Original Article

Assessment of the MicroRNA System in Salt-Sensitive Hypertension

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Most animal microRNAs are imperfectly complementary to their mRNA targets and inhibit protein synthesis through an unknown mechanism. MicroRNAs have been reported to play important roles in a number of biological processes. We assessed the microRNA system in Dahl salt-sensitive rats in order to investigate possible roles of microRNA in salt-sensitive hypertension. We constructed microRNA libraries from the kidneys of Dahl salt-sensitive and Lewis rats taking normal or high-salt diets (4 groups), and identified 91 previously reported and 12 new microRNAs expressed in the kidney. We then used Northern blotting to assess the expression levels of 118 microRNAs in the kidneys and heart ventricles. No significant differences in microRNA expression profiles were observed among the 4 groups. Thus, the microRNA system seemed to be unlikely to contribute to salt-sensitive hypertension in Dahl salt-sensitive rats. (*Hypertens Res* 2005; 28: 819–826)

***Key Words*: microRNA, hypertension, Dahl salt-sensitive rat, hypertrophy**

Introduction

MicroRNAs constitute a growing class of non-coding RNAs (*1*, *2*). Most animal microRNAs are imperfectly complementary to their mRNA targets and inhibit protein synthesis through an unknown mechanism (*3*). MicroRNAs have been reported to modulate hematopoietic lineage differentiation (*4*), adipocyte differentiation (*5*), insulin secretion (*6*) and HIV-1 transcription (*7*), thus indicating that they could play important roles in a number of biological processes. The high degree of phylogenetic conservation of pre-microRNA sequences also supports the importance of this biological system (*8*).

Dahl salt-sensitive rats (DS) are one of the most prevalently used animal models of salt-sensitive hypertension (*9–12*). Although more than 16 quantitative trait loci (QTLs) have been identified in this model (*13*), most of the genes responsible for hypertension have not yet been identified (*14*). The precise mechanism of the increased salt retention in the kidneys of DS remains to be elucidated (*15*, *16*).

As described above, the microRNA system has been recognized to play important roles in various biological processes. It is thus possible that irregularities in this microRNA system are involved in the pathogenesis of hypertension in DS. The purpose of the present study was to address this hypothesis.

A microRNA cDNA library was constructed in order to identify the major microRNAs expressed in the kidney. The expression levels of more than 100 microRNA species were then assessed in the kidneys of DS and Lewis rats (LW). Moreover, microRNA expression profiles in the heart, one of the target organs of hypertension, were also investigated. A similar strategy has been employed by Poy *et al*. (*6*). They have identified 67 different microRNAs in the miRNA library

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Table 1. MicroRNA (miRNA) Sequences Identified by Cloning from Kidney of Dahl Salt-Sensitive and Lewis Rats

miRNA[a]	Sequence (5′ to 3′)[b]	LW−[e]		LW+[e]		DS−[e]		DS+[e]	
		No. clones[c]	%[d]	No. clones[c]	%[d]	No. clones[c]	%[d]	No. clones[c]	%[d]
rno-let-7a	ugagguaguagguuguauaguu	6	1.6	16	4.0	7	1.9	4	1.0
rno-let-7b	ugagguaguagguuguugguu	17	4.5	8	2.0	20	5.6	9	2.4
rno-let-7c	ugagguaguagguuguaugguu	12	3.2	10	2.5	17	4.7	8	2.1
rno-let-7d	agagguaguagguugcauagu			3	0.8	2	0.6		
rno-let-7e	ugagguaggagguuguauagu			1	0.3	3	0.8		
rno-let-7f	ugagguaguagauuguauaguu	10	2.7	3	0.8	3	0.8	7	1.8
mmu-let-7g[f]	ugagguaguaguuuguacagu	5	1.3	11	2.8	5	1.4	6	1.6
rno-let-7i	ugagguaguaguuugugcu			1	0.3	3	0.8	1	0.3
rno-miR-10a	uacccuguagauccgaauuugug	8	2.1	11	2.8	7	1.9	6	1.6
rno-miR-10b	uacccuguagaaccgaauuugu	5	1.3	3	0.8	7	1.9	4	1.0
mmu-miR-15a[f]	uagcagcacauaaugguuugug	5	1.3	3	0.8	2	0.6	4	1.0
rno-miR-15b	uagcagcacaucaugguuuaca	1	0.3					1	0.3
rno-miR-16	uagcagcacguaaauauuggcg	2	0.5	5	1.3	7	1.9	5	1.3
rno-miR-19b	ugugcaaauccaugcaaaacuga	1	0.3					1	0.3
rno-miR-20	uaaagugcuuauagugcaggua							1	0.3
rno-miR-21	uagcuuaucagacugauguuga	18	4.8	19	4.8	21	5.8	44	11.5
rno-miR-22	aagcugccaguugaagaacugu			1	0.3	1	0.3	1	0.3
rno-miR-23a	aucacauugccagggauuucc			1	0.3	3	0.8	1	0.3
rno-miR-23b	aucacauugccagggauuaccac	1	0.3	3	0.8	1	0.3	1	0.3
rno-miR-24	uggcucaguucagcaggaacag	6	1.6	4	1.0	8	2.2	6	1.6
rno-miR-25	cauugcacuugucucggucuga			1	0.3	1	0.3		
rno-miR-26a	uucaaguaauccaggauaggcu	6	1.6	5	1.3	8	2.2	6	1.6
rno-miR-26b	uucaaguaauucaggauagguu	61	16.3	65	16.3	50	13.9	59	15.5
rno-miR-27a	uucacaguggcuaaguuccgc			2	0.5	2	0.6	3	0.8
rno-miR-27b	uucacaguggcuaaguucug	1	0.3	2	0.5	3	0.8	3	0.8
rno-miR-28	aaggagcucacagucuauugag	1	0.3						
rno-miR-29a	cuagcaccaucugaaaucgguu	21	5.6	32	8.0	17	4.7	26	6.8
rno-miR-29b	uagcaccauuugaaaucagugu	14	3.7	19	4.8	6	1.7	14	3.7
rno-miR-29c	uagcaccauuugaaaucgguua	9	2.4	7	1.8	8	2.2	9	2.4
rno-miR-30a-5p	uguaaacauccucgacuggaagc	5	1.3	6	1.5	8	2.2	3	0.8
rno-miR-30a-3p	cuuucagucggauguuugcagc	1	0.3	8	2.0	1	0.3	2	0.5
rno-miR-30b	uguaaacauccuacacucagc			2	0.5	2	0.6	1	0.3
rno-miR-30c	uguaaacauccuacacucucagc	11	2.9	6	1.5	10	2.8	8	2.1
rno-miR-30d	uguaaacaucccccgacuggaag	5	1.3	1	0.3	3	0.8	3	0.8
rno-miR-30e	uguaaacauccuugacugga	1	0.3			2	0.6	3	0.8
rno-miR-32	uauugcacauuacuaaguugc							2	0.5
rno-miR-33	gugcauuguaguugcauug	8	2.1	1	0.3	5	1.4	2	0.5
cel-miR-83[f]	uagcaccauauaaauucaguaa			1	0.3		0.0		
cel-miR-84[f]	ugagguaguauguaauauugu					1	0.3		
rno-miR-92	uauugcacuuguccggccug	2	0.5						
rno-miR-96	uuuggcacuagcacauuuuugcu			1	0.3			1	0.3
rno-miR-98	ugagguaguaaguuguauuguu					1	0.3		
rno-miR-99a	aacccguagauccgaucuugug					2	0.6	1	0.3
rno-miR-99b	cacccguagaaccgaccuugcg	2	0.5						
rno-miR-101	uacaguacugugauaacugaag	6	1.6	7	1.8	4	1.1	5	1.3
rno-miR-101b	uacaguacugugauagcugaag			1	0.3	1	0.3	1	0.3

Table 1. (Continued)

rno-miR-103	agcagcauuguacagggcuauga			3	0.8	1	0.3	1	0.3
rno-miR-106b	uaaagugcugacagugcagau			1	0.3				
rno-miR-125a	ucccugagacccuuuaaccugug	3	0.8	2	0.5	1	0.3	2	0.5
rno-miR-125b	ucccugagacccuaacuuguga			1	0.3	2	0.6	1	0.3
rno-miR-126	ucguaccgugaguaauaaugc	2	0.5	3	0.8	1	0.3	5	1.3
rno-miR-126*	cauuauuacuuuugguacgcg	60	16.0	60	15.0	59	16.4	44	11.5
rno-miR-130a	cagugcaauguuaaaagggc	2	0.5	3	0.8	1	0.3		
rno-miR-130b	ugcaaugaugaagguuau							1	0.3
rno-miR-135a	uauggcuuuuuauuccuauguga					1	0.3		
rno-miR-139	ucuacagugcacgugucu			1	0.3				
rno-miR-140	agugguuuuacccuaugguag	2	0.5	1	0.3	2	0.6	3	0.8
rno-miR-142-3p	uguaguguuuccuacuuuaugga	5	1.3	7	1.8	2	0.6	18	4.7
rno-miR-142-5p	cauaaaguagaaagcacuac	6	1.6			3	0.8	5	1.3
rno-miR-143	ugagaugaagcacuguagcuca	3	0.8	3	0.8	1	0.3	2	0.5
rno-miR-145	guccaguuuucccaggaaucccuu			1	0.3	1	0.3		
rno-miR-146	ugagaacugaauuccauggguu	3	0.8	7	1.8	5	1.4	10	2.6
rno-miR-150	ucucccaacccuuguaccagug					1	0.3		
rno-miR-151*	ucgaggagcucacagucuagua			1	0.3				
mmu-miR-182[f]	uuuggcaaugguagaacucaca					1	0.3		
rno-miR-183	uauggcacugguagaauucacug	1	0.3	1	0.3			1	0.3
rno-miR-185	uggagagaaaggcaguuc							1	0.3
rno-miR-186	caaagaauucuccuuuugggcuu			1	0.3			1	0.3
mmu-miR-189[f]	gugccuacugagcugauaucagu							1	0.3
rno-miR-191	caacggaaucccaaaagcagcu	1	0.3						
rno-miR-192	cugaccuaugaauugacagcc	2	0.5	3	0.8	2	0.6	3	0.8
rno-miR-193	aacuggccuacaaagucccag	4	1.1	4	1.0			2	0.5
rno-miR-194	uguaacagcaacuccaugugga	2	0.5	1	0.3	2	0.6	1	0.3
rno-miR-195	uagcagcacagaaauauuggc					1	0.3		
rno-miR-196a	uagguaguuucauguuguugg	5	1.3	4	1.0	3	0.8	1	0.3
mmu-miR-199a*[f]	uacaguagucugcacauugguu	1	0.3	5	1.3	1	0.3	2	0.5
rno-miR-200a	uaacacugucugguaacgaugu	13	3.5	6	1.5	8	2.2	5	1.3
rno-miR-200b	cucuaauacugccugguaaugaug	2	0.5	2	0.5	1	0.3	1	0.3
rno-miR-203	gugaaauguuuaggaccacuag	1	0.3	1	0.3				
rno-miR-206	uggaauguaaggaagugugugg					1	0.3		
rno-miR-210	cugugcgugugacagcggcug							1	0.3
rno-miR-214	acagcaggcacagacaggcag					2	0.6		
rno-miR-218	uugugcuugaucuaaccaugu	3	0.8	2	0.5	1	0.3	3	0.8
rno-miR-223	ugucaguuugucaaauacccc			1	0.3				
rno-miR-301	cagugcaauaguauugucaaagcau	2	0.5	1	0.3				
rno-miR-322	aaacaugaagcgcugcaaca			1	0.3	1	0.3	1	0.3
rno-miR-324-3p	ccacugccccaggugcugcugg							1	0.3
rno-miR-338	uccagcaucagugauuuuguuga			1	0.3				
rno-miR-342	ucucacacagaaaucgcacccguc					1	0.3	2	0.5
rno-miR-345	ugcugaccccuaguccagugc			1	0.3				
rno-miR-351	ucccugaggagcccuuugagccug					1	0.3		
Total		374	100	399	100	359	100	381	100

[a]miRNA genes are named according to the miRNA Registry from the Sanger Institute (http://www.sanger.ac.uk/Software/Rfam/). [b]The longest representative from each miRNA sequence is presented. [c]Number of clones found in each library. [d]Percentage of clones in each library. [e]Dahl salt-sensitive (DS) and Lewis rats (LW) were fed a 0.2% (–) or 8% (+) NaCl diet. [f]No homologous sequences were found in rat miRNA Registry, but identical sequences are present in other organisms (mouse, mmu; *C. elegance*, cel).

form murine pancreatic β-cell line MIN6 and murine pancreatic α-cell line TC1. And one of them, the islet-specific miR-375 (miR means microRNA), has been found to be involved in insulin secretion (*6*).

Methods

Animal Studies

DS and LW rats were obtained from Charles River Laboratories (Yokohama, Japan) and Oriental Yeast, Co., Ltd. (Tokyo, Japan), respectively. Rats were weaned at 4 weeks of age and then fed a 0.2% or 8% NaCl diet (Oriental Yeast, Co., Ltd.) for a period of 9 weeks (*14*, *17*). We compared the expression levels in DS and LW, since we have previously performed genetic linkage analyses in the F2 rats derived from DS and LW (*14*). All rats were killed by cervical dislocation following a brief period of ether anesthesia, and the kidneys and hearts were rapidly removed. Total RNA was extracted from the kidneys and the left ventricles using Trizol reagent (Invitrogen, Carlsbad, USA). The present study was conducted in accordance with current guidelines for the care and use of experimental animals of the National Cardiovascular Center.

Cloning of MicroRNAs

Cloning of microRNAs was essentially performed as described by Lagos-Quintana *et al*. (*18*). Total RNA (500 μg) was separated on a 15% denaturing polyacrylamide gel and RNA fractions of between 18 and 25 bases were selected. A 5′-phosphorylated 3′-adaptor oligonucleotide (5′-pUUUaac cgcgaattccagx: uppercase, RNA; lowercase, DNA; p, phosphate; x, 3′-Amino-Modifier C-7; Integrated DNA Technologies, Inc., Coralville, USA) and a 5′-adaptor oligonucleotide (5′-acggaattcctcactAAA: uppercase, RNA; lowercase, DNA) were directionally ligated to the small RNAs. Adaptor-ligated RNA was amplified by reverse transcription and polymerase chain reaction (RT-PCR) with a 3′-primer (5′-CAGCCAA CAGGCACCGAATTCCTCACTAAA) and a 5′-primer (5′-GACTAGCTTGGTGCCGAATTCGCGGTTAAA). Polymerase chain reaction (PCR) products were then digested with *Ban*1 and concatamerized using T4 DNA ligase. Concatamers ranging in size from 500 to 1,000 bp were separated on agarose gel and directly ligated in a pCR3.1-TOPO vector. Plasmid inserts were amplified by PCR using primers to vector sequences and were subjected to direct sequencing. Sequences from cloned microRNAs were grouped by sequence identity, and compared with a public database, the microRNA registry (http://www.sanger.ac.uk/Software/Rfam), in order to identify the clones. Unknown sequences, including sequences homologous to particular microRNAs, were searched using the Basic Local Alignment Sequence Tool (BLAST) algorithm, available at the National Center for Biotechnology Information (NCBI). The flanking sequences of the novel microRNAs were used to predict the secondary structure with the RNAfold program of the Vienna RNA package (http://www.tbi.univie.ac.at/). All of the novel microRNAs were submitted to the microRNA Registry website for official annotation.

Northern Blot Analysis

Fractionated total RNA was transferred to a Zetaprobe membrane (Biorad, Hercules, USA) and cross-linked to the membrane by UV irradiation. The resulting blots were then probed with StarFire probes corresponding to various microRNAs, which were labeled with [α-^{32}P]dATP. Prehybridization and hybridization were carried out as described previously (*8*). Equal amounts of RNA from the five rats in each group were pooled and these samples (10 μg) were used for first screening. We assessed the expression levels in the four groups: DS with high-salt diet (DS+), DS with normal-salt diet (DS−), LW with high-salt diet (LW+), and LW with normal-salt diet (LW−). When there was an apparent difference in expression among the four groups, the expression levels were reassessed with other Northern strips. Since the expression levels of several microRNAs (miR-30c, miR-151*, miR-214, miR-223, miR-322, Can-1, Can-7) were reconfirmed to differ among the four groups, the expression levels of these microRNAs were assessed in individual rats (n=5).

Results and Discussion

If the microRNA system were involved in the pathogenesis of hypertension, some microRNAs would be differentially expressed in the kidneys of hypertensive and normotensive rats. We initially investigated the microRNAs that were abundantly expressed in rat kidneys by constructing microRNA libraries. Four microRNA libraries were constructed from a DS given a high-salt diet (DS+), a DS given a normal diet (DS−), an LW given a high-salt diet (LW+) and an LW given a normal diet (LW−). About 400 tags for microRNAs were sequenced and identified in each library, as shown in Table 1.

We identified 84 previously reported microRNA species and 7 microRNAs that have not been reported in rats (mmu-let-7g, mmu-miR-15a, cel-miR-83, cel-miR-84, mmu-miR-182, mmu-miR-189, mmu-miR-199a*). We also identified 12 possible microRNA species, designated candidates (Can) 1 to 12 (Table 2). All of these new microRNA sequences were found in the rat genome. The sequences surrounding these potential microRNAs showed a stem-loop structure, which is a prerequisite for microRNA processing. Moreover, expression of these possible microRNAs was confirmed by Northern blotting (Fig. 1). Some species of microRNA (miR-21, miR-26b, miR-29a, miR-126*) were abundantly expressed (>5%) in the kidney. The number of tags corresponding to miR-21 in the DS+ rat seemed to be larger than those in the other rats. Although the Northern blot analysis confirmed that the expression level of miR-21 in this DS+ rat was indeed higher than that in the other rats, this was not consistently

Table 2. Candidate MicroRNAs (miRNAs)

Temporary name[a]	No. of clones[b] LW−	LW+	DS−	DS+[c]	Sequence (5′ to 3′)[d]	Harpin precursor[e]	Chromosome[f] Rat	Human	Mouse	Northern[g]
Can-1	6	2	2	1	acuggacuuggagucagaaggc	−c u guuacc 5′gggcu cugacuccaggucc gugu u 3′uccgg gacugagguucagg cacg c aa u auaaag	18	5	18	+
Can-2	3	2	2	2	cagcagcaauucauguuuugga	a aa guauu 5′ugcagc gc uucauguuuugga g 3′acgucg cg aaguacaaaacuu c − −− ggaac	X	X	X	+
Can-3	–	2	1	2	auauaauacaaccugcuaagug	au c u 5′augg auaauacaac ugcuaagugu c 3′uguu uauuauguug acgauucacg u au c a	X	X	X	+
Can-4	1	–	–	1	gucaacacuugcugguuucc	u g a −− g 5′ag gggggagccag aagu uugauguu ucu c 3′uc cuccuuugguc uuca aacugcga gga c u g c uu −	X	X	X	+
Can-5	1	–	–	1	uuaagacuugcagugauguuu	u ua a gcuccu 5′gc gu agacuugc gugauguuua c 3′cg cg ucugaacg cacuacaagu u u ug a guaccu	3	20	2	+
Can-6	–	–	–	1	ugaccgauuucuccugguguuc	5′cu ucc c gucu gaccgauuuc ugguguu aga g uuggcuaaag accacga ucu u 3′ua uuu − guuu	13	1	12	+
Can-7	1	–	1	1	aaaccguuaccauuacugag	a a 5′agg aaccguuaccauuacug g 3′ucu uugguaaugguaaugau u c u	10	17	11	+
Can-8	–	1	1	–	uauucauuuacuccccagccua	5′cu −− a ggcuggggga aaauga auagaaa c ccgaccccu uuuacu uaucuua a 3′au ca ---- u u	13	1	1	+
Can-9	–	1	–	–	aaucauucacggacaacacuu	a− ug uuua 5′aagaga guuguucgugg gauucgc c 3′uuuuuu caacaggcacu cuaagca u ca ua gugu	6	14	12	+
Can-10	–	1	–	1	cagcagcacacuguggguuugua	a− −−− −− uac ac 5′cc gc agcac ac uguggguuug ggc u 3′gg cg uuguggug acaccaaac ccg g ag aga uc cugca gu	10	17	11	+
Can-11	–	–	–	1	aguucuucaguggcaagcuuu	− a u ccu 5′aguaguucuucag uggca gcuuua gu g 3′uugucaagaaguu accgu cgaaau cg a g − − accc	10	17	11	+
Can-12	10	5	3	5	uaauacugucugguaaugccgu	au c ug cugga 5′gg gu uuaccagaca guuagau u 3′cu cg aauggucugu uaaucug g gc u ca ucuau	5	–	4	+

[a]miRNA candidates that were newly identified in this study are listed. [b]Number of clones found in each library. [c]Lewis (LW) or Dahl salt-sensitive rats (DS) were fed a 0.2% (−) or 8% (+) NaCl diet. [d]The longest representative from each miRNA sequence is presented. [e]Predicted structures of miRNA precursors. The miRNA sequences are underlined. [f]Chromosome number locating miRNA precursors are presented. [g]All miRNA candidates were detected by Northern blotting.

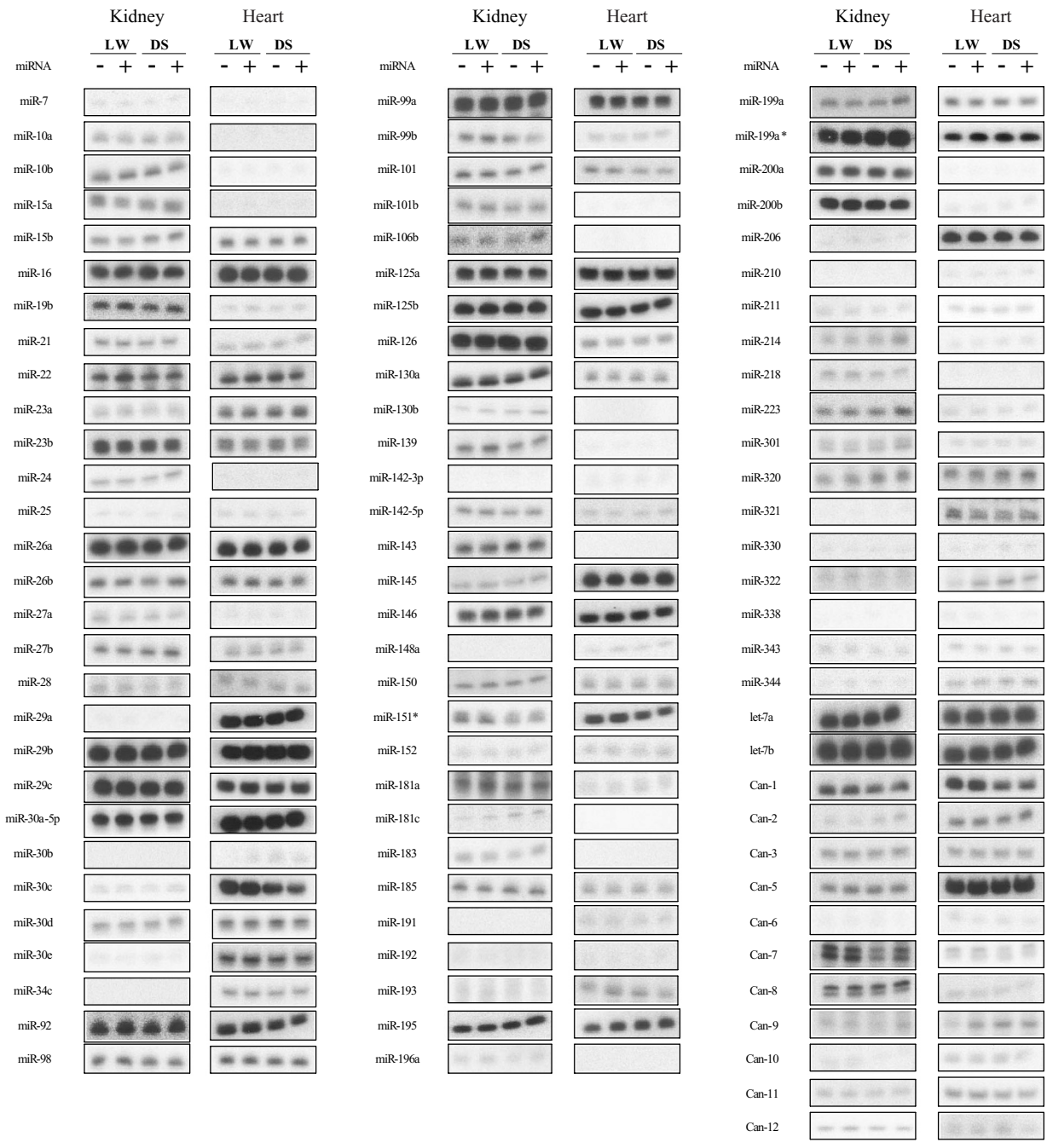

Fig. 1. *Northern blot analysis of microRNAs in kidneys and hearts of Dahl salt-sesitive (DS) and Lewis rats (LW). Male DS and LW fed a 0.2% (–) or 8% (+) NaCl diet were sacrificed, and the kidneys and hearts were excised. Total RNA was prepared with TRIzol reagent from excised whole tissues and was electrophoresed, blotted, fixed to membrane and hybridized to StarFire probes for each microRNA, as described in the Methods. A total of 29 microRNAs were undetectable in either the kidney or heart (data not shown). These were miR-9, 17-3p, 20, 30a-3p, 31, 32, 33, 34b, 96, 103, 126*, 135a, 135b, 182, 185, 189, 190, 194, 199b, 203, 221, 224, 324-3p, 328, 331, 345 and 351, and cel-miR-83 and 84.*

observed in the other DS+ rats.

We then assessed the expression levels of 118 microRNA species in the kidneys of DS+, DS–, LW+, and LW– by Northern blotting (Fig. 1). These 118 microRNAs included those present among libraries and microRNAs from the chromosome 1 QTL for blood pressure (miR-193, miR-142-3p, miR-142-5p, miR-150, miR-211, miR-301, miR-330, miR-343, miR-344). This chromosome 1 QTL is the most promi-

Table 3. Comparison of Body and Heart Weights, and Blood Pressure

Rat strain	Salt diet	BW (g)	HW (g)	SBP (mmHg)
LW	–	358.0±9.3	1.14±0.05	105.8±3.5
LW	+	357.8±3.2	1.12±0.06	110.6±3.2
DS	–	363.6±4.1	1.29±0.01	122.0±2.5
DS	+	370.0±4.5	1.74±0.04*	197.6±9.1*

Dahl salt-sensitive (DS) and Lewis rats (LW) were fed a 0.2% (–) or 8% (+) NaCl diet from 4 to 13 weeks of age. *$p < 0.01$, DS(+) compared to each other group ($n=5$). BW, body weight; HW, heart weight; SBP, systolic blood pressure.

nent QTL for blood pressure and 2–3 responsible genes are thought to be present in this region (*14*). To investigate whether microRNA genes in this region might contribute to blood pressure regulation, we measured the expression levels of the microRNAs in this region.

Expression of 76 microRNA species was detected in the kidney (Fig. 1). Some microRNA species appeared differentially expressed among the four groups, and the expression levels of these microRNA species were subjected to reconfirmation using other Northern blotting strips. Moreover, the expression levels of several microRNA species (miR-30c, miR-151*, miR-214, miR-223, miR-322, Can-1, Can-7) were assessed in individual rats ($n=5$). None of the microRNA species were found to be differentially expressed among the kidneys of DS+, DS–, LW+ and LW–. No significant differences in microRNA expression profiles were detected between the strains. Moreover, salt loading did not modulate microRNA expression.

Blood pressure levels of DS+, DS–, LW+ and LW– are shown in Table 3. Salt loading significantly increased blood pressure in DS+ rats, and the ventricles of DS+ rats were significantly hypertrophied (*19–21*).

Although the kidneys of DS+ rats exhibited sclerotic changes, as previously reported (*22*), the expression profiles of microRNAs were not markedly altered. Expression profiles of microRNAs have been reported to change dramatically during cellular differentiation (*23–25*) and in cancer cell growth (*26*, *27*), and it is likely that the expression profiles of microRNAs are not modulated without cellular differentiation.

We then investigated the expression profiles of microRNAs in the ventricles of the heart. The left ventricle of the heart is one of the target organs most affected by high blood pressure (*28*), and significant cardiac hypertrophy was observed in the DS+ rat (Table 3). It is well known that hypertrophy dramatically changes the expression profiles of mRNA from an adult to a fetal pattern (*29*, *30*). Although we did not construct libraries from the ventricles of the heart and heart-specific microRNAs may not be involved in the analysis, no significant differences in microRNA expression profiles were observed among the four groups, despite the marked cardiac hypertrophy in the DS+ (Fig. 1). This observation also strengthens the hypothesis that microRNA expression profiles are specific to each cell type, and may not be modulated without cellular differentiation.

The precise number of microRNAs has not been determined (*31*, *32*). Moreover, we were not able to detect microRNAs with low expression levels. The kidney comprises a variety of cells. Thus, some species of cell-specific microRNA may have been diluted to an undetectable level in total RNA derived from the whole kidney. Therefore, it may be premature to conclude that microRNA systems are not involved in the pathogenesis of hypertension in DS.

There were several limitations in the present study. We employed Northern blot analysis for the assessment of the expression levels. Although we used a StarFire oligo labeling method (Integrated DNA Technologies) that permits the generation of oligonucleotide probes which are 10-fold more sensitive than traditional ^{32}P end-labeled probes, the sensitivity of the Northern blot may not have been sufficiently high, in which case a substantial number of microRNAs would not have been detected in the present study. This limitation is currently being overcome by various experimental procedures, including a microarray system, a TaqMan probe-based real-time PCR method, and an RNase mapping method. Future investigation will require application of these methods.

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