Microarray expression profiling and gene ontology analysis of long non-coding RNAs in spontaneously hypertensive rats and their potential roles in the pathogenesis of hypertension

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Abstract. Long non-coding RNAs (lncRNAs) have been demonstrated to be significant in numerous biological processes. Hypertension is a form of cardiovascular disease with at least one billion cases worldwide. The present study sought to compare the differential expression profiles of lncRNAs in the renal cortex of spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats. The ipsilateral renal cortex was obtained from 15-week-old SHRs and WKY rats whose blood pressures had been monitored. Total RNA was extracted using TRIzol, and lncRNAs and messenger RNAs were profiled by microarray and validated using fluorescent quantitative reverse transcription-polymerase chain reaction. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were performed to predict the function of differentially expressed genes. Microarray analysis demonstrated that 145 lncRNAs were differentially expressed between SHRs and WKY rats. GO and KEGG pathway analysis indicated that these lncRNAs are involved in numerous biological processes. Thus, lncRNAs may contribute to the pathogenesis of hypertension.

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Abbreviations: miRNAs, microRNAs; siRNAs, short interfering RNAs; snRNAs, small nuclear RNAs; ncRNA, non-coding RNA; lncRNA, long non-coding RNA; GO, gene ontology; SHR, spontaneous hypertensive rat; WKY, normotensive Wistar-Kyoto rat

Key words: hypertension, long non-coding RNA, microarray

Introduction

More than 90% of the genome is transcribed into non-protein-coding RNAs, including micro- (mi), short interfering (si) and small nuclear (sn) RNAs. Another class of non-coding (nc) RNA, that does not fit into any of these categories, is >200 nt in length and has been defined as long ncRNA (lncRNA) (1). Recently, lncRNAs have gained widespread attention due to their notable regulatory roles in various pathophysiological changes, including differentiation, proliferation and apoptosis, as well as disease states such as hepatocellular carcinoma and tuberculosis (2,3). Thousands of lncRNA transcripts have been identified in humans, mice and pigs (2,3). Previous studies have indicated that lncRNAs are highly associated with cardiovascular disease, particularly with regard to cardiac development and myocardial fibrosis (2,3). In addition, certain studies have employed the hypertensive rat model; however, the role of lncRNAs in this type of model remains to be elucidated (4,5).

Hypertension is a cardiovascular disease associated with high morbidity and is increasing in prevalence worldwide (6). Multiple factors are implicated in the pathogenesis of high blood pressure, including the renin-angiotensin-aldosterone system, vascular smooth muscle and vascular endothelial dysfunction, as well as impaired platelet function and kidney-related factors. To further investigate renal-associated hypertension, and taking advantage of recent developments in microarray technology, the present study employed lncRNA microarray to detect and compare differentially expressed ncRNAs in the renal cortex of spontaneously hypertensive rats (SHRs) and normotensive Wistar-Kyoto (WKY) rats.

Materials and methods

Tissue collection and RNA extraction. SHRs demonstrating increased blood pressure levels at the age of 5-6 weeks, were first obtained by Okamoto and colleagues by inbreeding WKY rats with high blood pressure (7,8). Twelve-week-old SHR and WKY rats (weight, 250-330 g) were obtained from the Experimental Animal Research Center (Zhejiang Chinese Medical University,

Hangzhou, China) and maintained at 18-29˚C, 40-70% humidity, in a 12/12 h light/dark cycle, 3-5 rats/ cage. Blood pressure levels were monitored using tail cuffs until the rats were 15-weeks-old (ALCOTT-automated non-invasive blood pressure rat sphygmomanometer; Shanghai Alcott Biotech Co., Ltd., Shanghai, China). All rats were anesthetized with an injection of pentobarbital sodium (25 mg/kg) (Sigma-Aldrich, St. Louis, MO, USA) and perfusion with normal saline prior to the removal of the renal cortex and the heart. All surgical instruments, as well as the operating table, were sterilized using high temperature and high pressure. To remove the renal cortex, an abdominal incision was made, the intestines moved aside and the kidney removed. The kidney was bisected to expose the junction of the medulla and cortex, and the cortex was removed. All of the specimens were flash-frozen in liquid nitrogen and stored in freezing tubes (cat no. 430661; Corning Life Sciences, Corning, NY, USA) at -80˚C until use. Three pairs of rats with marked differences in blood pressure were selected as the hypertension and control groups. These were selected, from five pairs of rats with marked differences in blood pressure, following total RNA extraction, as the three selected pairs of samples exhibited the greatest density and purification of RNA. The animal study was approved by the animal welfare committee of Wenzhou Medical University (Wenzhou, China), according to state and institutional regulations.

Total RNA was extracted from the ipsilateral renal cortex (left side) of SHR and WKY rats with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The purity and yield of the RNA was evaluated by the ratio of absorbance at A_{260}-A_{280} with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) and 2% agarose gel electrophoresis (120V, 15 min) using 2% agarose (Biowest, Hong Kong, China), a gel imaging and analysis system (Bio-Rad, Hercules, CA, USA), and an electrophoresis system (Bio-Rad).

lncRNA and mRNA microarray expression profiling and quantitative reverse transcription-polymerase chain reaction (RT-qPCR) validation. The microarray contained ~10,000 rat lncRNAs that were derived from authoritative databases, including RefSeq (http://www.ncbi.nlm.nih.gov/refseq/), Ensemble and Ultra-conserved Region Encoding LncRNA (https://users.soe.ucsc.edu/~jill/ultra.html), lncRNAdb (http://www.lncrnadb.org/), ncRNA (http://www.ncrna.org/), and sequencing data from the Beijing Aerospace Control Center, which contained 30,367 mRNA probes. Total RNA was reverse transcribed to cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with random primers. Following fragmentation, the samples were applied to a custom rat lncRNA microarray, using a LowInput Quick-Amp Labeling kit, Gene Expression Wash kit, Gene Expression Hybridization kit and a RNA Spike-In kit (Agilent Technologies, Inc., Santa Clara, CA, USA). The cDNA fragments were hybridized with the lncRNA microarray chip at 65˚C for 17 h according to the manufacturer's instructions. Slides were scanned with the Agilent Microarray Scanner G2505C (Agilent Technologies, Inc.). The data extraction was performed following selection of the appropriate data to export to text, using Feature Extraction software, version 11.0.1.1 (Agilent Technologies, Inc.).

In addition, Feature Extraction software (version 11.0.1.1) was used to analyze the acquired array images of the results. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v12.0 software package (Agilent Technologies, Inc.). Microarray profiling and analysis was conducted by the OE Biotechnology Co. (Shanghai, China).

Six differentially expressed lncRNAs of interest were selected for validation. RT-qPCR was performed using a LightCycler® 480 Instrument II (Roche Diagnostics, Basel, Switzerland) with 10 ml PCR reaction mixture (1 ml cDNA, 5 ml 2X LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics), 0.2 ml forward primer, 0.2 ml reverse primer and 3.6 ml nuclease-free water). Reactions were incubated in a 384-well optical plate (Roche Diagnostics) at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec. Each sample was run in triplicate for analysis. Following the PCR cycles, melting curve analysis was performed specifically to validate the generation of the expected PCR product. The expression levels of sequences were normalized to GAPDH and were calculated according to the $2^{-\Delta\Delta Ct}$ methodology (9). The primer sequences were designed in the laboratory using Primer 6.0 and Oligo 5.0 and synthesized by Generay Biotech (Shanghai) Co., Ltd. (Shanghai, China) based on the ncRNA sequences obtained from the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/nucleotide/), as presented in Table I.

Gene ontology (GO) analysis, and functional predictions of mRNAs and lncRNAs. GO enrichment analysis was used to make preliminary predictions of the biological functions of the sequences of interest. GO is a common method for gene annotation and predominantly includes three fields: Biological processes, cellular components and molecular functions. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.kegg.jp/kegg/docs/statistics.html) is another synthetic pathway prediction tool that contains >200 pathways, and a collection of pathway maps representing information based on the molecular interaction and reaction networks for sequences (10).

The final gene list was submitted to the FunNet database (http://www.funnet.ws/) for in-depth analysis of the mRNA or associated lncRNA in the microarray. FunNet is described as an exploratory tool that performs relevance-based searching in transcriptional coexpression networks; the information in FunNet was extracted from genomic databases, converted to a comprehensive exploratory framework and then updated to the website (11). The results provide values to describe the association between the submitted genes and the terms in the database.

Results

lncRNAs are differentially expressed between SHRs and WKY rats. Microarray analysis indicated that 145 of 9,991 ncRNAs were differentially expressed in SHRs compared with WKY rats; of these, 93 ncRNAs were upregulated and 52 were downregulated (≥2-fold; Fig. 1). The included sequences were demonstrated to have functions that predominantly begin with 'NR', such as NR_038078.1, also referred to as small

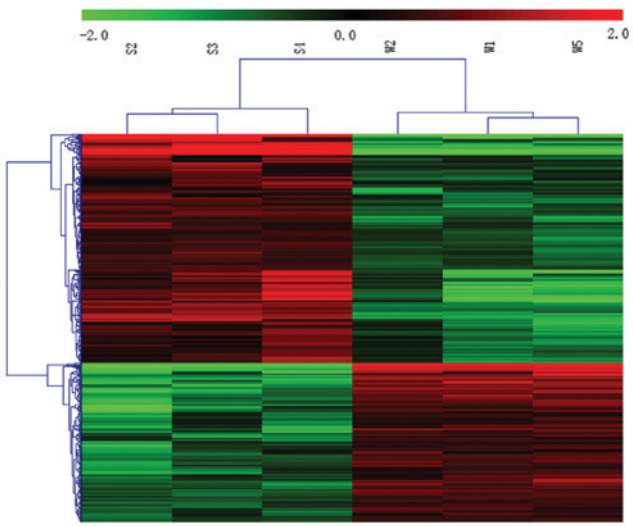

Figure 1. Heat map demonstrating differentially expressed lncRNAs between spontaneously hypertensive rat samples (S2, S3, S4) and Wistar-Kyoto rat samples (W1, W2, W5). Each row represents one lncRNA contained in the microarray and each column represents one tissue sample. The relative lncRNA expression level was downregulated. The red color indicates upregulation. Fold changes are represented by -2.0, 0 and 2.0 in the corresponding spectrum. lncRNA, long non-coding RNA.

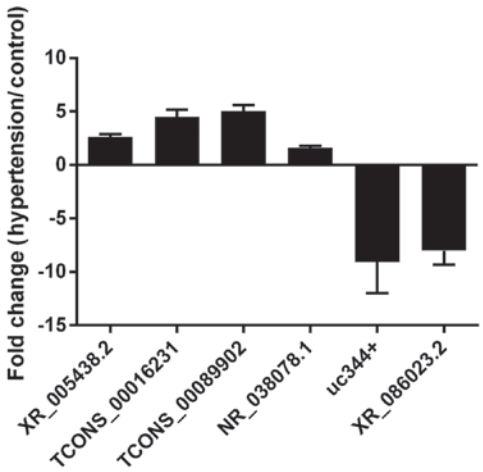

Figure 2. Verification of the expression of six downregulated long non-coding RNAs via quantitative reverse transcription-polymerase chain reaction.

nucleolar RNA host gene 4 (SNHG4), a gene which may be associated with the bystander effect in radiation biology (12). The included sequences were also functionally associated with miscellaneous RNAs, pseudogenes, ultraconserved elements and unclassified sequences (partial data are listed in Table II). Using the same criteria, 383 differentially coexpressed messenger RNAs were identified.

RT-qPCR validation of the differential expression of partial sequences. Six differentially expressed sequences were selected to validate the results of the microarray using RT-qPCR. Partial sequences indicated greater differences, however were not selected due to disparities between groups, for example, TCONS_00031872 demonstrated virtually no expression in the SHR group. The result of RT-qPCR validation is presented in Fig. 2.

Table I. Primer sequences.

Gene symbol	Primer sequence (5'-3')
XR_005438. 2	TGACTGTAGCTTCACAGGAAT
	TCCAGGACAGTTCAGGAT
TCONS_00016231	CAAAGTACCTCACCTTACCAG
	ACTTCCATGACTCTAGCCT
TCONS_00089902	TTATCGGGAGAGGCTCAAC
	GCTACATTGGATCATCTTGTCA
NR_038078. 1	CTGCTTTGTAAAAGCAAAGGT
	GCCAAAGACAGTACTAACAAC
XR_086023. 2	TGAGGTGAGGACCACTAGGAAGAC
	AGGTGCCATCGGAGGAATCATCT
Uc344+	CCAATATCCCTGCCTATAACA
	TAAGTCCAATCCGCCGTA

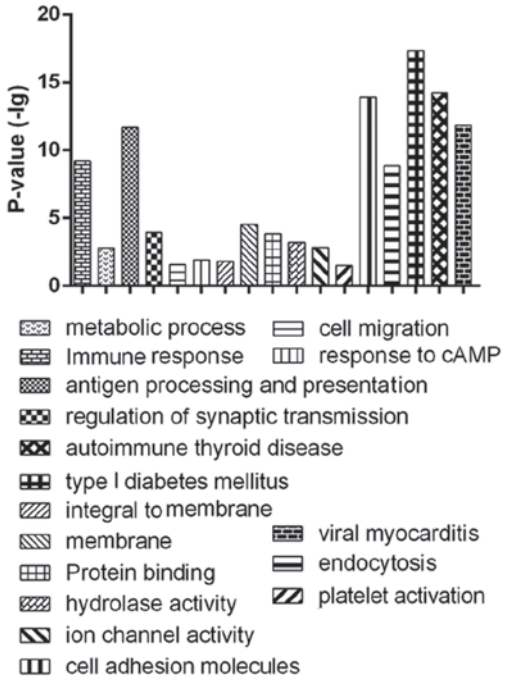

Figure 3. Partial terms of interest or high-scoring terms from GO and KEGG pathway analysis. Seventeen terms that were of interest or had high scores were collected from the GO and KEGG pathway analyses, and integrated into a bar chart; the Y-axis represents the P-value of the statistics provided by the FunNet database. GO, gene ontology; KEGG, Kyoto Encylopedia of Genes and Genomes; cAMP, cyclic adenosine monophosphate.

lncRNAs may serve roles in numerous biological processes and pathophysiological alterations. GO enrichment analysis is based on the knowledge of various biological elements, and KEGG annotations contain information on >200 pathways. Certain notable or high-scoring terms in the GO and KEGG pathway analyses were collected in a summary and are listed in Fig. 3. The GO functions identified were predominantly associated with isoprenoid biosynthetic processes, fatty acid metabolic processes, responses to cyclic AMP, cell migration in biological processes, the apical plasma membrane, microsomes, the external side of the plasma membrane, the perinuclear region of the cytoplasm (identified during cellular

Table II. Partial differentially expressed lncRNAs (abs ≥ 3-fold) between SHRs and control WKY rats.

Target_id	Chr	Regulation	FC (abs)
TCONS_00016233	1	Up	3.03
TCONS_00054808	16	Up	3.11
XR_085734. 1		Up	3.12
XR_086159. 2		Up	3.13
TCONS_00054032	16	Up	3.17
TCONS_00110995	6	Up	3.23
TCONS_00045352	14	Up	3.25
TCONS_00108373	6	Down	3.26
TCONS_00058983	17	Up	3.26
uc. 107+	3	Down	3.30
NR_038078. 1 (Snhg4)	18	Down	3.35
XR_086329. 2		Up	3.35
TCONS_00005805	1	Up	3.39
TCONS_00045737	14	Down	3.39
TCONS_00089821	3	Down	3.41
TCONS_00066490	2	Up	3.45
XR_146926. 1		Down	3.49
TCONS_00016688	1	Up	3.53
TCONS_00135006	9	Up	3.55
TCONS_00045364	14	Up	3.70
TCONS_00054225	16	Up	3.75
XR_085687. 1		Up	3.76
XR_147157. 1		Up	3.98
TCONS_00077817	20	Up	4.01
TCONS_00016690	1	Up	4.06
TCONS_00043934	14	Up	4.07
TCONS_00053182	16	Up	4.16
TCONS_00003762	1	Up	4.25
TCONS_00066377	19	Down	4.30
XR_085881. 1		Down	4.38
TCONS_00134998	9	Up	4.40
TCONS_00073324	2	Down	4.41
TCONS_00079171	20	Up	4.49
TCONS_00041050	14	Up	4.68
TCONS_00119346	7	Down	4.85
TCONS_00063145	19	Up	4.88
TCONS_00017712	10	Down	4.93
ENSRNOT00000010509	7	Down	4.94
TCONS_00016231	1	Up	5.00
XR_086204. 2		Up	5.03
uc. 101+	3	Up	5.43
TCONS_00134930	9	Up	5.47
TCONS_00058134	17	Up	6.20
TCONS_00089902	3	Up	6.36
ENSRNOT00000036466	X	Down	6.39
TCONS_00045074	14	Up	6.61
TCONS_00045348	14	Up	6.97
XR_085575. 1		Down	7.38
NR_003722. 1 (Gapdh-ps1)	3	Up	9.81
XR_006589. 2		Down	10.49
XR_006589. 2		Down	10.49
TCONS_00106261	5	Down	11.38
TCONS_00016693	1	Up	17.88
XR_006738. 3		Up	18.33
TCONS_00139993	X	Down	27.31
TCONS_00031872	11	Down	29.43
TCONS_00098342	4	Down	62.10

Chr, chromosome; lncRNAs, long non-coding RNAs; SHR, spontaneously hypertensive rats; WKY, Wistar-Kyoto; FC, fold change; abs, absolute.

component analysis), glycoprotein binding, phosphatase activity and isopentenyl-pyrophosphate δ-isomerase activity (identified during molecular function analysis). The KEGG pathway analysis demonstrated that lncRNA coexpressed genes predominantly mapped to three pathways, including the cytochrome p450-associated process, extracellular matrix-receptor interaction and viral myocarditis.

Discussion

These preliminary data indicate that numerous lncRNAs are differentially expressed between SHRs and normal (WKY) rats. These results were expected as high blood pressure (considered to be a multifactorial disease) involves complicated biological networks.

According to the GO and pathway analyses, numerous genes are associated with a variety of membrane functions, particularly including cell membrane permeability, transmembrane receptors and membrane potential. However, this finding contributes little guidance for follow-up experiments, as these physiological elements are involved in almost all biological processes. Although type I diabetes mellitus and viral myocarditis are associated with blood pressure or are involved in the cardiovascular system and received high scores in the pathway analysis, it is challenging to conduct further research on pathways with such a direct association to blood pressure.

One notable finding of the present study was the observation of altered cell migration and ion channel activity, which are two pathogenic factors associated with hypertension. Excessive activation or deactivation of ion channels may have a marked effect on vasomotion or blood pressure, and the target genes of lncRNAs may be associated with the Na^+/K^+ATP pump or Ca^+ channel (13,14). Changes in vascular smooth muscle cell proliferation and migration are considered to be pathogenic factors leading to high blood pressure, as these processes significantly contribute to angiogenesis and microcirculation blood volume (15).

A fundamental limitation of the present study was the disorganized database and unofficial analysis tool used for the identification of animal lncRNAs, including the use of trans- and/or cis-regulatory network analysis.

Although this is a common method to analyze associated protein-coding genes and transcription factors, as well as to predict biological pathways, these databases are based on the human genome and therefore cannot be used to determine sequence information from rats or mice. The above-mentioned GO and KEGG pathway analyses were used as an effective prediction tool for mRNAs; however, with this approach, the user is capable of predicting only the biological function of coexpressed lncRNAs indirectly. Furthermore, the number of studies and the database integrity for rat lncRNAs are markedly lower than those for human and mouse models. Other biological tools, such as ncFANS Function Annotation (http://www.bioinfo.org/ncfans/), are primarily based on data from humans and mice.

Additional limitations of the present study, as well as other ncRNA studies, are the result of the following factors. First, a number of ncRNAs are shorter than 200 nt, are functional, and cannot be grouped into miRNAs and structural RNA groups (16); the microarray in the present study contained only lncRNA probes longer than 200 nt. In addition, many ncRNAs are too similar to other mRNAs with regards to sequence. For example, the lncRNA, NR_003722.1 was upregulated in the SHR group in the current results; however, this represents a pseudogene of GAPDH and there is little difference in base sequence composition. Therefore, one of the main obstacles in investigating lncRNAs is designing a primer or siRNA that cannot be associated with similar mRNAs. Furthermore, siRNAs typically interfere with the expression of their target mRNA in the cytoplasm, whereas numerous lncRNAs have been suggested to be located intranuclearly, where the majority of siRNAs have difficulty entering (17). Further to design and synthesize, ensuring the function of the siRNA presents another challenge.

Recently, an RNA sequencing study reported that lncRNAs were differentially altered between the Brown Norway rat and the Dahl salt-sensitive rat, which is recognized as another classical animal model of hypertension. However, Wang *et al* (4) stated that one limitation of investigating lncRNAs in hypertension is the lack of systematic lncRNA characterization in rats. In another renal lncRNA study that performed sequencing and lncRNA transcriptome analysis, >3,000 transcripts were identified as rat lncRNAs; Gopalakrishnan *et al* (5) reported numerous lncRNAs that were differentially expressed between Dahl salt-resistant, and Dahl salt-sensitive rats and SHRs. The study also identified that certain mRNAs were coexpressed with lncRNAs and that the majority of lncRNAs were not predicted to have target genes (5). These studies contribute important information to the role of lncRNAs in hypertension in rats.

Previous studies have identified that genes associated with hypertension exert significant influences on lncRNAs. For example, angiotensin II (AngII), a classic hypertensive factor, has been demonstrated to regulate the lncRNA termed Lnc-Ang362 in vascular smooth muscle cells. Following treatment with AngII, the expression level of more than three lncRNAs was dynamically altered; specifically, upregulated lncRNAs increased and downregulated lncRNAs decreased at an early time-point following treatment. However, these levels all returned to baseline within 24 h (18). The growth of vascular endothelial cells was also indicated to be regulated by the recently described lncRNA, metastasis associated lung adenocarcinoma transcript 1 (MALAT1); in particular, silencing MALAT1 using siRNA was observed to induce various changes in endothelial cells with respect to angiogenesis (19).

Numerous studies have identified miRNAs as highly associated with blood pressure (13,20), and recent studies have highlighted the competing endogenous RNA reaction between miRNAs and lncRNAs. This reaction may represent a novel pathological process, as numerous miRNA-lncRNA or miRNA-mRNA interactions may be implicated in the multi-layered lncRNA-mRNA/protein crosstalk (21). Furthermore, these mechanisms have been repeatedly reported for the cardiovascular system (22). In a previous study, two miRNAs (miR-221 and miR-222) were verified to be associated with lncRNA in AngII-treated vascular smooth muscle cells, indicating that lncRNAs may serve as host genes for miRNAs (18). Recently, another study demonstrated the importance of the tripartite axis of lncRNA-miRNA-mRNA in the regulation of cardiovascular disease, which may present as a novel research direction in future (23).

It is not considered to be practical to investigate lncRNAs or miRNAs for the diagnosis of hypertension, as blood pressure measurements may be more readily obtained; however, RNAs may serve as valuable diagnostic tools for individuals with early genetic alterations that do not demonstrate any disease symptoms. For healthy individuals who live with anxiety, psychological stress, or poor living habits and who may be asymptomatic genetic carriers of the hypertensive trait, blood pressure measurements may not indicate abnormalities, whereas molecular biology may provide information on the likelihood of disease. Furthermore, a large number of studies have indicated that miRNAs are closely associated with hypertensive therapy (24). However, the development of essential therapeutic agents utilizing ncRNAs may require significant further research. In conclusion, with the identification of the first miRNA-targeted therapeutic agent and its application in volunteers (25), lncRNAs may present as a novel target for the treatment of hypertension and may potentially serve as molecular therapeutic strategies.

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