Transcribed ultraconserved region uc.242 is a novel regulator of cardiomyocyte hypertrophy induced by angiotensin II

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ABSTRACT

Cardiomyocyte hypertrophy is a response to stress or hormone stimulation and is characterized by an increase of cardiomyocyte size. Abnormal long non-coding RNA (lncRNA) expression profile has been identified in various cardiovascular diseases. Though some lncRNAs had been reported to participate in regulation of cardiac hypertrophy, the universal lncRNA profile of cardiomyocyte hypertrophy had not been established. In the present study, we aimed to identify the differentially expressed lncRNA-mRNA network in angiotensin II-stimulated cardiomyocytes, and screen the potential lncRNAs involved in regulation of cardiomyocyte hypertrophy. The hypertrophic cardiomyocytes were induced by angiotensin II (0.1 µmol/L) for 48 hours. High-throughput microarray analysis combined with quantitative real-time PCR assay were then performed to screen the differentially expressed lncRNAs and mRNAs. A total of 1577 lncRNAs and 496 mRNAs transcripts were identified differentially expressed in hypertrophic cardiomyocytes. Among them, 59 transcribed ultraconserved non-coding RNAs (T-UCRs) were found by evolutionary conservation analysis. Subsequently, the lncRNA-mRNA coexpression network was constructed based on Pearson's correlation analysis results, including 4 T-UCRs and 215 mRNAs. The results revealed that uc.242 was positively interacted with prohypertrophic genes (Hgf and Tnc). Functional study showed that inhibition of uc.242 dramatically decreased hypertrophic marker expression levels and cardiomyocyte surface area under the condition of angiotensin II stimulation. The expression of Hgf and Tnc was also decreased in cardiomyocytes after silencing of uc.242. Summarily, the present study provided crucial clues to explore therapeutic targets for pathological cardiac hypertrophy.

Significance of this study

What is already known about this subject?

► Cardiac hypertrophy is an adaptive reaction of the heart in response to various biomechanical and physiopathological stimuli, such as neurohumoral activation and pressure overload. It is characterized by increased cardiomyocyte size, heightened organization of sarcomere, and enhanced protein synthesis. The expression profile of abnormal long non-coding RNAs (lncRNAs) has been identified in various cardiovascular diseases.

What are the new findings?

► In the present study, first the hypertrophic cardiomyocytes were induced by angiotensin II (0.1µmol/L) for 48 hours. High-throughput microarray analysis combined with quantitative real-time PCR assay were then performed to screen the dysregulated lncRNAs and mRNAs. Subsequently, the lncRNA-mRNA coexpression network was constructed based on Pearson's correlation analysis results, including 4 transcribed ultraconserved non-coding RNAs and 215 mRNAs. Functional study showed that inhibition of uc.242 dramatically alleviated cardiomyocyte hypertrophy induced by angiotensin II. The results suggested that uc.242 might act as a novel regulator in cardiomyocyte hypertrophy.

How might these results change the focus of research or clinical practice?

► The results could help us better understand cardiomyocyte hypertrophy and provide crucial clues to find therapeutic targets for cardiovascular diseases.

INTRODUCTION

Cardiac hypertrophy is the enlargement of the heart, characterized by an increase in the size of cardiomyocytes and disarrangement of the sarcomeric structure. The heart responds effectively to hemodynamic and neurohumoral factors, which are associated with the release of hormones, chemokines, and peptide growth factors.[1 2] However, prolonged hypertrophy contributes to cardiac dysfunction, and ultimately leads to heart failure or sudden death.[3] Although advances have been made in identification of genes and signaling pathways involved in cardiac hypertrophy, the regulatory mechanisms of cardiac hypertrophy are still unknown.

Long non-coding RNAs (lncRNAs), whose transcripts are longer than 200 nucleotides without protein-coding capacity, regulate the epigenetic process associated with chromatin remodeling, mRNA processing, and elongation.[4 5] The abnormal expression of lncRNAs occurs in a large number of diseases, such as cancers, diabetes and cardiovascular diseases.[6–8] The role of lncRNAs has been demonstrated in several heart diseases.[9 10] Previous studies have identified the expression profile of lncRNAs in mouse and rat models of cardiac hypertrophy.[11 12] However, the overall profiles of differentially expressed lncRNAs in cardiomyocyte hypertrophy had not been investigated.

In the present study, we aimed to detect the expression profiles of lncRNAs and mRNAs in cardiomyocytes with or without angiotensin II stimulation by high-throughput microarray and quantitative real-time PCR (qRT-PCR) assays, and screen out the hypertrophy-related lncRNAs through coexpression analysis. The results could help us better understand cardiomyocyte hypertrophy and provide crucial clues to find therapeutic targets for pathological cardiac hypertrophy.

MATERIAL AND METHODS

Cardiomyocytes culture and treatment

Primary cardiomyocytes were isolated from male Sprague-Dawley rats, that were 1–2 days old, as previously described.[13] Cardiomyocyte hypertrophy was induced by angiotensin II (0.1 μmol/L, Sigma, USA) for 48 hours. The cell surface area was evaluated by Image J software based on the result of α-actinin immunofluorescence.

Total RNA extraction

Total RNA was extracted from cardiomyocytes by mirVana RNA Isolation Kit (Thermo Scientific, USA) according to the manufacturer's instructions. RNA integrity was assessed by Agilent Bioanalyzer 2100 (Agilent Technologies, USA). RNA quality was identified according to absorbance and agarose gel electrophoresis.

Quantitative real-time PCR

SYBR Green-based qRT-PCR assay was performed for detection of lncRNA and mRNA expression as described previously.[14] Three technical replicates were set for each sample in the qRT-PCR assay. The $2^{-\triangle\triangle CT}$ method was applied to analyze the relative expression levels of lncRNAs and mRNAs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Microarray data analysis

Microarray assay (OE Biotech, China) was used to screen the differentially expressed lncRNAs and mRNAs. The raw microarray data scanned by Agilent Scanner G2505C (Agilent Technologies, USA) were normalized with the quantile algorithm and analyzed by Transcriptome Analysis Console and Genespring software. Normalized data were analyzed by t-test with Bonferroni's correction, and differentially expressed lncRNAs and mRNAs were identified by the threshold values of ≥twofold change and a t-test p value of ≤0.05.

Gene ontology and pathway analysis

Gene ontology (GO) analysis and pathway analysis were performed using FunRich software. Enrichment of biological processes, cellular components and molecular functions was achieved by GO function analysis, while pathway enrichment was achieved based on the Kyoto Encyclopedia of Genes and Genomes database. A value of $p<0.05$ was used as the reference value.

LncRNA-mRNA coexpression analysis

Coexpression analysis was performed on differentially expressed transcribed ultraconserved non-coding RNAs (T-UCRs) and all the dysregulated mRNAs. Pearson's correlation coefficients between lncRNA and differential expression of mRNA were compared and the degree was calculated by CentiScaPe (V.2.2). The coexpression network was constructed by Cytoscape (V.3.6.1) based on Pearson's correlation coefficients with the reference of correlation >0.80 and $p<0.01$.

Small interfering RNA transfection

Small interfering RNAs (siRNAs) targeting uc.242 (si-uc.242) and negative control (si-NC) were synthesized by Ribobio Technology (China). Cardiomyocytes were transfected with si-uc.242 or si-NC at a final concentration of 50 nmol/L according to the manufacturer's protocol of riboFECT CP Transfection (Ribobio, China).

Western blot

Western blot assay was performed as described previously.[14] Equal amounts of protein samples (20 μg) were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to the polyvinylidene-fluoride membrane. After blocking with 5% non-fat milk for 1 hour, the membrane was incubated with diluted primary antibodies (Hgf, 1:1000 dilution. Tnc, 1:1000 dilution. GAPDH, 1:3000 dilution. Boster, China) at 4°C overnight followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive bands were visualized by ECL (enhanced chemiluminescence) Kit (Boster, China) according to the manufacturer's instructions. GAPDH was used as the loading control.

Statistical analysis

All statistical analyses were performed using SPSS V.22.0. Quantitative data were expressed as mean±SD, and analyzed by Student's t-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Differentially expressed lncRNAs and mRNAs in cardiomyocyte hypertrophy

After angiotensin II (0.1 μmol/L) stimulation for 48 hours, the cardiomyocyte surface area was significantly enlarged (figure 1A,B), and the expression of hypertrophic marker genes (Nppa and Nppb) was also significantly increased (figure 1C). The results showed that the hypertrophic cardiomyocytes could be successfully developed after 48h-angiotensin II stimulation. Then a microarray assay was applied to detect the expression profiles of lncRNAs and mRNAs in normal and hypertrophic cardiomyocytes. The

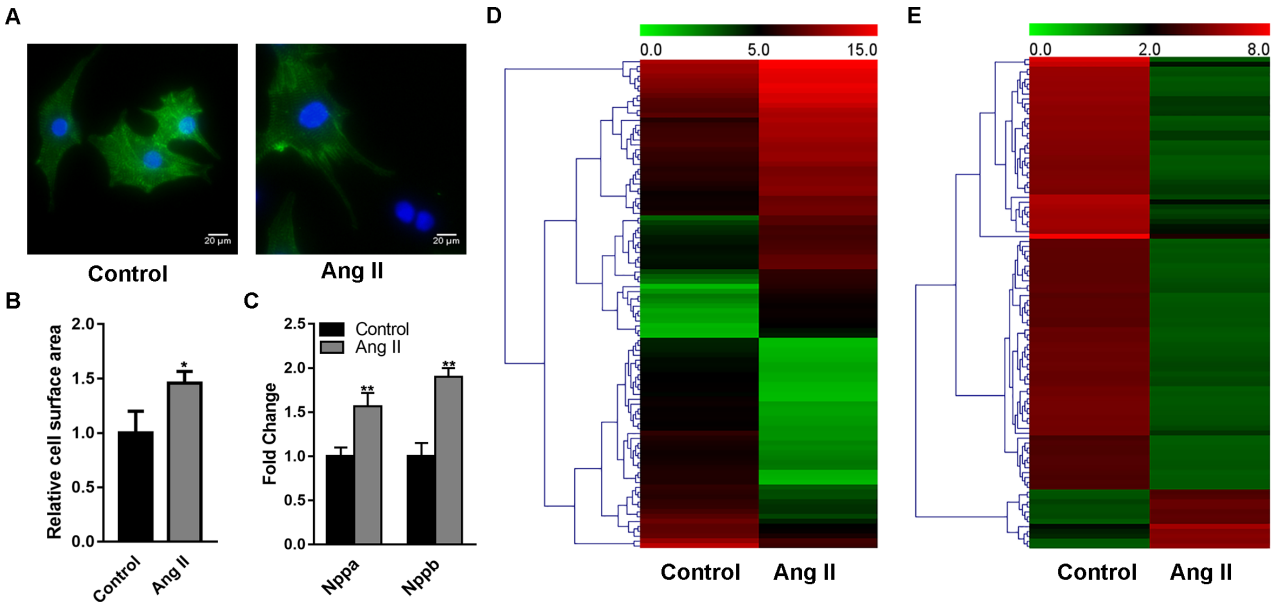

Figure 1 Differentially expressed lncRNAs and mRNAs in cardiomyocyte hypertrophy induced by angiotensin II. Primary neonatal rat cardiomyocytes were successfully isolated, and then treated with angiotensin II for 48 hours. (A) Representative images of α-actinin immunofluorescence staining in cardiomyocytes stimulated by angiotensin II. Magnification × 400. Scale bars=20 µm. (B) Quantitative analysis of cardiomyocyte surface area. (C) The relative expression of Nppa and Nppb in cardiomyocytes after angiotensin II stimulation. GAPDH was used as an internal control. Each sample was arranged with three replicates. **p<0.01 versus control group. (D,E) The top 100 dysregulated lncRNAs and mRNAs in control and hypertrophic cardiomyocytes, respectively. The heatmap was constructed at normalized intensity of lncRNAs and mRNAs. Red represented the highest value and green represented the lowest value. lncRNA, long noncoding RNA.

results showed that a total of 1577 lncRNAs was identified differentially expressed in hypertrophic cardiomyocytes, including 870 upregulated lncRNAs and 707 downregulated lncRNAs (figure 1D and online supplemental table S1). Meanwhile, a total of 496 dysregulated mRNA transcripts was also detected, with 129 being upregulated and 367 being downregulated (figure 1E and online supplemental table S2).

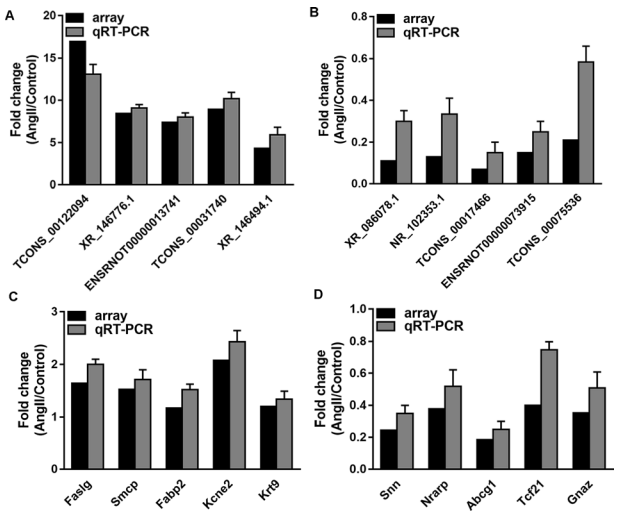

Figure 2 The differential expression of lncRNAs and mRNAs validated by qRT-PCR. (A,B) Comparison between microarray and qRT-PCR results of 10 upregulated and downregulated lncRNAs. (C,D) Validation of 10 differentially expressed genes between microarray and qRT-PCR. Each sample was a set of three replicates, and GAPDH was used as an internal control. lncRNA, long noncoding RNA; qRT-PCR, quantitative real-time PCR.

Confirmation of microarray results by qRT-PCR

To confirm the reliability of microarray results, 10 lncRNAs were randomly selected for verification. qRT-PCR analysis showed that the expression of TCONS_00122094, XR_146776.1, ENSRNOT00000013741, TCONS_00031740, and XR_146494.1 was significantly increased in cardiomyocytes after angiotensin II stimulation, while the expression of TCONS_00017466, XR_086078.1, NR_102353.1, ENSRNOT00000073915, and TCONS_00075536 was significantly decreased (figure 2A,B). These lncRNA expression changes were consistent with microarray results. Consistent results could also be found on expression changes of 10 randomly selected mRNAs, including five upregulated mRNAs (Kcne2, Smcp, Faslg, Fabp2, and Krt9), and five downregulated mRNAs (Abcg1, Snn, Tcf21, Nrarp, and Rbp4).

Classification and subgroup analysis of differentially expressed lncRNAs

Through chromosome location analysis, the differentially expressed lncRNAs were found to be located on each of the chromosomes, except chromosome Y. These dysregulated lncRNAs were mainly located on chromosomes 1, 2, and 3 (figure 3A). Classification of lncRNAs was helpful to predict their expression regulation mechanism. As shown in figure 3B, the profiling data indicated that most of the differentially expressed lncRNAs were intergenic. T-UCRs, a new class of lncRNAs, are 100% conserved among orthologous regions of human, mouse, and rat genomes.

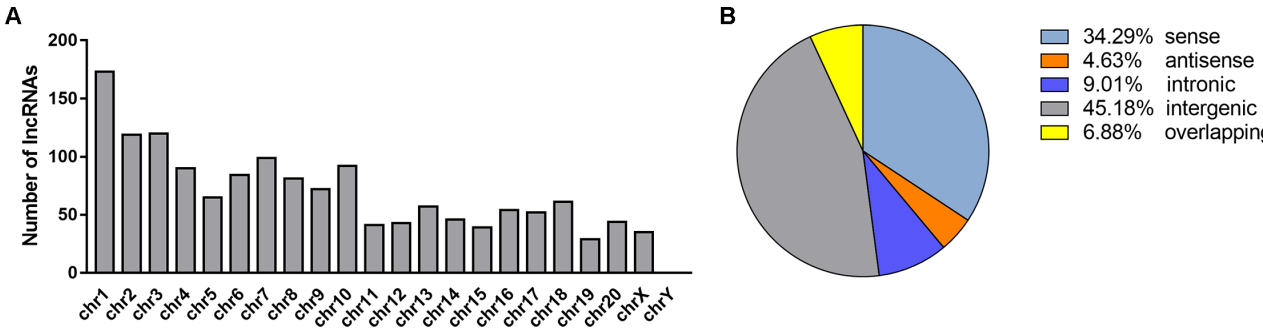

Figure 3 Classification and subgroup analysis of differentially expressed lncRNAs. (A) Chromosome location of dysregulated lncRNAs. The majority of differentially expressed lncRNAs were located on chromosomes 1, 2, and 3. (B) Distribution of dysregulated lncRNAs. Most of the differentially expressed lncRNAs were intergenic and sense. Dysregulated lncRNAs classified as intergenic and sense accounted for 45.18% and 34.29%, respectively. lncRNA, long noncoding RNA.

Of the 481 identified T-UCRs, 59 T-UCRs were found differentially expressed in angiotensin II-stimulated cardiomyocytes, including 27 upregulated T-UCRs and 32 downregulated T-UCRs (online supplemental table S1).

GO and pathway enrichment analysis of differentially expressed mRNA

GO and pathway enrichment analysis are important tools for functional analysis of the screened differentially expressed genes. GO enrichment analysis showed that these dysregulated genes were mainly enriched in extracellular and plasma membranes for cellular components (figure 4A), enriched in cell adhesion molecule activity and receptor activity for molecular function (figure 4B), and enriched in cell communication and signal transduction for biological process (figure 4C). Biological pathway analysis showed these dysregulated genes were enriched in epithelial-to-mesenchymal transition and regulation of insulin secretion (figure 4D).

Construction of the lncRNA-mRNA coexpression network

LncRNA and mRNA coexpression networks could uncover the possible modulating mechanism of lncRNAs in cardiomyocyte hypertrophy. Using results of Pearson's correlation analysis, a coexpression network with 4 dysregulated T-UCRs and 215 mRNAs was constructed, including 136 positive interactions and 188 negative interactions (figure 5A). Among them, uc.242 positively interacted with both Hgf and Tnc, which had been identified as prohypertrophic genes.[15 16] It could be found that uc.88 positively interacted with Hgf, while uc.411 negatively interacted with Tnc. Then, qRT-PCR and western blot assays confirmed that expression of Hgf and Tnc was significantly increased in cardiomyocytes after angiotensin II treatment (figure 5B,C). Meanwhile, increased uc.242 and uc.88 levels as well as decreased uc.411 level were also detected in angiotensin II-treated cardiomyocytes (figure 5D).

Inhibition of uc.242 attenuated cardiomyocyte hypertrophy

A synthetic siRNA (si-uc.242) was applied to explore the role of uc.242 on cardiomyocyte hypertrophy. After transfection in cardiomyocytes, the expression of uc.242 was significantly reduced (figure 6A). The surface area of cardiomyocytes was dramatically decreased after silencing of uc.242 (figure 6B,C). Meanwhile, qRT-PCR assay found the expression of hypertrophic markers (Nppa and Nppb) significantly reduced in cardiomyocytes after si-uc.242 transfection under angiotensin II stimulation condition (figure 6D). The mRNA and protein expression levels of Hgf and Tnc were found to be decreased after inhibition of uc.242 (figure 6E,F).

DISCUSSION

High-throughput screening analysis has been widely applied to establish the universal profiles of lncRNAs in diverse diseases, which could provide valuable data for exploration of disease pathogenesis and novel drug therapy options. In the current study, we identified the differentially expressed lncRNAs involved in cardiomyocyte hypertrophy, and the functional study revealed that silencing of uc.242 could alleviate cardiomyocyte hypertrophy induced by angiotensin II. The results suggested that uc.242 might act as a novel regulator in cardiomyocyte hypertrophy.

Increasing evidence had shown that abnormal expression of lncRNAs was found in heart development and diseases.[17 18] Zhu *et al* had identified 31 423 lncRNAs in the normal development of fetal mouse heart, and revealed that lncRNA AK011347 might regulate heart development by mediating Map3k7.[19] Besides, there was a total of 313 dysregulated lncRNAs detected in the heart from patients with dilated cardiomyopathy. The top upregulated dilated cardiomyopathy related IncRNA was involved in regulation of dilated cardiomyopathy.[20] The present work has established the lncRNA profile in angiotensin II-stimulated hypertrophic cardiomyocytes. These differentially expressed lncRNAs might also play an important role in regulating cardiomyocyte hypertrophy.

Cardiac hypertrophy is a complex pathological process, accompanied by alterations in heart metabolism.[21] Bioinformatics analysis reveals that the differentially expressed mRNAs could be involved in the regulation of metabolism and energy pathways in biological processes. It has been reported that pathological hypertrophy is interrelated with changes in energy metabolism and contributes to damaged energy supplements in a failing heart.[22] However, it is still not clear whether these metabolic alternations lead to or result from the development of cardiac hypertrophy.

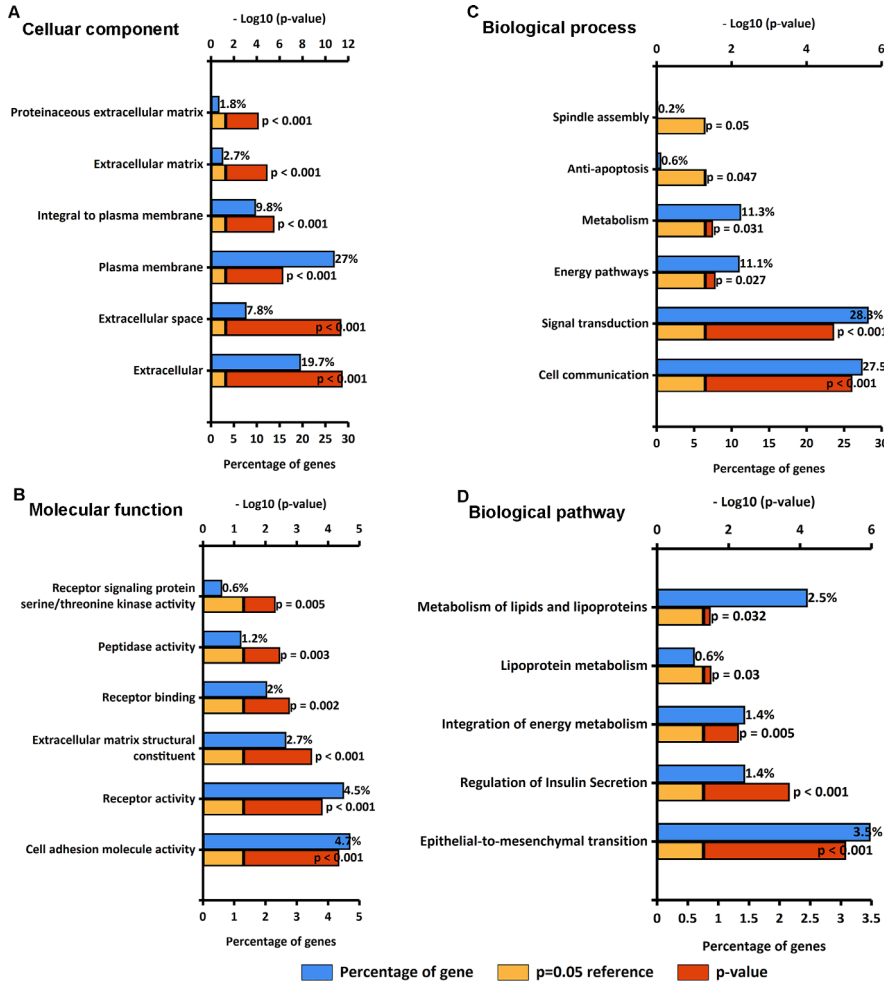

Figure 4 GO and pathway functional enrichment analysis. GO functional analysis and pathway analysis were performed by using FunRich analysis software. (A–C) Top 6 GO enrichment analyses of dysregulated genes in cellular components, molecular function and biological process. (D) Top six enriched pathways of differentially expressed genes. p<0.05 was employed as a reference value. GO, gene ontology.

A recent study has proved that lncRNA is involved in the regulation of cardiac hypertrophy.[23] Nuclear-enriched abundant transcript 1 (NEAT1) was first identified as a nuclear-enriched abundant transcript in human fibroblast cells and lymphoblastoids,[24] and was then found to regulate the assembly and function of paraspeckles during human embryonic stem cell differentiation.[25] Sun *et al* found that the highly expressed NEAT1 could promote cardiomyocyte hypertrophy by binding with miR-19a to upregulate SMYD2 expression.[23] However, the differential expression of NEAT1 was not screened in our study. Moreover, chromosome Y-derived lncRNAs were also not detected by microarray. The probes for lncRNA detection were designed based on the Noncode, RefSeq, Ensembl, and Unigene databases. However, the lncRNA databases of rats were not as sufficiently complete as those of humans, which led to a lack of corresponding probes in the current microarray. Therefore, the present results could not indicate the disproportional regulation of lncRNAs between chromosomes. Other improved technologies, like transcriptome sequencing, would provide more data and support for identification and analysis of lncRNA in future.

Coexpression network analysis had been largely used to exploit the functions of non-coding RNAs on the basis of genes with similar expression patterns.[26] The lncRNA-mRNA coexpression network is a critical method for bioinformatics prediction of lncRNAs functions. Coexpression network analysis results show that three T-UCRs (uc.242, uc.411, and uc.88) interact with protein-coding genes that regulate cardiac hypertrophy. Our data reveal that these T-UCRs might be crucial for further exploration of the cardiomyocyte hypertrophy mechanism.

It is generally considered that most of the lncRNAs in various species are highly tissue-specific and are poorly conserved in sequence among mouse, rat and human genomes. T-UCRs, a novel category of lncRNAs, are absolutely conserved between the orthologous regions of human, rat, and mouse genomes.[27 28] Recent reports have shown that T-UCRs might serve as markers for diagnosis and prognosis of cancers. The alterations of uc.73 and uc.388 expression levels were found to be correlated with the development of colorectal cancer.[29] Besides, uc.4 was found to be involved in the development of the heart of zebrafish and regulate cell differentiation by inhibiting the

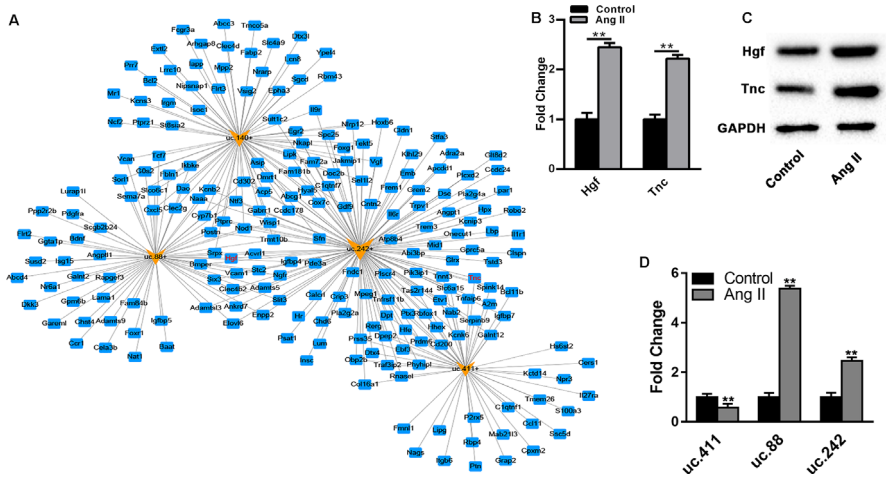

Figure 5 Construction of coexpression network between lncRNAs and mRNAs. (A) Coexpression network of T-UCRs and mRNAs. Yellow represents dysregulated lncRNAs and blue represents dysregulated mRNAs. (B,C) mRNA and protein expression changes the detection of Hgf and Tnc in cardiomyocytes with or without angiotensin II treatment. QRT-PCR and western blot assays confirmed that the expression of Hgf and Tnc were significantly increased after angiotensin II treatment for 48 hours. (D) Expression changes detection for uc.411, uc.88, and uc.242. Expression of uc.411 was significantly decreased after angiotensin II treatment, whereas expression of uc.88 and uc.242 were significantly increased. GAPDH was employed as an internal control. Three replicates were set for each sample. **p<0.01 versus control group. T-UCR, lncRNA, long non-coding RNA; T-UCR, transcribed ultraconserved non-coding RNA.

TGF-β signaling pathway.[30] In our present research, inhibition of uc.242 attenuated cardiomyocyte hypertrophy induced by angiotensin II. The intensive research on dysregulated T-UCRs, which have a high conservative sequence, could broaden our view of cardiomyocyte hypertrophy.

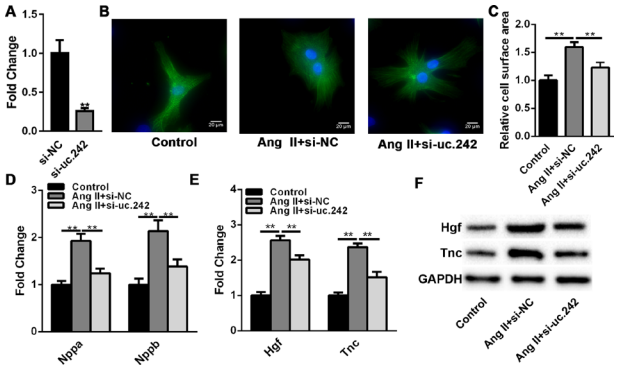

Figure 6 Role of uc.242 in cardiomyocyte hypertrophy stimulated by angiotensin II. (A) Relative expression of uc.242 in cardiomyocytes treated with angiotensin II followed by siRNA transfection. GAPDH was set as an internal control. Three replicates were set for each sample. **p<0.01 vs si-NC group. (B) Representative images of α-actinin immunofluorescence staining in cardiomyocytes stimulated by angiotensin II with or without si-uc.242 transfection. Magnification × 400. Scale bars=20 µm. (C) Quantitative analysis of cardiomyocyte surface area. **p<0.01. (D) Expression change of Nppa and Nppb in cardiomyocytes treated with angiotensin II followed by siRNA transfection. **p<0.01. (E,F) Relative expression of Hgf and Tnc mRNA and protein levels in cardiomyocytes stimulated by angiotensin II with or without si-uc.242 transfection. Three replicates were set for each sample. **p<0.01. siRNA, small interfering RNA. si-uc.242, siRNAs targeting uc.242; si-NC, siRNAs targeting negative control.

It is reported that lncRNAs could regulate downstream target genes by acting as microRNA sponges through a competitive endogenous RNA mechanism.[31 32] In the present work, uc.242 positively interacted with both Hgf and Tnc. Inhibition of uc.242 in cardiomyocytes reduced expression levels of Hgf and Tnc. The previous study had revealed that Hgf alone could strongly induce cardiomyocyte hypertrophy, and combination of Hgf and angiotensin II had an enhanced effect on cardiomyocyte hypertrophy.[15] Similarly, Tnc-deficient mice displayed damaged heart function when subjected to pressure overload and aggravated cardiac fibrosis by promoting inflammatory reactions.[16] Though the direct interactions between uc.242 and the two prohypertrophic genes were not disclosed, there could be a probable hypothesis that uc.242 might mediate Hgf and Tnc by interacting with microRNAs in cardiomyocyte hypertrophy.

An important limitation of the present study was that we used neonatal rat cardiomyocytes as hypertrophic cell models. The small rodent cells could not recapitulate the distinct expression patterns of humans. Although neonatal rat cardiomyocytes were commonly used in cardiac hypertrophy and signal transduction experiments, it would be more meaningful to explore the differential lncRNA expression profile in human cardiomyocytes. It would also be more direct and better for the development of drugs targeting lncRNAs, because most of lncRNA sequences (except T-UCR) were not found to be conserved among species.

In conclusion, our study identified the expression profile of lncRNAs and mRNAs in cardiomyocyte hypertrophy. Although the regulatory mechanism of these dysregulated lncRNAs was unclear, the current research would enrich our knowledge about cardiomyocyte hypertrophy, and provide new ideas for further study and treatment options of cardiovascular diseases. The profile-based diagnostic strategy of

lncRNAs and lncRNA-targeted drug therapy would be good application prospects in future.

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Contributors GW and LM designed the experiments. YG, BZ and YY performed the experiments. YG, FY and YX analyzed the data. YY and WC performed the statistical analysis. GW and YG wrote the manuscript. WW and GW revised the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval All animal procedures in the present study were approved by the Animal Ethics Committee of Naval Medical University. All experiments were performed in strict compliance with requirements of the Animal Ethics Procedures and Guidelines of China.

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request.

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