterns with that of the predicted targets by RNA22 and miRWALK algorithms. Key microRNAs plotted with transcripts predicted to be a target of its respective microRNA. A) miR-135a and its predicted target Il1rn show opposite behavior in SHR NTS as the Pre and Onset stages. B) miR-135a shows anti-correlated behavior with target Agtrap in SHR in NTS and RVLM at the Pre-Onset Stage, and in RVL WKY at the Pre-Onset Stage. C) miR-376a shows opposite expression levels than target PTGR1. D) miR-376a show inverse correlated behavior with target Agtrap in SHR NTS at the Pre-Onset stage.

Figure 8. Network representation of microRNA – putative target (A) mapped to pathway annotations derived from literature and gene ontology. (B,D) Key microRNAs at the hypertension onset stage in NTS of miR-135a regulatory network (B) and miR-376a regulatory
network (D). (C,E). Key microRNAs at the prehypertension stage in RVLM of miR-135a regulatory network (C) and miR-376a regulatory network (E). Edges are mapped to correlation data differences of SHR-WKY. Blue: positive, Red:negative. Line connections are present if the transcript is a predicted target of a microRNA with line thickness representing relative strength of correlation subtraction [-2,2].

Figure 9. Literature-pruned subset of larger data-driven microRNA regulatory network depicted previously captures the interactions relevant to well-established SHR literature focusing on Angiotensin II and inflammatory signaling. The network relates key microRNAs either persistently up-regulated or showing a larger rise in SHR than in WKY during the developmental phase of hypertension. A common feature of this network is that the two key microRNAs are predicted by a consensus of bioinformatics tools to target negative regulators of pathways that are amplified in SHR during the development of hypertension.

Table Captions:

Table 1. Summary of predicted binding sites of key pair of microRNAs in human and rat

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Gene Target</th>
<th># predicted sites</th>
<th>Homo Sapiens</th>
<th>Rattus Norvegicus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Region</td>
<td>Transcript Location</td>
</tr>
<tr>
<td>miR-376a</td>
<td>Agtrap</td>
<td>2</td>
<td>5' UTR 53-74</td>
<td>3' UTR 1646-1668</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR 936-959</td>
<td></td>
</tr>
<tr>
<td>miR-135a</td>
<td>Agtrap</td>
<td>5</td>
<td>3' UTR 714-726</td>
<td>3' UTR 1125-1151</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR 749-770</td>
<td></td>
</tr>
<tr>
<td>miR-135a</td>
<td>Ptgr1</td>
<td>2</td>
<td>3' UTR 839-852</td>
<td>3' UTR 1509-1531</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR 906-918</td>
<td></td>
</tr>
<tr>
<td>miR-135a</td>
<td>Il1m</td>
<td>2</td>
<td>5' UTR 573-586</td>
<td>CDS 82-104</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR 1215-1232</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDS 544-561</td>
<td></td>
</tr>
</tbody>
</table>

Supplementary Dataset Captions:

Table S1. Putative miRNA:mRNA target network including all predicted interactions from bioinformatics target prediction grouped by signaling pathway.

Table S2. Raw nanoString miRNA expression data annotated by sample type.

Table S3. Raw high-throughput qPCR data of mRNA putative target expression measurements.
Table S4. Normalized NTS transcript expression data.

Table S5. Normalized RVLM transcript expression data.

Table S6. qPCR primer design used for high-throughput qPCR experiments.
A) NTS

B) RVLM

C) NTS

D) RVLM

Relative expression

Stage
<table>
<thead>
<tr>
<th>microRNA</th>
<th>Gene Target</th>
<th># predicted sites</th>
<th>Region</th>
<th>Transcript Location</th>
<th># predicted sites</th>
<th>Region</th>
<th>Transcript Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-376a</td>
<td>Agtrap</td>
<td>2</td>
<td>5' UTR</td>
<td>53-74</td>
<td>1</td>
<td>3' UTR</td>
<td>1646-1668</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR</td>
<td>936-959</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-135a</td>
<td>Agtrap</td>
<td>5</td>
<td>3' UTR</td>
<td>714-726</td>
<td>2</td>
<td>3' UTR</td>
<td>1125-1151</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR</td>
<td>749-770</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR</td>
<td>839-852</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR</td>
<td>906-918</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR</td>
<td>961-980</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-135a</td>
<td>Ptg1</td>
<td>2</td>
<td>CDS</td>
<td>573-586</td>
<td>1</td>
<td>CDS</td>
<td>82-104</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3' UTR</td>
<td>1215-1232</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-135a</td>
<td>Il1rn</td>
<td>2</td>
<td>5' UTR</td>
<td>14-33</td>
<td>1</td>
<td>3' UTR</td>
<td>1785-1807</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CDS</td>
<td>544-561</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>