Original Article

Profiles of long noncoding RNAs in hypertensive rats: long noncoding RNA XR007793 regulates cyclic strain-induced proliferation and migration of vascular smooth muscle cells

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Background: Long noncoding RNAs (lncRNAs) are being discovered in multiple diseases at a rapid pace. However, the contribution of lncRNAs to hypertension remains largely unknown. In hypertension, the vascular walls are exposed to abnormal mechanical cyclic strain, which leads to vascular remodelling. Here, we investigated the mechanobiological role of lncRNAs in hypertension.

Methods and results: Differences in the lncRNAs and mRNAs between spontaneously hypertensive rats and Wistar–Kyoto rats were screened using a gene microarray. The results showed that 68 lncRNAs and 255 mRNAs were upregulated in the aorta of spontaneously hypertensive rats, whereas 167 lncRNAs and 272 mRNAs were downregulated. Expressions of the screened lncRNAs, including XR007793, were validated by real-time PCR. A coexpression network was composed, and gene function was analysed using Ingenuity Pathway Analysis. *In vitro*, vascular smooth muscle cells (VSMCs) were subjected to cyclic strain at a magnitude of 5 (physiological normotensive cyclic strain) or 15% (pathological hypertensive cyclic strain) by Flexcell-4000T. A total of 15% cyclic strain increased XR007793 expression. XR007793 knockdown attenuated VSMC proliferation and migration and inhibited coexpressed genes such as signal transducers and activators of transcription 2 (*stat2*), LIM domain only 2 (*lmo2*) and interferon regulatory factor 7 (*irf7*).

Conclusion: The profile of lncRNAs was varied in response to hypertension, and pathological elevated cyclic strain may play crucial roles during this process. Our data revealed a novel mechanoresponsive lncRNA-XR007793, which modulates VSMC proliferation and migration, and participates in vascular remodelling during hypertension.

Keywords: cyclic strain, hypertension, long noncoding RNA, vascular smooth muscle cells

Abbreviations: cdca8, cell division cycle associated 8; CS, cyclic strain; irf7, interferon regulatory factor 7; lmo2, LIM domain only 2; lncRNA, long noncoding RNA; SHR, spontaneously hypertensive rat; stat2, signal transducers and activators of transcription 2; VSMC, vascular smooth muscle cell; WKY, Wistar–Kyoto rat

INTRODUCTION

Hypertension is a major risk factor for many target organ vascular diseases, including diseases involving the heart, brain, retinopathy, renal function and large arteries [1,2]. Clinical trials show that the artery wall suffers hypermechanical stretch (strain) during hypertension, that is the magnitude of the cyclic strain is above 15% in the brachial artery of hypertensive patients. In contrast, cyclic strain is normally approximately 5–10% [3]. Persistent elevation of cyclic strain is strongly associated with vascular remodelling, which is characterized by migration of vascular smooth muscle cells (VSMCs) from tunica media into the subendothelial layer and abnormal proliferation [4–6]. Many in-vitro studies have demonstrated that cyclic strain modulated VSMC morphology, migration, proliferation and cell cycle through various signalling pathways [7,8]. However, the underlying mechanobiological mechanism is still unclear. In particular, the relevance of a large group of noncoding RNAs to hypertension and their role in cyclic strain-induced VSMC functions remains nearly completely unexplored. The significance of noncoding RNAs in general is being increasingly recognized in hypertension research. However, much of the work in this area is focused on microRNAs, but not long noncoding RNAs (lncRNAs) [9,10].

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LncRNAs are nonprotein-coding transcripts that are longer than 200 nucleotides, which is an arbitrary cut-off value that distinguishes these transcripts from other small RNAs. Unlike the well established mechanism of microRNA action, the mode of function for lncRNAs is not fully understood. Increasing evidence shows that lncRNAs modulate gene expression via a multilevel-regulated pathway. For example, some lncRNAs exert their functions through interacting with chromatin to silencing multiple genes, whereas other lncRNAs recruit promoters to their target regions to activate genes. In the cytoplasm, a few lncRNAs have been implicated in posttranscriptional gene regulation through controlling processes such as RNA maturation and transport, mRNA stability and protein synthesis [11,12]. Given their large number and complicated functional modes, lncRNAs are emerging as important regulators of a variety of cellular responses, developmental processes and diseases [13,14]. Although the relevance of lncRNAs in cancer, neurological diseases and heart diseases is increasingly being investigated, there are few reports to date on the role of lncRNAs in hypertension and cyclic strain-induced vascular remodelling.

Therefore, this study sought to characterize the profiles of lncRNAs and mRNAs in spontaneously hypertensive rats (SHRs) and Wistar–Kyoto rats (WKYs) using gene microarrays, construction gene coexpression networks and analysis of gene function using Ingenuity Pathway Analysis (IPA) (QIAGEN Redwood City, California, USA). We also examined the role of the screened lncRNAs in modulating the proliferation and migration of VSMCs undergoing cyclic strain. Illuminating the role of lncRNAs in hypertension-induced vascular remodelling may provide deeper insight into the mechanobiological mechanism underlying hypertension.

MATERIALS AND METHODS

Measurement of blood pressure

The animal care and experimental protocols used in the current study conformed to the Animal Management Rules of China (Documentation 55, 2001, Ministry of Health, China). The blood pressure (BP) of male SHR and normotensive control rats (WKY) was recorded using the tail-cuff method with a programmable sphygmomanometer (BP-98A; Softron; Tokyo, Japan) [15]. At week 18, the SBP of the SHR was maintained between 180 and 200 mmHg for more than 1 week. The thoracic aortas from three SHRs and three WKYs were harvested for microarray analysis, and the other four animals' tissues were used for quantitative real-time PCR (qRT-PCR).

Microarray and computational analysis

Briefly, total RNA from each sample was amplified and transcribed into fluorescent cRNA using the Quick Amp Labelling Kit, One-Colour (Agilent p/n 5190-0442; Agilent Technologies, Palo Alto, California, USA). The labelled cRNAs were hybridized to the Rat LncRNA Array v2.0 (4×44 K, Arraystar; Rockville, Maryland, USA). After washing, the arrays were scanned using the Agilent Microarray Scanner (Agilent p/n G2565BA; Agilent Technologies). Agilent Feature Extraction software (version 11.0.1.1) was used to analyse the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies). Differentially expressed lncRNAs/mRNAs with statistical significance were identified through Volcano Plot filtering. A P value was calculated using Student's t test, with further analysis of corrected P value and the false discovery rate (FDR). The threshold set for upregulated and downregulated genes was a fold change more than 2.0 and a P value less than 0.05.

TABLE 1. Gene-specific primer sequences and annealing temperatures

Genes		Primers sequences, 5′–3′	Tm (°C)
XR007793	FWD	CATAACCCAAGCGTCAAAGG	60.0
	REV	CATGAAGGCAGGTAAGAAAACAC	60.0
cdca8	FWD	TCCCGGTCTCATTCGCTAAC	60.0
	REV	CTCGCGGTCGAAGTCCTTTA	60.0
lmo2	FWD	TGGACTCTTCCTGGGCACTA	60.0
	REV	TCTCCTAGGGCTGGTCCTTT	60.0
stat2	FWD	GGCATTACCTGCTCTTGGGT	60.0
	REV	ATAGAGGAAGCGGAGTGGGT	60.0
Irf7	FWD	TCTGCTTTCTGGTGATGCTG	60.0
	REV	GGAAGGTGTTCTTGCTCCTG	60.0
Gapdh	FWD	GATGGTGAAGGTCGGTGTGA	60.0
	REV	TGAACTTGCCGTGGGTAGAG	60.0

cdca8, cell division cycle associated 8; FWD, forward primer; irf7, interferon regulatory factor 7; lmo, LIM domain only 2; REV, reverse primer; stat2, signal transducers and activators of transcription 2; Tm, temperature.

Coexpression network and Ingenuity Pathway Analysis

To identify interactions among genes, gene coexpression networks were built according to the normalized signal intensity of different expressed genes. A Pearson correlation coefficient (PCC) between the specific lncRNA and mRNA (not including lncRNA and lncRNA or mRNA and mRNA) was calculated. Only the strongest correlated ($PCC \geq 0.95$) genes were presented in the coexpression network in which the specific lncRNA and mRNA are connected by an edge indicating either positive or negative correlation.

The functions of the coding genes in the coexpression network were analysed through the use of QIAGEN's IPA (http://www.ingenuity.com/products/ipa). IPA software is used to model, analyse and understand the complex biological and chemical systems at the core of life science research. For our studies, the core analysis tool was used. The top canonical pathways, molecular functions and networks were presented as generated by the software.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific; Waltham, Massachusetts, USA), and qRT-PCR was performed using the Quanti-Tect SYBR Green PCR Kit (Bio-Rad, Waltham, Massachusetts, USA) on a StepOne plus PCR system (Thermo Fisher Scientific). The gene-specific primers and annealing temperatures are listed in Table 1. The results were normalized to GAPDH expression levels, and relative gene expression was measured using the $2^{-\Delta\Delta Ct}$ method.

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Cell culture and cyclic stretch loading

VSMCs were isolated from the thoracic aortas of male Sprague–Dawley rats and SHR rats using an explant method as previously described [16]. VSMCs between passages 4 and 8, with cell populations of more than 95% purity, were used in all experiments. For cyclic strain loading, VSMCs were seeded in 6-well Bioflex plates (Flexcell International; Burlington, North Carolina, USA) at a density of 2×10^5 cells/well. After achieving 70% confluence, the cells were serum-starved in DMEM for 24 h and then subjected to a cyclic strain of 5 and 15% elongation at 1.25 Hz for a designated time course using a Flexcell Tension Plus System (FX-4000T, Flexcell International).

Cell proliferation detection using the water-soluble tetrazolium assay

VSMC proliferation was assessed *in vitro* using a WST-1 reagent (Cell Proliferation Reagent WST-1; Roche; Basel, Basel-Stadt, Switzerland). VSMCs were incubated with water-soluble tetrazolium (WST) for 30 min prior to harvest, which was subsequently converted into a coloured dye by mitochondrial dehydrogenase enzymes. The absorbance at 450 nm was measured in an ELISA plate reader (Bio-Rad 680; BIO-Rad, Hercules, California, USA). The colorimetric analysis reflected the proliferation and metabolic activity of cells.

Cell migration detection using a wound healing assay

A monolayer wound healing cell migration assay was performed as previously described [17]. Once 95% confluent, a line was scratched across a monolayer of cells using a sterile 200-μl pipette tip. Images of the scratched line were immediately photographed and photographed again after 24 h (IX-71; Olympus, Tokyo, Japan). The distance of cell migration was measured using Image-ProPlus 4.5.1 software (Media Cybernetics Inc., Rockville, Maryland, USA).

RNA interference

For the RNA interference experiment, VSMCs were transfected with 100 nmol/l of XR007793 small interfering RNA (siRNA) fragments or control nonsilencing siRNA (GenePharma; Shanghai, China) for 48 h using Lipofectamine 2000 transfection reagent (Invitrogen) in Opti-MEM medium (Gibco; Grand Island, New York, USA) according to the manufacturer's instructions. The sequences of the siRNA oligos were as follows:XR007793 siRNA: sense 5′-GCAAUCCUAUGGUGUUCUATT-3′ and antisense 5′-UAGAACACCAUAGGAUUGCTT-3′.Nonsilencing siRNA: sense 5′-UUCUCCGAACGUGUCACGUTT-3′ and antisense 5′-ACGUGACACGUUCGGAGAATT-3′.

Western blot assays

Protein extracts were separated using 10% SDS-PAGE. The proteins were detected using primary antibodies specific to STAT2 (Cell Signalling, Danvers, Massachusetts, USA; 1 : 500), IRF7 (Abcam, Cambridge, UK; 1 : 1000), LMO2 (Proteintech; Chicago, Illinois, USA; 1 : 500) and GAPDH (Proteintech; 1 : 500). After incubation with alkaline phosphatase-conjugated secondary antibodies (Jackson ImmunoResearch; Baltimore, Pennsylvania, USA), the signals were visualized with nitroblue tetrazolium-5′ bromo-4′chloro-3′-indolyl phosphate (Bio Basic, Inc., Markham, Ontario, Canada) and quantified with Quantity One software (Bio-Rad).

Statistical analysis

Each experiment was performed at least in triplicate, and all values are expressed as the mean ± SD. Student's t test was used to compare two groups. Values of P less than 0.05 were accepted as significant.

RESULTS

Long noncoding RNA expression profiles in the aortas of spontaneously hypertensive rats and Wistar–Kyoto rats

The microarray analysis identified 68 lncRNAs and 255 mRNAs that were upregulated, whereas 167 lncRNAs and 272 mRNAs were downregulated in the aortas of SHR compared with WKY [fold change >2.0 and a P value <0.05, Supplementary data (Sup.) 1–4, http://links.lww.com/HJH/A743]. Hierarchical clustering showed systematic variations (fold change >4, $P < 0.05$) in the differentially expressed lncRNAs (Fig. 1a) and protein-coding RNAs (fold change >7, $P < 0.05$) (Fig. 1b) between SHR and WKY using a gene microarray. Figure 1c and d showed the Volcano plots that were constructed using fold-change values and P values and revealed the relationship between fold change and statistical significance. The vertical lines correspond to 2.0-fold change (both up and down, respectively), and the horizontal line represents a P value of 0.05. The red points in the plot represented the differentially expressed lncRNAs and mRNAs with statistical significance.

To validate the microarray analysis, we selected 14 lncRNAs among the differentially expressed lncRNAs for qRT-PCR analysis based on the fold change, P value, raw intensity, the function of the coding gene within 100 kb of the lncRNA and the lncRNA length. The expressions of seven lncRNAs were consistent with the data of the gene microarray (Fig. 1e), and seven other lncRNAs had no significant difference compared with the WKY group (Fig. 1f).

The lncRNA XR007793 was remarkably higher in SHR compared with WKY (fold change = 59.73, $P = 5.906\text{E}-06$, FDR = 0.0069), which was also validated by qRT-PCR (fold change = 4.27, $P = 0.03$, $P < 0.05$). Interestingly, there were no significant differences in the expression of XR007793 in the aorta between 3-week-aged SHRs and WKYs, which appeared similar in BP between the two rat strains (Sup. 5, http://links.lww.com/HJH/A743).

LncRNA XR007793 is located on chromosome 8 in the rat according to the database of NCBI (rattus norvegicus genome in the strain brown Norway and Sprague–Dawley rats). In addition, its sequence is referred to in RefSeq_XR and was identified in microarray by the probe, TCTTAAATCACTACAGGTGTTTTCTTACCTGCCTTCATGAGCACCTTCTGGATTTTGTTT. There are no coding genes nearby lncRNA XR007793; thus, a coexpression network was constructed to investigate the function of lncRNA XR007793.

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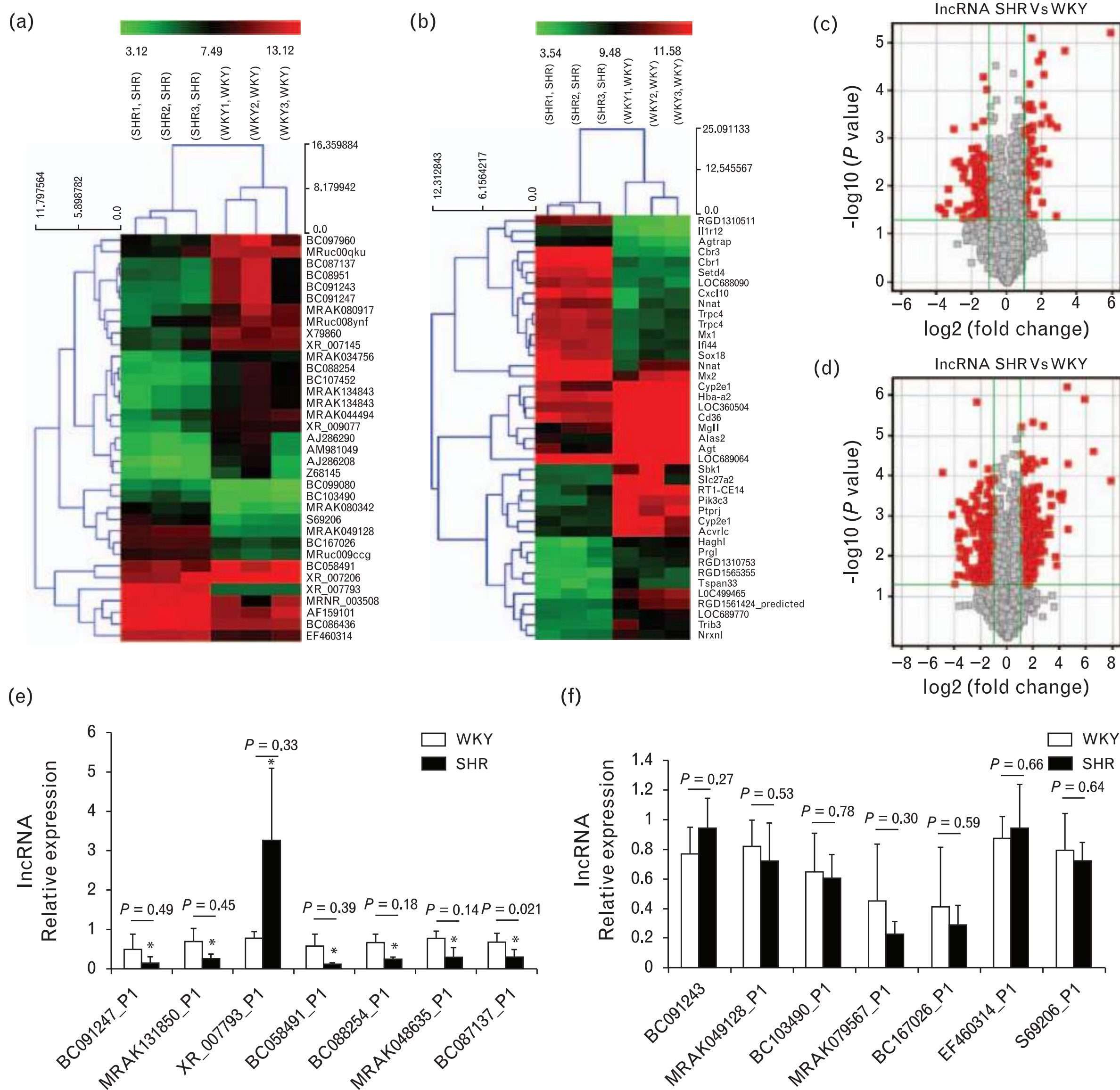

FIGURE 1 (a) The differential expression of long noncoding RNA in microarray. Hierarchical clustering analysis is shown for 35 long noncoding RNAs (fold change >4, $P < 0.05$) in the aorta of spontaneously hypertensive rat compared with Wistar–Kyoto rat using gene microarray analysis. Expression values are represented in shades of red and green to indicate expression to the left and right of the median expression value, respectively, across all samples (log scale 2, from 3.12 to 13.12), $n = 3$. (b) The differential expression of mRNA in the microarray analysis. Hierarchical clustering analysis is shown for 42 mRNA (fold change >7, $P < 0.05$) in the aorta of spontaneously hypertensive rat compared with Wistar–Kyoto rat using gene microarray analysis. Expression values are represented in shades of red and green to indicate the expression to the left and right of the median expression value, respectively, across all samples (log scale 2, from 3.54 to 11.58), $n = 3$. (c and d) the Volcano plots. The vertical lines correspond to 2.0-fold up and down, respectively, and the horizontal line represents a P value of 0.05. So the red point in the plot represents the differentially expressed long noncoding RNAs and mRNA with statistical significance. (e and f) The differential expression of long noncoding RNA in the aorta of spontaneously hypertensive rat compared with Wistar–Kyoto rat was validated using quantitative real-time PCR. The values shown are the mean ± SD for each group from independent experiments, $^{*}P < 0.05$, $n = 4$.

Coexpression network and gene function analysis

According to the raw intensity of lncRNA XR007793 and the normalized signal intensities of the differentially expressed coding genes in the microarray, a sub coexpression network including lncRNA XR007793 and coding genes were created. Figure 2a shows that 44 coding genes were correlated with lncRNA XR007793 ($PCC \geq 0.95$). The blue lines between two nodes indicate positive correlations between genes, and the orange dashed lines indicate negative correlations. Notably, these relationships do not indicate a direct interaction between the two molecules.

The 44 coding genes were analysed using IPA. The summary report (Sup. 6, http://links.lww.com/HJH/A743) revealed that the top five canonical pathways were zymosterol biosynthesis; cholesterol biosynthesis I, II and III; and a super pathway for cholesterol biosynthesis. The top five diseases and disorders were development disorder, gastrointestinal disease, hereditary disorder, immunological disease and infectious diseases. The top five molecular and cellular functions were cell-to-cell signalling and interaction, cellular assembly and organization, cellular development, cellular growth and proliferation and lipid metabolism.

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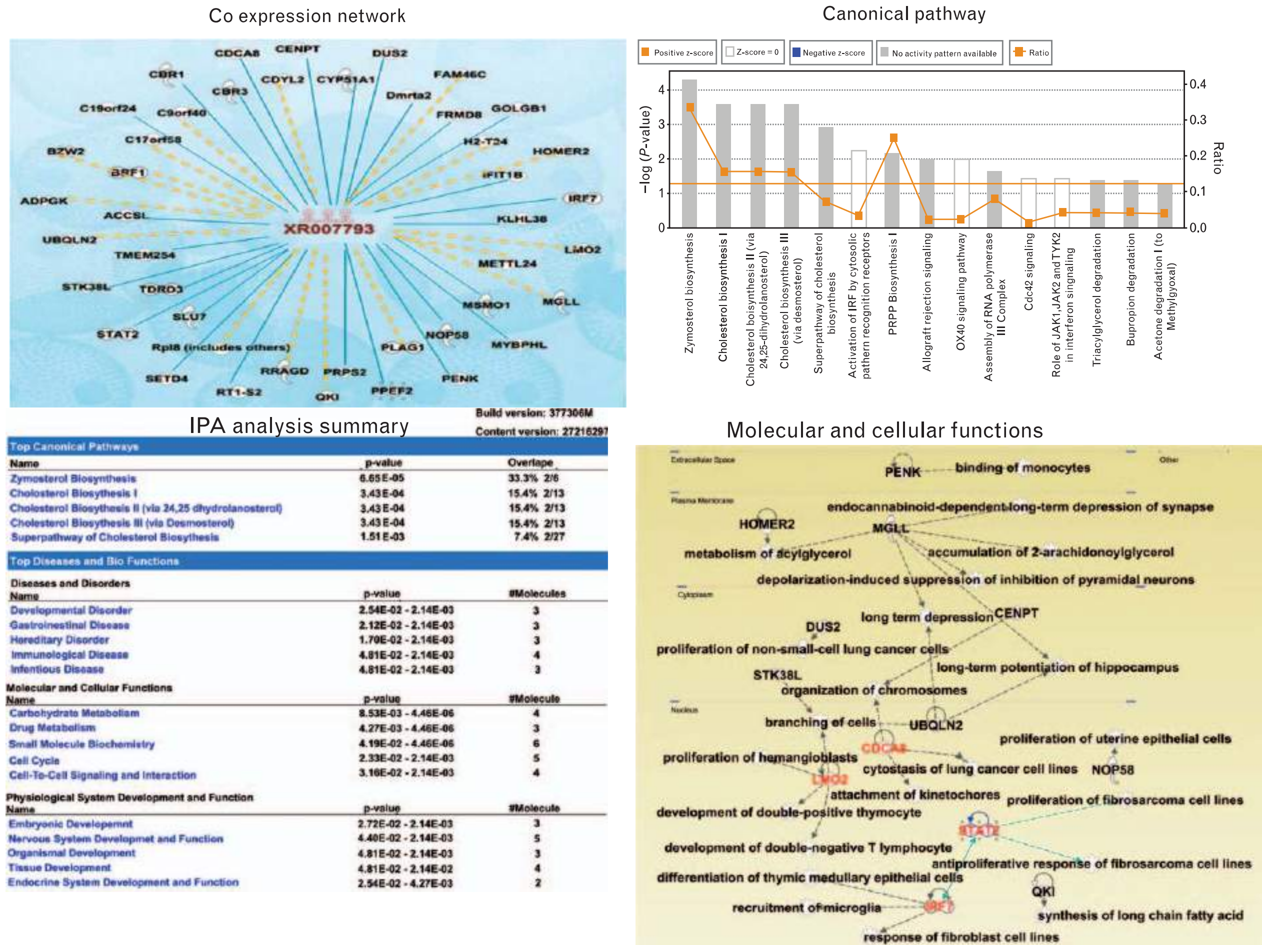

IPA analysis summary

Build version: 377306M
Content version: 27216297

Top Canonical Pathways

Name	p-value	Overlape
Zymosterol Biosynthesis	6.65E-05	33.3% 2/6
Cholosterol Biosythesis I	3.43E-04	15.4% 2/13
Cholosterol Biosythesis II (via 24,25 dhydrolanosterol)	3.43E-04	15.4% 2/13
Cholesterol Biosythesis III (via Desmosterol)	3.43E-04	15.4% 2/13
Superpathway of Cholesterol Biosythesis	1.51E-03	7.4% 2/27

Top Diseases and Bio Functions

Diseases and Disorders

Name	p-value	#Molecules
Developmental Disorder	2.54E-02 - 2.14E-03	3
Gastroinestinal Disease	2.12E-02 - 2.14E-03	3
Hereditary Disorder	1.70E-02 - 2.14E-03	3
Immunological Disease	4.81E-02 - 2.14E-03	4
Infentious Disease	4.81E-02 - 2.14E-03	3

Molecular and Cellular Functions

Name	p-value	#Molecule
Carbohydrate Metabolism	8.53E-03 - 4.46E-06	4
Drug Metabolism	4.27E-03 - 4.46E-06	3
Small Molecule Biochemistry	4.19E-02 - 4.46E-06	6
Cell Cycle	2.33E-02 - 2.14E-03	5
Cell-To-Cell Signaling and Interaction	3.16E-02 - 2.14E-03	4

Physiological System Development and Function

Name	p-value	#Molecule
Embryonic Developemnt	2.72E-02 - 2.14E-03	3
Nervous System Developmet and Function	4.40E-02 - 2.14E-03	5
Organismal Development	4.81E-02 - 2.14E-03	3
Tissue Development	4.81E-02 - 2.14E-02	4
Endocrine System Development and Function	2.54E-02 - 4.27E-03	2

FIGURE 2 (a) Long noncoding RNA XR007793 coexpression network. This subnetwork consists of long noncoding RNA XR007793 (centre) and its 44 direct neighbours. The blue lines between two nodes indicate positively correlated interactions, and the orange dashed lines indicate negatively correlated interactions. (b) Ingenuity Pathway Analysis report. Shown are the 44 coding genes involved in the top five canonical pathways, and the top five diseases, molecular functions and physiological systems. (c) Canonical pathway. The 44 coding genes involved in 12 canonical pathways are shown. (d) Molecular and cellular functions.

The canonical pathways that were identified also included activation of IRF by cytosolic pattern recognition receptors, 5-phosphoribosyl 1-pyrophosphate (PRPP) biosynthesis I and allograft rejection signalling, among others. The molecular and cellular functions analysis revealed the involvement of IRF7, proenkephalin and monoglyceride lipase in cell assembly or cell-to-cell signalling and interaction. STAT2, cell division cycle associated 8 (CDCA8) and LMO2, among other molecules, were involved in cell proliferation or cellular development. The functions of the coding genes that showed correlated expression with lncRNA XR007793 led us to propose that lncRNA XR007793 might modulate cell proliferation and migration. Abnormal VSMC proliferation and migration is the pathological basis of the vascular remodelling that is induced by many cardiovascular diseases including hypertension. Thus, we hypothesized that lncRNA XR007793 might be involved in cell proliferation and migration.

The effect of cyclic strain on vascular smooth muscle cell proliferation and expression of long noncoding RNA XR007793 and related coding genes

To explore how the hypertensive cyclic strain affects VSMC function and lncRNA XR007793, the VSMCs were subjected to different magnitudes of cyclic strain using a tension system *in vitro*. VSMC proliferation was evaluated using a WST assay after loading cyclic strain for 12 h. As shown in Fig. 3a, the proliferation of VSMCs exposed to 15% cyclic strain stimulation was higher than that of VSMCs exposed to 5% cyclic strain stimulation ($P < 0.05$) at 12 h. In our previous work, VSMC proliferation was evaluated by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporation in newly synthesized DNA after loading cyclic strain for 24 h, and VSMC proliferation was evaluated in a transwell assay [16]. Together, these results suggest that pathological hypertensive cyclic strain might be a pathogenic factor for vascular homeostasis.

Figure 3b shows the effect of different magnitudes of cyclic strain on the expressions of lncRNA XR007793 at 3, 6 and 12 h. Compared with the 5% cyclic strain stimulation, 15% cyclic strain increased the expression of lncRNA XR007793 at 3 and 6 h, whereas there was no significant effect at 12 h. These results indicated that 15% cyclic strain stimulation *in vitro* increased the short-term expression of lncRNA XR007793 and recovered the long-term expression.

Parallel experiments were performed to examine the expression of the coding genes that are related to cell proliferation and migration, including *stat2*, *cdca8*, *lmo2* and *irf7*, after loading cyclic strain for 12 h. As shown in

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