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Original Article

Expression profiling of long noncoding RNAs and the dynamic changes of lncRNA-NR024118 and Cdkn1c in angiotensin II-treated cardiac fibroblasts

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Abstract: A growing body of evidence shows that long non-coding RNAs (lncRNAs) are involved in multiple human diseases than previously realized. However, no information is available now about lncRNAs in cardiac fibroblasts. The expression profile of lncRNAs was analyzed in Ang II-treated cardiac fibroblasts using lncRNAs arrays. The analysis showed that 282 of 4376 detected lncRNAs demonstrated >2-fold differential expression in response to the treatment with Ang II (100 nm) for 24 h. Among of them, 22 lncRNAs showed a greater than 4-fold changes. Meanwhile, Ang II also induced a widely expression changes in protein-coding genes in cardiac fibroblasts. Quantitative real time PCR confirmed the changes of six lncRNAs (AF159100, BC086588, MRNR026574, MRAK134679, NR024118, AX765700) and mRNAs (IL6, RGS2, PRG4, TIMP1, Cdkn1c, TIMP3, Col I, Col III and Fibronectin) in cardiac fibroblasts. Bioinformatic analysis indicated the process of cell proliferation. Further studies revealed that the down-regulating of Ang II on the expression of lncRNA-NR024118 was time-dependent, that the level of NR024118 was lowest at 24 h and back at 48 h. Ang II also dynamically down regulated the expression of Cdkn1c in cardiac fibroblasts. Ang II at a range from 10^{-9} M to 10^{-6} M induced a decrease of NR024118 and Cdkn1c in cardiac fibroblasts. In conclusion, the expression profile of lncRNAs was significantly altered in the Ang II-treated cardiac fibroblasts and Ang II dynamically regulated the expression of lncRNA-NR024118 and Cdkn1c in cardiac fibroblasts, indicating the potential role of NR024118 in cardiac fibroblasts.

Keywords: Angiotensin П, cardiac fibroblasts, long non-coding RNA

Introduction

Cardiac fibrosis is the excess accumulation of extracellular matrix in the heart, which is closely associated with numerous cardiovascular diseases [1, 2]. Cardiac fibroblasts play a pivotal role in the development of cardiac fibrosis through the synthesis of extracellular matrix (ECM) proteins, the degradation of ECM by producing matrix metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs), and the secretion of cytokines including interleukin (IL)-6 [3, 4]. Angiotensin II (Ang II) is considered to be a major factor in the pathogenesis of cardiac remodeling [5, 6]. Ang II has been shown to induce cardiac fibrosis by stimulation of cell proliferation, ECM synthesis and cytokines secretion in cardiac fibroblasts [7, 8]. At the present time, the molecular mechanisms underlying the effects of Ang II on cardiac fibroblasts are still not completely understood.

Currently, it is known that only ~2% of the mammalian genome encodes proteins, and that the over-whelming majority of the remaining genome is transcribed into noncoding RNAs (ncRNAs). These ncRNAs can be divided into housekeeping RNAs and regulatory ncRNAs, which are further grouped into short and long ncRNAs [9]. MicroRNAs (miRNA), as the short ncRNAs, have been demonstrated to be involved in the development of various diseases [10]. Several miRNAs have been confirmed to be closely associated with cardiac fibrosis [4]. The long ncRNAs (lncRNAs) are defined as a kind of ncRNAs which are longer than ~200 nucleotides and lacking of protein-encoding capacity. The ratio of lncRNAs in total ncRNAs is beyond

Table 1. Primers sequences for long non-coding RNAs and protein-coding RNAs

Gene name	Primer sequences
GAPDH	Forward: 5'GGGAAACTGTGGCGTGAT3'
	Reverse: 5'GAGTGGGTGTCGCTGTTGA3'
AF159100	Forward: 5'GTTGCTCCTCGCTGGTTTC3'
	Reverse: 5'CAGCTGCCTTTATTCAGATGTA3'
BC086588	Forward: 5'CCATAGTAGAAACAGGCAGGAC3'
	Reverse: 5'CCAGGCAACAATCAAATCAG3'
MRNR026574	Forward: 5'GGCCACCTGCCTTACCTAC3'
	Reverse: 5'AGCCCACGGGACCACAAC3'
MRAK134679	Forward: 5'ACCATGAGGCGGGACTGAC3'
	Reverse: 5'TCTGGTTAAACGAAAGGCAAAT3'
NR024118	Forward: 5'GCTGCCCACCTCACTCAC3'
	Reverse: 5'CTTTATTGCTCCATTTCCCTC3'
AX765700	Forward: 5'TTCCGAGCAGCCATTGACA3'
	Reverse: 5'CATCATCTAGCTCAGGGTTTCC3'
IL6	Forward: 5'GCCTATTGAAAATCTGCTCTGGT3'
	Reverse: 5'GTCTTGGTCCTTAGCCACTCCT3'
RGS2	Forward: 5'TCTGGTTGGCTTGCGAAGAC3'
	Reverse: 5'TCTCTTTGGGAGCTTCCTTC3'
PRG4	Forward: 5'CGGGACGTTAGTTGCATTCG-3',
	Reverse: 5'TCAGTGATTCTGCGTGGTGGA-3'
Cdkn1c	Forward: 5'CCCCCACACATTCATCTTCA3'
	Reverse: 5'GGGCAGTACAGGAACCATTTT3'
TIMP3	Forward: 5'CCTTTGGCACTCTGGTCT3'
	Reverse: 5'TCAGCAGGTACTGGTATTTGT3'
Fibronectin	Forward: 5'GTGAAGAACGAGGAGGATGTG3'
	Reverse: 5'GTGATGGCGGATGATGTAGC3'
TIMP1	Forward: 5'GTTGCTCCTCGCTGGTTTC3'
	Reverse: 5'CAGCTGCCTTTATTCAGATGTA3'
Collagen I	Forward: 5'GTTGCTCCTCGCTGGTTTC3'
	Reverse: 5'CAGCTGCCTTTATTCAGATGTA3'
β-actin	Forward: 5'CCTGTACGCCAACACAGTGC3'
	Reverse: 5'ATACTCCTGCTTGCTGATCC3'

80% but is the least well-understood ncRNAs now [10]. Although initially thought to be transcriptional noise, recent evidence suggests that the expression of lncRNAs is cell- and developmental stage-specific and regulated by common transcription factors [11-13].

Although lncRNAs have been studied in different types of human cancer and neural diseases, the research of lncRNAs in cardiovascular disease is clearly in its infancy [14]. Only several lncRNAs were reported in cardiovascular system. LncRNA-MIAT has been identified to confer risk of myocardial infarction and lncRNA-ANRIL was considered to be a risk factor of coronary artery diseases [14, 15]. LncRNA-AK143260 (Braveheart) was reported to be necessary for cardiac development [16]. Recently, lncRNAs were reported to be able to be regulated by Ang II in vascular smooth cells [17]. However, no information is available now about lncRNAs in cardiac fibroblasts. In this study, we found that Ang II (100 nm) for 24 h simultaneously induced widely changes of lncRNAs and protein-coding RNAs in adult rat cardiac fibroblasts. Bioinformatic analysis indicated the process of cell proliferation. Further studies revealed that Ang II dynamically downregulated the expression of lncRNA-NR024118, companying the decrease of Cdkn1c in cardiac fibroblasts. Ang II at a range from 10^{-9} M to 10^{-6} M induced a decrease of NR024118 and Cdkn1c in cardiac fibroblasts. Our current studies indicated the potential role of NR024118 in cardiac fibroblasts.

Methods

Materials and animals

Collagenase, trypsin and Ang II were obtained from Sigma Chemical (St Louis, MO, USA); Dulbecco's modified Eagle's medium (DMEM) and TRIzol were obtained from Life Technologies (Invitrogen, Carlsbad, CA, USA). Rat 4x44K LncRNA expression arrays were purchased from Arraystar (Rockville, USA). Sprague-Dawley (SD) rats were supplied from the Experimental Animal Center of Xian Jiaotong University (Xian, China). The animal experiments were approved by the University Committee of Laboratory Animal Care and Use and followed the guidelines of the National Animal Research Center.

Isolation and culture cardiac fibroblasts

Cardiac ventricular fibroblasts were obtained from hearts of adult male SD rats weighing 250~300 g as described previously [18]. In brief, following rapid excision of the hearts, the fibroblasts were prepared by enzymatic digestion with a collagenase/trypsin solution. After a 2h period of attachment to uncoated culture plates, the cells which were weakly attached or unattached were rinsed free and attached cells

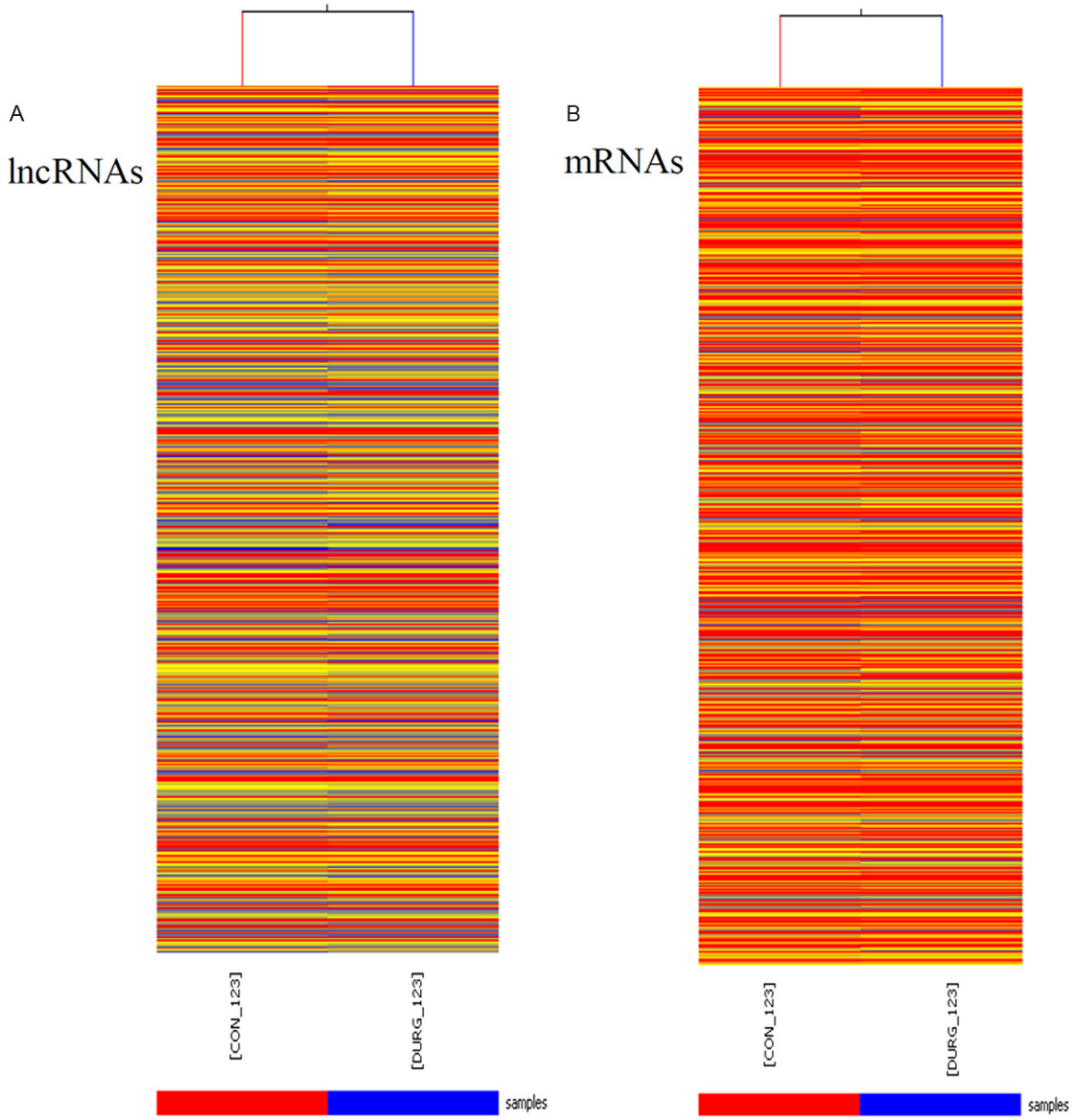

Figure 1. Heat map presentation of the expression profile of lncRNAs and mRNAs in angiotensin II-treatment and control cardiac fibroblasts. "Red" indicates high relative expression, and "blue" indicates low relative expression. CON-123 indicates cardiac fibroblasts. DRUG-123 indicates cardiac fibroblasts treated by angiotensin II (100 nM) for 24 h.

(mostly fibroblasts) were washed and grown in DMEM with 10% fetal bovine serum. The cardiac fibroblasts (passages 3~5) were grown to 80-90% confluence and serum starved for 24 h before treatment.

Preparation of RNA

Following 24 h serum starvation, cardiac fibroblasts were treated with Ang II (100 nM) for 24 h. Total RNAs were extracted using the TRIZOL reagent as previously described and RNAs were dissolved in RNase-free water [18]. The RNA quantity was determined spectrophotometrically as A260 and A260/A280 ratio using NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and RNA quality were checked by electrophoresis on a 1.2% agarose/formaldehyde gel. Isolated RNAs were stored at -70°C prior to lncRNAs arrays analysis and real time-PCR.

Table 2. Differentially expressed long non-coding RNAs in cardiac fibroblasts (fold change >4)

Probe Name	Expression	chromosome	strand	Start	End	Fold change
MRAK034346	up	chr2	+	120115104	120115164	4.25
AF167308	up	chr16	-	73031874	73031931	4.26
XR007499	up	chr13	-	39625623	39625683	4.26
XR006412	up	chr12	+	1021078	1021138	4.61
U57362	up	chr8	-	84641434	84641494	4.71
U57361	up	chr8	-	84643465	84643525	4.74
BC158638	up	chr3	-	113780927	113801831	5.15
U78517	up	chr3	+	54716706	54716766	5.40
AF239157	up	chr10	-	46291442	46291502	5.92
BC086588	up	chr2	-	186049351	186049411	8.92
MRNR026574	up	chr1	+	159013775	159013835	12.20
AF159100	up	chr16	-	70640308	70640368	47.16
MRAK134679	down	chr3	-	62648034	62648094	-8.34
NR_024118	down	chr20	-	4128070	4128130	-7.97
AX765700	down	chr9	+	60980297	60980357	-7.17
MRAK053938	down	chr15	+	109052626	109052699	-6.42
MRAK031289	down	chr16	+	64128370	64128430	-6.26
AY973245	down	chr16	+	64124051	64124111	-5.46
AJ005396	down	chr2	+	210193318	210193378	-5.33
MRAK162711	down	chr5	+	28340911	28340971	-5.15
MRAK132609	down	chr2	+	210192978	210193038	-5.09
NR_027324	down	chr1	-	202822980	202823040	-4.63

Microarray analysis of long ncRNAs and mRNAs expression

The rat LncRNA 4x44K Arrays from Arraystar (Rockville, USA) were used to analyze the expression profile of long non-coding RNAs and mRNAs in adult rat cardiac fibroblasts. The array contains probes of lncRNAs (~9300) and protein-coding genes (~15,200). The microarray hybridization was performed based on the manufacturer's standard protocols [19]. Briefly, total RNAs from three pairs of control cardiac fibroblasts and Ang II-treated cardiac fibroblasts were extracted and pooled. Next, 1 μg of total RNAs were amplified and transcribed into fluorescent cRNA using Agilent's Quick Amp Labeling protocol (version 5.7, Agilent Technologies). The labeled cRNAs were hybridized onto the Rat LncRNA 4x44K Array (Arraystar, Rockville, USA), washed and the microarrays scanned using an Agilent Scanner G2505B. Agilent Feature Extraction software (version 10.7.3.1) were used to analyze acquired array images. Median normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Agilent Technologies).

Quantitative real time-PCR

Quantitative real time-PCR (qPCR) was performed to quantify the levels of lncRNAs and mRNAs as previously described [18]. Briefly, total RNAs of cardiac fibroblasts were extracted using TRIzol Reagent. cDNAs were synthesized using the First Strand cDNA Synthesis kit (Fermentas Life Science, Burling, ON, Canada). Reactions were incubated for 60 mins at 42°C, 5 mins at 70°C, and then stored at -20°C. Quantitative PCR was then performed by using SYBR Premix Ex Taq™ II (TaKaRa, Ohtsu, Shiga, Japan) in iQ5 realtime PCR detection system (Bio-Rad, Hercules, CA, USA). PCR reactions were performed at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 30 s. The specificity of PCR products was assessed by melting curve analysis. Primers sequences of lncRNAs and mRNAs for qPCR are listed in **Table 1**. Gene expression in each sample was normalized to GAPDH and actin expression. Relative quantitation of lncRNAs and mRNAs

Table 3. Differentially expressed protein-coding RNAs in cardiac fibroblasts (fold change >10)

Probe Name	Gene Symbol	Description	Fold change
CUST3948	Rgs2	Rattus norvegicus regulator of G-protein signaling 2	10.16
CUST10456	Nefh	Rattus norvegicus neurofilament, heavy polypeptide	10.38
CUST8969	Spp1	Rattus norvegicus secreted phosphoprotein 1	10.48
CUST12405	Gch1	Rattus norvegicus GTP cyclohydrolase 1	11.92
CUST4425	Cd55	Rattus norvegicus decay accelerating factor 1 (Daf1)	12.69
CUST12794	Ccdc19	Rattus norvegicus coiled-coil domain containing 19	13.079
CUST14423	Pde2a	phosphodiesterase 2A isoform 1	13.219
CUST15179	Pde2a	phosphodiesterase 2A, cGMP-stimulated	13.28
CUST4963	Gjb2	gap junction membrane channel protein beta 2	13.46
CUST2183	Elf5	E74-like factor 5	13.51
CUST11179	Rnase1	Rattus norvegicus ribonuclease, RNase A family, 1	13.73
CUST1306	Ptprn	Rattus norvegicus protein tyrosine phosphatase, receptor type, N	14.33
CUST11361	Hspb7	cardiovascular heat shock protein	14.65
CUST1958	Esm1	Rattus norvegicus endothelial cell-specific molecule 1	15.10
CUST10736	Cldn3	Rattus norvegicus claudin 3	17.50
CUST11657	Prg4	proteoglycan 4	18.11
CUST14921	Lcn2	Rattus norvegicus lipocalin 2	19.78
CUST10982	Hp	Rattus norvegicus haptoglobin	20.20
CUST6098	Ccl11	Rattus norvegicus chemokine (C-C motif) ligand 11	21.15
CUST5899	Gja5	gap junction membrane channel protein alpha 5	22.59
CUST6613	RGD1562551	hypothetical protein LOC311760	24.421
CUST10878	Pnoc	Rattus norvegicus prepronociceptin	25.39
CUST3944	Cldn11	Rattus norvegicus claudin 11	25.89
CUST11937	Star	Rattus norvegicus steroidogenic acute regulatory protein	31.11
CUST5850	Slco4a1	Rattus norvegicus solute carrier organic anion transporter family, member 4a1	47.80
CUST10972	Il6	Rattus norvegicus interleukin 6	49.94
CUST13799	Slc16a3	Rattus norvegicus solute carrier family 16, member 3	55.36
CUST7261	Cilp	cartilage intermediate layer protein, nucleotide	-30.32
CUST7758	Ces1d	Rattus norvegicus carboxylesterase 3	-20.97
CUST4333	Adh7	Rattus norvegicus alcohol dehydrogenase 7 (class IV)	-15.41
CUST7819	Cdkn1c	Rattus norvegicus cyclin-dependent kinase inhibitor 1C	-13.86
CUST8965	Timp3	Rattus norvegicus tissue inhibitor of metalloproteinase 3	-13.85
CUST8211	Arhgap20	Rattus norvegicus Rho GTPase activating protein 20	-13.49
CUST10750	Olfml2a	olfactomedin-like 2A	-10.41
CUST9855	Flrt3	fibronectin leucine rich transmembrane protein	-10.29
CUST4116	Mtss1	metastasis suppressor 1	-10.15

expression was evaluated by the $2^{(-\Delta\Delta Ct)}$ methods.

Bioinformatic analysis

The Gene Ontology project provides a controlled vocabulary to describe gene and gene product attributes in any organism (http://www.geneontology.org). The ontology covers three domains: Biological Process, Cellular Component and Molecular Function. Fisher's exact test is used to find if there is more overlap between the DE list and the GO annotation list than would be expected by chance. The *p*-value denotes the significance of GO terms enrichment in the DE genes. The lower the *p*-value, the more significant the GO Term (p-value ≤ 0.05 is recommended). Pathway analysis is a functional analysis that maps genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (http://www.genome.jp/kegg/). The

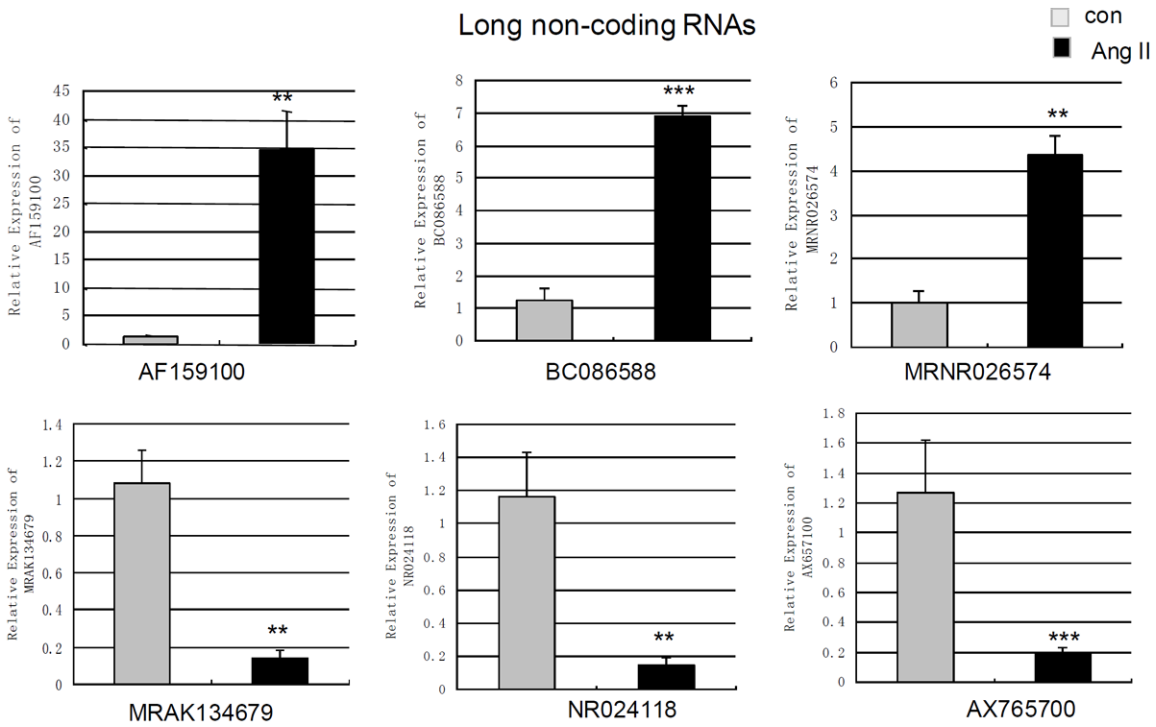

Figure 2. Measurement of changes in long non-coding RNAs using qPCR. The expression levels of AF159100, BC086588, MRNR026574, MRAK134679, NR024118, AX765700 in cardiac fibroblasts treated by angiotensin II-treatment (100 nm 24 h) and control cells were measured by qPCR. Expression of lncRNAs was normalized to GAPDH expression. Data are the mean ± SEM (n=6). *P*<0.05 was considered to indicate a statistically significant difference compared with control fibroblasts.*p<0.05, **p<0.01 and ***p<0.001.

P-value (Fisher-*P* value) denotes the significance of the Pathway correlated to the conditions. Lower the *P*-value, more significant is the Pathway (The recommend *P*-value cut-off is 0.05).

Statistical analysis

Data were presented as the means ± SEM. The Student's *t*-test was used to compare data between the two groups and one-way ANOVA for more than three groups. *P*<0.05 was considered to indicate a statistically significant difference. *p<0.05, **p<0.01 and ***p<0.001.

Results

Arrays analysis of lncRNAs and mRNAs expression in cardiac fibroblasts

Initial studies determined the overall numbers and quantity of lncRNAs and mRNAs that could be detected using an Arraystar microarray (Rockville, USA). This showed that 4376 (~47%) of the 9300 lncRNAs could be detected in untreated cells which is lower fraction than the protein coding mRNAs, for which 9553 (~63%) of the 15200 could be detected. The average intensity of 4376 lncRNAs was 2841 while the average intensity of 9553 protein-coding genes was 5467. These results are consistent with other studies showing that lncRNAs were generally expressed at lower levels than protein-coding genes (**Figure 1**) [20].

To gain further insights into the putative biological relevance of lncRNAs in cardiac fibroblasts, we compared the levels of lncRNAs and mRNAs in cardiac fibroblasts with/without Ang II-treatment for 24 h. We found that 282 of 4376 detected lncRNAs demonstrated >2-fold differential expression with 178 lncRNAs showing up-regulated and 106 lncRNAs showing down-regulated. When the cut-off was set at 4-fold, 12 lncRNAs were up-regulated while 10 lncRNAs were down-regulated (**Table 2**). Meanwhile, 882 mRNAs showed beyond a 2-fold differential expression in cardiac fibroblasts when compared to control cells. 521 mRNAs were up-regulated while 361 mRNAs were down-regulated. When the cut-off was set at 10-fold, 27

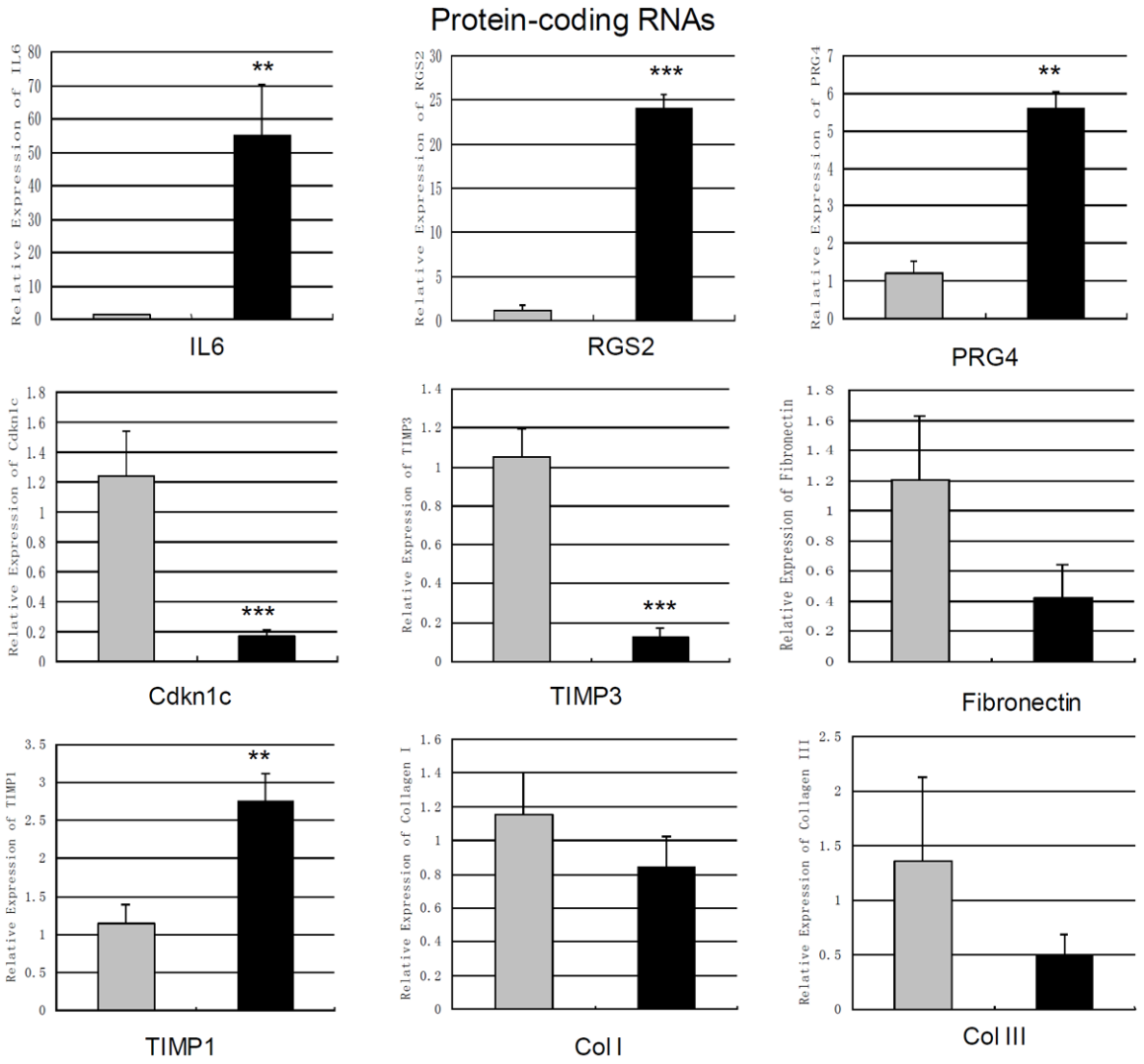

Figure 3. Measurement of changes in protein coding mRNAs using qPCR. The expression levels of IL6, RGS2, PRG4, TIMP1, Cdkn1c, TIMP3, Col I, Col III and Fibronectin in cardiac fibroblasts treated by angiotensin II-treatment (100 nm 24 h) and control cells were measured by qPCR. Expression of mRNAs was normalized to GAPDH expression. Data are the mean ± SEM (n=6). *P*<0.05 was considered to indicate a statistically significant difference compared with control fibroblasts.*p<0.05, **p<0.01 and ***p<0.001.

mRNAs were up-regulated while 9 mRNAs down-regulated (**Table 3**).

Quantitative real time-PCR analysis of lncRNAs and mRNAs expression

Quantitative real time PCR was used to re-measure the abundance of six lncRNAs (AF159100, BC086588, MRNR026574, MRAK134679, NR-024118 and AX765700) and 9 mRNAs associated with fibrosis (IL6, RGS2, PRG4, TIMP1, Cdkn1c, TIMP3, Col I, Col III and Fibronectin). qPCR analysis revealed that the levels of AF159100, BC086588 and MRNR026574 in Ang II-treated cells were up-regulated to 27.42 fold (p=0.0041), 5.50 fold (p<0.001) and 4.37 fold (p=0.0058) compared to control cells (**Figure 2**). qPCR showed the levels of MR-AK134679, NR024118 and AX765700 were decreased to 7.59 fold (p=0.0057), 8.05 fold (p=0.004) and 6.36-fold (p=0.001) compared to control cells (**Figure 2**).

The levels of 8 mRNAs were also verified by qPCR. It was revealed that the levels of IL6, regulator of G-protein signaling 2 (RGS2) and proteoglycan 4 (PRG4) were increased to 46.93 fold (p=0.0057), 19.28 fold (p=0.001) and

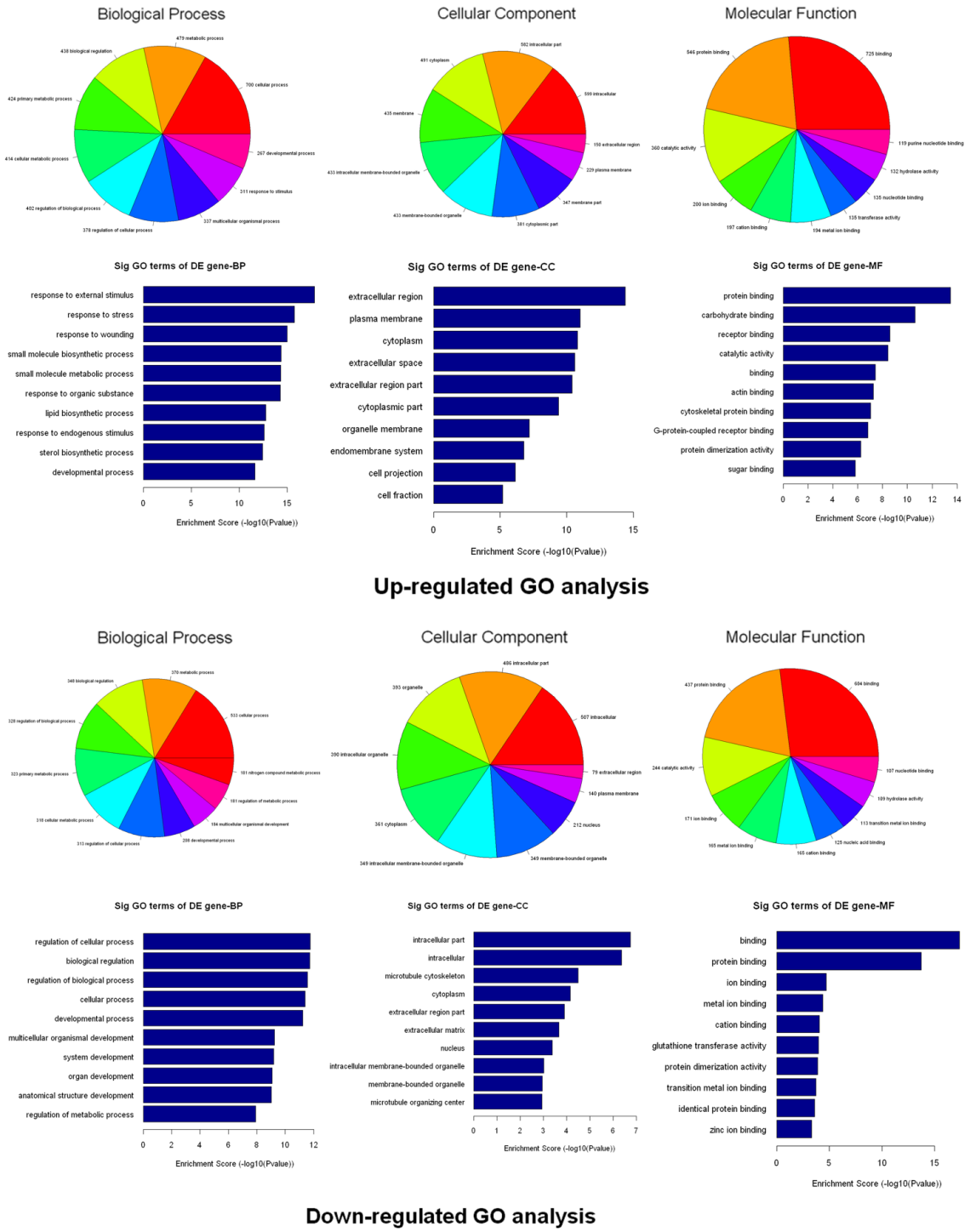

Figure 4. Bioinformatic analysis of the differentially expressed genes. The Gene Ontology (GO) analysis provides a controlled vocabulary to describe differentially expressed transcript attributes in all organisms. The ontology covers three domains: Biological Process, Cellular Component and Molecular Function. The *p*-value denotes the significance of GO terms enrichment in the DE genes. The lower the *p*-value, the more significant the GO Term (*p*-value ≤0.05 is recommended).

4.69-fold (p=0.0044) in cardiac fibroblasts treated by Ang II (**Figure 3**). qPCR showed that Ang II decreased the levels of cyclin-dependent kinase inhibitor 1C (Cdkn1c) and TIMP3 to 7.22

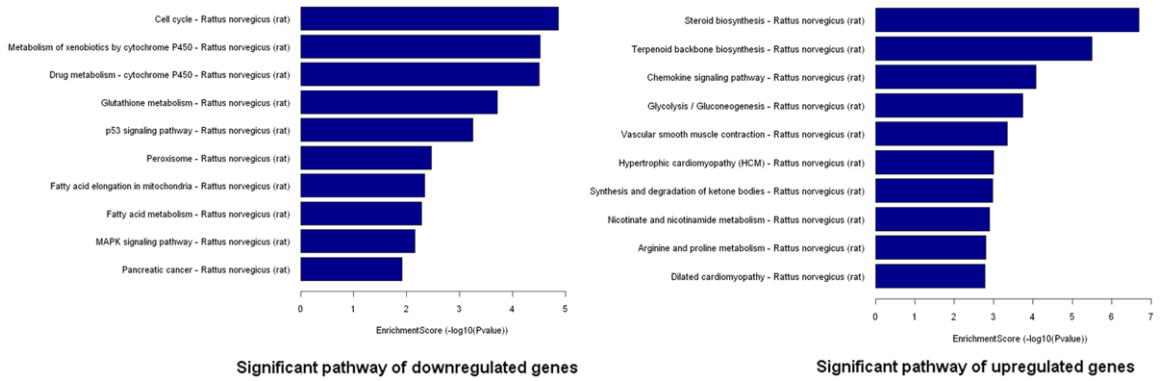

Figure 5. Pathway analysis of the differentially expressed genes. Pathway analysis is a functional analysis that maps genes to KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (http://www.genome.jp/kegg/). The *P*-value (Fisher-*P* value) denotes the significance of the Pathway correlated to the conditions. Lower the *P*-value, more significant is the Pathway (The recommend *P*-value cut-off is 0.05).

fold ($p<0.001$) and 8.16-fold ($p=0.001$) in cardiac fibroblasts compared to control (**Figure 3**). qPCR also revealed that Ang II up-regulated the level of TIMP1 to 2.41 fold ($p=0.0048$) (**Figure 3**). However, the changes of collagen I, collagen III and Fibronectin did not show statistical significance using qPCR (**Figure 3**).

Go analysis and pathway analysis

The number (Top ten) of genes associated with GO term and the significance of GO term (Top ten) were shown (**Figure 4**). The upregulated genes were involved in 843 biological process, 110 cellular components and 165 molecular functions. In the biological process category, the most significant term was the response to external stimulus (p=1.33509E-18). In the cellular component category, the most represented GO term was the extracellular region (p=4.20796E-15). Within the molecular component category, protein binding (p=3.38671E-14) as the most highly represented term. The downregulated genes were involved in 501 biological process, 40 cellular components and 85 molecular functions. In the biological process category, the regulation of cellular process was enriched most. Within the cellular component category, intracellular part was the most represented GO terms. Among the various molecular functions, binding were most highly represented term.

Following Go analysis, KEGG was used to do a pathway enrichment analysis. The downregulated genes were involved in 18 pathways while upregulated genes were involved in 30 pathways. The top 10 pathways of downregulated and upregulated genes were shown (**Figure 5**). The pathways of downregulated genes include cell cycle, P53 signaling pathway, MAPK signaling pathway, indicating the activation of cell proliferation. Among the ten pathways, the most significant pathway was the Cell cycle pathway (p=0.000013705).

The regulation of Ang II on the expression of NR024118 and Cdkn1c

In order to investigate how Ang II regulate the expression of lncRNA-NR024118 and Cdkn1c, we determined the levels of NR024118 and Cdkn1c in cardiac fibroblasts when treated by Ang II at different time and different concentration using quantitative real time PCR. We found that the level of NR024118 was gradually decreasing with the exposure time to Ang II in cardiac fibroblasts. The level of NR024118 reached to the lowest at 24 h but back at 48 h in cardiac fibroblasts (**Figure 6A**). Meanwhile, the level of Cdkn1c in cardiac fibroblasts was gradually decreasing as the extension of time of treatment by Ang II with the lowest level at 48 h (**Figure 6B**). All of treatment by Ang II at a range from 10^{-9} M to 10^{-6} M induced a decrease of both NR024118 and Cdkn1c in cardiac fibroblasts (**Figure 6C** and **6D**).

Discussion

The current literature on lncRNAs mainly focused on cancer and neural diseases [21-25]. Up to now, only a few lncRNAs were reported in cardiovascular system. In our study, the

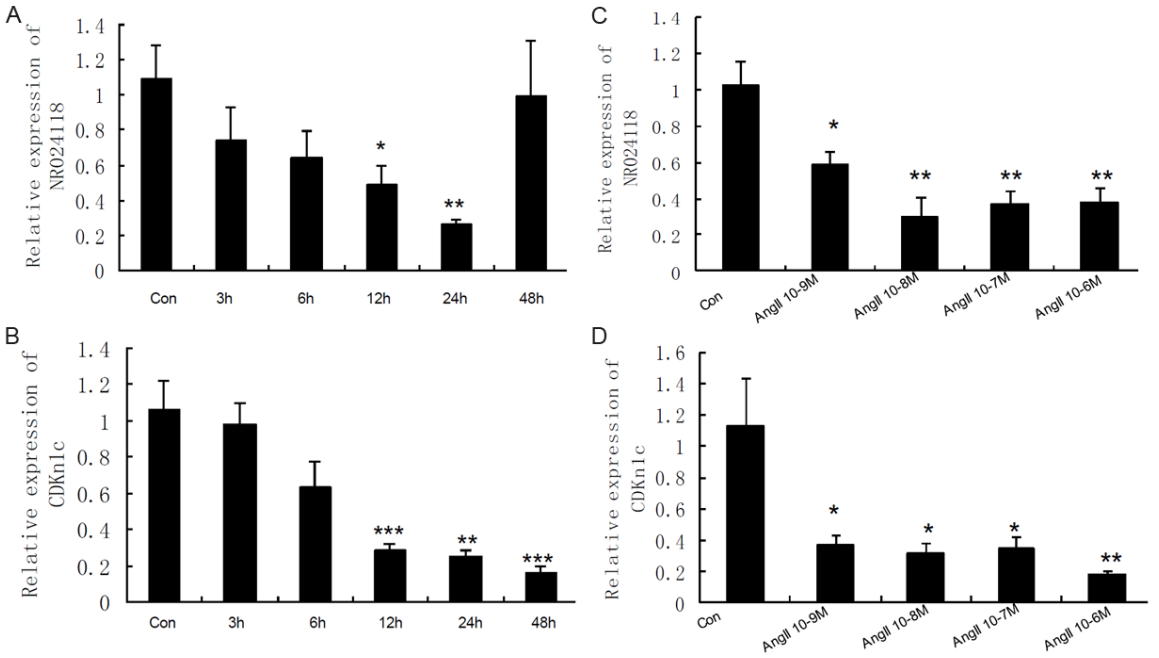

Figure 6. The regulation of angiotensin II on the expression of NR024118 and Cdkn1c in cardiac fibroblasts. The expression of NR024118 and Cdkn1c in cardiac fibroblasts treated by angiotensin II (Ang II 100 nm) at different time and at different concentration for 24 h was measured by qPCR. Expression of transcripts was normalized to actin expression. Data are the mean ± SEM (n=6). *P*<0.05 was considered to indicate a statistically significant difference compared with control fibroblasts. *p<0.05, **p<0.01 and ***p<0.001.

clear changes of lncRNAs (AF159100, BC086588, MRNR026574, MRAK134679, NR024118, AX765700) in adult cardiac fibroblasts in response to Ang II were associated with the changes of several protein-coding RNAs. We suspected that the decrease of Cdkn1c indicated that cell proliferation. The upregulated PRG4 induced by Ang II, an extracellular matrix molecule, indicated the enhancing of the synthesis of extracellular matrix. The changes of TIMP1 and TIMP3 showed that the degradation of ECM might be affected the activity of MMPs. The obvious increase of IL 6 indicated the inflammation mechanism of cytokines in the developing of cardiac fibrosis. It was reported that production of IL6 in cardiac fibroblasts will lead to TGF-β1 production and stimulates cardiac fibrosis that induced by Ang II [26]. RGS2 has been showed to be a regulator of Ang II-effects in cardiac fibroblast that might have a role in Ang II-induced fibrosis [8].

Go analysis indicated cell response to external stimulus and cell division. Pathways analysis showed several significant pathways related to cell proliferation including cell cycle, P53 signaling pathway and MAPK signaling pathway. Further studies revealed that Ang II at a range from 10^{-9} M to 10^{-6} M decreased the expression of NR024118 and Cdkn1c in cardiac fibroblasts. Moreover, the regulation of Ang II on the expression of NR024118 and Cdkn1c was in a time-dependent pattern. Moreover, Cdkn1c (p57/KIP2) was common down-regulated in different human cancers, indicating the role of Cdkn1c in cell proliferation [32]. Ang II dynamically down regulated the level of NR024118 and Cdkn1c in cardiac fibroblasts, strongly suggesting the potential role of NR024118 in cardiac fibroblasts.

Bioinformatic analysis reveal that LncRNA-NR024118 (793 bp), located in chromosome 20, is defined as Rattus norvegicus tenascin XA, pseudogene 1 (Tnxa-ps1), non-coding RNA. The protein-coding RNA of tenascin X has been reported to facilitate myocardial fibrosis through transforming growth factor-β1 and peroxisome proliferator-activated receptor γ [27]. Pseudogenes have long been neglected because of being considering nonfunctional. However, recent advances have established that the RNA transcribed from a pseudogene can have diverse functions not only to their

parental genes but also to unrelated genes [28]. Pseudogenes are considered not to be pseudo any more now [29]. The pseudogene (PTENP1) of the tumor suppressor PTEN has been reported to regulate the expression of PTEN in mRNA and protein levels [30]. Recently, it was reported that a pseudogene lncRNAs network (PTENpg1 α and β) regulates PTEN transcription and translation in human cells [31]. We anticipate that the next functional studies of Tnxa-ps1 will reveal the roles of Tnxa-ps1 in cardiac fibroblasts.

In conclusion, our current studies showed that the expression profile of lncRNAs was significantly altered in the Ang II-induced cardiac fibroblasts and Ang II dynamically regulated the expression of lncRNA-NR024118 and Cdkn1c in adult cardiac fibroblasts, strongly indicating the potential role of NR024118 in adult cardiac fibroblasts.

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Disclosure of conflict of interest

None.

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