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RESEARCH ARTICLE

Long non-coding RNA SAP30-2:1 is downregulated in congenital heart disease and regulates cell proliferation by targeting HAND2

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Abstract **Congenital heart disease (CHD) is the most common birth defect worldwide. Long non-coding RNAs (lncRNAs) have been implicated in many diseases. However, their involvement in CHD is not well understood. This study aimed to investigate the role of dysregulated lncRNAs in CHD. We used Gene Expression Omnibus data mining, bioinformatics analysis, and analysis of clinical tissue samples and observed that the novel lncRNA SAP30-2:1 with unknown function was significantly downregulated in damaged cardiac tissues from patients with CHD. Knockdown of lncRNA SAP30-2:1 inhibited the proliferation of human embryonic kidney and AC16 cells and decreased the expression of heart and neural crest derivatives expressed 2 (HAND2). Moreover, lncRNA SAP30-2:1 was associated with HAND2 by RNA immunoprecipitation. Overall, these results suggest that lncRNA SAP30-2:1 may be involved in heart development through affecting cell proliferation via targeting HAND2 and may thus represent a novel therapeutic target for CHD.**

Keywords congenital heart disease; Gene Expression Omnibus; lncRNA SAP30-2:1; cell proliferation; RNA immunoprecipitation; HAND2

Introduction

Congenital heart disease (CHD) is the most common defect among live births, accounting for 0.8%–1.2% of all birth defects worldwide [1]. CHD is also the leading noninfectious cause of death in the first year of life [1]. Although the development of novel surgical and catheter interventions has decreased the mortality of CHD, such developments cannot resolve all the cardiac problems, and postoperative patients still face risks, such as those of arrhythmia, heart failure, and neurodevelopmental deficit [2]. Thus, the etiology and pathogenesis of CHD must be further explored to improve its prevention and treatment.

CHD involves morphological, structural, functional, and metabolic abnormalities of the heart and large blood vessels caused by cardiovascular dysplasia during the embryonic period. This condition is a multifactorial, complex disease affected by environmental and genetic factors [3, 4], with genetic factors playing a leading role in its occurrence and development [5]. Cardiac development involves the orderly proliferation, migration, and differentiation of a variety of cells as a result of the tightly regulated spatiotemporal expression of a network of genes. The faulty regulation of gene expression could thus underpin the development of CHD [6]. Although several genes encoding transcription factors, chromatin regulatory factors, and signaling molecules are currently known to alter the expression of genes related to cardiac development, increasing evidence suggests that non-coding transcripts that affect gene expression regulation may also be involved in cardiogenesis [6–8]. Long non-coding

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RNAs (lncRNAs), which consist of over 200 nucleotides, are non-coding transcripts that play important roles in regulating cellular processes controlled by gene expression [9]. Unlike the functional mechanism of microRNAs, which silences their target genes by base pairing the complementary mRNA sequences, lncRNAs regulate gene expression via diverse and complex mechanisms [10]. Dysregulated lncRNAs are related to many diseases [11], and previous studies have identified hundreds of lncRNAs involved in mammalian cardiogenesis and the pathogenesis of related diseases [6, 12, 13]; however, their function in CHD remains largely unclear.

In this study, we performed Gene Expression Omnibus (GEO) data mining and bioinformatics analysis to characterize the profiles of lncRNAs and mRNAs in human fetal and adult heart tissues. We then focused on the novel lncRNA SAP30-2:1 with unknown function and discovered that it was significantly upregulated in fetal hearts and was predicted to adjust the expression of heart and neural crest derivatives expressed 2 (HAND2) as a vital transcription factor related to cardiac development. We further demonstrated that lncRNA SAP30-2:1 expression was markedly decreased in damaged heart tissue from patients with CHD. Downregulation of lncRNA SAP30-2:1 significantly suppressed cell proliferation, whereas knockdown of lncRNA SAP30-2:1 reduced HAND2 protein expression by binding to it.

Materials and methods

Online data mining and bioinformatics analysis

Data mining was performed online on the GEO database using the keywords lncRNA, human, heart development, and CHD. The differential expressions of lncRNAs were analyzed using the DESeq Package, and differentially expressed mRNAs related to heart development or CHD were screened in accordance with the Wikipathway database. A coding and noncoding co-expression (CNC) network was established based on the correlations between the differentially expressed lncRNAs and mRNAs, with a correlation coefficient $\geqslant 0.9$ or $\leqslant -0.9$ and $P \leqslant 0.05$. At the same time, mRNAs adjacent to the lncRNAs in the CNC network (distance $\leqslant$ 20 kbp on the genome) were annotated. The target lncRNA was selected in accordance with the following requirements: (1) it is in the CNC network; (2) its adjacent mRNA is differentially expressed in relation to heart development or CHD; (3) its adjacent mRNA is co-expressed with it.

Study subjects and samples

This study was reviewed and approved by the Institutional Research Ethics Committee of the Children's Hospital of Fudan University (2016-56). Cardiac tissue samples were obtained from 53 patients with CHD at the Children's Hospital of Fudan University, Shanghai, China. The age of patients undergoing cardiac operations ranged from 1 month old to 156 months old. The patients had one of the three types of CHD: tetralogy of Fallot, ventricular septal defect, and double-chambered right ventricle. None of the patients included in the study had been diagnosed with extracardiac anomalies nor had any common chromosomal anomalies such as 22q11 microdeletion. Normal cardiac tissue samples were acquired from 11 subjects aged 20–204 months who died as a result of traffic accidents and who showed no abnormal cardiac structures during the autopsy (Department of Forensic Medicine, Fudan University). All tissue samples for RNA extraction were maintained in RNAlater® RNA Stabilization Solution (Thermo Fisher Scientific, Inc., Waltham, MA, USA) after surgery or autopsy and were stored at −80 °C.

RNA extraction and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from cardiac tissues and cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The quantity and quality of RNA were determined with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Inc.) and agarose gel electrophoresis. RNA was reverse-transcribed into cDNA using a PrimeScript RT Reagent Kit (Takara, Tokyo, Japan). qPCR was conducted using SYBR Premix Ex Taq™ (Takara, Tokyo, Japan) on a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.). Gene expression was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and analyzed in accordance with the relative quantification method ($2^{-\Delta\Delta Ct}$). Three independent experiments were performed.

The primers used were as follows:

lncRNA SAP30-2:1-F: TAGGTGGGTGCCCAAGAGA;

lncRNA SAP30-2:1-R: AAGTTGCCCTGGAGTACTGG;

GAPDH-F: GGGAGCCAAAAGGGTCAT;

GAPDH-R: GAGTCCTTCCACGATACCAA;

U1-F: GACGGGAAAAGATTGAGCGG;

U1-R: GCCACGAAGAGAGTCTTGAAGG;

Actin-F: CATGTACGTTGCTATCCAGGC;

Actin-R: CTCCTTAATGTCACGCACGAT.

Cell culture

Human embryonic kidney (HEK293T) cells and AC16 cells were cultured in Dulbecco's Modified Eagle's

Medium with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel) and 1% penicillin/streptomycin antibiotics at 37 °C and 5% CO_2. All cell culture dishes and plates were purchased from Hangzhou Xinyou Biotechnology Co., Ltd. (China).

Construction of eukaryotic expression vector and transient transfection

Human lncRNA SAP30-2:1-pcDNA 3.1 was synthesized by GeneRay (Shanghai, China). The entire plasmid was sequenced and was in line with the sequence published by the National Center for Biotechnology Information. An empty vector (pcDNA 3.1) was used as a negative control (NC). The HEK293T cells and AC16 cells were grown to a density of about 75% in 6 cm dishes and then transfected with lncRNA SAP30-2:1 and NC plasmids using Lipofectamine 3000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The cells were harvested 48 h after transfection.

Construction of lentiviral interference vector and stable cell line

Three short hairpin RNAs targeting lncRNA SAP30-2:1 were designed and cloned into the lentiviral vector PGMLV-SC5-GFP by Genomeditech (Shanghai, China). Lentiviruses were amplified in HEK293T cells and concentrated using polyethylene glycol (System Biosciences, CA, USA). The interference efficiency was verified by qPCR. The HEK293T cells and AC16 cells infected by the most interference-efficient lentivirus were used to establish stable cell lines with puromycin (Sigma-Aldrich, St. Louis, MO, USA) selection.

Cell Counting Kit-8 (CCK8) assay

Cell proliferation was measured with CCK-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The HEK293T cells and AC16 cells (1×10^4 per well) were incubated in 96-well plates for adherence. Three hours before the measurement, 10 μL CCK8 reagent was added to each well and incubated at 37 °C. Cell viability was assessed as a measure of proliferation and evaluated by the absorbance at 450 nm at 40, 56, and 72 h after transfection or incubation. All samples were prepared in triplicate and normalized to a blank control.

EdU assay

Cell proliferation was also measured using an EdU Labeling Kit (RiboBio, Shanghai, China). A total of 5×10^3 cells per well were seeded in 96-well plates for adherence. EdU labeling media were added to the plates and incubated for 2 h at 37 °C. After treatment with 4% paraformaldehyde and 0.5% Triton X-100, the cells were stained with anti-EdU working solution, followed by Hoechst nuclear staining. The images were measured by fluorescence microscope.

Cell apoptosis assay

HEK293T and AC16 cells (1×10^6) were collected and stained using an Annexin V-APC/PI Kit (RiboBio, Shanghai, China) following the manufacturer's instructions. The cells were stained successively with 5 μL Annexin V-APC reagent and 5 μL propidium iodide and incubated in the dark for 15 min at room temperature after each treatment. Flow cytometry analysis was performed with a FACSCalibur (BD Biosciences, San Jose, CA, USA), and cell apoptosis was analyzed using FlowJo software.

Subcellular localization analysis

Nuclear and cytoplasmic fractions of HEK293T cells and AC16 cells were isolated using a PARIS kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's instructions. Actin was used as a cytoplasmic control and U1 as a nuclear control. The RNA levels of lncRNA SAP30-2:1, actin, and U1 in the cytoplasm and nuclear components were detected by qPCR.

RNA immunoprecipitation (RIP) assay

RIP assay was performed using an EZ-Magna RIP Kit (EMD Millipore, Billerica, MA, USA) following the manufacturer's instructions. In brief, HEK293T cells were lysed in RIP lysis buffer and then incubated with RIP buffer containing magnetic beads conjugated with human anti-HAND2 (Proteintech, Chicago, IL, USA) or NC mouse anti-IgG antibody (Proteintech). RNase-free DNase I (Promega, Madison, WI, USA) and proteinase K (Yeasen, Shanghai, China) were used to digest extra DNAs and proteins, respectively. Finally, purified RNA was used for qPCR analysis of lncRNA SAP30-2:1.

Western blot (WB) analysis

HEK293T cells were lysed by RIPA buffer (Yeasen, Shanghai, China) containing a protease inhibitor cocktail (Sigma-Aldrich), and protein determination was performed with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The same amounts of protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Pall, NY, USA). The primary antibodies were anti-HAND2 (1:1000), anti-tubulin (1:5000), and anti-actin

(1:5000), and the secondary horseradish peroxidase-conjugated antibody was anti-mouse IgG (1:5000) (all Proteintech). The proteins were visualized on X-ray films using a Clinx ChemiScope (Clinx Science Instruments, Shanghai, China).

Statistical analysis

All statistical analyses were performed by paired two-tailed Student's *t*-tests with GraphPad Software (Inc. La Jolla, CA, USA). $P < 0.05$ was considered significant.

Results

Selection of lncRNA SAP30-2:1 by data mining and bioinformatics analysis

To gain insight into the roles of lncRNAs in heart development and CHD, we performed data mining on GEO, identified lncRNAs that were differentially expressed in four human fetal and three adult hearts by RNA-seq, and subjected them to further bioinformatics analysis (Fig. 1A). The ages of the four fetal samples were 13, 16, 17, and 17 weeks, those of the two adult samples were 73 and 24 years old, and that of another adult sample was unavailable in the database. A total of 277 lncRNAs and 47 mRNAs related to heart development or CHD were differentially expressed between the fetal and adult heart tissues (Tables S1 and S2). Analysis of the CNC network, including 19 lncRNAs and 26 mRNAs meeting the required standards, indicated that lncRNAs and mRNAs may have regulatory relationships and play important roles in cardiogenesis and CHD (Fig. 1B). Among the 19 lncRNAs, lncRNA SAP30-2:1 was significantly upregulated in fetal heart tissue (Fig. 1C) and was selected based on the requirements set above. Furthermore, lncRNA SAP30-2:1 may act on its nearby gene *HAND2* (Fig. 1D and 1E).

lncRNA SAP30-2:1 was downregulated in damaged CHD heart tissues.

To validate whether lncRNA SAP30-2:1 is related to CHD or heart development, we detected its expression in 53 samples of damaged heart tissue from patients with CHD and 11 normal heart tissue samples using qPCR. lncRNA SAP30-2:1 was significantly downregulated in CHD compared with normal heart tissue (Fig. 2).

Downregulation of lncRNA SAP30-2:1 inhibited cell proliferation *in vitro*

Aberrant cell proliferation and apoptosis are key features of CHD. We therefore examined the effects of lncRNA SAP30-2:1 on the proliferation and apoptosis of HEK293T and AC16 cells. lncRNA SAP30-2:1 was effectively downregulated and upregulated in HEK293T cells and AC16 cells by knockdown and overexpression, respectively (Figs. 3A, 3B, 4A, and 4B). CCK-8 assay indicated that HEK293T and AC16 cell proliferation was suppressed by lncRNA SAP30-2:1 knockdown, consistent with the tendency in cells overexpressing lncRNA SAP30-2:1 (Figs. 3C, 3D, 4C, and 4D). The result was further confirmed by EdU assay of HEK293T cells (Fig. 3E–3H). However, flow cytometry analysis showed that the dysregulation of lncRNA SAP30-2:1 caused no effect on HEK293T and AC16 cell apoptosis (Figs. 3I, 3J, 4E, and 4F).

lncRNA SAP30-2:1 regulated the expression of HAND2 protein *in vitro* by binding to it

We further explored the mechanism by which lncRNA SAP30-2:1 affects cell proliferation during heart development by detecting its subcellular localization, which in turn determines its mode of action. Following nucleocytoplasmic separation, lncRNA SAP30-2:1 was mainly located in the nucleus of HEK293T and AC16 cells, with small amounts detected in the cytoplasm (Fig. 5A and 5B). Previous bioinformatics prediction indicated that its adjacent gene *HAND2*, encoding a cardiac transcription factor, may be co-expressed with lncRNA SAP30-2:1. We therefore detected the expression level of HAND2 in HEK293T cells with downregulated or upregulated lncRNA SAP30-2:1 by WB. Relative HAND2 protein levels were significantly downregulated by lncRNA SAP30-2:1 knockdown (Fig. 5C) and upregulated by lncRNA SAP30-2:1 overexpression (Fig. 5D). We clarified the mechanism by which lncRNA SAP30-2:1 affected HAND2 expression by RIP with a specific anti-HAND2 antibody in HEK293T cells. lncRNA-SAP30-2:1 enrichment was detected by qPCR in HAND2 RNA precipitates, which indicated the physical interaction between lncRNA-SAP30-2:1 and HAND2 (Fig. 5E and 5F). These results suggest that the downregulation of lncRNA-SAP30-2:1 might inhibit the expression of HAND2 via this interaction.

Discussion

Abnormal cardiogenesis in early embryonic development results in CHD. Heart development relies on the correct regulation of gene expression. Understanding the gene expression profile in human heart tissue, especially the fetal heart, and identifying genes that are aberrantly expressed in CHD may thus be crucial for improving the diagnosis and therapeutic interventions of CHD.

Thousands of lncRNAs have been identified by genomic

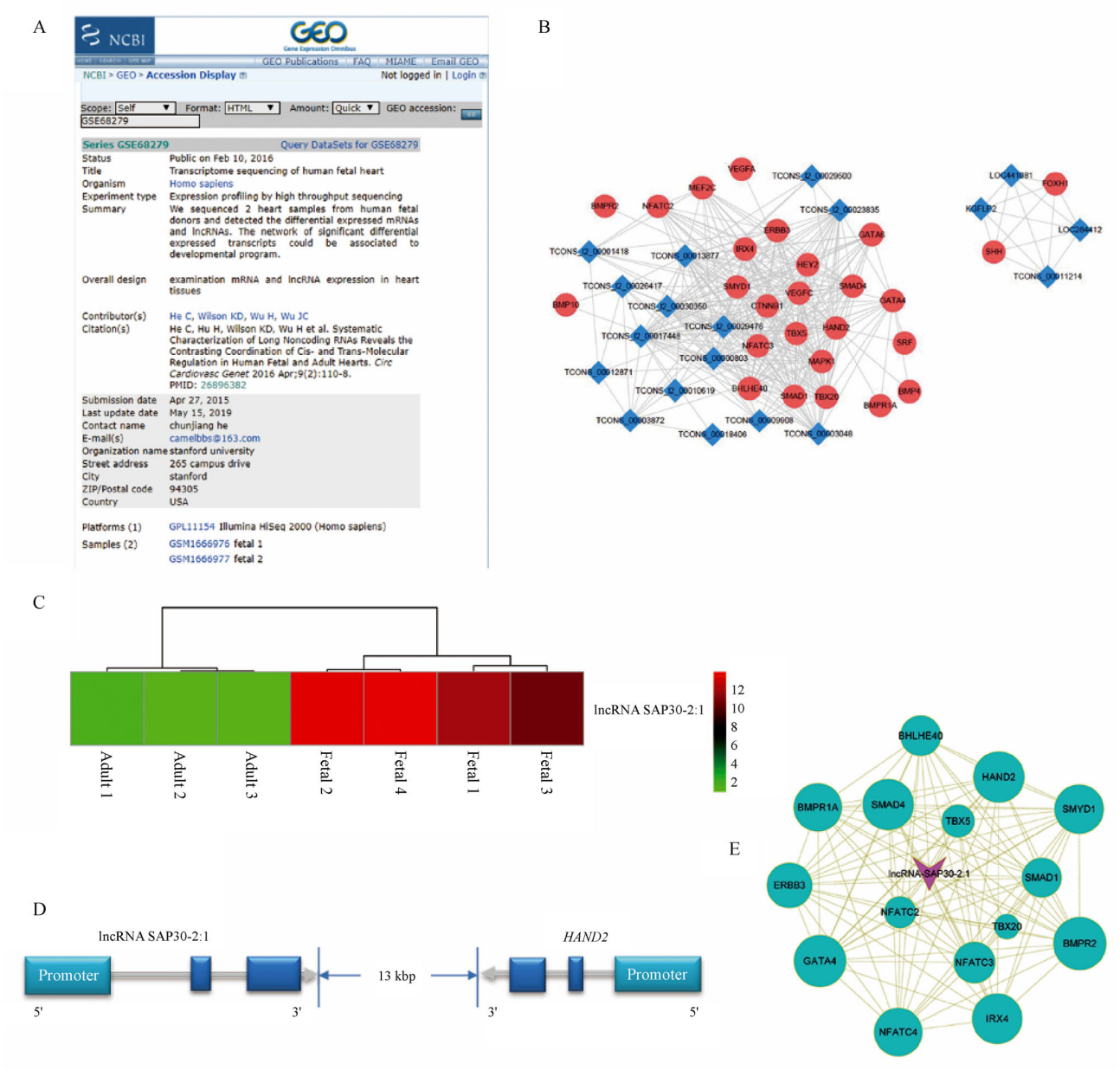

Fig. 1 lncRNA SAP30-2:1 was screened by data mining of GEO database and bioinformatics analysis. (A) RNA-seq data for human fetal and adult hearts were extracted from the GEO database. (B) CNC network of 19 lncRNAs and 26 mRNAs with a correlation coefficient $\geqslant 0.9$ or $\leqslant -0.9$ and $P \leqslant 0.05$. Blue diamonds represent lncRNAs, red circular nodes denote mRNAs, and lines indicate the gene co-expression relationship between lncRNAs and mRNAs. (C) Higher expression of lncRNA SAP30-2:1 in fetal hearts compared with those of adults. Expression values presented in red (above median) or green (below median). (D) Relative positions of lncRNA SAP30-2:1 and its nearby gene *HAND2* on the chromosome. *HAND2* is located at about 13 kbp downstream of lncRNA SAP30-2:1. (E) CNC network of lncRNA SAP30-2:1 and 15 mRNAs. Rose V represents lncRNA SAP30-2:1; green circular nodes denote mRNAs; node size indicates the expression level of a gene (large dot indicates high expression level). Lines represent the gene co-expression relationship between lncRNA SAP30-2:1 and mRNA (full lines represent positive correlations; dashed lines indicate negative correlations).

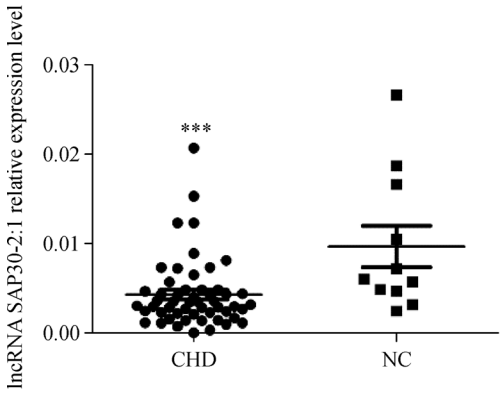

Fig. 2 lncRNA SAP30-2:1 was significantly downregulated in damaged cardiac tissue samples from CHD patients compared with NC based on qPCR analysis. ***$P < 0.001$.

transcription noise to play a pivotal role in various physiological and pathological processes regulated by gene expression [14]. The unique effect and tissue-specific expression of lncRNAs *in vivo* suggest that they may represent a novel class of molecules regulating heart development, with a role in the formation of mammalian cardiac structures. Many lncRNAs are related to cardiac development and diseases [6, 15], but most of these lncRNAs were identified in mouse models and studied in mouse cell lines [16–18]. The conservation of lncRNAs between humans and mice is low [19, 20]; several studies have investigated the role of lncRNAs in CHD [21, 22]. We therefore focused on lncRNAs from human heart tissue and their abnormal expression in injured heart tissue of patients with CHD. We then searched the gene expression profile of human heart in the GEO repository and mined the RNA-seq data for fetal and adult heart tissues. We identified 277 lncRNAs and 42 mRNAs related to heart development or CHD that were differentially expressed between fetal and adult hearts by bioinformatics analysis. The regulatory effects of specific lncRNAs on several mRNAs may thus be involved in heart development.

Among the differentially expressed lncRNAs, we focused on lncRNA SAP30-2:1 because it is a novel lncRNA with a relative higher foldchange in fetal heart compared with the adult group according to RNA-seq analysis, and bioinformatics analysis predicted that its target gene could be *HAND2,* an important transcription factor related to CHD. lncRNA SAP30-2:1 was downregulated in injured heart tissue from CHD patients. We therefore carried out *in vitro* experiments to clarify the molecular mechanism underlying this clinical phenomenon. However, no human myocardial cell lines were available, and lncRNA-SAP30-2:1 has no homologous sequence in mice. Thus, we used the tool cell line HEK293T, in line with previous research on CHD and human heart development [23–25]. A part of *in vitro* experiments was repeated using AC16, a cell line similar to human cardiomyocytes. Cell experiments showed that lncRNA SAP30-2:1 was mainly located in the nuclei. lncRNAs located in the nucleus play a regulatory role as either cis-acting or trans-acting factors [26]. Low-expression lncRNA SAP30-2:1 downregulated HAND2 protein expression by binding to it, whereas the knockdown of lncRNA SAP30-2:1 significantly inhibited HEK293T and AC16 cell proliferation. HAND2 regulates the development of cardiovascular structures and acts as a transcription factor to control cardiomyocyte production [27, 28]. Furthermore, mutations or abnormal expression of *HAND2* can increase the risk of CHD [29–32], whereas non-coding transcripts influence heart development by targeting *HAND2* [33, 34]. Cardiogenesis is a complex developmental process including cell proliferation and contributes to cardiac growth and regeneration [35, 36]; HAND2 also plays critical regulatory roles in cardiomyocyte proliferation [30]. The relationship between the novel non-coding transcript lncRNA SAP30-2:1 and CHD may be as follows: lncRNA SAP30-2:1 may positively regulate the expression level of HAND2, the molecular mediator of CHD, by joining with it to affect cell proliferation. Thus, lncRNA SAP30-2:1 may be involved in the process of cardiogenesis. However, the detailed mechanisms by which lncRNA SAP30-2:1 modulates cardiac development and its function require further studies.

In summary, our study identified the downregulation of the novel lncRNA SAP30-2:1 in damaged heart tissue of patients with CHD and partly revealed its function and mechanism in cardiac development by data mining and *in vitro* experiments. The findings will provide a new diagnostic and therapeutic target for CHD.

Acknowledgements

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Compliance with ethics guidelines

Jing Ma, Shiyu Chen, Lili Hao, Wei Sheng, Weicheng Chen, Xiaojing Ma, Bowen Zhang, Duan Ma, and Guoying Huang declare that they have no conflict of interest. All the human tissues used in the present study were obtained with written informed consent. This study was approved by the Institutional Research Ethics Committee of the Children's Hospital of Fudan University (2016-56).

Electronic Supplementary Material Supplementary material is available in the online version of this article at https://doi.org/10.1007/s11684-020-0778-5 and is accessible for authorized users.

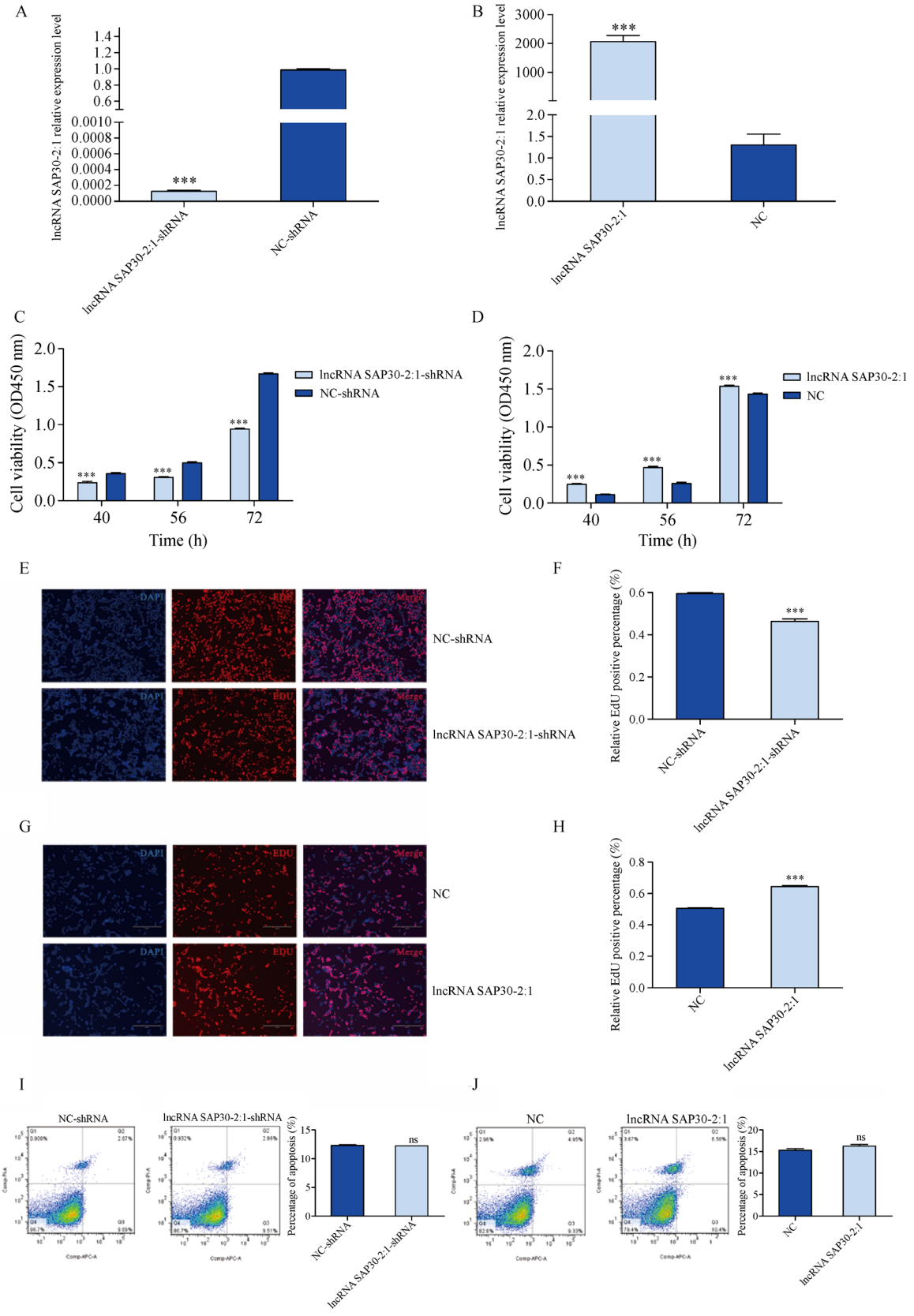

Fig. 3 Changes in lncRNA SAP30-2:1 affected the proliferation of HEK293T cells. (A,B) Efficiencies of upregulating and downregulating lncRNA SAP30-2:1 in HEK293T cells were detected by qPCR. (C, D) Positive regulation of cell proliferation by lncRNA SAP30-2:1 was revealed by CCK8 assay. (E–H) Positive regulation of cell proliferation by lncRNA SAP30-2:1 was revealed by EdU assay. (I, J) lncRNA SAP30-2:1 had no effect on cell apoptosis as shown by flow cytometry analysis. ***$P < 0.001$.

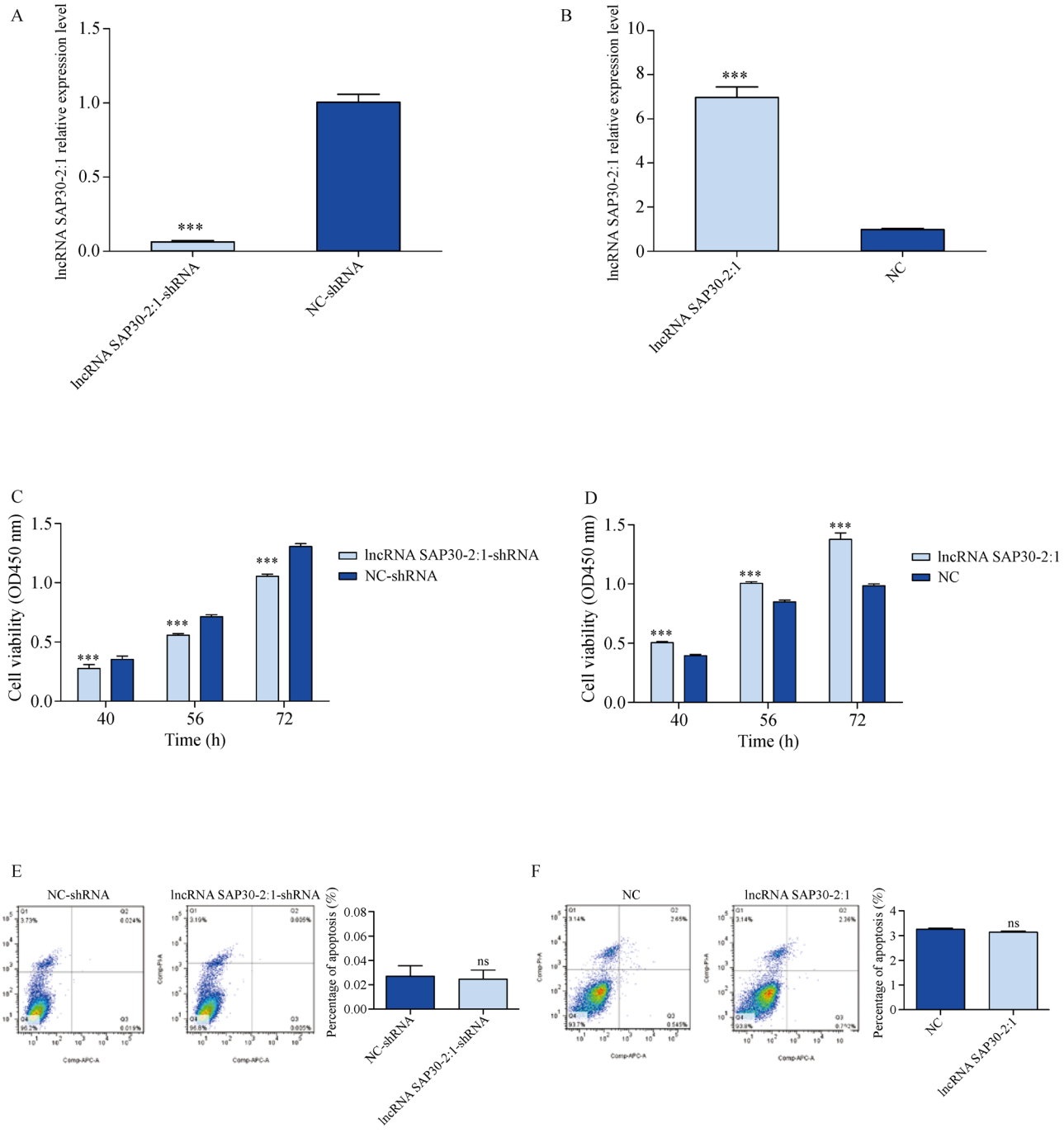

Fig. 4 Changes in lncRNA SAP30-2:1 affected proliferation of AC16 cells. (A, B) Efficiencies of upregulating and downregulating lncRNA SAP30-2:1 in AC16 cells were detected by qPCR. (C, D) Positive regulation of cell proliferation by lncRNA SAP30-2:1 was revealed by CCK8 assay. (E, F) lncRNA SAP30-2:1 had no effect on cell apoptosis as shown by flow cytometry analysis. ***$P < 0.001$.

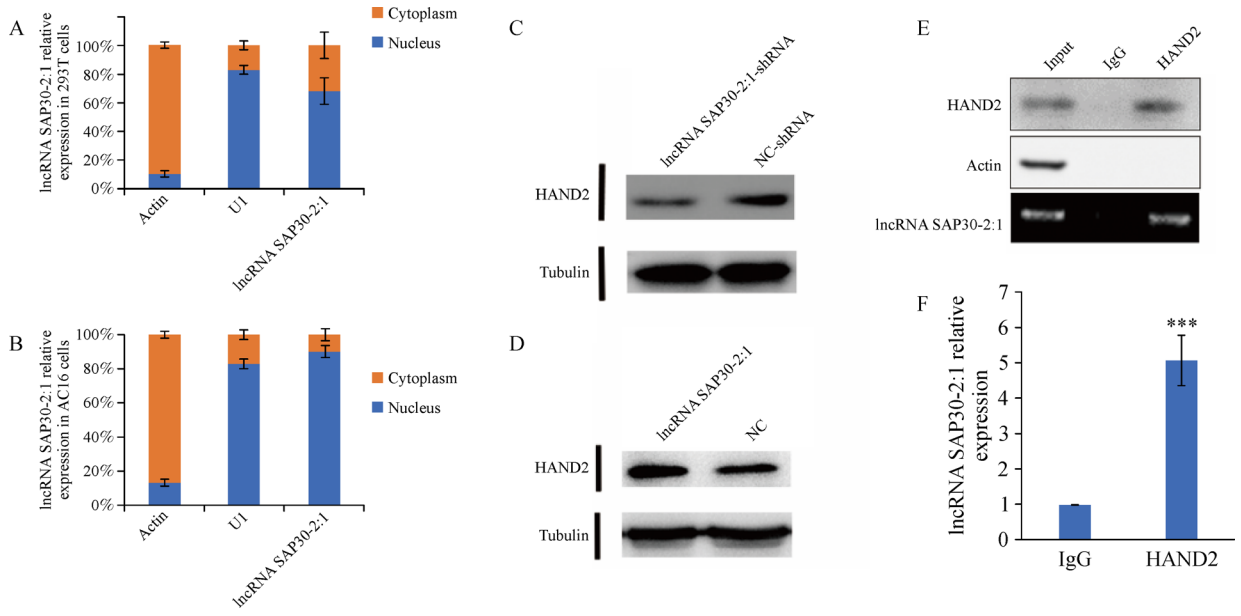

Fig. 5 lncRNA SAP30-2:1 regulated HAND2 expression *in vitro* by binding to it. (A, B) lncRNA SAP30-2:1 was mainly located in the nucleus of HEK293T and AC16 cells, similar to U1. (C, D) Dysregulation of lncRNA SAP30-2:1 positively regulated HAND2 protein levels according to WB analysis. (E, F) RIP assay of lncRNA SAP30-2:1 with anti-HAND2 antibody. lncRNA SAP30-2:1 and HAND2 interaction in HAND2-RNA precipitates was revealed by qPCR analysis.

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