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14. ABSTRACT

Familial Hypercholesterolemia (FH) is a genetic disorder within the FY21 PRMRP Topic Areas. Whether long non-coding RNAs (lncRNAs) have the therapeutic potential in FH remains unclear. In our preliminary study, we identified a lncRNA as a regulator of cholesterol metabolism and a potential therapeutic target for FH. During the research period, we have further confirmed its critical role in cholesterol metabolism in hepatocytes. Moreover, we have found that this lncRNA regulates cholesterol metabolism through the transcription factor SREBP2.To study the function of this lncRNA in vivo, we have produced adenovirus vector for overexpression of this lncRNA. We found that overexpressing this lncRNA in mouse liver suppresses the expression of SREBP2 pathway genes. Consistent with this, the liver and blood cholesterol levels were decreased. We also found that the overexpression of this lncRNA suppresses the transcription of the SREBP2 pathway genes by performing CHIP-PCR analysis.

15. SUBJECT TERMS

Familial Hypercholesterolemia, lncRNA, Cholesterol metabolism, SREBP2, liver

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1. Introduction

This study is to determine the therapeutic potential of a lncRNA for FH treatment.

2. Keywords

Familial Hypercholesterolemia, lncRNA, Cholesterol metabolism, SREBP2, liver

3. Accomplishments

What were the major goals of the project?

Major Task 1: Determine whether this lncRNA regulates the transcription of SREBP2 pathway genes

Major Task 2: Determine the binding between this lncRNA and SP1

Major Task 3: Determine whether this lncRNA functions through SP1

Major Task 4: prepare animal protocol and adenovirus

Major Task 5: Mouse studies using mouse homologue of this lncRNA

Major Task 6: Mouse studies using this human lncRNA

What was accomplished under these goals?

We have finished 3 out 6 major tasks listed above: Major Tasks 1, 4, and 5. In addition, we have validated key findings using primary human hepatocytes in response to the reviewers' concern about the findings in animal models of familial hypercholesterolemia (FH) limits the significance to human cholesterol metabolism and disease. Please find our detail findings below.

This lncRNA (named as LNCRNA1 in this report) negatively correlates with cholesterol metabolism gene expression in the human liver. To identify novel human lncRNA regulators in cholesterol metabolism in the human liver, we searched the GEO database and found that there are no publicly available RNA-seq datasets from liver samples obtained from patients with atherosclerotic disease. However, increased accumulation of cholesterol was reported in the livers of patients with non-alcoholic steatohepatitis (NASH). Therefore, we analyzed an RNA-seq dataset (GSE126848) with liver samples from healthy individuals and those with NASH. We identified that the expression of a human lncRNA LNCRNA1 is negatively correlated with genes involved in cholesterol metabolism such as LDLR, HMGCS1, and SQLE (Fig. 1).

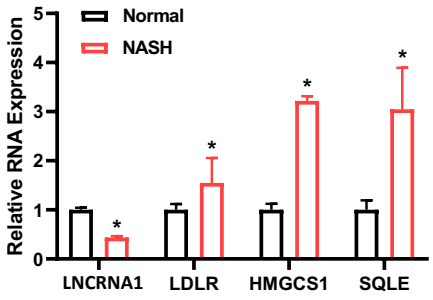

Fig. 1. RNA levels of LNCRNA1 and SREBP2 pathway genes in the liver samples from healthy (n = 14) and NASH (n = 16) individuals. Error bars are SEM, * $p < 0.05$.

LNCRNA1 is responsive to cholesterol signaling in hepatocytes. To test whether LNCRNA1 is regulated by cholesterol, we first treated the human hepatocyte cell line HepG2 cells with water soluble cholesterol for different time periods and found that 8hr is ideal for

cholesterol responsive genes. Next, we treated HepG2 and primary human hepatocytes (PHHs) with varying doses of cholesterol for 8 h. Results showed that cholesterol treatment suppressed the expression of SREBP2 pathway genes while increasing LNCRNA1 expression (Fig. 2A, B). These results reinforced our above findings that the expression of LNCRNA1 is negatively correlated with SREBP2 pathway genes, suggesting that LNCRNA1 contributes to the regulation of cholesterol signaling in human hepatocytes.

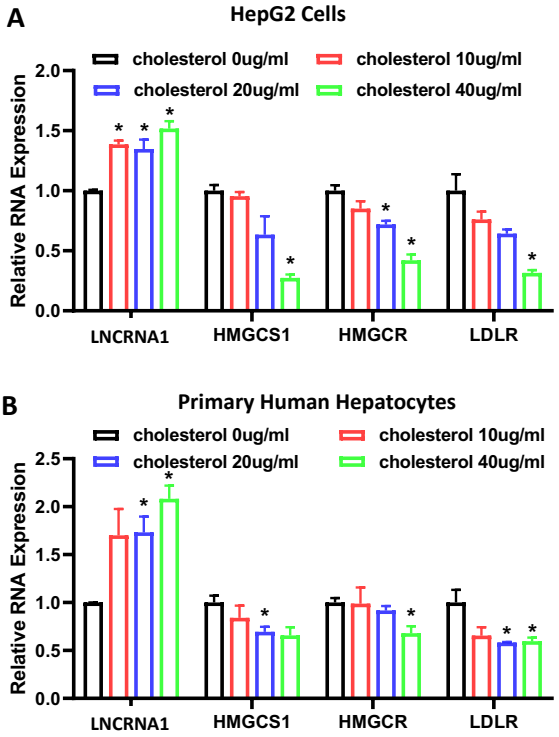

Fig. 2. The regulation of LNCRNA1 by cholesterol levels. (A-B) qRT-PCR analysis of LNCRNA1 and the SREBP2 pathway genes in control and cholesterol treated (A) HepG2 or (B) PHHs. n=3, Error bars are SEM, * $p < 0.05$.

The SREBP2 transcription factor suppresses LNCRNA1. The negative correlation between LNCRNA1 and SREBP2 pathway genes in the human liver and hepatocytes prompts us to further test the relationship between LNCRNA1 and SREBP2, the transcription factor responsible for the expression of SREBP2 pathway genes. We overexpressed SREBP2 (the active form is used for the entire proposal) in HepG2 cells or PHHs and measured the expression levels of LNCRNA1 and SREBP2 downstream genes. SREBP2 overexpression (OE) significantly increased its canonical downstream targets such as HMGCR, HMGCS1, and LDLR (Fig. 3A-C). Interestingly, the expression level of LNCRNA1 was decreased upon SREBP2 OE. Consistently, SREBP2 knockdown (KD) increases LNCRNA1 expression (Fig. 3D). These results suggested that LNCRNA1 expression is regulated by SREBP2.

LNCRNA1 is a suppressor of SREBP2 pathway gene expression. LncRNAs regulated by transcription factors often play a feedback role in the pathways these

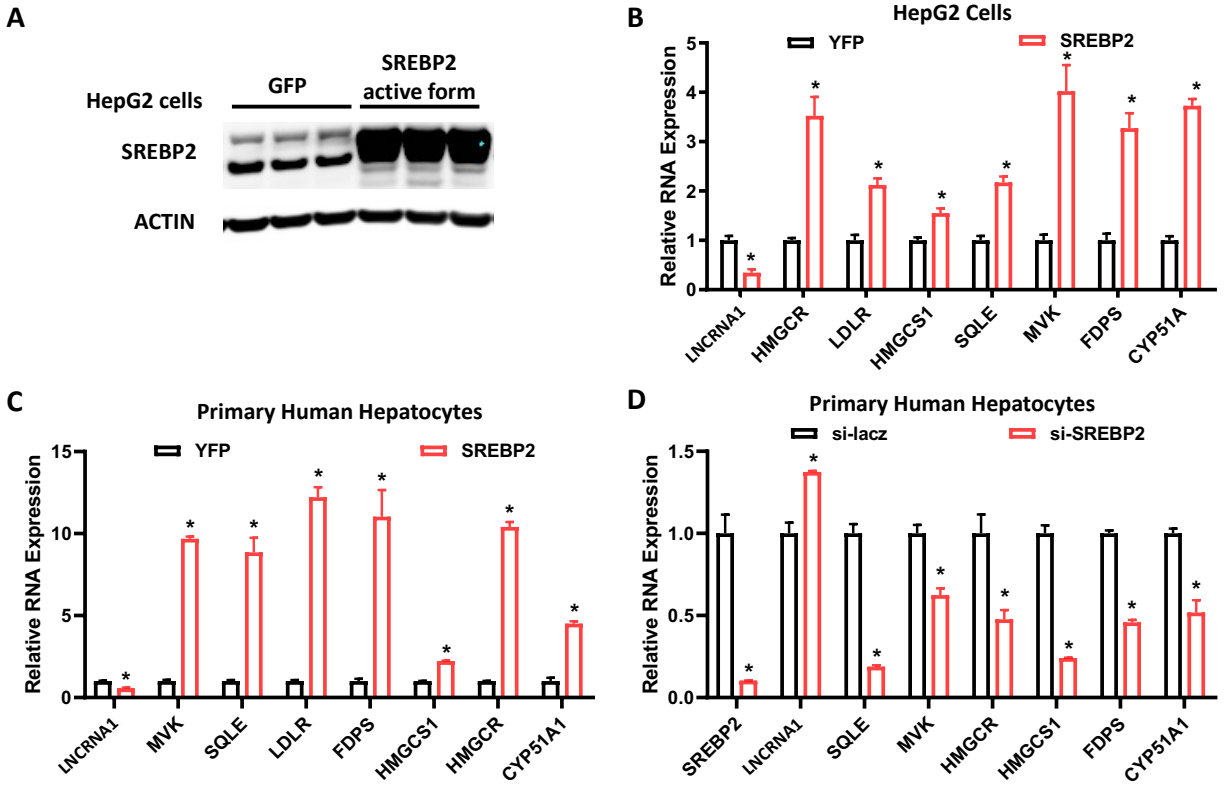

Fig. 3. The regulation of LNCRNA1 by SREBP2. (A) Western blot analysis of SREBP2 active form and ACTIN. (B-D) qRT-PCR analysis of LNCRNA1 and the SREBP2 pathway genes in control or mature form of SREBP2 OE or SREBP2 KD in (B) HepG2 or (C-D) PHHs. n=3, Error bars are SEM, * $p < 0.05$.

transcription factors regulate. To determine the role of LNCRNA1 in the SREBP2 pathway, we knocked down LNCRNA1 and measured the expression levels of SREBP2 pathway genes. Three different LNCRNA1 siRNAs (si-LNCRNA1) and control siRNA (si-lacz) were transfected into HepG2 cells for 48 hours. RT-PCR results indicated that the expression level of LNCRNA1 was successfully reduced by LNCRNA1 siRNAs (Fig. 4A). Interestingly, the expression levels of SREBP2 and its downstream cholesterol metabolic genes were increased by LNCRNA1 knockdown (KD) (Fig. 4A, B). Consistently, LNCRNA1 KD increases SREBP2 and its downstream genes in PHHs (Fig. 4C). Next, we examined cellular cholesterol content using filipin staining. We found that HepG2 cells or PHHs with LNCRNA1 KD have more cholesterol contents than the control (Fig. 5A, B). We also examined cellular cholesterol content by extracting lipids from the cells. Consistently, there are more cholesterols in LNCRNA1 KD cells using this lipid extraction method (Fig. 5C, D). Taken together, these data suggested that LNCRNA1 plays a feedback role in the SREBP2 pathway and serves as an important regulator in cholesterol metabolism.

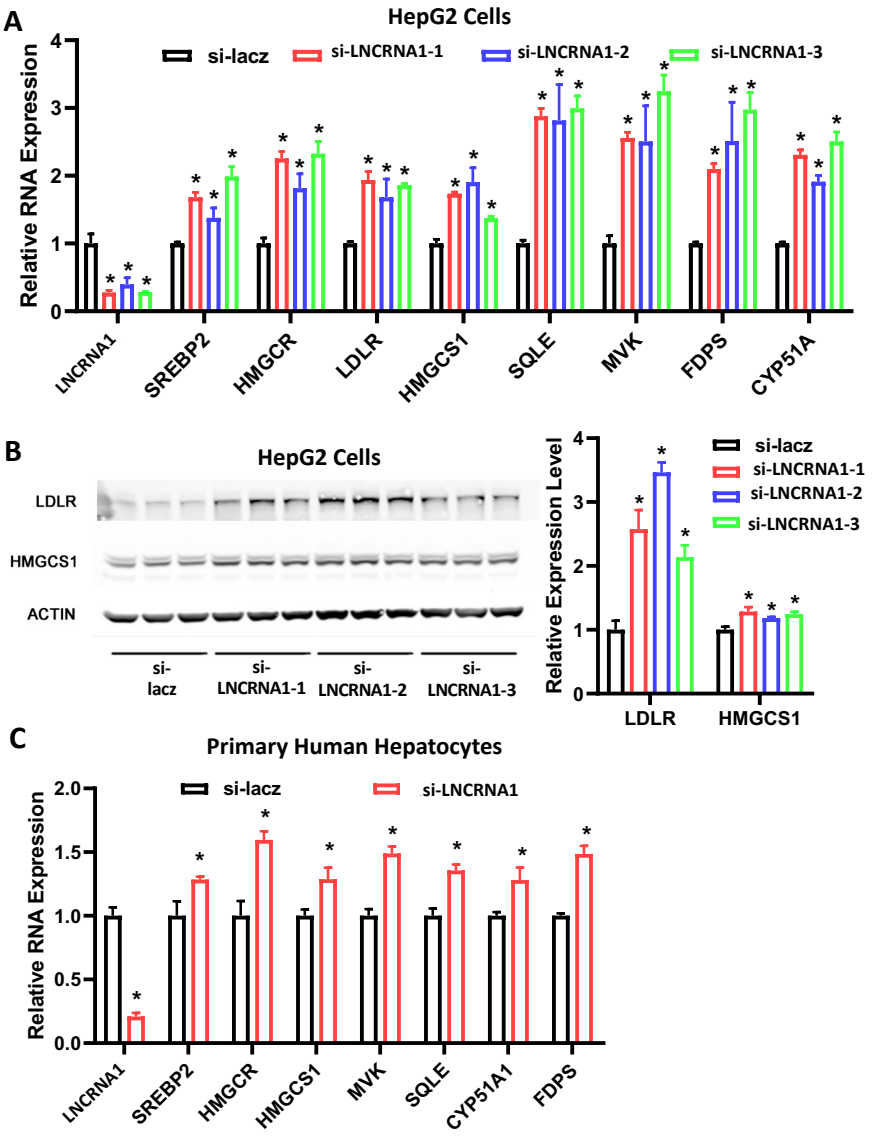

Fig. 4. LNCRNA1 KD increases the expression of the SREBP2 pathway genes. (A) qRT-PCR analysis of LNCRNA1 and the SREBP2 pathway genes in control (si-lacz) and LNCRNA1 KD HepG2 cells. (B) Western blot analysis of the protein levels of the SREBP2 pathway genes in HepG2 cells. Quantification is shown in the right panel. (C) qRT-PCR analysis of LNCRNA1 and the SREBP2 pathway genes in control (si-lacz) and LNCRNA1 KD (pooled siRNA1/2/3) PHHs. n = 3, Error bars are SEM, * $p < 0.05$.

LNCRNA1 inhibits the SREBP2 pathway genes at the transcriptional level. To determine whether LNCRNA1 regulates the transcription of SREBP2 pathway genes, we first performed an RNA stability assay using the transcription inhibitor actinomycin D. Control or LNCRNA1 KD HepG2 cells were treated with 2 μg/ml actinomycin D for 0, 3, and 6 hours. At the indicated time points, the RNA expression levels of SREBP2 pathway genes were determined by RT-PCR. Results showed that the RNA degradation rate of cholesterol synthesis genes such as HMGCR, HMGCS1, and MVK has no significant differences between the control and LNCRNA1 KD groups. This result suggested that LNCRNA1 regulates SREBP2 pathway genes through transcription rather than affecting their RNA stability (Fig. 6). Next, we used an RNA polymerase II (Pol II) Chromatin immunoprecipitation (ChIP) assay (Sigma) to determine the transcription levels of SREBP2 pathway genes since Pol II transcribes SREBP2 pathway genes. We performed a CHIP assay using an anti-Pol II antibody in HepG2 cells transfected with si-lacz or si-LNCRNA1. Mouse IgG has been used as a negative control antibody. The results showed that more DNA fragments from the transcription region of SREBP2 pathway genes were pulled down by Pol II protein in the si-LNCRNA1 group (Fig. 7), suggesting that LNCRNA1 KD increases the transcription of SREBP2 pathway genes. These results indicated that LNCRNA1 is a suppressor of the transcription of SREBP2 pathway genes.

LNCRNA1 OE in the livers of wild-type (WT) mice reduces the liver and plasma cholesterol levels. To investigate whether hepatic LNCRNA1 modulates cholesterol levels in vivo, we overexpressed human LNCRNA1 in WT mouse liver using adenovirus since adenovirus almost exclusively targets the liver. Fourteen days after the adenovirus injection, mice were sacrificed, and the liver tissue and blood were collected for analysis. As shown in Fig. 8A, human LNCRNA1 was successfully expressed in mouse liver. Next, the expression levels of mouse Srebp2 pathway genes were measured. The results showed that human LNCRNA1 OE suppressed the expression of Srebp2 pathway genes (Fig. 8B, C). Moreover, CHIP results showed that in the LNCRNA1 OE sample, fewer DNA fragments

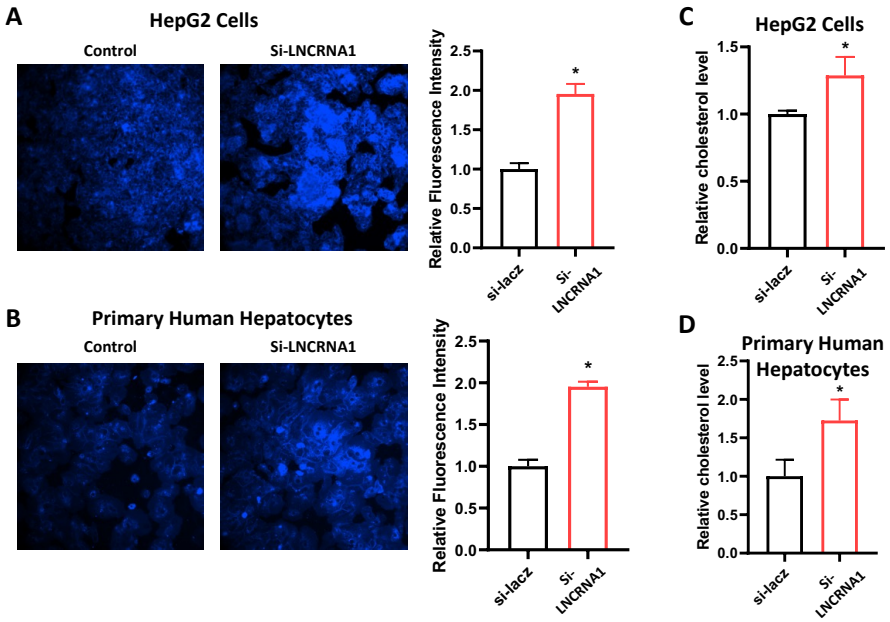

Fig. 5. LNCRNA1 KD increases cholesterol content in HepG2 and human primary hepatocytes. (A-B) Representative image of filipin staining of cholesterol contents in control or LNCRNA1 KD (A) HepG2 or (B) PHHs. The quantification results are shown on the right. (C-D) The cellular cholesterol levels in control or LNCRNA1 KD (C) HepG2 or (D) PHHs measured by extracting lipids from the cells.

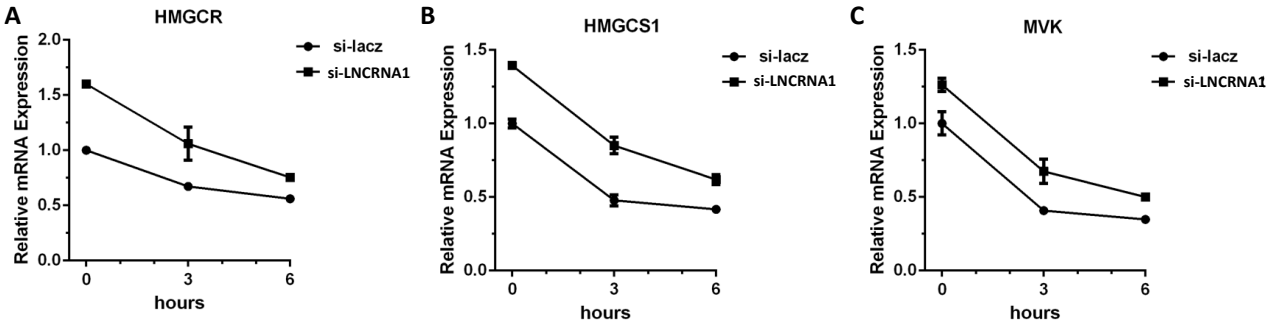

Fig. 6. LNCRNA1 does not regulate the RNA stability of the SREBP2 pathway genes. (A-C) qPCR analysis of HMGCR (A), HMGCS1 (B), and MVK (C) in control (si-lacz) and LNCRNA1 KD HepG2 cells treated with actinomycin D for 0, 3, and 6 hours. n = 3, Error bars are SEM, * $p < 0.05$.

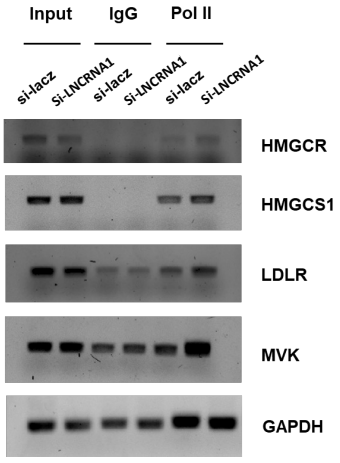

Fig. 7. LNCRNA1 KD promotes the transcription of the SREBP2 pathway genes. ChIP-PCR analysis of the promoter regions of the SREBP2 pathway genes in control (si-lacz) and LNCRNA1 KD HepG2 cells using an anti-pol II antibody.

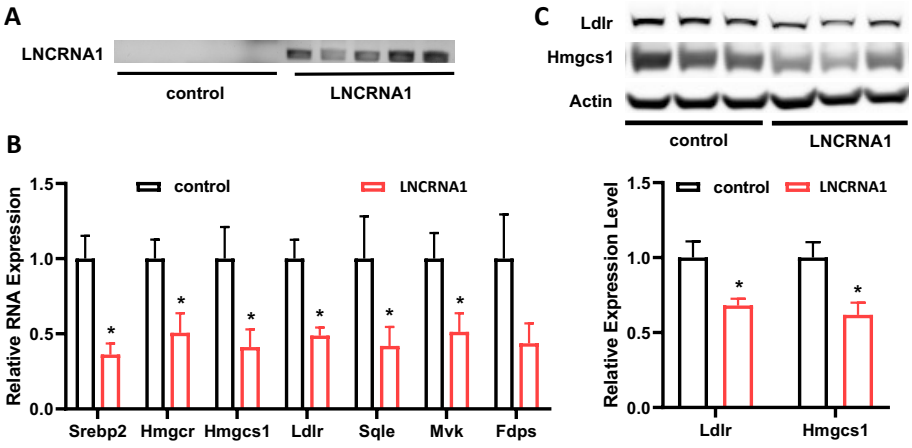

Fig. 8. LNCRNA1 suppresses the expression of the Srebp2 pathway genes in mouse liver. (A-B) qRT-PCR analysis of human LNCRNA1 (A), and mouse Srebp2 pathway genes in control and human LNCRNA1 OE mouse liver (n=7) (B); and (C) Western blot analysis of the protein levels of the Srebp2 pathway genes in control and human LNCRNA1 OE mouse liver. Quantification is shown in the bottom panel. Error bars are SEM, * $p < 0.05$.

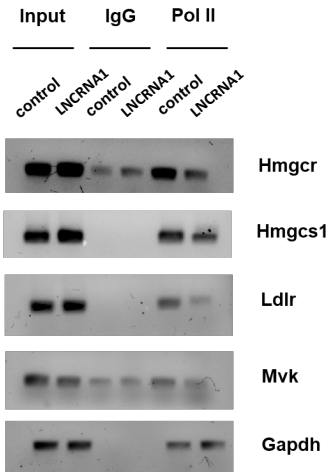

Fig. 9. LNCRNA1 OE suppresses the transcription of the SREBP2 pathway genes. ChIP-PCR analysis of the promoter regions of the SREBP2 pathway genes in control and human LNCRNA1 OE mouse liver using an anti-pol II antibody.

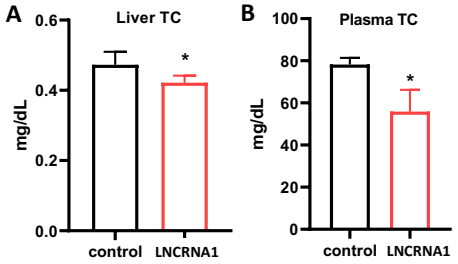

Fig. 10. LNCRNA1 reduces the liver and plasma cholesterol levels. (A-B) The total cholesterol levels of liver (A) and plasma (B) in control and human LNCRNA1 OE mice. n = 7, Error bars are SEM, * $p < 0.05$.

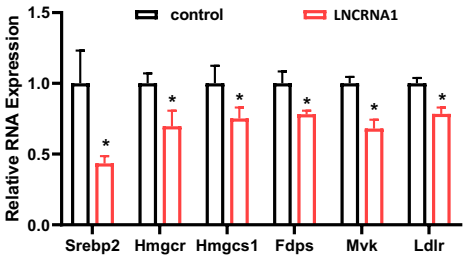

Fig. 11. LNCRNA1 suppresses the expression of the Srebp2 pathway genes in vitro. qRT-PCR analysis of mouse Srebp2 pathway genes in control and human LNCRNA1 OE AML12 cells (n=3) Error bars are SEM, * $p < 0.05$.

from the transcription region of SREBP2 pathway genes were pulled down by Pol II protein (Fig. 9), suggesting that LNCRNA1 OE inhibits the transcription SREBP2 pathway gene, and this function of LNCRNA1 in vivo is consistent with its function in vitro. Furthermore, the total cholesterol (TC) level in the liver and plasma was measured. Our results showed that the level of TC was decreased in LNCRNA1 OE liver and plasma (Fig. 10A, B). These results suggested that LNCRNA1 may be a key regulator in cholesterol metabolism in vivo. Consistently, LNCRNA1 OE inhibits the expression of SREBP2 pathway gene in cultured mouse hepatocyte cell line AML12 cell (Fig. 11).

In summary, our data suggest that LNCRNA1 plays a central role in the SREBP2 regulatory network of cholesterol metabolism. This further indicates the importance of LNCRNA1 in maintaining cholesterol homeostasis and the therapeutic potential of this lncRNA in Familial Hypercholesterolemia.

What opportunities for training and professional development has the project provided?

Nothing to Report.

How were the results disseminated to communities of interest?

Nothing to Report.

What do you plan to do during the next reporting period to accomplish the goals?

We will finish the rest of 3 major tasks listed above: Major Tasks 2, 3, and 6.

4. Impact

Nothing to Report.

5. Changes/Problems

Nothing to Report.

6. Products

Nothing to Report.

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

(1) PDs/PIs

Name:	*Ling Yang*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*0000-0002-7586-3922*
Nearest person month worked:	*3.6*
Contribution to Project:	*Dr. Yang directed and overseed all the aspects of the proposed studies*
Funding Support:	*This grant and Dr. Yang's start-up funding from Temple University*

(2) Each person

Name:	*Qingchun Lu*
Project Role:	*Postdoc fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Lu performed the proposed experiments, collect and analyze the data.*
Funding Support:	*This grant*

Name:	*Qian Guo*
Project Role:	*Postdoc fellow*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Guo helped with proposed experiments*
Funding Support:	*This grant and Dr. Yang's start-up funding from Temple University*

Name:	*Mingyang Xin*
Project Role:	*Graduate student*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*1*

Contribution to Project:	*Mingyang helped with proposed experiments*
Funding Support:	*Dr. Yang's start-up funding from Temple University*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. Special Reporting Requirements

None

9. Appendices

None