Transcriptome analysis reveals key pathways that vary in patients with paroxysmal and persistent atrial fibrillation

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Abstract. The present study evaluated mRNA and long non-coding RNA (lncRNA) expression profiles and the pathways involved in paroxysmal atrial fibrillation (ParoAF) and persistent atrial fibrillation (PersAF). Nine left atrial appendage (LAA) tissues collected from the hearts of patients with AF (patients with ParoAF=3; and patients with PersAF=3) and healthy donors (n=3) were analyzed by RNA sequencing. Differentially expressed (DE) mRNAs and lncRNAs were identified by \(|\text{Log}_2 \text{fold change}|>2 \text{ and } P<0.05\). Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes pathway enrichment, protein-protein interaction network and mRNA-lncRNA interaction network analyses of DE mRNA and mRNA at the upstream/downstream of DE lncRNA were conducted. A total of 285 and 275 DE mRNAs, 575 and 583 DE lncRNAs were detected in ParoAF and PersAF samples compared with controls, respectively. PI3K/Akt and transforming growth factor-β signaling pathways were significantly enriched in the ParoAF_Control and the calcium signaling pathway was significantly enriched in the PersAF_Control. Cis and trans analyses revealed some important interactions in DE mRNAs and lncRNA, including an interaction of GPC-AS2 with dopachrome tautomerase, and phosphodiesterase 4D and cAMP-specific with XLOC_110310 and XLOC_137634. Overall, the present study provides a molecular basis for future clinical studies on ParoAF and PersAF.

Introduction

Atrial fibrillation (AF) is the most common type of arrhythmia and afflicts numerous individuals worldwide. AF increases the probability of thromboembolism, ischemic stroke, congestive heart failure, psychological distress and death (1). AF impairs patient quality of life (2-4), especially those who require open-heart surgery (5). AF occurs alone or concomitantly with other cardiovascular diseases including valvular heart, coronary artery disease, hypertension and congestive heart failure (6).

AF usually begins in a self-terminating paroxysmal form (ParoAF, defined by episodes lasting <7 days). Over time, the AF pattern often evolves to become persistent (PersAF, duration of episodes >7 days) and non-terminating within 7 days (7). Significant differences exist between ParoAF and PersAF in terms of clinical features, responsiveness to antiarrhythmic drugs and ablation therapy (8). However, the underlying molecular mechanism of the occurrence and development of AF is poorly understood, as well as factors regulating the progression from ParoAF to PersAF.

Gene expression changes are associated with the progression from ParoAF to PersAF (9,10). Studies have primarily focused on the association between microRNAs (miRNAs/miRs) and AF. Several have revealed that miRNAs regulate AF or other cardiovascular diseases by promoting electrical or structural remodeling of the atrium (11-13). Dawson et al (11) reported that miR29 likely regulates atrial fibrotic remodeling and may represent a biomarker and/or therapeutic target. In recent years, long non-coding RNAs (lncRNAs), which are longer than 200 nt in length with a nonprotein-coding function, and mRNA expression have gained interest among researchers (14,15). lncRNA plays a central role in many processes during heart development and various heart diseases, including cardiac hypertrophy, cardiac fibrosis, AF and heart failure (16-21). lncRNA AK055347 was upregulated in AF and shown to regulate mitochondrial energy production in myocardiocytes (18). Several mRNAs were demonstrated to be contributed to ParoAF pathogenesis via the gonadotropin releasing hormone receptor and p53 pathways (22). However, these molecular markers were investigated either on patients with ParoAF or PersAF. Few studies have been performed on the differences in the molecular mechanism of patients with ParoAF and PersAF.

In the present study, RNA sequencing (RNA-Seq) technology was used to identify mRNAs and IncRNAs associated with ParoAF and PersAF and to explore the underlying disease mechanisms. Differentially expressed mRNAs (DE mRNAs) and IncRNAs (DE IncRNAs) in ParoAF and
Peroxisome proliferator-activated receptor γ coactivator 2, alpha (PGC-2 alpha) and phosphodiesterase 4D, cAMP-specific (PDE4D) were identified and compared with controls. The putative function of the DE mRNAs and lncRNAs were determined by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses. A co-expression network for lncRNA-mRNA was subsequently constructed. The results provide valuable molecular markers associated with the occurrence and development of ParoAF and PersAF.

**Materials and methods**

**Patients and tissue collection.** This study was permitted by the Human Ethics Committee of the First Affiliated Hospital of Nanjing Medical University (approval number: 2020-SRFA-340). All patients with AF and healthy donors provided written informed consent for the use of their tissue in this study. The informed consent of the donors was signed by one of their legal relatives.

Patients who underwent heart transplantation in the Department of Cardiovascular Surgery at the First Affiliated Hospital of Nanjing Medical University (Nanjing, China) were recruited. Patients categorized as ParoAF and PersAF met the following criteria: i) Over 18 years old; ii) exhibited obvious symptoms of AF as confirmed by electrocardiogram and had a clear medical history; iii) minimally invasive radiofrequency ablation performed for the first time; and iv) one-lung ventilation implemented. The exclusion criteria were: i) Below 18 years old or over 70 years old; ii) unclear medical history data; iii) combined with other cardiac surgery or undergoing secondary surgery; and iv) with psychiatric illness. The donor criteria included: i) Age <50 years old, with some marginal donors <60 years old; ii) estimated cold ischemia time <8 h; iii) no long-term or repeated history of cardiopulmonary resuscitation; iv) serological examination without hepatitis B/C, acquired immunodeficiency syndrome; v) no existing bacteremia; and vi) no malignant tumors other than primary brain tumors. The exclusion criteria for donors were: i) Brain death due to cardiac arrest; ii) heart contusion; iii) intractable ventricular arrhythmia; iv) long-term or repeated cardiopulmonary resuscitation; v) past heart disease, particularly congenital heart malformations; vi) after actively optimizing the before and after load, the support of a super-large dose of inotropic drugs still needed; vii) echocardiography findings of severe heart wall motion abnormalities and reduced sustained left ventricular ejection fraction (after optimized afterload, positive muscle support and other treatments, remained 40% lower); viii) severe left ventricular hypertrophy, with ventricular septum >13 mm and accompanied by electrocardiogram manifestations of left ventricular hypertrophy.

Left atrial appendage (LAA) tissues collected from the hearts of patients with AF (patients with ParoAF=3 and patients with PersAF=3) and donors (n=3) were used for RNA-Seq analysis. All healthy donors exhibited a sinus rhythm and were in good condition. The LAA tissues were selected from patients with ParoAF and PersAF, who underwent maze surgery. The tissues were quickly cut into pieces, placed into liquid nitrogen for at least 4 h, and then stored at -80°C for further use.

**RNA extraction.** Total RNA was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions and quantified using a Qubit 3.0 (Invitrogen; Thermo Fisher Scientific, Inc.). RNA integrity (RIN) was evaluated with an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.). Samples with OD_{260/280} values >1.9 and RIN ≥7 were used for library construction.

**Whole transcriptome library preparation and sequencing.** Ribosomal RNA (rRNA) was removed from the total RNA samples using the Ribo-Minus kit (Thermo Fisher Scientific, Inc.). Next, each RNA sample was quantified and used for library preparation using the TruSeq RNA Library Preparation kit version 2 (Illumina, Inc.). All libraries were loaded into one lane on the Illumina HiSeq X ten (Illumina, Inc.) platform, followed by 2x150 bp pair-end sequencing.

**Bioinformatics analysis.** FastQC (v0.11.5; http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to process the raw sequencing data. The clean reads were mapped onto the *Homo sapiens* (assembly GRCh38. P12) sequence using TopHat (v2.0.12; https://ccb.jhu.edu/software/tophat/index.shtml), followed by assembly using Cufflinks (v2.2.1; http://cufflinks.cbcb.umd.edu). Transcripts with reads per kilobase of exon per million reads mapped (RPKM>0) were retained for further analyses. EdgeR (http://www.biocductor.org/packages/release/bioc/html/edgeR.html) was utilized to identify the DE lncRNAs and mRNAs by pairwise comparisons, using thresholds of |Log₂ fold change|>2 and P<0.05.

As reported, IncRNAs usually work through *cis-* and *trans*-elements (23). In the present study, the mRNAs detected by *cis* function for the DE IncRNAs were analyzed by GO (http://www.Geneontology.org/) and KEGG (http://www.genome.jp/kegg/) with a corrected P<0.05 considered as significantly enriched for terms and pathways, respectively. *Trans* function targeted mRNAs were predicted by correlations ≥0.9 or ≤-0.9.

**mRNA-lncRNA co-expression network.** The DE mRNAs upstream or downstream of the DE IncRNAs were identified and their associations were visualized using Cytoscape software (v3.6.1, https://cytoscape.org/). The DE mRNAs targeted by DE IncRNA were also calculated, and the co-expression network was displayed by Cytoscape software (v3.6.1).

**Protein-protein interaction (PPI) network.** The association between DE mRNAs was also revealed using a PPI network. The STRING database (https://string-db.org/cgi/input.pl) was used for an interaction correlation study and correlations of r>0.7 were displayed.

**Reverse transcription-quantitative PCR (RT-qPCR).** RT-qPCR was used to validate the sequencing analysis results for the DE IncRNAs and mRNAs. Expression levels of collagen, type I, alpha 1 (*COL1A1*); collagen, type I, alpha 2 (*COL2A1*); collagen, type VI, alpha 1 (*COL6A1*); dopachrome tautomerase (*DCT*); phosphodiesterase 4D, cAMP-specific (*PDE4D*); *RPI1-428C19.4*; *GPC-AS2*; and *XLOC_110310* were measured in ParoAF Control and PersAF Control samples and normalized by β-actin expression. RNA samples were prepared as aforementioned. The primers were designed

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Table I. Clinical characteristics of subjects used for the sequencing.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control, n=3</th>
<th>ParoAF, n=3</th>
<th>PersAF, n=3</th>
<th>P-value</th>
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<tr>
<td>Age, years</td>
<td>39.67±1.86</td>
<td>53.67±3.76</td>
<td>57.33±3.76</td>
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<tr>
<td>BMI, kg/m²</td>
<td>21.70±1.62</td>
<td>23.07±0.55</td>
<td>25.17±0.44</td>
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<td>LA, mm</td>
<td>35.67±1.20</td>
<td>36.33±3.48</td>
<td>44.33±3.38</td>
<td>0.086*</td>
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<td>LVDD, mm</td>
<td>44.33±2.73</td>
<td>46.67±1.20</td>
<td>45.33±1.33</td>
<td>0.042*</td>
</tr>
<tr>
<td>LVSD, mm</td>
<td>27.33±1.76</td>
<td>32.00±2.00</td>
<td>29.67±0.88</td>
<td>0.097*</td>
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<tr>
<td>Ventricular rate</td>
<td>56.00±4.16</td>
<td>91.33±8.41</td>
<td>73.67±8.09</td>
<td>0.083*</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>125.0±5.51</td>
<td>114.0±6.47</td>
<td>121.3±8.05</td>
<td>0.287*</td>
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<tr>
<td>Diastolic blood pressure</td>
<td>78.05±6.89</td>
<td>80.24±5.11</td>
<td>80.46±4.10</td>
<td>0.617*</td>
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<tr>
<td>CHA2DS2-VASc score</td>
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<td>3.280±0.85</td>
<td>2.962±0.88</td>
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<tr>
<td>HASBLED score</td>
<td>–</td>
<td>2.852±0.61</td>
<td>2.846±0.74</td>
<td>0.988*</td>
</tr>
<tr>
<td>EHRA</td>
<td>–</td>
<td>I(1)/II(2)</td>
<td>I(1)/II(2)</td>
<td>–</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. P≤0.05 was considered as statistically significant. *Difference was analyzed using non-parametric Kruskal-Wallis H test followed by Dunn's test. **Difference was analyzed using non-parametric Mann-Whitney U test followed by Dunn's test. CHA2DS2-VASc contains congestive heart failure/LV dysfunction, hypertension, age ≥75, diabetes mellitus, stroke/TIA/TE, vascular disease, age=65-74, and sex category. HAS-BLED contains parameters of hypertension, abnormal renal function, abnormal liver function, previous stroke, bleeding history or predisposition, history of labile international normalized ratio, age ≥65, concomitant aspirin or nonsteroidal anti-inflammatory drug therapy, and substantial alcohol intake. BMI, body mass index; LA, left atrium; LVDD, left ventricular diastolic dimension; LVSD, left ventricular systolic diameter; EHRA, European Heart Rhythm Association; PersAF, persistent atrial fibrillation.

according to the sequences provided by the RNA-Seq data and synthesized by Genewiz Inc. (https://www.genewiz.com.cn/). The primers were provided in Table S1. RT-PCR was performed using HiScript II One Step RT-PCR Kit (Vazyme Biotech Co., Ltd.) kit according to the manufacturer's instruction. The RT-qPCR reaction was performed using the AceQ qPCR SYBR Green Master Mix (Vazyme Biotech Co., Ltd.) with a 20 µl PCR reaction systems, including 0.5 µl of each primer (10 µM), 2 µl of cDNA, 10 µl of AceQ qPCR SYBR Green Master Mix and 7 µl of RNase free H₂O. The mixture was put on the ABI 7500 system real-time PCR instrument (Applied Biosystems, Thermo Fisher Scientific, Inc.), and the amplification conditions were as follows: Pre-denaturation at 95°C for 30 sec; denaturation at 95°C for 5 sec, annealing at 60°C for 30 sec (39 cycles); extension at 95°C for 10 sec. All the experiments were repeated in triplicate. The mRNA and IncRNA expression levels were calculated using the 2⁻ΔΔCq method (24).

Statistical analysis. All statistical analyses were performed using IBM SPSS Statistics v22.0 (IBM Corp.) or GraphPad Prism 8 (GraphPad Software, Inc.) software. Normal data are shown as means ± standard error of means. Comparison among three groups was done using the non-parametric Kruskal-Wallis H test followed by Dunn's test. Comparisons between two groups were performed using the non-parametric Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical characteristics. There were no significant differences in age, body mass index, left ventricular diastolic dimension (LVDD), left ventricular systolic diameter, ventricular rate, systolic blood pressure and diastolic blood pressure across control donors or patients with ParoAF and PersAF (P>0.05; Table I). The CHA2DS2-VASc score (that considers congestive heart failure/LV dysfunction, hypertension, age ≥75, diabetes mellitus, stroke/TIA/TE, vascular disease, age=65-74 and sex category), HASBLED score (that considers parameters of hypertension, abnormal renal function, abnormal liver function, previous stroke, bleeding history or predisposition, history of labile international normalized ratio, age ≥65, concomitant aspirin or nonsteroidal anti-inflammatory drug therapy and substantial alcohol intake) and European Hearth Rhythm Association showed no significant differences between the ParoAF and PersAF groups.

Differentially expressed IncRNAs and mRNAs. A total of 285 (116 upregulated and 169 downregulated) and 275 (110 upregulated and 165 downregulated) DE mRNAs were identified in patients with ParoAF and PersAF compared with the control group, respectively. A total of 575 (276 upregulated and 299 downregulated) and 583 (330 upregulated and 253 downregulated) DE IncRNAs were detected in the ParoAF_Control and PersAF_Control samples, respectively. The volcano map of DE mRNAs and IncRNAs in the ParoAF_Control and PersAF_Control samples are shown in Fig. 1A-D. In addition, the expression levels of DE mRNA and IncRNAs in the ParoAF_Control and PersAF_Control samples are provided in Tables SII and SIII, respectively, whereas the DE mRNAs and IncRNAs in the PersAF_Control samples are listed in Tables SIV and SV, respectively.
GO and KEGG pathway analysis of DE mRNAs. There were two GO terms for ‘extracellular matrix’ and ‘platelet-derived growth factor binding’ that were significantly enriched in the DE mRNA profile of the ParoAF_Control sample. These two GO terms were represented by COL1A2 (upregulated), COL6A1 (upregulated), COL3A1 (upregulated), COL2A1 (downregulated) and so forth. Moreover, COL1A2 (upregulated), COL6A1 (upregulated), and COL3A1 (upregulated) were also significantly enriched in the GO terms ‘collagen type I’ and ‘platelet-derived growth factor binding’ in the PersAF_Control samples (Table II).

The top five enriched pathways were ‘ECM-receptor interaction,’ ‘protein digestion and absorption,’ ‘PI3K-Akt signaling pathway Focal adhesion Amoebiasis,’ ‘TGF-beta signaling pathway,’ and ‘Amoebiasis’ in the ParoAF_Control samples (Fig. 2A). Upreregulated DE mRNAs, such as COL1A1, COL1A2 and laminin β2 (LAMB2), were associated with most of the top ten pathways (Fig. 2C). Downregulated COL2A1 was associated with most of the top ten pathways. Decorin (DCN), SMAD specific E3 ubiquitin protein ligase 2 (SMURF2), and interferon, γ (IFNG) are known to participate in the TGF-β signaling pathway.

The top five enriched pathways in the PersAF_Control were ‘insulin resistance,’ ‘protein digestion and absorption,’ ‘type I diabetes mellitus,’ ‘herpes simplex infection,’ and ‘antigen processing and presentation dorso-ventral axis formation.’ They were enriched in IFNG (upregulated), major histocompatibility complex class I C (HLA-C; upregulated), and interferon regulatory factor 9 (IRF9; downregulated) (Fig. 2B and D).

Function analysis of DE lncRNAs through cis. The upstream mRNAs of the DE lncRNAs were enriched in ‘phosphoric diester hydrolase activity,’ ‘regulation of nucleotide metabolic process,’ and ‘cytoskeleton.’ The GO terms ‘sequence-specific DNA binding RNA polymerase II transcription factor activity,’ ‘response to dietary excess,’ and ‘positive regulation of gene expression’ were enriched among the mRNAs downstream of the DE lncRNAs. The top ten GO terms of the upstream and downstream mRNAs of the DE lncRNAs are listed in Table III.

In addition, 23 and 47 KEGG pathways were enriched in upstream and downstream mRNAs respectively. The upstream mRNAs were mainly enriched in the ‘Ras signaling pathway,’ ‘MAPK signaling pathway,’ and ‘PPAR signaling pathway’ (Fig. 3A), whereas the downstream mRNAs were involved in ‘Arrhythmogenic right ventricular cardiomyopathy,’ ‘Signaling pathways regulating pluripotency of stem cells,’ and ‘Glycine, serine and threonine metabolism’ (Fig. 3B). Mitogen-activated protein kinase 10 (MAPK10) and phospholipase A2, group IVA (PLA2G4A) were involved in more pathways compared with the others for upstream DE mRNAs (Fig. 3C). For downstream mRNAs, calcium channel, voltage-dependent, L-type, alpha 1C subunit (CACNA1C), ATPase, Ca++ transporting, cardiac muscle, slow twitch 2 (ATP2A2), catenin (cadherin-associated protein), beta 1, 88 kDa (CTNNB1),
phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic
subunit alpha (PIK3CA), and protein kinase, cAMP-dependent,
catalytic, γ (PRKACG) involved in no less than four pathways
(Fig. 3D).

**mRNA-lncRNA co-expression network.** The DE mRNAs
nearby the DE lncRNAs were identified and are show in
Fig. 4A and B. The association between DE mRNAs and
lncRNAs in the ParoAF_Control were as follows: DCT
was downstream of GPC5-AS2, methyltransferase like 15
(METTL15) at the downstream of BDNF-AS (Fig. 4A). The
association between DE mRNAs and DE lncRNAs in PersAF,
including DCT at the downstream of GPC5-AS2, chromosome 21 open reading frame 90 (C21orf90) at the
downstream of TSPEAR-AS1, and Mdm1 nuclear protein
chimera class II DQ alpha 1 (HLA-DQA1), cell division
cycle 25A (CDC25A), β-2-microglobulin (B2M), COL6A1,
COL3A1, SPARC, COL1A1, and COL1A2, and downregulated
DE mRNAs, including cyclin-dependent kinase 2 (CDK2),
kelch repeat and BTB (POZ) domain containing 13 (KBTBD13),
histone deacetylase 1 (HDAC1), split hand/foot malformation
(ectrodactyly) type 1 (SHFM1), F-box and leucine-rich repeat
protein 19 (FBXL19), and ring finger protein 19A, RBR E3
ubiquitin protein ligase (RNF19A) are essential genes that
may interact with many other DE mRNAs in the ParoAF_Control network (Fig. 5A).

In PersAF_Control, upregulated genes for protein tyrosine
phosphatase nonreceptor type 11 (PTPN11), major histocompat-
ibility complex class II DQ alpha 1 (HLA-DQA1), cell division
cycle 25A (CDC25A), β-2-microglobulin (B2M), COL6A1,
COL3A1, SPARC, COL1A1 and COL1A2, and downregulated
de genes, including breast cancer 1 early onset (BRCA1)
and COL2A1 are key genes that may interact with many other DE mRNAs
in the ParoAF_Control network (Fig. 5A).

**PPI network analysis.** The PPI network in ParoAF_Control
and PersAF_Control is shown in Fig. 5A and B. Fig. 5A shows
that COL3A1, COL1A1, secreted protein acidic cysteine-rich
(SPARC), COL6A1, COL1A2, DCN, and COL2A1 exhibited
a strong interaction with another. Downregulated genes,
including breast cancer 1 early onset (BRCA1) and COL2A1
are key genes that may interact with many other DE mRNAs
in the ParoAF_Control network (Fig. 5A).

Next, the possible target mRNAs of the DE lncRNAs
(Trans prediction) was assessed and a total of 709 and
1,275 mRNAs were identified in ParoAF_Control and
PersAF_Control, respectively. DE mRNAs that were also
targeted by DE lncRNAs were selected, which are shown
in Fig. 4C and D. There were 12 and 31 DE lncRNAs and
DE mRNAs co-expressed in ParoAF and PersAF samples
compared with the control, respectively.

The selected mRNAs were then used for an mRNA-lncRNA
network analysis. The mRNA-lncRNA co-expression network
of ParoAF_Control revealed that anoctamin 3 (ANO3) exhibit-
ed a coconitant downregulation trend with XLOC_002352,
XLOC_110321, XLOC_098657 and XLOC_11033. Churchill
domain containing 1 (CHURC1) showed downregulated
expression consistent with RP11-428C19.4 (Fig. 4E). ANO3
and CHURC1 co-expression were also detected in the
PersAF_Control. Furthermore, CHURC1 was also found to be
co-expressed with XLOC_108610. The upregulated trend was
also detected, as cardiolipin synthase 1 (CRLS1) was associated
with RP11-380f14.2, carnitine palmitoyltransferase 1C
(CPT1C) with RP11-498P14.4, and NF1L with RP11-121C2.2
(Fig. 4F).

**Validation of differentially expressed lncRNAs and mRNAs
using RT-qPCR.** The expression of the DE mRNAs and
lncRNAs measured by RT-qPCR exhibited a similar trend
in expression consistent with that of RNA-Seq (Fig. 6).
Moreover, mRNA-lncRNA co-expression interactions of
cyclin cycle 25A (CyclinD25A), β-2-microglobulin (B2M), COL6A1,
COL3A1, SPARC, COL1A1 and COL1A2, and downregulated
de mRNAs, including cyclin-dependent kinase 2 (CDK2),
kelch repeat and BTB (POZ) domain containing 13 (KBTBD13),
histone deacetylase 1 (HDAC1), split hand/foot malformation
(ectrodactyly) type 1 (SHFM1), F-box and leucine-rich repeat
protein 19 (FBXL19), and ring finger protein 19A, RBR E3
ubiquitin protein ligase (RNF19A) are essential genes that
can interact with other DE mRNAs (Fig. 5B).
AF is the most prevalent heart disease worldwide, with different subtypes causing different clinical features. Therefore, these subtypes should be treated differently. In recent years, the molecular mechanism of AF has been studied in a variety of ways. However, the studies on the differences of lncRNAs and mRNAs between ParoAF and PersAF have not been sufficient. In the present study, ParoAF, PersAF and healthy donors samples were sequenced, and found that DE mRNAs and lncRNAs vary considerably. The putative function of DE mRNAs and DE lncRNA in ParoAF_Control and PersAF_Control were also different.

**COL1A2**, was associated with platelet-derived growth factor binding in the comparison of both ParoAF_Control and PersAF_Control. Moreover, **COL1A2** and **COL2A1** were also associated with most of the top ten KEGG pathways in patients with ParoAF. In a study by Zhou *et al* (25), upregulated **COL1A2** in patients with AF were observed when compared with a control, suggesting that it may participate in the occurrence and development of AF. Gambini *et al* (26) demonstrated that TGF-β1 could induce the upregulation of **COL1A2** in human cardiac mesenchymal progenitor cells from PersAF specimens. The expression level of **COL1A2** in these two studies was consistent with that of the present results. In the study by Dawson *et al* (11), it was reported that **COL1A2** was a target of miR29, which may play a role in atrial fibrotic remodeling and is considered to be a biomarker or therapeutic target. In the present study, **COL1A2** also exhibited a strong connection with other recombinant collagen type I, III and IV family proteins, as determined by PPI analysis. These results suggest an important role for **COL1A2** in the pathophysiology of both patients with ParoAF and PersAF.

The PI3K/Akt and TGF-β signaling pathways were significantly enriched in the ParoAF_Control, whereas the calcium signaling pathway was significantly enriched in the PersAF_Control. Studies have demonstrated that some herbs can...
Table III. Top ten GO terms for cis target genes of upstream and downstream DE lncRNAs.

A, Upstream

<table>
<thead>
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<th>GO Terms</th>
<th>Term name</th>
<th>Corrected P-value</th>
<th>Input genesa</th>
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<tbody>
<tr>
<td>GO:0008081_MF</td>
<td>Phosphoric diester hydrolase activity</td>
<td>5.73x10^-6</td>
<td>PLCXD3, PDE7B, GPCPD1, ENPP2, PDE5A, PDE3A, LOC101928269, PLCH1, CHRM3, PLCD1</td>
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<tr>
<td>GO:0006140_BP</td>
<td>Regulation of nucleotide metabolic process</td>
<td>1.6x10^-4</td>
<td>ITGB1, ADRB2, MCF2L2, RIN3, ARHGAP24, NF1, CRHR1, BPGM, RASA2, GRM3</td>
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<td>GO:0005856_CC</td>
<td>Cytoskeleton</td>
<td>3.9x10^-4</td>
<td>GRM3, HSPB7, ATP6V1D, BCL10, MPLKIP, CTNNB1, PCGF5, RLPL1, NFE2L2, FRMD4A</td>
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<td>GO:0042995_CC</td>
<td>Cell projection</td>
<td>5x10^-4</td>
<td>RILPL1, CTNNB1, SPG11, TIA2, FAM49A, GRM3, ATP6V1D, NF1, ARHGAP24, UNC13B</td>
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<td>GO:1900542_BP</td>
<td>Regulation of purine nucleotide metabolic process</td>
<td>5x10^-4</td>
<td>HTR1B, FBXO8, BCAS3, AB2, MYO9A, BCAR3, RAP1B, ARF4, NRG4, NTRK2</td>
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<td>GO:0043005_CC</td>
<td>Neuron projection</td>
<td>5.9x10^-4</td>
<td>APC, AMFR, NTRK2, PDE5A, PRSS23, ANK3, KCNIP1, SEMA6A, CAMK1G, DLG2</td>
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<td>GO:1901701_BP</td>
<td>Cellular response to oxygen-containing compound</td>
<td>7.1x10^-4</td>
<td>TGFBI, PIK3CA, CALCRL, PDE4D, RORA, WDFC1, ATP6V1D, CTNNB1, FZD4, CMKP2</td>
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<td>GO:0008092_MF</td>
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<td>1.05x10^-3</td>
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<td>GO:0045202_CC</td>
<td>Synapse</td>
<td>1.41x10^-3</td>
<td>CLSTN1, GRM1, CAMK2D, SYNE1, SLC30A3, UNC13B, RIMS4, CACNA1C, ITGB1, SPG11</td>
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<td>GO:0048731_BP</td>
<td>System development</td>
<td>1.63x10^-3</td>
<td>ARHGAP24, ADAMS18, MAPK10, UNC13B, ITGB1, SPG11, SYCP2, COL18A1, PLK5, FGF14</td>
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B, Downstream

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<th>GO Terms</th>
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<tr>
<td>GO:0000981_MF</td>
<td>Sequence-specific DNA binding RNA polymerase II transcription factor activity</td>
<td>3.12x10^-5</td>
<td>TGIF1, ZSCAN5A, RORA, NFE2L2, ERG, GATA6, CSRNP3, RHDI3, NFIB, ZBTB16</td>
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<tr>
<td>GO:0002021_BP</td>
<td>Response to dietary excess</td>
<td>1x10^-4</td>
<td>BMP8A, TBL1XR1, ADRB2, RMI1, CCKAR, PPARGC1A, MC4R</td>
</tr>
<tr>
<td>GO:0010628_BP</td>
<td>Positive regulation of gene expression</td>
<td>2.2x10^-4</td>
<td>HIF1A, FOXD2, MED12L, NF1A, GTF2F2, TFAP2C, HIPK2, MAPK10, FGF7, TLR2</td>
</tr>
<tr>
<td>GO:1902531_BP</td>
<td>Regulation of intracellular signal transduction</td>
<td>6.5x10^-4</td>
<td>C12orf60, PDGFD, TAOK3, MTA3, FOXM1, MCF2L2, EPHB1, MUL1, OTUD7A, TBPL1</td>
</tr>
<tr>
<td>GO:0045893_BP</td>
<td>Positive regulation of transcription, DNA-dependent</td>
<td>2.33x10^-3</td>
<td>ARF4, GATA6, TET2, BCL9, TBL1XR1, BCL10, BCAS3, NFE2L2, SMARCA2, ZBTB17</td>
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<tr>
<td>GO:1902532_BP</td>
<td>Negative regulation of intracellular signal transduction</td>
<td>2.42x10^-3</td>
<td>SORL1, SPRED2, NF1, VGL4, RORA, NFE2L2, RDH13, VDAC2, CNKSR3, TIMP3</td>
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<tr>
<td>GO:0000977_MF</td>
<td>RNA polymerase II regulatory region sequence-specific DNA binding</td>
<td>4.8x10^-3</td>
<td>NHLH2, ZNF486, ZNF812, AEBP2, E2F8, NFIA, FOXD2, ZNF736, MTA3, ZNF98</td>
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<tr>
<td>GO:0001012_MF</td>
<td>RNA polymerase II regulatory region DNA binding</td>
<td>5.22x10^-3</td>
<td>FBXO16, ZNF506, ZBTB16, NFIB, SMARC2, RORA, ZNF708, NFE2L2, TGIF1, GATA6</td>
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<tr>
<td>GO:0045944_BP</td>
<td>Positive regulation of transcription from RNA polymerase II promoter</td>
<td>6.37x10^-3</td>
<td>RDH13, CSRN3, CTNNB1, ADRB2, TMEM173, TGF6B, ATXN7, SMARCA2, NFIB, TET2</td>
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<tr>
<td>GO:0001071_MF</td>
<td>Nucleic acid binding transcription factor activity</td>
<td>8.72x10^-3</td>
<td>HIF1A, C12orf60, NFIA, FOXD2, TBX20, EPAS1, MTA3, ZNF98, AEBP2</td>
</tr>
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</table>

*aList genes no more than ten. GO, Gene Ontology; CC, cellular component; MF, molecular function; BP, biological process; DE, differentially expressed; ParoAF, paroxysmal atrial fibrillation; PersAF, persistent atrial fibrillation."
decrease the incidence of AF through the PI3K/Akt signaling pathway (27, 28). Other studies have revealed that activating this pathway may reverse atrial remodeling and inhibit the occurrence of AF (29, 30). Most collagen family genes detected in the present study were associated with enriched pathways of both the ParoAF_Control and PersAF_Control. Of note, COL2A1 was only downregulated in the ParoAF_Control and is involved in PI3K‑Akt signaling pathway. However, there have been no reports describing any associations among COL2A1, ParoAF and the PI3K/Akt signaling pathway.

TGF-β signaling is involved in the course of atrial structural remodeling in AF and is associated with the occurrence and maintenance of AF (31). Fu et al (32) showed that overexpressed Gal-3 in AF could subsequently activate the TGF-β1/α-SMA/Col I pathway in cardiac fibroblasts, thus strengthening atrial fibrosis. Moreover, suppressing the overexpression of TGF-β could prevent atrial remodeling (33-35). TGF-β has also been demonstrated to be a promising therapeutic target for decreasing cardiac fibrosis (36). In the present study, the TGF-β signaling pathway was significantly enriched in the ParoAF_Control
along with SMURF2 and DCN. SMURF2, which was upregulated in the present study, can induce proteasomal degradation of Smad7 and activate the TGF-β signaling pathway (37,38). DCN, a proteoglycan that can bind to collagen fibrils in the ECM (extracellular matrix), interacts with many growth factors, and inhibits TGF-β activity (37). Moreover, DCN exhibited a strong connection with other DE mRNAs in the PPI analysis in the present study. Taken together, it was hypothesized that the TGF-β signaling pathway and DCN may play essential roles in AF occurrence and maintenance. Moreover, TGF-β may represent an inhibitor for preventing AF occurrence.

In the present study, the calcium signaling pathway was significantly enriched in the PersAF_Control. Calcium signaling pathway was considered to play a vital role in electrical remodeling and promoting the recurrence of AF (39). Tan et al (38) have demonstrated that the lncRNA HOTAIR was involved in the modulation of calcium homeostasis in human cardiomyocytes. It was also confirmed that HOTAIR inhibited intracellular Ca²⁺ content by regulating L-type calcium channels. In a study on mice with ParoAF and PersAF (40), PersAF mice exhibited a phenomenon of enhanced diastolic Ca²⁺ release, marked conduction abnormalities and atrial enlargement. The absence of PLN increased Ca²⁺ transient amplitude and a faster Ca²⁺ decay rate (39). The combined results of these studies with the present study indicate that the calcium signaling pathway may play an essential role in the processing
Figure 5. The protein-protein interaction network of ParoAF_Control and Pers_Control. (A) The protein-protein interaction network of ParoAF_Control. (B) The protein-protein interaction network of Pers_Control. The more lines connected, the stronger the interaction between the two mRNAs. ParoAF, paroxysmal atrial fibrillation; PersAF, persistent atrial fibrillation.
of ParoAF to PersAF. Moreover, it was hypothesized that developing inhibitors to this pathway may inhibit the ParoAF to PersAF transition. Unfortunately, the study did not provide detailed information regarding ParoAF and PersAF and was not conducive for the comparison analysis.

lncRNAs play essential roles in ParoAF and PersAF through cis-elements. In the present study, DCT interacted with GPC5-AS2, and METTL15 interacted with BDNF-AS in the ParoAF_Control. DCT interacted with GPC5-AS2, C21orf90 interacted with TSPEAR-AS1, and MDM1 interacted with RP11-42H2C9.4 in the PersAF_Control. These DE lncRNAs may perform their function through nearby DE mRNAs. Levin et al (41) revealed that adult mice lacking DCT display normal cardiac development but an increased susceptibility to atrial arrhythmias. This suggests that lncRNA GPC-AS2 inhibits the expression of DCT to influence atrial arrhythmias, which warrants further study. Through our trans analysis, mRNAs that were differentially expressed and targeted DE lncRNAs were identified. These DE mRNAs exhibited co-expression with DE lncRNAs. PDE4D, a biomarker of myocardial infarction and heart failure (42), was downregulated in both ParoAF_Control and PersAF_Control and was targeted by the DE lncRNAs of XLOC_110310 and XLOC_137634. Further studies are needed to understand the molecular mechanism of DE mRNA and lncRNA interactions.

The function of DE lncRNAs in ParoAF_Control and PersAF_Control was analyzed through cis-elements. As a nearby gene of DE lncRNAs, CACNA1C was enriched in both the MAPK and cAMP signaling pathways. Zhang et al (43) revealed that the MAPKs/TGF-β1/TRA6 signaling pathways participate in atrial fibrosis in patients with rheumatic heart disease, causing the occurrence of AF following cardiac surgery. Inhibiting the MAPK pathways can prevent atrial parasympathetic remodeling and the occurrence of AF (44). The induction of AF and structural remodeling was associated with MAPK expression and the decrease in collagenase activity (15). CACNA1C is enriched in the MAPK signal pathway which is the direct target gene of miR-29a-3p. Zhao et al (45) found that decreased expression of CACNA1C caused by overexpression of microRNA-29a underlies the pathogenesis of AF. Thus, it was assumed that miR-29a-3p may be a potential therapeutic target in AF. These studies combined with the present result indicate that the MAPK signaling pathway is an important pathway that participates in AF occurrence by preventing atrial parasympathetic remodeling.

There were some limitations in the present study. Firstly, the original intention was to study the molecular mechanism of all AF types including permanent AF, ParoAF and PersAF. However, the permanent AF samples were difficult to collect. Secondly, the sample number used in the present study was low, as more than five would have been better. Thirdly, the present study lacked functional verification of the mRNAs and lncRNAs, which will be the subject of a future study.

The present study analyzed the DE mRNAs and lncRNAs and their putative roles in ParoAF and PersAF. It was found that PI3K/Akt and TGF-β signaling were significantly enriched in the ParoAF_Control, and the calcium signaling pathway was significantly enriched in the PersAF_Control. The cis and trans analyses revealed some important interactions between DE mRNAs and lncRNAs including GPC-AS2 with DCT, and PDE4D with XLOC_110310 and XLOC_137634. In summary, the present study provided molecular theoretical data for further clinical studies involving ParoAF and PersAF.

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Not applicable.

Availability of data and materials
The raw data were deposited on the NCBI Sequence Read Archive (SRA), with the SRA accession number of PRJNA531935.
Authors' contributions

HS designed the study, collected the samples, conducted the experiment, analyzed the data, and wrote the manuscript. YS equally designed the study, analyzed the data, provided the foundation and revised the manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was permitted by the Human Ethics Committee of the Jiangsu People Hospital, (approval number, 2020-SRFA-340). All patients with AF and control patients have been informed of writing consent to use their tissue for this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


