Journal of Human Hypertension (2015) **29,** 459–467

www.nature.com/jhh

REVIEW

The emerging role of non-coding RNA in essential hypertension and blood pressure regulation

FZ Marques, SA Booth and FJ Charchar

Unravelling the complete genetic predisposition to high blood pressure (BP) has proven to be challenging. This puzzle and the fact that coding regions of the genome account for less than 2% of the entire human DNA support the hypothesis that genetic mechanism besides coding genes are likely to contribute to BP regulation. Non-coding RNAs (ncRNAs) are emerging as key players of transcription regulation in both health and disease states. They control basic functions in virtually all cell types relevant to the cardiovascular system and, thus, a direct involvement with BP regulation is highly probable. Here, we review the literature about ncRNAs associated with human BP and essential hypertension, highlighting investigations, methodology and difficulties arising in the field. The most investigated ncRNAs so far are microRNAs (miRNAs), small ncRNAs that modulate gene expression by posttranscriptional mechanisms. We discuss studies that have examined miRNAs associated with BP in biological fluids, such as blood and urine, and tissues, such as vascular smooth muscle cells and the kidney. Furthermore, we review the interaction between miRNA binding sites and single nucleotide polymorphisms in genes associated with BP. In conclusion, there is a clear need for more human and functional studies to help elucidate the multifaceted roles of ncRNAs, in particular mid- and long ncRNAs in BP regulation.

Journal of Human Hypertension (2015) **29,** 459–467; doi:10.1038/jhh.2014.99; published online 13 November 2014

INTRODUCTION

The human genome was mapped in its near entirety in 2003, and since then there has been many noteworthy discoveries but many challenges remain. Unravelling the complete genetic predisposition to high blood pressure (BP) has proven to be one of these challenges, perhaps because of its phenotypic heterogeneity,[1] but it is more likely that there are many mysteries that our genome has not yet revealed. Only up to 2.2% of inter-individual variance in BP may be explained by common single nucleotide polymorphisms (SNPs) associated with BP/hypertension identified by the largest genome-wide association meta-analysis.[2] This finding and the fact that coding regions of the genome account for less than 2% of the entire human DNA[3] support the hypothesis that other mechanisms besides coding genes are likely to contribute to BP regulation. In the past decade, non-coding RNAs (ncRNA, that is, RNA which does not code for a protein) have emerged as key players in transcription regulation in both health and disease states. In contrast to coding regions highly conserved between species, ncRNAs are usually stage-, tissue- and species-specific,[4–6] and contribute to the complexity and variety of the transcriptome in humans. Here, we review the literature concerning ncRNAs associated with human BP and essential hypertension and ask the question—do transcripts that are not transcribed into protein play a role in BP regulation?

Search strategy

Owing to word constraints, we have included studies in humans only. The search methodology is described in Figure 1. Briefly, we used relevant keywords to search the free-engine PubMed (www.pubmed.com) database of references, and then manually excluded those articles that were not appropriate for inclusion, based on the exclusion criteria described in Figure 1. No article was excluded based on year.

Small and mid-size ncRNAs

In the last decade, several types of small (<50 base pairs) and mid-size (<200 base pairs) ncRNAs have been described (summarised in Figure 2). Small ncRNAs include PIWI-interacting RNAs ($>23\,000$ so far detected in the human genome), transcription initiation RNAs (>5000) and the most studied type, microRNAS (miRNAs, >1800).[6] Mid-size ncRNAs include small nucleolar RNAs (>300 so far detected in the human genome), promoter upstream transcripts (the number of these is not known), transcription start sites (TSS)-associated RNAs ($>10\,000$) and promoter-associated small RNAs ($>10\,000$).[6] Besides miRNAs, these other types of small and mid-size ncRNAs have not been investigated in relation to BP or hypertension, so they will not be further discussed here.

MiRNAs have been described as master gene regulators as they can regulate downstream gene expression by posttranscriptional mechanisms, specifically by binding to the 3′ untranslated region (UTR) of a messenger RNA (mRNA). This leads to mRNA degradation or repression of translation.[7] The regulatory roles of miRNAs binding to promoters and 5′UTR has also been described, but remains mostly unclear at present.[8] On the basis of a search conducted on 11 September 2014 (http://www.mirbase.org/), a total of 1881 miRNAs has been identified in the human genome,[9] and each miRNA can regulate several hundred genes.[6] Even though the pace of research into miRNAs and cardiovascular disease has been increasing in the past decade, our search resulted in only 18 original articles in miRNAs and human hypertension and/or BP, as discussed below.

School of Applied and Biomedical Sciences, Faculty of Science and Technology, Federation University Australia, Victoria, Australia. Correspondence: Professor FJ Charchar, School of Health Sciences, Federation University Australia, Y Building, University Drive, Mt Helen, 3350, Victoria, P.O Box 663, Ballarat, Victoria 3353, Australia.
E-mail: f.charchar@federation.edu.au
Received 27 March 2014; revised 19 September 2014; accepted 7 October 2014; published online 13 November 2014

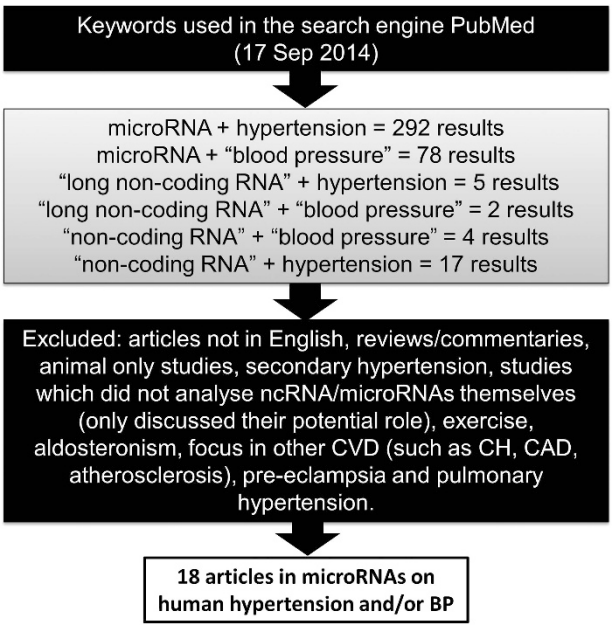

Figure 1. Flow chart of methodology used to select the original investigations included in this review. Searches for other types of ncRNAs resulted in no original research. CVD, cardiovascular disease; CH, cardiac hypertrophy; CAD, coronary artery disease.

Biological fluid and circulating miRNAs

Biological fluid miRNAs can be isolated from urine or circulation, such as plasma, serum and blood cells (including vascular endothelial and peripheral blood mononuclear cells). Plasma, serum and urine miRNAs are protected from endogenous RNase activity because they are carried in extracellular vesicles (such as apoptotic bodies and exosomes), RNA-binding proteins and lipoprotein complexes.[10] These miRNAs are unique in that they are highly stable even when exposed to storage at room temperature or extreme conditions, such as multiple freeze–thaw cycles, boiling and extreme pH.[11] The most common technique used to measure circulating miRNA concentrations is relative quantification by quantitative real-time polymerase chain reaction (qPCR). In relative quantification, genes involved in basic cell maintenance (referred to as housekeeping genes) are usually used as a reference to the quantity of expression of the gene of interest. Housekeeping genes are expressed at similar levels in both health and disease states.[12]

Although body fluids are easily obtainable, measuring circulating levels of miRNAs in these samples remains challenging. One of the main difficulties is low miRNA concentration that hinders accurate quantification and sometimes requires the pooling of samples. In the measurement of circulating miRNAs, there are also technical difficulties due to the lack of accepted standards in normalising the data. There are discussions in favour of exogenous (a RNA transcript specific to other species introduced during the RNA extraction, termed 'spike-in') and endogenous miRNAs or

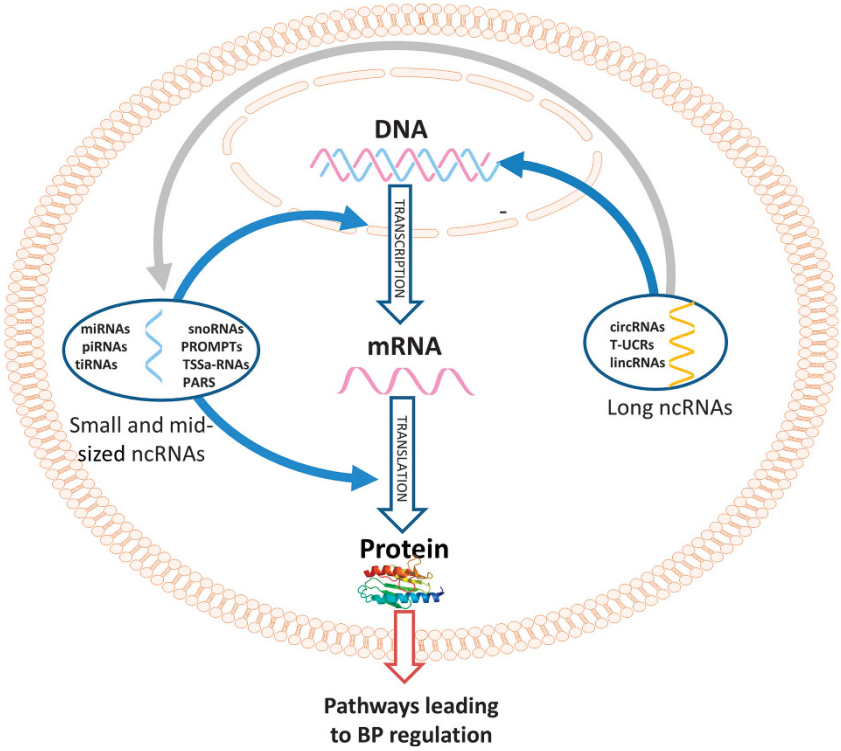

Figure 2. The role of ncRNAs in the 'central dogma'. NcRNAs have changed the view of the 'central dogma', that all DNA was transcribed into RNA and then translated into protein. Although protein is the link between genotype and phenotype, many factors determine the presence and abundance of proteins, and hence, their function. NcRNAs have a fundamental role in regulating protein levels by modulating transcription and translation to either increase or decrease protein levels. Long ncRNAs are mostly known to modulate chromatin complexes (blue arrow), and thus change DNA condensation, where more condensation results in less transcription. Small and mid-size ncRNAs mostly regulate transcription, posttranscription and translation (blue arrows). Moreover, some long ncRNAs have a function in regulating the levels of miRNAs (grey arrow). Small ncRNAs include PIWI-interacting RNAs (piRNAs), transcription initiation RNAs (tiRNAs) and miRNAs; mid-size ncRNAs include small nucleolar RNAs (snoRNAs), promoter upstream transcripts (PROMPTs) and TSS-associated RNAs (TSSa-RNAs); and long ncRNAs include circular RNAs (circRNAs), transcribed ultra-conserved regions (T-UCRs) and large intergenic ncRNAs (lincRNAs).

small RNAs (such as RNU6, produced by the cells), or the use of both as normalisers. We refer the reader to a review on methods used for normalisation during the measurement of miRNAs in body fluids.[10] Below, we discuss those studies focused on biological fluid miRNAs and BP, summarised in Table 1.

A genome-wide miRNA study in plasma compared miRNA expression in 13 hypertensive patients and 5 normotensive Chinese subjects by microarray.[13] The authors found 27 differentially expressed miRNAs, 9 miRNAs were upregulated and 18 miRNAs that were downregulated in hypertensives.[13] Three of these miRNAs—hcmv-miR-UL112, miR-296–5p and let-7e—were validated using qPCR in a larger cohort of 127 patients and 67 control subjects.[13] The fold changes for these miRNAs were 2.5, 0.5 and 1.7, respectively, in the hypertensive cohort. The miRNA hcmv-miR-UL112 is a human cytomegalovirus (HCMV)-encoded miRNA. Using *in vitro* transfection of human embryonic kidney 293 (HEK293) cells with reporter gene constructs, the authors demonstrated that levels for the interferon regulatory factor 1 transcript could be regulated by hcmv-miR-UL112. The authors also found higher titres of human cytomegalovirus in hypertensives. This led to the suggestion that cytomegalovirus is responsible for the differential expression of hcmv-miR-UL112 in circulating vascular endothelial cells.[13] The interesting, yet highly speculative, conclusion from this study is that cytomegalovirus could contribute to the elevation in BP.

In a study aimed to analyse metabolic syndrome and its risk factors, miRNAs were measured in healthy controls compared with those with metabolic syndrome, type 2 diabetes, hypercholesterolemia or hypertension.[14] Differentially expressed miRNAs were identified in both blood and exosomes isolated from serum.[14] MiR-150, miR-192 and miR-27a were downregulated in subjects with hypercholesterolaemia or hypertension.[14] MiR-130a, miR-195 and miR-92a were upregulated in hypertensives and those with metabolic syndrome. MiR-130a and miR-195 in blood were also positively correlated with BP.[14] Only miR-92a, predicted to target the angiotensin II (Ang II) receptor type I (AGTR1) mRNA, was present in exosomes. Similarly, miR-27a was downregulated in aortas of the spontaneously hypertensive rat (SHR) compared with Wistar–Kyoto controls. Furthermore, exercise as a form of BP-reducing intervention was able to normalise miRNA expression to Wistar–Kyoto levels.[15] This, however, is contradictory to findings in mice deficient in miR-92a which do not exhibit changes in BP.[16]

The expression of urine miRNAs, isolated from exosomes, were investigated in a cohort of 10 salt-sensitive, inverse salt-sensitive (that is, subjects whose BP decreases in response to high salt intake) and salt-resistant subjects (that is, subjects whose BP increases in response to high salt intake).[17] The authors reported 45 differentially expressed miRNAs secreted from the kidney in salt-sensitive and salt-resistant individuals or between the inverse salt-sensitive and salt-resistant subjects. The only miRNA that

Table 1. Biological fluid and circulating microRNAs in blood pressure regulation and essential hypertension

Reference	*Body fluid*	*Sample size and characteristics*	*Technique used*	*Normalised against*	*Main findings in hypertension and blood pressure*
Li *et al.*[13]	Plasma	Discovery: 13 HT and 5 NT, validation 1: 24 HT and 22 NT, validation 2: 194 HT and 97 NT	Discovery: microarrays, validation: qPCR	RNU6	↑ hcmv-miR-UL112, ↑ hsa-let-7e, ↓ hsa-miR-296–5p
Karolina *et al.*[14]	Blood and exosomes from serum	Discovery: $n=17$ NT, 18 MS, 21 T2D, 41 HCL and 14 HT, validation: $n=29$ NT, 32 MS, 29 T2D, 48 HCL and 16 HT	Discovery: microarrays, validation: qPCR	RNU6	Changes unique to HT and MS in blood: ↑ hsa-miR-130a, hsa-miR-195, hsa-miR-92a, changes unique to HT and HCL in blood: ↓ hsa-miR-150, hsa-miR-192, hsa-miR-27a. Changes unique to HT and MS in exosomes: ↑ hsa-miR-92a
Gildea *et al.*[17]	Exosomes in urine	$n=3$ salt-sensitive, $n=4$ salt-resistant (control group) and $n=3$ inverse-salt sensitive	Discovery: microarrays, validation: qPCR	hsa-miR-320a	hsa-miR-4516 ↓ in inverse-salt-sensitive and ↑ in salt-sensitive.
Kontaraki *et al.*[18]	Peripheral blood mononuclear cells	60 HT, 29 NT		RNU6	↓ hsa-miR-143, hsa-miR-145 and hsa-miR-133a, and ↑ hsa-miR-21, and hsa-miR-1. –ve correlation between DBP and hsa-miR-143, hsa-miR-145 and hsa-miR-21, and +ve between DBP and hsa-miR-133a.
Mandraffino *et al.*[20]	Peripheral blood mononuclear cells (CD34+)	94 untreated essential hypertension, divided into those with ($n=41$) or without ($n=53$) carotid intima-media thickness and left ventricular hypertrophy	qPCR	18SrRNA	↑ ROS, CRP, fibrinogen, CD34+ cells, hsa-miR-221 and hsa-miR-222
Kontaraki *et al.*[19]	Peripheral blood mononuclear cells	29 NT and 60 untreated HT	qPCR	RNU6	↓ hsa-miR-9 and hsa-miR-126 in HT. hsa-miR-9 was positively correlated with left ventricular mass and pulse pressure, and miR-126 with pulse pressure.

Abbreviations: 18SrRNA, 18S ribosomal RNA; *CCL3,* chemokine (C-C motif) ligand 3; CD34+, cells expressing CD34; CRP, C-reactive protein; DBP, diastolic blood pressure; discovery, discovery phase; HCL, hypercholesterolaemia; HT, hypertensive subjects; *IL1B,* interleukin 1 β; miRNA, microRNA; MS, metabolic syndrome; *NFKBIA,* nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α; NT, normotensive subjects; qPCR, real-time PCR; RNU6, U6 small nuclear RNA; ROS, reactive oxygen species; T2D, type 2 diabetes; *TNF,* tumor necrosis factor gene; validation, validation phase of the study.

could differentiate the two extremes (salt sensitivity or inverse salt sensitivity) vs the salt-resistant group was hsa-miR-4516. Although the findings have not been replicated in a larger cohort, this study demonstrates that miRNAs could be altered or even used as biomarkers for aberrant sodium regulatory pathways in hypertension.

To gain insights into molecular mechanisms by which vascular smooth muscle cells (VSMC) affect vascular resistance, Kontaraki and colleagues[18] compared the expression of miR-143, miR-145, miR-21, miR-133 and miR-1 in peripheral blood mononuclear cells between 60 patients with essential hypertension and 29 healthy normotensive subjects. All of the hypertensive patients underwent 24 h ambulatory BP monitoring. The specific miRNAs were chosen based on their previously published role in VSMC phenotype and differentiation. Hypertensives had lower hsa-miR-143, hsa-miR-145 and hsa-miR-133a, and higher hsa-miR-21 and hsa-miR-1. Twenty-four hour mean diastolic BP (DBP) was negatively correlated with hsa-miR-143, hsa-miR-145 and hsa-miR-21, whereas mean DBP and hsa-miR-133a were positively correlated. Although these miRNAs were highly expressed in VSMC, the authors did not determine whether these miRNAs were truly derived from VSMC or whether they may have originated from other sources. Further investigations are required to determine the role of these miRNAs in inflammation.

Another study from the same group focused on the expression levels of hsa-miR-9 and hsa-miR-126 in peripheral blood mononuclear cells in normotensive compared with hypertensive subjects.[19] Both miRNAs were significantly lower in those with high BP, and positively correlated with pulse pressure. Mir-9 was also positively correlated with left ventricular mass. This supports a role for these miRNAs as markers of advanced target-organ damage in hypertension.

A recent study investigated the expression of hsa-miR-221 and hsa-miR-222 in circulating progenitor cells (also termed pro-angiogenic haematopoietic cells), selected by CD34 surface antigen. The authors compared cells from normotensive and hypertensive subjects, the latter being sub-divided into those with carotid intima-media thickening but without left ventricular hypertrophy, and those with left ventricular hypertrophy but without carotid intima-media thickening.[20] The miRNAs were chosen based on their association with previously reported regulatory roles in angiogenesis, cell proliferation and vascular inflammation. Hypertensive subjects had increased reactive oxygen species, C-reactive protein, fibrinogen and a higher number of CD34+ cells. These heightened inflammation parameters were associated with an increase in hsa-miR-221 and hsa-miR-222 expression. In the hypertensive group with left ventricular hypertrophy, these miRNAs were negatively correlated with the number of CD34+ cells and positively correlated with reactive oxygen species. The higher levels of hsa-miR-221 and hsa-miR-222 are consistent with increased reactive oxygen species production and inflammatory markers, but a direct involvement with impairment of circulating progenitor cells is yet to be established.

In summary, there is still little evidence for the use of circulating miRNA as biomarkers for hypertension. The field is still in its infancy and this may change over the next few years. Larger sample sizes and improvements in methodology, such as the identification of markers of exosomal membrane that distinguish tissues and different cell types, will be useful. This can be used to pinpoint where the circulating exosomes, and thus the miRNAs, are coming from and their potential roles. The study of circulating exosomes would highly improve currently limited knowledge of the interaction between compartments and cell-to-cell signalling. Another limitation is related to qPCR technology: miRNA sequences are amplified using oligonucleotides specific for small nucleic acids, and the amount of miRNA is very low. The amplification step may lead to artefacts if not properly performed with rigorous controls. Recent advances such as digital PCR (which can measure a single copy of a miRNA) or the use of small RNA sequencing (requiring as little as 5 ng as described in Williams *et al.*[21]) are improving the precision of miRNA measurement.[22] Researchers still need to consider whether pre-amplification is used or not, what housekeeping miRNAs (if endogenous or exogenous, or preferably both) are chosen, and the number of exosomes and cells collected to allow for accurate assessment of each miRNA.

Tissue miRNAs

The studies that analysed miRNAs in tissues relevant to BP and hypertension are summarised below and in Table 2.

We have previously described genome-wide changes in genes and miRNAs measured by microarrays in the renal cortex and medulla of hypertensive ($n=5$ and $n=9$, respectively) and normotensive ($n=3$ and $n=5$, respectively) European subjects who underwent elective unilateral nephrectomies because of non-invasive renal cancer.[23] This cohort, collected specifically to study the molecular aspects of human cardiovascular disease,[24,25] might provide further insights about renal mechanisms which contribute

Table 2. Tissue microRNAs in blood pressure regulation and essential hypertension

Reference	*Tissue*	*Sample size and characteristics*	*Technique used*	*Normalised against*	*Main findings in hypertension and blood pressure*
Marques *et al.*[23]	Kidney	Discovery: 5 HT and 3 NT in cortex, 9 HT and 5 NT in medulla, validation: 22 HT and 16 NT	Discovery: microarrays, validation: qPCR	RNU6	↓ hsa-miR-181a and ↓ hsa-miR-663 bind to and regulate ↑ renin mRNA.
Eskildsen *et al.*[28]	Animals: heart, aortic wall, kidney; humans: internal mammalian artery	Rats treated with Ang II: 6–7/group, treated with endothelin-1: 3–5/group), mice treated with Ang II: 3/group, humans: 16 treated with AGTR1 blockers and 9 treated with β-blockers.	Discovery: microarrays, validation: qPCR	Heart: miR-17 and miR-191; aorta: miR-103 and miR-191; kidney: miR-17 and miR-191; and plasma, miR-17 and miR-103	↑ miR-132 and miR-212 validated in the heart, artery and kidney of the 10 days And II or endothelin-1 treated rats. Both miRNAs were ↓ in patients treated with AGTR1 blockers.
Santovito *et al.*[29]	Atherosclerotic plaques	15 HT and 7 controls	qPCR	RNU6	↑ hsa-miR-145 in HT subjects

Abbreviations: Ang II, angiotensin II; AGTR1, Ang II receptor type 1; *ATM,* ataxia telangiectasia mutated gene; discovery, discovery phase; HT, hypertensive subjects; miRNA, microRNA; mRNA, messenger RNA; NT, normotensive subjects; qPCR, real-time PCR; RNU19, U19 small nuclear RNA; RNU6, U6 small nuclear RNA; validation, validation phase of the study.

to BP regulation. The RNA samples were derived from a pole of kidney unaffected by the neoplastic process. In the renal medulla, 12 genes and 11 miRNAs were differentially expressed, and in the cortex, 46 genes and 13 miRNAs were differentially expressed. These miRNAs and genes were further investigated in 22 hypertensives and 16 normotensives. In the renal medulla samples, we validated by qPCR the miRNAs hsa-miR-638 and hsa-let-7c, and in the cortex samples hsa-miR-21, hsa-miR-126, hsa-miR-181a, hsa-miR-196a, hsa-miR-451, hsa-miR-638 and hsa-miR-663. Two under-expressed miRNAs, hsa-miR-181a and hsa-miR-663, were of particular interest because *in silico* analyses had shown they could target specific sequences in the human renin 3′UTR mRNA. Furthermore, by means of co-transfection experiments *in vitro* using HEK293 cells, we showed that hsa-miR-181a and hsa-miR-663 bind to renin, and also revealed that these miRNAs were able to regulate endogenous renin levels. These results suggest that a change in these miRNAs could explain the overexpression of renin mRNA observed in the hypertensive kidneys. Moreover in the Schlager BHP/2J mouse, a neurogenic model of hypertension with marked circadian elevation of BP,[26] lower levels of miR-181a and higher renin mRNA was reported during the active period,[27] supporting this mechanism.

Owing to the well-recognised role of the renin–angiotensin–aldosterone system (RAAS) in BP regulation, miRNAs involved in the mechanisms of action of Ang II have also been investigated in mice, rats and humans. A study by Eskildsen and colleagues[28] examined global miRNA expression in the left ventricle of Sprague-Dawley rats treated with a constant intravenous infusion of Ang II for 10 days that lead to cardiac hypertrophy and fibrosis. In the heart, aorta and kidneys of Ang II-treated rats, the authors identified that the cluster composed of the miRNAs miR-132 and miR-212 was upregulated, and suggested that this may mediate Ang II-induced hypertension.[28] These miRNAs were also over-expressed in rats treated with endothelin-1 for 10 days, but no fibrosis or cardiac hypertrophy was evident. Moreover, the expression of these miRNAs was attenuated in internal mammalian artery (also known as internal thoracic artery, which supplies blood for the anterior chest wall) of patients treated with AGTR1 blockers compared with those treated with β-blockers.[28] Although the authors speculate the involvement of Gαq, in addition to the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), a thorough investigation of miR-132 and miR-212 gene targets is needed to fully elucidate their contribution to BP regulation.

Santovito and colleagues[29] measured the expression of hsa-miR-145, a miRNA that is involved in VSMC proliferation and vascular tone in human atherosclerotic plaques from 22 subjects with and without essential hypertension. The authors found that this miRNA was overexpressed in hypertension, especially in those treated with Ang II receptor blockers.[29] The mRNA targets of miR-145, however, and thus the genetic mechanisms involved are still unknown.

In summary, there is emerging evidence that miRNAs may be involved in the development of hypertension. Large tissue banks of human kidneys[24,25] are now being studied and will have a crucial part in this. *In vivo* studies in animal models, such as the SHR, where these miRNAs can be knocked out or overexpressed, could play an important role in determining whether miRNAs regulate BP. It is clear, however, that there is a need for more research using larger human cohorts and relevant tissues to fully understand the involvement of miRNAs in BP regulation.

SNPs creating or modifying miRNA-binding sites

SNPs can create or disrupt binding sites for miRNAs (summary of findings in Table 3). If a miRNA binds preferentially to one allele of an mRNA 3′UTR, it results in lower levels of the mRNA or protein. Sethupathy *et al.*[30] conducted a study on hsa-miR-155 and SNPs in the angiotensin II receptor, type 1 gene. They found that miRNA hsa-miR-155 could bind more efficiently to the *A* allele than to the *C* allele (more prevalent in essential hypertension) at position +1166 of the SNP rs5186 in the 3′UTR of the *AGTR1* mRNA. The binding of hsa-miR-155 has the potential to suppress the level of *AGTR1* mRNA and thus cause the pressor effect of Ang II in subjects with the *A* allele.[30] Further evidence for this is that protein levels of AGTR1 in untreated essential hypertension subjects homozygous for the *C* allele of rs5186 were also positively correlated with systolic BP and DBP. AGTR1 protein levels were also negatively correlated with the expression levels of hsa-miR-155, and the miRNA levels were lower in those with the *CC* genotype.[31] These findings are consistent with those in animal models showing that miR-155 was downregulated in aortas of the SHR compared with Wistar–Kyoto, and that exercise as a BP-lowering intervention was able to normalise miRNA expression to Wistar–Kyoto levels.[15]

The modulation of expression of genes in the RAAS by the preferential binding of miRNAs to SNPs in the 3′UTR region has been predicted by two studies,[32,33] of which only one proceeded to experimental validation *in vitro*.[33] Four out of 10 SNPs within eight genes in the RAAS were associated with changes in BP. The authors also demonstrated that SNPs in the arginine vasopressin 1A receptor (*AVPR1A*) gene, bradykinin 2 receptor (*BDKRB2*) gene and thromboxane A2 receptor (*TBXA2R*) gene changed the binding site for several miRNAs.[33]

Besides the RAAS, other mechanisms commonly known to influence BP have been associated with SNPs that lead to the creation or disruption of miRNA-binding sites. The minor *T* allele of the SNP ss52051869 in the 3′UTR of the gene for L-arginine transporter (*SLC7A1*) was previously associated with endothelial and nitric oxide dysfunction, and increased predisposition to hypertension.[34] The *T* allele of this SNP was later associated with the presence of a longer 3′UTR with more binding sites for the miRNA miR-122 (four vs three sites in the shorter 3′UTR associated with the *C* allele).[35] This allele also disrupts the binding of the transcription factor specificity protein 1 (SP1).[35] Another example is the SNP A+1050G in the 3′UTR of the neuropeptide Y1 receptor (NPY1R) mRNA that is important for adrenergic activity and BP control.[36]

The gene for the vacuolar H^+-ATPase subunit (*ATP6V0A1*), an ATP-driven proton pump, was previously associated with BP.[37] The *C* variant of the SNP rs938671 in the 3′UTR of this gene was associated with the downregulation *ATP6V0A1* mRNA levels by Wei *et al.*[38] The group found that having the *C* allele created preferential binding to miRNA hsa-miR-637 and as a result a decrease in *ATP6V0A1* mRNA and protein levels.[38]

The SNP rs5068 in the 3′UTR of the atrial natriuretic peptide (*NPPA*) gene was previously identified to be associated with essential hypertension and BP in a meta-analysis of genome-wide association studies.[2] Recently, miR-425 was shown to be produced in human atria and ventricles, and to bind to the *A* allele, but not to the *G* allele, of rs5068. Therefore, this miRNA could also regulate *NPPA* mRNA levels *in vitro* only when the *A* allele was present.[39]

One investigation by Hanin *et al.*[40] analysed the impact of the preferential binding of miR-608 to the major *C* allele in comparison with the minor *A* allele of the 3′UTR SNP rs17228616 in the acetylcholinesterase (*ACHE*) mRNA. Subjects carrying the *A* allele had reduced serum cortisol and higher BP. The *A* allele presented with 65% higher ACHE hydrolytic activity than the *C* allele. In the presence of the *A* allele, and thus less miRNA-mRNA binding, there was a higher availability of miR-608, resulting in the suppression of other mRNA targets of this miRNA.

Several of the studies described here lacked *in vitro* analyses with the overexpression or inhibition of miRNAs and a direct effect on the expression of mRNAs in a specific cell line. Together these findings highlight the importance of the interaction between miRNAs and polymorphisms at the mRNA level, and future studies

Table 3. Single nucleotide polymorphisms affecting microRNA-binding sites in blood pressure regulation and essential hypertension

Reference	*SNPs*	*miRNAs*	*Sample size and characteristics*	*Tissue*	*miRNA normalised against*	*Main findings in hypertension and blood pressure*
Sethupathy *et al.*[30]	rs5186 in *AGTR1*	hsa-miR-155	N/A	Only *in vitro* experiments		hsa-miR-155 binds more efficiently to the *A* allele. The *C* allele is associated with HT.
Ceolotto *et al.*[31]	rs5186 in *AGTR1*	hsa-miR-155	$n=64$ (25 AA, 20 AC and 19 CC genotypes)	Peripheral blood mononuclear cells	RNU48	hsa-miR-155 expression was negatively correlated with AGTR1 protein and was lower in subjects with *CC* genotype.
Nossent *et al.*[33]	rs11174811 in *AVPR1 A*, rs5225 and rs2069591 in *BDKRB2*, rs13306046 in *TBXA2R*	hsa-miR-526, hsa-miR-578, hsa-miR-34a, hsa-miR-34b, hsa-miR-449, hsa-miR-765	$n=1246$ used for SNP genotyping only	Only *in vitro* experiments	N/A	hsa-miR-526 and hsa-miR-578 bind to the *G* allele of rs11174811, associated with lower BP. hsa-miR-34a, hsa-miR-34b, and hsa-miR-449 bind to the *T* allele of rs5225, associated with lower BP. hsa-miR-765 binds to the *C* allele of rs13306046, associated with higher BP.
Yang and Kaye[35]	ss52051869 in *SLC7A1*	hsa-miR-122	Genotyping: 391 NT and 240 HT, sequencing: 3 x 7 pooled samples of each genotype	Buffy coat (blood), *in vitro* experiments	N/A	The *T* allele, associated with hypertension, is linked to longer 3'UTR (with 4 binding sites for hsa-miR-122). Together with the transcription factor SP1, miR-122 could regulate *SLC7A1* expression depending on the allele present at this SNP.
Wang *et al.*[36]	A+1050G in *NPY1R*	hsa-miR-511	Discovery: 80 subjects, validation: 376 twins/siblings of European ancestry and 936 subjects (426 high DBP)	*In vitro* experiment (but not specific for hsa-miR-511)	N/A	The miRNA hsa-miR-511 was predicted by bioinformatics to bind preferentially to the *G* allele, associated with higher BP.
Wei *et al.*[38]	rs938671 in *ATP6V0A1*	hsa-miR-637	N/A	*In vitro* experiments	N/A	hsa-miR-637 reduces *ATP6V0A1* expression by binding preferentially to the *C* allele associated with hypertension.
Arora *et al.*[39]	rs5068 in *NPPA*	hsa-miR-425	Genotyping: 699, sodium studies: 31, *NPPA* mRNA in blood and genotyping: 2246, tissue miRNA studies: 15 in total	Whole blood for *NPPA* mRNA, atria and ventricles for miRNA, *in vitro* experiments	RNU6	hsa-miR-425 binds to and regulate *NPPA* mRNA in presence of the *A* but not the *G* allele. *AA* genotype associated with higher predisposition to hypertension. Individuals with at least one *G* allele had↑*NPPA* mRNA in blood and↑NPPA plasma levels after sodium consumption.
Hanin *et al.*[40]	rs17228616 in *ACHE*	miR-608	$n=172$ African-Americans and $n=289$ European descendants	Entorhinal cortices, blood, *in vitro* and *in vivo* experiments	N/A	Carrying the *A* allele was associated with reduced serum cortisol and higher BP. miR-608 binds preferentially to the major *C* allele over the minor *A* allele. In the presence of the minor allele, more miR-608 is available to suppress other mRNA targets.

Abbreviations: 3'UTR, 3' untranslated region; ACHE, acetylcholinesterase; AGTR1, angiontensin II receptor type 1; *ATP6V0A1*, vacuolar H^+-ATPase subunit gene; *AVPR1A*, arginine vasopressin 1A receptor gene; *BDKRB2,* bradykinin 2 receptor gene; DBP, diastolic blood pressure; miRNA, microRNA; mRNA, messenger RNA; *NPPA*, atrial natriuretic peptide gene; *NPY1R*, neuropeptide Y1 receptor; RNU48, U48 small nuclear RNA; RNU6, U6 small nuclear RNA; *SLC7A1,* L-arginine transporter gene; SP1, specificity protein 1 transcription factor; *TBXA2R,* thromboxane A2 receptor gene. If a miRNA binds preferentially to one allele of an mRNA 3'UTR than to other allele(s), it results in lower levels of the mRNA or protein.

could gain more insight by exploring these interactions at the whole-genome level. Once again the difficulty will be to acquire relevant tissues, as miRNAs are tissue-specific, and large cohorts would be necessary to ensure sufficient power.

Polymorphisms in miRNA sequences

Some of the SNPs identified in the genome-wide association meta-analysis for BP and hypertension were not in the coding regions,[2] and thus could be in regions containing sequences for

ncRNAs. So far none of these SNPs are in sequences known to contain ncRNAs. Fu *et al.*[41] conducted an association study in 156 hypertensives and 187 normotensives between SNPs in miRNA sequences (miR-143) and essential hypertension. They found that the frequency of the minor *C* allele of SNP rs4705342 in the promoter of miR-143 was lower in the hypertensive group (39% controls vs 27.9% cases), and that it was associated with a lower risk of hypertension.[41] The authors did not investigate whether the presence of this SNP resulted in changes in the expression of miR-143. This genetic association needs to be replicated in a larger, independent cohort.

Long ncRNAs

Long ncRNAs (lncRNAs, >200 base pairs) include circular RNAs (>1900 so far detected in the human genome),[42] transcribed ultra-conserved regions (>350), large intergenic ncRNAs (>1000) and others,[6] summarised in Figure 2. The classic example of a lncRNA is *XIST*, which is responsible for silencing one copy of the X chromosome in female cells.[6]

So far, no studies have been reported on lncRNAs and human hypertension and/or BP. One study using *ex vivo* experiments offered important insights into these emerging regulatory elements of the transcriptome. Here, Leung and colleagues[43] identified, through RNA-seq, 466 lncRNAs expressed in VSMC isolated from rats and 24 novel lcnRNAs in Ang II-treated VSMC.[43] One lcnRNA in particular, *Lnc-Ang362*, is located in the genome near two miRNAs, miR-221 and miR-222, and all were involved in VSMC proliferation. Interestingly, Leung *et al.*[43] suggested that the transcriptional start site for *Lnc-Ang362* is enriched for histone H3-lysine-4 trimethylation (H3K4me3), pointing to an epigenetic control. This highlights the complexity and difficulty of investigating the function of lncRNAs. The field, however, is still in its infancy and little is known about the roles and regulation of lncRNAs. A summary of ncRNAs reviewed here is shown in Figure 3.

Challenges of studying ncRNAs in BP

Changes in the expression of any ncRNAs, as well as in mRNAs, could either contribute to or be a secondary effect of hypertension. When the sequence of the ncRNA is conserved between humans and rodents, the use of animal models might assist in providing physiological support for a putative causative mechanism inferred from molecular and *in vitro* findings. No tissues, besides kidneys and arteries (including VSMC), are currently available in humans, thus restricting research on the function of ncRNAs. In this regard, the availability of tissue engineering may be of use in the future.[44]

There are several algorithms and tools to predict the interaction between ncRNAs and ncRNAs, and ncRNAs with protein-coding mRNAs and DNA; but these predictions need validation by time-consuming *in vitro* techniques, such as luciferase assays for miRNAs and mRNAs. Although well-studied in other areas of human disease, deciphering interactions between RNA-RNA and RNA-DNA has been under-researched in BP regulation. Such studies can be challenging, because the effects of ncRNAs may only take place under certain conditions, such as in response to stressors or during specific developmental stages, which might further complicate findings. Furthermore, circular RNAs can act as efficient miRNAs sponges and may have several sites that bind to a specific miRNA thereby counteracting its molecular actions.[45] MiRNAs have also been found to bind to and regulate large intergenic ncRNA levels in a similar way as they regulate mRNAs.[46]

Clinical implications

The therapeutic use of miRNAs is currently being explored through the approaches of overexpression and inhibition in many diseases, including cardiovascular diseases. In hypertension, one example of therapeutic use of a miRNA is miR-22, which targets chromogranin A (Chga) mRNA.[47] SHR treated with four doses of miR-22 inhibitor at 25 mg kg^{-1} exhibited a decrease in BP of

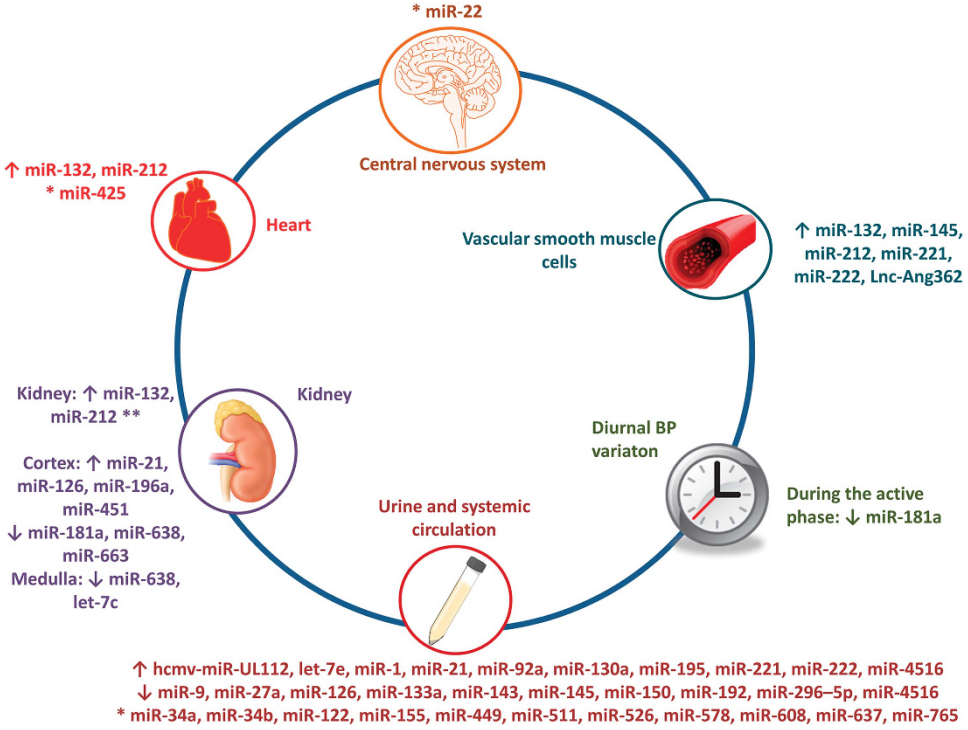

Figure 3. Schematic figure of ncRNAs likely to be involved in the regulation of BP and/or predisposition to hypertension, acting in different tissues in the human body. ↓ indicates downregulated and↑upregulated ncRNAs, whereas * indicates SNPs in the binding site of miRNA in BP-associated genes. ** No division between renal medulla and cortex was made in the study, so miRNAs were grouped together as 'kidney'. Lnc, long ncRNA.

18 mm Hg in 9 days.[47] Polymorphisms in the gene for *CHGA* and plasma concentrations of CHGA have been associated with human hypertension.[48] A polymorphism in the 3'UTR, which increases the binding of miR-22, was identified in the SHR.[47] There have been few studies to date that have assessed the use of miRNAs for anti-hypertensive therapy, but there are several examples in other cardiovascular diseases. For example, the inhibition of miR-25 in an established model of heart failure improved cardiac contractility and function.[49]

CONCLUSIONS

The recent advent of whole-genome sequencing and (small) RNA sequencing technologies has made more information about the human genome and transcriptome available. Both have also revealed the incredibly complex and malleable nature of integrations that exist between gene, RNA and protein. As summarised in Figure 3, there are currently a small number of studies that support a role of ncRNAs in BP regulation and essential hypertension (especially those involving miRNAs and inflammation or RAAS pathways). NcRNAs are, however, a relatively new area of investigation in hypertension research. Advances have lagged behind those made in other fields such as cancer and heart failure. Research into the individual role of ncRNAs and the interaction between different types of ncRNAs, DNA polymorphisms and environment is needed in hypertension. Studies using larger cohorts and tissues besides biological fluids should assist in a better understanding of the multifaceted roles of ncRNAs. Considering many studies have revealed that miRNAs are critical regulators of cardiovascular development[50] and ageing,[51] there are still important opportunities in the field to further understand how ncRNA affects hypertension.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

FZM and FJC are supported by grants from the National Health & Medical Research Council of Australia (NHMRC), the National Heart Foundation and the Federation University Australia 'Self-sustaining Regions Research and Innovation Initiative', an Australian Government Collaborative Research Network (CRN). FZM is supported by NHMRC (APP1052659) and National Heart Foundation (PF12M6785) co-shared Early Career Fellowships. SAB is supported by an Australian Postgraduate Award scholarship.

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