Chapter 10

Renin-Angiotensin System MicroRNAs, Special Focus on the Brain

Jose Gerardo-Aviles, Shelley Allen and Patrick Gavin Kehoe

Additional information is available at the end of the chapter

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Abstract

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression with important roles in cancer, cardiovascular and neurological disorders. Present in the brain, they play numerous regulatory roles shaping the proteome in an orchestrated manner with other non-coding RNAs. An independent brain-specific renin-angiotensin system (RAS) exists that is subject to miRNA remodelling. The brain RAS regulates cerebral blood flow and electrolytic balance and is involved in neurotransmitter signalling and cognitive processes. Circulating microRNAs allow interaction between systemic and local RAS in the heart and the brain. Their screening and manipulation may be valuable towards understanding pathophysiology and development of treatments for various systemic and central nervous system diseases.

Keywords: non-coding RNAs, microRNAs, brain renin-angiotensin system, cerebrovascular disease, circulating microRNAs, biomarkers

1. Introduction

High blood pressure, leading to cardiovascular and cerebrovascular disorders, is the principal cause of morbidity and mortality worldwide [1–3]. The renin-angiotensin system (RAS) is a major regulator of cardiovascular function and pharmaceutical compounds targeting the RAS are frontline treatments to control high blood pressure [4, 5]. In addition, lifestyle risk factors such as obesity, insulin resistance, high alcohol and salt intake and ageing promote the development of hypertension through epigenetic mechanisms [6–9]. These mechanisms have attracted attention because of their reversibility by environmental and lifestyle modi-

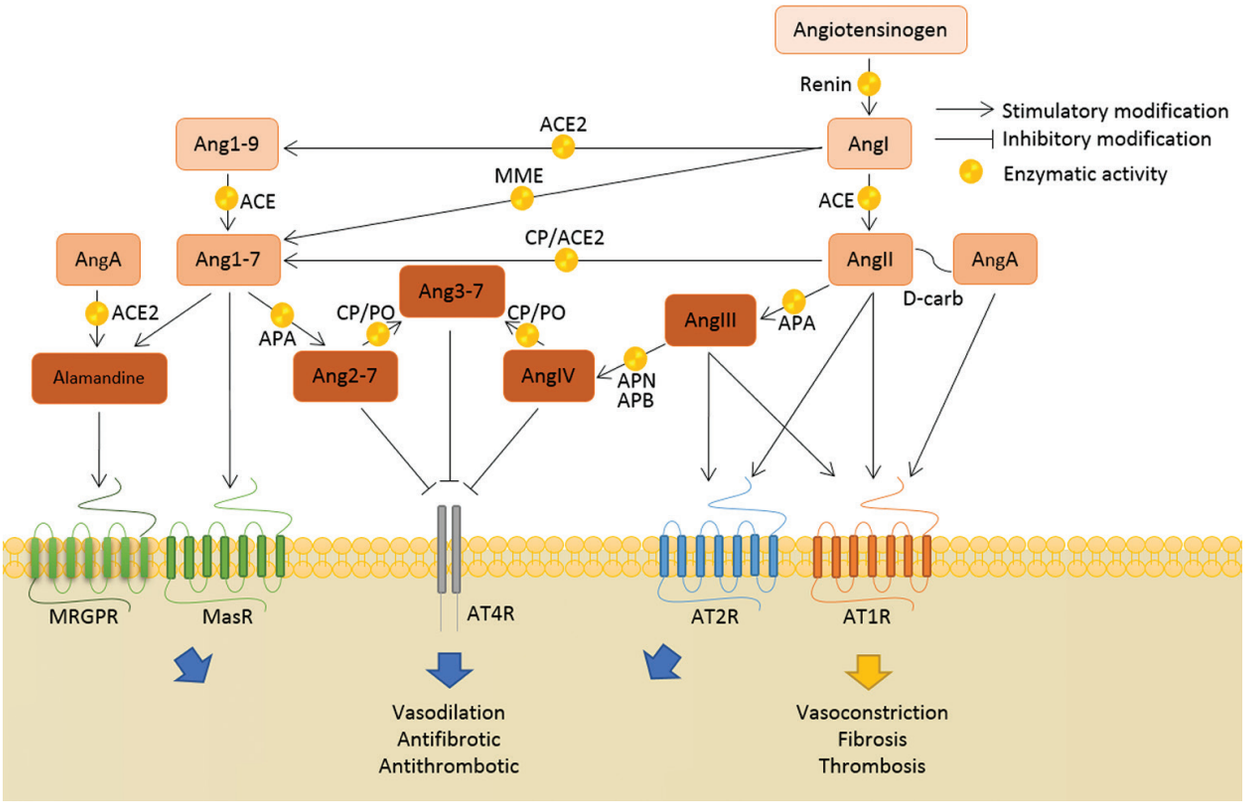

Figure 1. The renin-angiotensin system (RAS) and its components. This schematic depicts angiotensin ligands, receptors and the main enzymes involved; other peptidases and cathepsins also participate although to a lesser extent. All of the components of the RAS are present in the brain. RAS has two main axes: the pressor axis (tending towards an increase in blood pressure) comprising Ang II, ACE and AT1Rs and the counter-regulatory axis comprising Ang(1–7), ACE2 and MasR. Angiotensinogen is a substrate for renin to produce angiotensin I (Ang I), which is the inactive precursor of all angiotensin peptides. Conversion of Ang I to its most active ligand in the pressor axis, angiotensin II (Ang II), results from ACE-mediated hydrolysis [22]. Ang II is then sequentially converted to angiotensin III (Ang III) and angiotensin IV (Ang IV) by aminopeptidase A (APA) and aminopeptidase N (APN) respectively, which can be further cleaved by carboxypeptidase P (CP) and prolyl oligopeptidase (PO) to form angiotensin 3–7 (Ang3–7). Alternatively, Ang II can be converted, via the counter-regulatory axis to angiotensin 1–7 (Ang1–7) by carboxypeptidase P (CP) or ACE2, while both angiotensin A and Ang1–7 can be converted to alamandine by an ACE-mediated decarboxylation reaction [22–27]. Notably, angiotensin ligands acting on AT4R (also called insulin-regulated aminopeptidase (IRAP)) can have agonist or antagonist effects depending on whether or not they bind in the IRAP peptidase domain.

fications, making them important in the detection and treatment of multifactorial diseases such as hypertension [7, 10].

Some of those epigenetic modifications are mediated by miRNAs, defined as single-stranded, non-coding RNA sequences approximately 21–23 nucleotides in length, expressed under physiological and pathological conditions [11, 12]. Deletion of complexes involved in miRNA biogenesis resulted in deleterious and non-viable phenotypes, highlighting their necessary involvement in the cellular development and differentiation [13, 14]. To date, 28,645 miRNAs have been reported in miRbase, a widely used resource for miRNA cataloging and nomenclature [15]. As epigenetic regulators of gene expression, functions of miRNAs include RNA degradation, inhibition of protein expression, regulation of methylation and histone modification on DNA [12, 14, 16]. miRNAs perform these functions by complementary base pairing

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to the target mRNAs through a seed-pairing region of 6–8 nucleotides at the 5′ end of the miRNA. They also interact with other non-coding RNAs and mediate proteome remodelling. Non-coding RNAs represent 98% of the genome, comprising transfer and ribosomal RNA, small nuclear (snRNA) and nucleolar RNA (snoRNA), small interference RNA (siRNA), Piwi-interacting RNA (piRNA) and long non-coding RNAs (lncRNA) [14, 17, 18].

Dysregulation of miRNAs is associated with cancer, cardiovascular and neurodegenerative disorders. The RAS, with important signalling roles in numerous organs and regulatory pathways and being subject to miRNA-mediated remodelling, is a potential factor in many disorders. Thus, the presence of miRNAs that have the capacity to shift the balance between prominent and deleterious functions of the RAS to beneficial roles is interesting, particularly new advances in methods that allow the detection of circulating miRNAs. Exosomes and their role in cellular transport provide a source for miRNA profiling and the presence of miRNAs in the peripheral circulation suggests that they work in an autocrine, paracrine and also endocrine manner, allowing widespread distribution of miRNAs through the entire body. Therefore, screening of miRNA in biological fluids like a serum and cerebrospinal fluid is relevant for an understanding of normal function as well as pathophysiology with a view to potential novel treatments for the disease.

The discovery of local independent but interacting RAS systems, including the brain, which also interacts with systemic RAS [3], has helped to change the original view that the RAS was solely an endocrine system important in regulating blood pressure, electrolytic homeostasis, vascular injury and repair [19]. The brain RAS discussed here (**Figure 1**) is multifunctional including regulation of cerebral blood flow, electrolyte balance, neurotransmitters, learning and memory, many of which may be associated with certain neurological disorders [20, 21].

2. Biogenesis and function of miRNAs

Canonical miRNA biogenesis starts with transcription of the primary miRNA sequence by RNA polymerase II and III [14]. Approximately half (52%) of human miRNAs are located in intergenic regions, 40% in intronic and 8% in exonic [28]. Intergenic miRNAs are independently expressed through promoter elements; yet related miRNAs that often have overlapping targets can be located on different chromosomes and expressed under different conditions. Intronic and exonic miRNAs that are clustered within 50 kilobases from each other show similar expression, while those spaced further apart tend not to [29]. However, there are some exceptions. Some miRNAs separated by more than 50 kilobases retain high correlation, likely as a result of co-expression [30]. The differential localization and expression of miRNAs suggest an evolutionary response to environmental insults and specific cell responses, a theory supported by observed higher numbers of miRNAs expressed in organisms of higher complexity [31–33].

Figure 2A–F summarizes the process of miRNA biogenesis. Primary miRNAs are cleaved in the nucleus by a nuclear microprocessor complex comprised of the RNase III endoribonuclease DROSHA and its double-stranded RNA-binding protein DGCR8—DiGeorge Critical

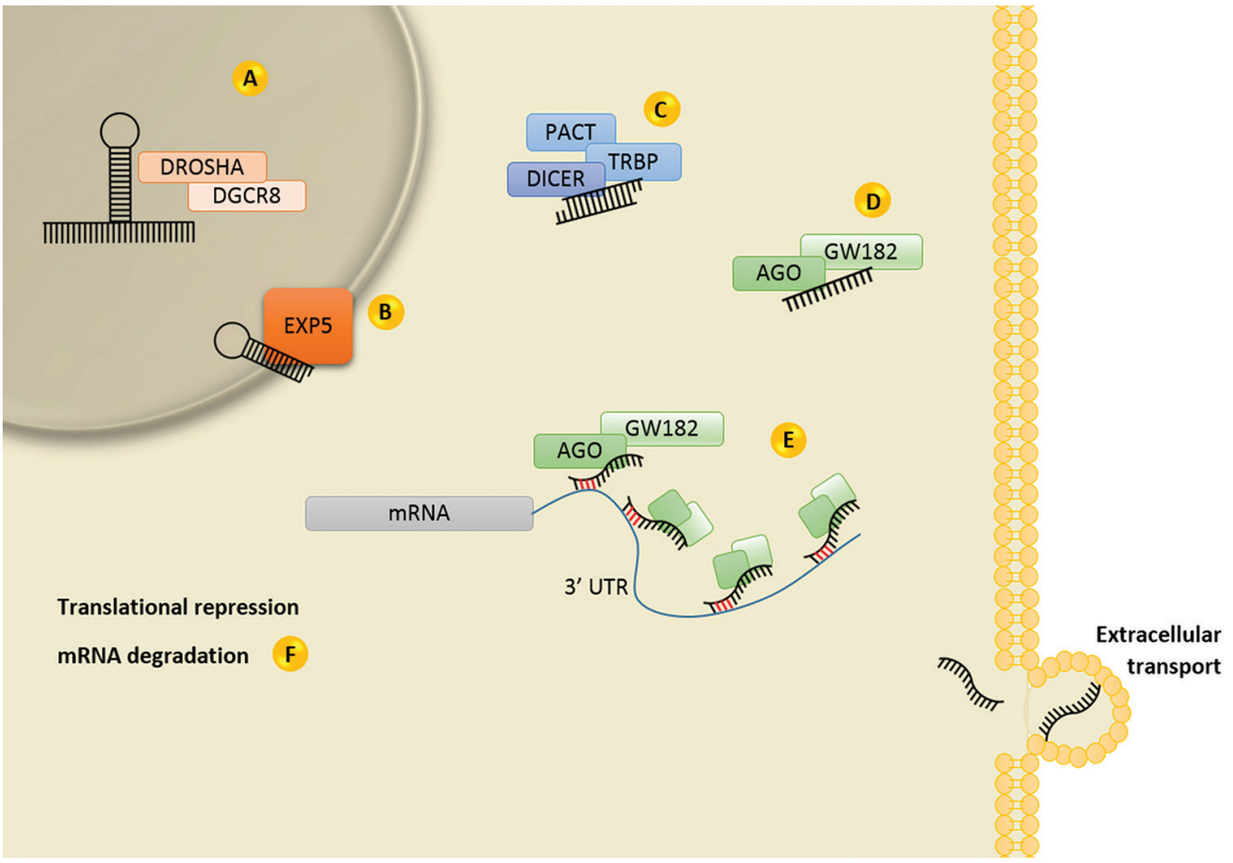

Figure 2. MicroRNA biogenesis and function. (A) Primary miRNAs are cleaved in the nucleus by the RNase III endoribonuclease DROSHA and DGCR8. (B) Once the primary miRNA is cleaved, the nuclear transport receptor exportin 5 binds the 3′ overhang structure of the pre-miRNA to export it to the cytoplasm. (C) The RNase III enzyme Dicer and TRBP and PACT target the pre-miRNA through the 3′ overhang, converting it into mature miRNA, liberating a duplex nucleotide structure with two nucleotides protruding at the 3′ end. (D) The guide strand is loaded into the RNA-induced silencing complex (RISC), and the passenger strand is degraded by RNases. (E) Complementary pairing with the seed region to mRNAs determines target binding and guides argonaute proteins to stop translation. Accumulation of untranslated mRNA in the cytoplasm allows recruitment of members of the GW182 protein family. (F) Deadenylase complexes cause destabilization of the transcript and further degradation by RNase activity.

Region 8, **Figure 2A** [34]. This cleavage by DROSHA/DGCR8 produces a 60 nucleotide stem-loop structure with a 3′ overhang, the pre-miRNA [11, 34, 35]. The primary miRNA can also be further subjected to RNA editing by ADARs (adenosine deaminases acting on RNA) that modify adenosine to inosine producing miRNA isoforms called isomiRs [36].

Exportin 5 allows export of the pre-miRNA to the cytoplasm, **Figure 2B** [36], where Dicer and substrate stabilizing binding partners, TRBP (trans-activation response RNA-binding protein) and PACT (protein activator of RNA-activated protein kinase) facilitate conversion into mature miRNA, **Figure 2C** [12, 14]. Two strands result from the unwinding of the duplex, the guide (3p) and passenger (5p) strands. Most of miRNA effects are mediated by the 3′ form; the 5′ form comprises <10% of all miRNA reads in humans [36]. The guide strand is loaded into the RNA-induced silencing complex (RISC) and the passenger strand is degraded by RNases, **Figure 2D**. IsomiRs can also be produced at this step by trimming and capping of the mature miRNA.

Non-canonical miRNA biogenesis is independent of DROSHA/DGCR8 processing in the nucleus. Such biogenesis arises if an intron is spliced lacking the sequences ordinarily flanking the stem region of a primary miRNA and it is of sufficient size to generate a pre-miRNA and it can be exported to the cytoplasm and further processed as a pre-miRNA to form a mirtron. Alongside mirtrons, other RNA sequences derived from transfer RNA and small nucleolar RNA are loaded into an RISC complex and act as miRNAs [13, 14, 29].

The RISC is a ribonucleoprotein complex that mediates mRNA degradation, destabilization or translational inhibition, whatever the biogenesis mechanism and comprises the miRNA guide strand and argonaute proteins, **Figure 2E**. The complementary base pairing of the miRNA seed region (2nd to 8th position on the 5′ end) to mRNAs determines target binding and guides argonaute proteins [28, 37, 38]. miRNA levels are dependent on argonaute proteins [39, 40] that are also present in the nucleus and currently, only miRNA-29b has been shown to translocate and localize in the nucleus [14, 39, 41]. In humans, argonaute 2 (also called eukaryotic translation initiation factor 2C) cleaves target mRNAs [29] but can also block other translation initiator factors and ribosomal subunits [42].

After pairing of the miRNA seed region, protein translation can be inhibited, **Figure 2E**. Accumulation of untranslated mRNA in the cytoplasm allows argonaute 2 to recruit members of the GW182 protein family, which are enriched in cytoplasmic areas called processing bodies (p-bodies) [42]. Here, the mRNA is destabilized by deadenylase complexes and further degraded by RNases [43–45]. Finally, the effect miRNAs have on protein or mRNA levels depends on the position where the miRNA binds and five different classes of miRNA binding have been determined [12, 46, 47]. Most miRNA effects are mediated by binding at the 3′ UTR of mRNA and further processing as described previously, non-canonical binding sites represent <1% [48].

3. MicroRNAs as autocrine, paracrine and endocrine molecules

miRNAs not only shape the intracellular proteome within specific cell types in response to microenvironment stimuli and cues, but can also mediate intercellular effects by means of nanotubes, exosomes and binding proteins, all mechanisms of intercellular communication [49]. Moreover, extracellular vesicles, including exosomes, microvesicles and apoptotic bodies, also participate in paracrine and endocrine signalling, as well as an intercellular transfer of miRNAs [50–52]. Exosomes, in particular, which are nanovesicles derived from endosomes are involved in cell-to-cell communication [53], contain significant amounts of miRNAs and are resistant to changes in temperature, pH and the effect of RNases making them reliable sources for screening [51, 54, 55]. miRNAs are transported by RNA-binding proteins and are taken up into intraluminal vesicles during the formation of multivesicular bodies in endosomes [56]. Upon fusion of the endosome to the plasma membrane, the intraluminal vesicles are released as exosomes and due to their lipid composition and size, they can easily transfer genetic material across lipid membranes [55, 56]. Several miRNAs are transferred in vivo and in vitro between fibroblasts, cardiomyocytes, human umbilical endothelial cells, mesenchymal stem cells, cardiac and cerebral endothelial cells [57, 58], while atheroprotective communication has been found between endothelial and smooth muscle cells through miRNAs [59].

miRNA transfer both propagates deleterious effects and helps recover cells from insults and prevent apoptosis. For example, miR-133 is increased in people with cardiovascular disease and is transferred through exosomes from multipotent mesenchymal stromal cells to astrocytes and neurons that promote recovery after stroke [60–63]. Furthermore, remote ischaemic conditioning, a technique of small cycles of ischaemia/reperfusion in distal extremities, was protective for cardiac and cerebrovascular effects in animal experiments and human clinical trials, with effects mediated by miRNAs such as miR-1 [64–69].

Exosomal circulating miRNAs have many properties that arguably make them ideal biomarkers, including their presence in peripheral blood, detection in many biological fluids, their stability in RNase-rich body fluids and their tissue-specific expression patterns. These have been described in cardio-cerebrovascular disorders, diabetes, dyslipidemia and neurodegenerative disorders [1, 70–76]. Furthermore, human exosomes can be used therapeutically as a gene delivery vector to provide cells with heterologous miRNAs [53].

4. Regulation of RAS by associated microRNAs

Given that there is further discussion of the biochemical functions of the RAS in other chapters, the discussion henceforth will focus on some of the most important components of the brain RAS and the miRNAs targeting them.

Since the vital function of the brain results in high physiological demands (i.e. requiring 20% of total cardiac output and a 10-fold higher oxygen and energy demand than other tissues), it requires strict coordination between blood flow and neuronal activity, a phenomena known as functional hyperaemia [77]. Cerebral blood flow is regulated by vasomotor, metabolic and neurogenic mechanisms, but can be modulated by vasoconstrictors such as Ang II and endothelin, vasodilators such as bradykinin, adenosine and other angiotensin ligands, while blood vessel capacity may be reduced or impeded by plaques of cholesterol, amyloid or fibrotic deposits.

Analysis by TargetScan [48], a software that predicts miRNA binding sites, suggests that 368 different miRNA families target RAS elements, the majority of which share transcripts. **Table 1** summarizes the total number of miRNAs and unique miRNAs with respect to RAS elements, as they have other targets outside the RAS. Angiotensin 4 receptor (also known as AT4R or IRAP) has 252 miRNA families associated with it, making it the highest amongst the RAS and approximately fivefold and threefold as many as that for arguably its better known receptors AT1R and AT2R. Notably, 88% of IRAP-associated miRNAs also regulate other RAS transcripts, suggesting its susceptibility to changes elsewhere in the RAS. In particular, IRAP has 28 miRNA families exclusively associated with it (also the most for RAS components), hinting at having high functional importance. Indeed, aminopeptidase B and dipeptidyl peptidase, necessary for Ang IV conversion, do not have exclusive miRNAs and thus may be subject to many regulatory effects.

A more in-depth examination of RAS-associated microRNAs, according to their functional impact in the RAS physiology is shown in **Table 2**. MiR-3163 targets the greatest number of RAS transcripts (N = 8) and may provide an over-arching level of regulation for the pathway

RAS element	Gene symbol	Total miRNA families	Unique miRNA families
Angiotensinogen	AGT	85	11
Angiotensin 1 receptor (AT1R)	AGTR1	46	1
Angiotensin 2 receptor (AT2R)	AGTR2	78	5
Angiotensin 4 receptor (AT4R/IRAP)	LNPEP	252	28
Mas receptor	MAS1	5	1
Angiotensin converting enzyme	ACE	59	4
Angiotensin converting enzyme 2	ACE2	54	4
Renin	REN	21	2
Neprilysin	MME	151	8
Aminopeptidase B	RNPEP	29	0
Aminopeptidase N	ANPEP	31	4
Aminopeptidase A	ENPEP	158	13
Dipeptidyl peptidase	DPP3	32	0

The total number of miRNAs represents miRNA families with binding sites at the 3′UTR region based on TargetScan [48]. Unique miRNAs are those considered solely with respect to other RAS elements.

Table 1. miRNA families targeting RAS elements.

as a whole, for example, in response to an external stimulus. miR-125-5p with five targets in common may function in a similar way, particularly since two of the targets are principal enzymes in RAS biochemistry. Yet, they make an ideal combination to block Ang II/AT1R and Ang IV/AT4R pathways and also shift the conversion of Ang I to Ang (1–7) via neprilysin and other peptidases to act on MasR.

In terms of RAS function, a group of microRNAs that can shift a predominant role of, for example, the Ang II/AT1R axis to opposing axes such as Ang(1–7)/MasR or Ang IV/AT4R could change cerebral blood flow, response to hypoxia and perhaps influence cognition and vice versa. Indeed, a panel of 17 miRNA families target aminopeptidase A and IRAP that could potentiate the formation of Ang IV (since aminopeptidase A converts Ang I and Ang II to Ang III, the Ang IV precursor for Ang IV). Thus, upregulation of those 17 miRNAs could modulate Ang III and IRAP to a greater extent than just one miRNA, such as miR-125. The net effect of reducing both ligand and receptor means that the function of the Ang IV/AT4R axis might be completely inhibited with likely deleterious effects on blood flow and cognitive performance. By contrast, downregulation of these miRNAs would increase the Ang IV/AT4R axis. The following section will discuss the effect of some specific miRNAs and their regulatory effects in RAS in the brain in health and in disease states.

RAS components	microRNA families in common	
ACE ACE2 ANPEP ENPEP LNPEP	1	**miR-125-5p**
ANPEP DPP3 ENPEP LNPEP	1	miR-670-3p
AGTR2 DPP3 LNPEP MME	1	miR-17-5p/20-5p/93-5p/106-5p/519-3p
ACE2 ENPEP LNPEP MME	3	miR-9-5p, miR-200-3p/429, miR-942-5p
AGTR2 LNPEP MAS1	1	miR-23-3p
ENPEP LNPEP MME	17	miR-26-5p, miR-30-5p, **miR-132-3p/212-3p**, miR-194-5p, miR-204-5p/211-5p, miR-216-5p, miR-376-3p, miR-376c-3p, miR-378-3p, miR-450b-5p, miR-518d-5p/519-5p, miR-522-3p, miR-580-3p, miR-653-5p, miR-1269, miR-3942-5p, miR-4766-3p
ACE2 ENPEP LNPEP	4	miR-374-5p/655-3p, miR-543, miR-4424, miR-1306-5p
ACE2 LNPEP MME	3	miR-374a-3p, miR-3194-3p, miR-5691
DPP3 LNPEP MME	5	miR-146-5p, miR-183-5p.1, miR-589-5p, miR-876-5p, miR-2355-5p
ACE2 DPP3 LNPEP	1	miR-329-3p/362-3p
ACE2 ENPEP MME	1	miR-140-3p.1
ENPEP LNPEP	17	miR-9-3p, miR-19-3p, miR-29-3p, miR-34b-5p/449c-5p, miR-105-5p, miR-122-5p, miR-144-3p, miR-320, miR-323-3p, miR-323b-3p, miR-382-3p, miR-494-3p, miR-514a-5p, miR-515-5p/519e-5p, miR-642a-5p, miR-3146, miR-5579-3p
DPP3 ENPEP	1	let-7-5p/98-5p
MAS1	1	**miR-143-3p**
ENPEP	13	**miR-133**, miR-142-3p.2, miR-219-5p, miR-371a-3p, miR-409-5p, miR-451, miR-496.1, miR-508-3p, miR-526b-5p, miR-877-5p, miR-1185-5p, miR-5094
ACE AGTR1 DPP3	1	**miR-34-5p/449-5p**
AGTR1	1	**miR-1-3p/206**
AGT AGTR1 AGTR2 DPP3 ENPEP LNPEP MME RNPEP	1	miR-3163

From 164 combinations of overlapping targets and miRNAs in common, only 14 are included here, 12 which if increased would favour vasoconstriction and 2 would increase vasodilation. Others tend to influence multiple RAS pathways, an example, miR-3163, is given at the bottom of the table. miRNAs in bold are described further in the text.

Table 2. A summary of the subgroups of miRNAs according to their functional effect in the RAS.

5. miRNAs and RAS: cerebrovascular regulation and cognitive function

5.1. MiR-1/206

The miR-1/206 family has been suggested to exclusively target AT1R in the RAS; however, it has an estimate of 790 other transcripts regulating other systems [48]. MiR-1 and miR-206 are located in chromosomes 20 and 6, respectively and share homology in the seed region.

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An evaluation of biochemical, cardiovascular and performance indexes of aerobic exercise activity showed that some miRNAs were significantly increased. Specific correlations were found between miR-1, miR-133a and miR-206 and performance parameters, with miR-206 having the strongest positive correlation [78]. MiR-1 was also found to be decreased 1.4-fold in post-mortem cardiac tissue from acute myocardial infarction patients [79]. In contrast, elevated plasma miR-1 levels were reported to predict heart failure after acute myocardial infarction although they returned to basal levels after medication [80].

In conditions of hypoxia such as infarcts, oxygen/glucose deprivation or with ischaemia/reperfusion intervals, miR-1 is highly expressed [79, 80]. Under less stressful and non-life-threatening situations, miR-206 is transcribed [78], both of them targeting AT1R to decrease Ang II-mediated vasoconstriction and in doing so increasing the supply of oxygen and glucose to cells to prevent apoptosis.

MiR-1 overexpression inhibits contractility and proliferation of human vascular smooth muscle cells (VSMCs) in vitro in a negative feedback loop [81, 82]. MiR-1 is downregulated in VSMCs from spontaneous hypertensive rats and its overexpression in vivo inhibits the proliferation of VSMCs by targeting insulin-like growth factor 1 (IGF1) [83]. By contrast, miR-1 upregulation enhances angiogenic differentiation of human cardiomyocyte progenitor cells [84]. The opposite effects of miRNA in different cell types may be explained by its cell-specific expression. Indeed, even if the miRNA is expressed under physiological conditions, variations to this will depend on local gene expression in a time- and cell type-dependent manner.

Evidence of peripheral and central roles for miRNAs was seen in a transgenic mouse model of cardiac-specific overexpression where miR-1 levels were increased not only in the heart but also in the hippocampus and peripheral blood. Furthermore, the mice showed cognitive impairment by downregulation of brain-derived neurotrophic factor (BDNF), a target of miR-1 [85], providing strong evidence for a role in endocrine signalling and association between vascular disorders and cognitive impairment. Nevertheless, it is unlikely that the response depends exclusively on miR-1 and it is not known as to whether the associations are primary or secondary in nature.

Collectively, miR-1 may serve to support protective mechanisms to adapt to adverse hypoxic insults and remodel the proteome as a result. Indeed, as mentioned above, remote ischaemic conditioning showed a high correlation between ischaemia/reperfusion intervals and the levels of miR-1 in rats independent of BDNF mRNA and protein levels [69]. Hence, the miR-1/206 family is likely important in cardioprotection, prevention of stroke and consequently cognitive impairment. Already it is used in screening for myocardial infarction, monitoring and response to therapy and also has a tentative therapeutic use for increasing vasodilation and angiogenesis [86, 87].

However, a solitary miRNA or miRNA-target interaction, such as between miR-1/206 and AT1R, is unlikely to be able to explain a complete physiological response. Inherent properties between miRNA transcription, interactions between their targets, the timing of their expression and subcellular localization provide a more likely explanation. A panel of dysregulated miRNAs is likely to cause an imbalance in targets, proteins and pathways involved. Such a characteristic combination of altered miRNAs may be useful as diagnostic tools. For example, a diagnosis of cholangiocarcinoma can now be made with 100% accuracy in the presence of a 30-miRNA signature, three of them are useful for prognosis and monitoring and one of which has already entered a Phase I clinical trial as a potential treatment [88–91].

5.2. MiR-143

The Mas receptor (MasR) has the lowest number of associated miRNAs, implying steady and tightly regulated homeostatic expression, although other post-transcriptional modifications are also likely to be involved in its regulation. In addition, miR-143 is exclusive to MasR in the RAS and interestingly, it has been found to be dysregulated in vascular disorders [92]. MiR-143 is enriched in cardiac stem cells before becoming localized to smooth muscle cells, including neural vascular smooth muscle cells (VSMC) in mice and its expression was found to be dependent on heartbeat rate in zebrafish [93, 94]. In human peripheral blood mononuclear cells, miR-143 was upregulated in patients with essential hypertension and decreased in aortic aneurysms [95, 96]. Previous studies have focused on other targets of miR-143 in hypertension, yet the potential effect of miR-143 via the MasR remains elusive. Due to the small number of miRNAs attributed to the regulation of MasR, fluctuations in just one of them might have a significant effect on MasR protein levels.

5.3. MiR-132/212

Ang II regulated the miR-132/212 family in hypertensive rats and humans [97, 98] and this family has been attributed with both cardiovascular and brain-specific properties [99–103]. MiR-132/212 was initially thought to directly target AT1R with experimental studies demonstrating a prevalent effect in the RAS, but new advances and criteria in miRNAs have shown that the effect was due to various downstream second messengers of AT1R activation. miRNA-132/212 has multiple targets including Ang II and endothelin-1 (ET-1) signalling [99]. Thus, miRNA-132/212 might be relevant in hypoxic conditions to control the vasoconstrictor effects of Ang II and ET-1. Indeed, transplantation of pericyte progenitor cells from human adult vena safena (Bristol pericytes) induced pro-angiogenic activity in endothelial cells, mediated by pericyte-produced miR-132 in response to hypoxia and taken up by endothelial cells passing through exosomes [104–106].

MiR-132 expression is also regulated by CREB [107, 108], enhances the frequency and amplitude of excitatory potentials in neurons and increases dendritic length and arborization by targeting the brain-enriched GTPase-activating protein p250GAP [109, 110]. MiR-132 triggered marked increases in dendritic spine density, while either underexpression or overexpression of miR-132 caused cognitive impairment in supra-physiological conditions [100, 111]. Similarly, BDNF is regulated by CREB and a negative feedback interaction between the previously described miRNA-1/206 and miRNA-132/212 regulates BDNF expression in the brain [112]. Notably, miRNA-132/212 is also involved in the brain-immune axis and miR-132 mediates an anti-inflammatory effect by targeting acetylcholinesterase, thus increasing acetylcholine that reduces cytokine production [113, 114]. Furthermore, projections from basal forebrain neurons to cortical microvessels (nervi vasorum) and astrocytes containing primarily acetylcholine and nitric oxide synthase (NOS) have contributed to increased cerebral blood flow [77].

5.4. MiR-29

Another miRNA family dysregulated in cerebrovascular disorders and regulated by Ang II is miR-29 [74, 98]. The miR-29 family is linked to cardiac and vascular ageing and

counteracts fibrosis by regulating extracellular matrix metallopeptidases [115]. Ang II increased miR-29b in cardiac fibroblasts with no effect in myocytes [116]. In the renal cortex of spontaneously hypertensive rats and in renal tubular epithelial cells, Ang II decreased the expression of miR-29b [117]. Notably, ET-1 decreased miR-29a expression in cardiac myocytes in vitro [118]. MiR-29b is increased in rat brain after focal ischaemia in vivo and in primary neurons exposed to oxygen/glucose deprivation in vitro [119]. Treatment of rats with peroxisome proliferator-activated receptor gamma (PPARγ) agonists protected against ischaemia-reperfusion injury by decreasing miR-29a and miR-29c levels; correspondingly, apoptosis was induced by overexpressing miR-29 [120]. However, mouse models of middle cerebral artery occlusion have inconsistently demonstrated increased and reduced miR-29 levels [119, 121–123]. These conflicting findings have a number of possible explanations including animal age and species, as well as techniques and biosamples used, or other factors discussed below.

Despite the inconsistent evidence, a meta-analysis of microRNAs induced by aerobic exercise in humans evaluated left ventricle hypertrophy and proposed miR-29 family to be antihypertrophic and miR-34 family to be prohypertrophic [124]. MiR-34 was increased in patients with cardiovascular disorders in response to stress [125], which promotes apoptosis and cardiac autophagy [102]. By contrast, myocardial hypertrophy induced by Ang II/AT1R activation in rats is antagonized by miR-34 and its inhibition stimulated Ang II signalling via atrial natriuretic peptide [126]. AT1R activation increased intracellular calcium levels producing vasoconstriction in vascular smooth muscle cells. In addition, in endothelial cells, elevation of intracellular calcium levels contributes to the inhibition of nitric oxide production by atrial natriuretic peptide [127].

5.5. MiR-34

MiR-34 is involved in cardiac and endothelial senescence, characterized by decreased production of the vasodilator nitric oxide by endothelial nitric oxide synthase, inflammation and resultant endothelial dysfunction [128]. MiR-34 promotes endothelial senescence by downregulating the histone deacetylase sirtuin-1 [129] and regulates cardiac contractile function during ageing and after acute myocardial infarction, as a result of inducing DNA damage and telomere attrition [130]. Transplantation of bone marrow-derived mononuclear cells from patients with cardiovascular disease induced cell death, while inhibition of the elevated levels of miR-34a ex vivo improved the functional benefit of transplanted bone marrow-derived mononuclear cells in mice after acute myocardial infarction in vivo [131]. Inhibition of miR-34 also attenuated ischaemia-induced cardiac remodelling, atrial enlargement and improved systolic function [125, 132].

By contrast, miR-34 promoted differentiation of mouse embryonic neural stem cells to postmitotic neurons by targeting sirtuin-1 [133]. Along with miR-132/212, miR-34 was upregulated in human epilepsy screenings and pilocarpine-induced status epilepticus in rats [134–139], suggesting neuronal activity-based regulation. MiR-34 expression in the amygdala is also linked to repression of stress-induced anxiety [140], modulates ageing and neurodegeneration in Drosophila [141] and is associated with cognitive impairment [142].

The miRNA families described are functionally relevant in the development of cardiovascular and cerebrovascular disorders, some of which appear to link cerebral ischaemia, endothelial dysfunction and cognitive impairment. Current therapy for cerebral ischaemia is limited to the use of recombinant tissue-plasminogen activator (tPA). Endogenous tPA is primarily expressed in endothelial cells and interactions between tPA and low-density lipoprotein receptor-related protein (LRP) are important for the hippocampal activity-dependent strengthening of synapses known as long-term potentiation (LTP) [143]. AT1R activation causes increased expression of tPA inhibitor (tPA-I), which binds to LRP and blocks its interaction with other ligands, including apolipoprotein E and alpha 2-macroglobulin [144]. Furthermore, tPA-I limits the maturation of proBDNF to BDNF and impedes protein synthesis-dependent late-phase LTP and hippocampal plasticity, mechanisms for learning and memory [145]. Chronic administration of tPA improved cognition in a APPswe/PS1 transgenic mice [146]. MiR-34 has two different binding sites at the 3′UTR of tPA-I, one of which has the highest probability of binding amongst the 108 miRNAs for this transcript. LRP1 is subject to regulation by 22 miRNAs, including miR-125 with one binding site and miR-212 with two binding sites [48].

There has been a recent consensus view on the roles of microRNAs, platelet and endothelial dysfunction in vascular disease and inflammation [147]. MiR-132/212 and miR-29 families target some proteins involved in endothelial dysfunction, such as the actin-related protein 2/3 complex, platelet-derived growth factor and aquaporin 4 [48]; the latter two are particularly relevant in the maintenance of blood-brain barrier (BBB) integrity [148, 149]. Factors involved in BBB disruption include chronic hypertension, ischaemia, trauma, infections and inflammation. Throughout the life course, these factors are likely to cause epigenetic modifications including miRNA fluctuations, leading to reduced protein translation and degradation of mRNA transcripts necessary for BBB integrity. BBB disruption is relevant in understanding the spectrum of clinical manifestations resulting from cerebrovascular disorders.

6. miRNAs challenges and considerations

More than 200 miRNAs have been found to be dysregulated in cerebrovascular disorders, with some inconsistency between studies [74–76, 150–152]. Inconsistencies likely relate partly to the size of the investigated cohorts, particularly since miRNAs may reflect the presence of comorbidities and hence statistical power and specificity would be lessened. Increasing the number of individuals and adding additional specificity (e.g. identifying disease-specific miRNAs as controls) might enable discrimination between the effects of dysregulated miRNAs. For instance, the ability to differentiate between changes in miRNAs associated with haemorrhagic and ischaemic cerebrovascular disorders and in the presence or absence of amyloid deposition or dementia, would be useful. Equally, changes in miRNA signatures could also explain pathophysiological processes in common, such as endothelial disruption and hypoxia due to hypoperfusion.

A second important factor in interpreting data across studies is that of methods used. miRNA detection with high sensitivity and specificity is demanding. The target sequence is present in

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the primary transcript, the precursor and the mature miRNA; some miRNAs within the same family differ by just a single nucleotide [153, 154]. Profiling can be achieved via three major methods: amplification using quantitative real-time polymerase chain reaction (qRT-PCR), hybridization based on microarrays and sequencing by next-generation sequencing (NGS) technologies [153, 155]. Due to the small size of miRNAs, guanidine-cytosine (GC) content and similar target sequence, hybridization-based methods lack specificity. NGS technologies have provided a considerable aid to advance the field of miRNA, elucidating new miRNAs and applying new criteria for the RNA sequences to be recognized as miRNAs. Studies evaluating sensitivity, specificity, quantification accuracy and reproducibility of different assays have shown that miRNA levels were dependent on the nature of the technique and also with differences between commercial kits [154, 156, 157]. Despite the advantages of NGS, a validation method is highly recommended for those dysregulated miRNAs in large-scale screenings. Although there is no specific consensus paper, qRT-PCR has been widely cited as the gold standard in miRNA research, providing specificity between isomiRs and using stem-loop primers for discrimination from primary miRNAs, pre-miRNAs and degraded mRNA [153, 158].

Another factor is the handling and sample source of miRNAs that are cell type specific and thus, the proportion of different cells contained in a sample can vary. In addition, blood contains high levels of RNase activity; while miRNAs are protected from RNase under normal conditions, their extraction causes immediate degradation if extracted and spiked back to plasma [153]. Other pre-analytic variables might also affect its profiling, such as centrifugation [159]. Collection and handling procedures are relevant to reliably detect dysregulated miRNAs. Exosomal RNA is protected by RNase A treatment and exosomes provide a consistent source of miRNA for disease biomarker detection [160]. Sources like formalin-fixed tissue have been found to be highly reliable [79, 153].

In studies of disease, the pathological stage of the disease, post-mortem status and the agonal state prior to death should also be considered as miRNAs measured could represent causal and/or responsive mechanisms. Thus, there is a need to discriminate between miRNAs produced under normal conditions in different cell types for effective comparisons with those regulated by an environmental insult (e.g. hypoxia), those regulated by the activation of a receptor (e.g. AT1R) or by a common downstream regulator (e.g. CREB). Indeed, during the natural history of a disease, microRNAs will likely fluctuate and their final signature might represent a retrospective picture of various protective mechanisms and aberrant dysregulations.

Finally, the effects of miRNAs on their targets should be viewed in the context of a whole functional analysis [161]. For instance, renin-sensitive microRNAs correlate with atherosclerosis plaque progression [162]. It is conceivable that only a specific combination of microRNAs produces a relevant physiological response. Several outcomes in miRNA research appear to be the result of well-defined miRNA-target-related effects. Nevertheless, the impact of a single miRNA via a specific target is related to the total number of different transcripts it targets and also by the number of other miRNAs that share the same target. It is reasonable to attribute a functional characteristic to a miRNA, based on the experimental outcome, such as in luciferase assays. However, luciferase assays are not able to differentiate between canonical

and non-canonical binding sites, neither if the effects are a result of direct miRNA binding to the transcript or by modifying transcription factors.

Furthermore, the experimental outcome will depend on the mRNAs expressed in that cell at that time. For instance, 213 miRNAs can bind at the 3'UTR of the anti-apoptotic protein BCL-2, whereby one could assume that those 213 miRNAs are pro-apoptotic by downregulating BCL-2. However, one of those 213 miRNAs alone could have several hundred targets, some of which promote apoptosis and others favouring survival. Thus, examination of the complete array of targets is needed to provide a functional analysis including an assessment of overlapping targets between miRNAs [161, 163]. Another consideration is the probability rate by which a miRNA binds to the 3′ UTR. Agarwal et al. developed a score based on 14 features (total context score) to allow determination of the probability of miRNA binding and categorization of miRNAs into percentiles based on the total context score [48]. Finally, it is prudent to consider the number of copies of a miRNA expressed. Some miRNAs, such as miR-124 and miR-128, are highly expressed up to 30,000–50,000 copies per neuron, while others can be as low as 1–2 copies per neuron [29]. Therefore, the biological impact of miRNAs relies on the combinatorial signature, the number of miRNA copies expressed, their affinity for different transcripts and the existing mRNA environment accessible for remodelling.

7. Conclusions

In summary, miRNAs are essential for cell fate and differentiation and their effects depend on the mRNA environment expressed, which can be transient over time and subject to dysregulation that may lead to disease. As a highly dynamic and interactive process, epigenetics and particularly miRNAs play a significant role in cognition [164, 165]. Drosha and Dicer are expressed throughout the brain with a higher expression in the hippocampus and dentate gyrus [166]. Functional analysis through bioinformatics and the use of next-generation sequencing could reveal a miRNA signature that helps to explain the effects on pathways and the fluctuations seen over the development of a specific disease. This could allow identification of a small group of miRNAs that are determinant in the clinical manifestation and therefore potential targets for diagnosis and therapeutic intervention. These would have a great advantage as therapies due to their small size and lipidic transport across the BBB, direct intracellular interaction with the transcriptome and may be able to facilitate regeneration while obviating the consequence of a degenerative microenvironment.

Author details

Jose Gerardo-Aviles*, Shelley Allen and Patrick Gavin Kehoe

*Address all correspondence to: jose.gerardo-aviles@bristol.ac.uk

Dementia Research Group, Institute of Clinical Neurosciences, School of Clinical Sciences, University of Bristol, Southmead Hospital, Bristol, UK

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