Original Research

FUS regulates the alternative splicing of cell proliferation genes related to atherosclerosis

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Impact Statement

FUS plays a significant role as an RNA-binding protein in several cellular processes, including RNA splicing, DNA repair, and transcriptional regulation. However, the RNA-binding capacity of FUS in atherosclerosis is unclear. This study showed that FUS regulated the transcriptional and AS levels of genes closely involved in cell apoptosis and proliferation of the HUVECs transcriptome, which indicates new knowledge of the mechanisms of FUS associated with atherosclerosis.

Abstract

FUS plays a significant role as an RNA-binding protein in several cellular processes, including RNA splicing, DNA repair, and transcriptional regulation. However, the RNA-binding capacity of FUS in atherosclerosis is unclear. We aimed to study the functions of FUS in inflammatory regulation through the role of the splicing factor. We knocked down FUS with siRNA to further study the overall transcriptional level and select alternative splicing (AS) of FUS regulation in human umbilical vein endothelial cells (HUVECs) by RNA sequencing. The results suggested that the knockdown of FUS significantly affected gene expression in HUVECs. In addition, the knockdown of FUS resulted in 200 differentially expressed genes (DEGs) that were highly related to apoptotic process, signal transduction, multicellular organism development, cell adhesion and regulation of transcription, and DNA-templated pathways. Importantly, FUS extensively regulated 2870 AS events with a significant difference. Functional analysis of its modulated AS genes revealed they were highly enriched in cell cycle and cell population proliferation pathways. The qRT-PCR and RNA-seq data showed consistent results. Our findings suggested new knowledge of the mechanisms of FUS associated with atherosclerosis.

Keywords: FUS, RNA-seq, RNA-binding protein, alternative splicing, atherosclerosis

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Introduction

Atherosclerosis is a chronic inflammatory process of the cardiovascular system with spectral pathophysiological features, including abnormal lipid metabolism, endothelial dysfunction, vascular inflammation, and apoptosis or proliferation of vascular smooth muscle cells (VSMCs).[1] Atherosclerosis is characteristic of lipid deposition and inflammation in the intima of many middle-sized and large arteries.[2] In the early stage of the lesion, lipid deposition in the vascular intima, oxidized low-density lipoprotein (ox-LDL), and cholesterol in elevated lipoprotein lead to functional injury to the arterial intima. Dysfunctional endothelial cells create chemokines and growth factors to induce the migration, accumulation and proliferation of monocytes and VSMCs in the intima. After the circulating monocyte migrates to the endothelial cells, it is stimulated by lipoprotein, which is gradually divided and differentiated into macrophages, phagocytize ox-LDL through scavenger receptors and transform into foam cells, which initiate the formation of fatty streaks.[3,4] With the increase of foam cells, fatty streaks develop into atherosclerotic plaques under the action of cytokines secreted by macrophages.[4] As the fibrous tissue and surrounding smooth muscle proliferate, the atherosclerotic plaques grow continuously, which narrows the lumen of the vessel and reduces the blood flow.[5] Deposition of connective tissue and calcium leads to arteriosclerosis, plaque enlargement leads to blocking the arterial lumen, and the tissue or organ supplied by the artery becomes ischemia or necrosis, which can lead to a variety of cardiovascular diseases including myocardial infarction, claudication, stroke, and heart failure.[6] RNA-binding proteins (RBPs) are a significant protein family involved in RNA regulation and metabolism, which mediate RNA maturation, splicing, transport, localization, and translation through interacting with the 3′-UTR of target mRNAs.[7] Several studies have demonstrated that RBPs have a direct bearing on atherosclerosis. Ramírez *et al*. found that the expression of HuR protein (an RBP) was increased in macrophages of atherosclerotic plaques. HuR may make a valuable contribution to atherosclerosis by post-transcriptional regulation of ABCA1 to increase cholesterol efflux and raise circulating HDL-C levels.[8]

Table 1. The sequence table for siRNAs and negative control.

Name	Forward	Reverse
siRNA1	GCCAAGAUCAAUCCUCCAUTT	AUGGAGGAUUGAUCUUGGCTT
siRNA2	CGUGGUGGCUUCAAUAAAUTT	AUUUAUUGAAGCCACCACGTT
siRNA3	CCACCUGUGAGAAUAUGAATT	UUCAUAUUCUCACAGGUGGTT
Control	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT

FUS is a nucleocytoplasmic shuttle protein with multifunctional and multiple domains, pertaining to the TET family of RBPs.[9] It is mainly expressed in the nucleus, yet it could also shuttle between the nucleus and cytoplasm. The N-terminus of FUS plays as a transcriptional activation portion, while the C-terminal portion can bind to RNA. FUS plays a vital role in many cellular processes, including regulation of transcription, RNA splicing, DNA repair, transport, and damage response.[10] Aberrant expression of FUS leads to the occurrence of various human tumors and is associated with neurodegeneration. FUS-CHOP is formed after fusing the N-terminal portion of FUS with the pro-apoptotic transcription factor CHOP, which can lead to the occurrence of myxoid liposarcoma.[11] Studies showed that FUS in Ewing family tumors fused with ERG through at (16; 21) (P11; q24), as well as fused with FEV through at (2;16) (q35; p11) translocation.[12,13] The mutation of FUS could result in amyotrophic lateral sclerosis, in which motor neurons that control muscle movement slowly degenerate and die, and the muscle gradually shrinks.[14] Moreover, a recent study reported that FUS was highly enriched in the cardiomyocyte nucleus by immunofluorescence staining and subcellular separation analysis and was associated with the promotion of cardiomyocyte apoptosis after myocardial infarction.[15] Wang *et al.*[16] found that FUS inhibits the proliferation and migration of HUVECs and reduces the inflammatory response in atherosclerosis. However, the RNA-binding ability of FUS in atherosclerosis remains so far unknown. To determine the underlying functions of FUS in regulating gene expression and AS that may be involved in atherosclerosis, this study knocked down FUS expression in HUVECs and obtained the regulated transcriptomes by RNA sequencing. Then, we performed qRT-PCR to verify FUS-regulated differentially expressed genes (DEGs) and AS events. In conclusion, this study demonstrated that RNA-binding protein FUS acts as a key regulator of differential splicing in atherosclerosis.

Materials and methods

FUS cloning and plasmid construction

This study designed all three siRNA duplexes (Table 1) from Gemma (Suzhou, China). The final selected siRNA targeting FUS (siFUS): 5′-CCACCUGUGAGAAUAUGAATT-3′ (sense). PIRES-hrGFP-1a vector was digested with *Eco*RI and *Xho*I (NEB) for 2-3 h, and then gel electrophoresis was performed on 1% agarose gel, purified with Qiagen column kit (Qiagen. Inc, Valencia, CA, USA). This study utilized TRIzol reagent (15596-018, Ambion, Texas, USA) to extract total RNA from HUVECs and synthesized cDNA with oligonucleotide primers. The inserted fragment was amplified through the PCR strategy. We used the ClonExpress II One Step Cloning Kit (C112, Vazyme, Nanjing, China) to add the vector and the inserted fragment into PCR microtubes and introduced plasmids into *Escherichia coli* strains through chemical transformation. We used universal primers to screen colonies through PCR (28 cycles). The insertion sequence was verified through Sanger sequencing.

Cell culture and transfections

The HUVECs line (Angio-Proteomie, Boston, MA, USA) was cultured in Ham's F-12 K at 37℃ with 5% CO_2. Following the manufacturer's instructions, We insert siRNA into a cell by Lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA, USA). The transfected cells were harvested after 48 h for qRT-PCR and western blotting.

Assessment of gene expression

This study utilized GAPDH to evaluate the effects of FUS knockdown. qRT-PCR was conducted on the Bio-Rad S1000 with HieffTM qPCR SYBR® Green Master Mix (Low Rox Plus 11202ES08, Yeasen, Wuhan, China). Supplementary Table S1 shows the information of primers used in this study. Then, we normalized the gene expression data to GAPDH mRNA levels through $2^{-\Delta\Delta CT}$ method.[17] Comparing the differences between the two results using the paired Student's *t*-test through the GraphPad Prism software (San Diego, CA, USA).

Western blot

HUVECs were cracked in ice-cold Wash Buffer supplemented with a protease inhibitor cocktail (Roche) and incubated on ice for 30 min. We used 1× SDS sample buffer to boil the sample in boiling water for 10 min and separated it on 10% SDS-PAGE. At room temperature, incubate the membranes with primary antibody for 1 h using TBST buffer (20 mM Tris-buffered saline and 0.1% Tween-20) containing 5% nonfat milk powder, followed by HRP-conjugated secondary antibody, FUS antibody (1:1000, A5921, ABclonal, Wuhan, China), GAPDH (1:5000, ATPA00013Rb, AtaGenix, Wuhan, China). Finally, we used the enhanced chemiluminescence (170506, BioRad, California, USA) to detect the binding secondary antibody (HRP Goat Anti-Mouse IgG (H+L) (1:5000, AS003, ABclonal, Wuhan, China).

RNA extraction and sequencing

About 1 × 105 cells were provided for RNA-seq library construction for each sample. We used TRIzol reagent (Invitrogen, cat. No 15596026) to extract total RNA from HUVEC according to the method of Chomczynski and Sacchi[18] We used RQ1 DNase (M6101, Promega, Wisconsin,

USA) to remove DNA from total RNA. The quality and quantity of the purified RNA were determined by measuring the absorbance at 260 nm/280 nm (A_{260}/A_{280}) using NanoPhotometer N50 (Implen, Munich,Germany). The integrity of RNA was further verified by 1.0% agarose gel electrophoresis. VAHTS® Universal V8 RNA-seq Library Prep Kit for Illumina (NR605,Vazyme,Nanjing,China) was used for the stranded RNA sequencing library with 1 μg total RNA per sample. Finally, the libraries were prepared following the manufacturer's instructions and applied to Illumina Novaseq 6000 system for 150 nt paired-end sequencing.

RNA-seq raw data clean and alignment

This study first discarded more than 2 N bases in raw data and used the FASTX toolkit (version 0.0.13) to filter the adapter and low-quality bases (<20). After that, HISAT software was utilized to align quality-filtered reads onto the GRCH38 genome, allowing no more than four mismatches.[19] The gene expression levels were estimated as fragments per kilobase per million reads according to the previously reported method.[20]

DEGs analysis

This study screens the DEGs by the "DESeq2" package (Version 1.34.0) of R software (Version 4.1.0).[21] The screening criteria are P value <0.01 and fold change >1.5.

Alternative splicing analysis

This study defined and quantified alternative splicing events (ASEs) and regulatory alternative splicing events (RASEs) between samples by the ABLas pipeline.[22,23] ABLas could detect ten types of ASE on the basis of splice junctions, including exon skipping (ES), alternative 5′ splice site (A5SS), and alternative 3′ splice site (A3SS). We assessed the significance of changes in AS event ratios by Student's t-test. Those events with significant significance at the cutoff point of P value (corresponding to the cutoff point of 5% false discovery rate (FDR)) were regarded as RBP-regulated ASEs.

Functional enrichment analysis

Gene Ontology (GO) and KEGG pathways were identified by KOBAS 2.0 server to explore the functional enrichment categories of DEGs.[24] We defined the enrichment for each term by the hypergeometric test and the Benjamini-Hochberg FDR with criteria as $P<0.05$ and FDR <0.25.

Results

FUS regulates gene expression in HUVECs

In order to explore the molecular machinery of FUS-mediated gene expression, we performed the siRNA-mediated gene-silencing method to knock down FUS expression in HUVECs. Moreover, high-quality RNA-seq sequencing experiments were conducted to investigate the molecular mechanisms of FUS-mediated transcriptional and posttranscriptional regulation in AS. After treatment with HUVECs for 48 hours, the inhibitory effect of predesigned siRNAs targeting FUS was determined by qRT-PCR. The results demonstrated that FUS siRNAs (siFUS) inhibited the expression of FUS at the level of mRNA and protein (Figure 1(A) and (B)). Principal component analysis (PCA) was conducted on all samples according to the gene expression levels. The closer the distance, the higher the similarity between samples. The results showed that the samples in the siFUS group and control group were obviously separated (Figure 1(C)), indicating that there was a certain difference in gene expression composition between the two groups. A total of 29,678 genes were identified based on the RNA-seq data from the siFUS and control samples. Differential expression analysis indicated that 200 DEGs (142 upregulated, 58 downregulated) might be regulated by FUS at the level of transcription (Figure 1(D)). Cluster analysis of DEGs expression in the siFUS and control samples showed that the gene expression pattern was highly in accordance with FUS-mediated transcription in the two groups (Figure 1(E)). These results suggest that FUS widely regulates gene expression.

FUS-regulated expression of cell apoptosis genes in HUVECs

This study performed GO enrichment analysis of DEGs after silencing FUS, and the results indicated that upregulated genes were primarily enriched in "apoptotic process," "signal transduction," "ion transport," "multicellular organism development," and other terms. While downregulation of DEGs was predominantly involved in "cell adhesion" and "regulation of transcription, DNA-templated" pathways. Among them, the "apoptotic process" is closely related to atherosclerosis.[25] The most representative GO terms of up or down-regulated DEGs were shown in Figure 2(A). The top 25 DEGs were screened according to the expression level based on up and down-regulated gene lists. The expression levels of six genes related to atherosclerosis were considerably different between the siFUS and control samples. FUS actively regulates the expression of IFIT2, IDO1, CCL5, and NEAT1 and oppositely modulates the expression of FERMT2 and AKT3 in HUVECs. We validated the reliability of DEGs from RNA-seq data by qRT-PCR strategy, as illustrated in Figure 2(B), the results of qRT-PCR were consistent with RNA-seq data.

FUS regulates alternative splicing of genes in HUVECs

A key objective of our study was to analyze the RASEs of FUS in HUVECs. We utilized ABLas software to perform the global changes in the AS profiles after the FUS knockdown. A total of 2780 FUS-regulated ASEs were detected, including 180 3pMXE1, 232 5pMXE1, 545 A3SS, 92 A3SS&ES, 725 A5SS, 127 A5SS&ES, 316 ES, 173 MXE, and 390 cassette exons, which indicated that FUS could globally regulate AS events of HUVECs (Figure 3(A)). We then performed GO enrichment analyses on the RASEs-associated genes (regulated alternatively spliced genes [RASGs]). We found that genes whose AS levels were regulated by FUS were highly enriched in "viral process," "protein transport," "cell cycle," "mRNA transport," "cellular response to DNA damage

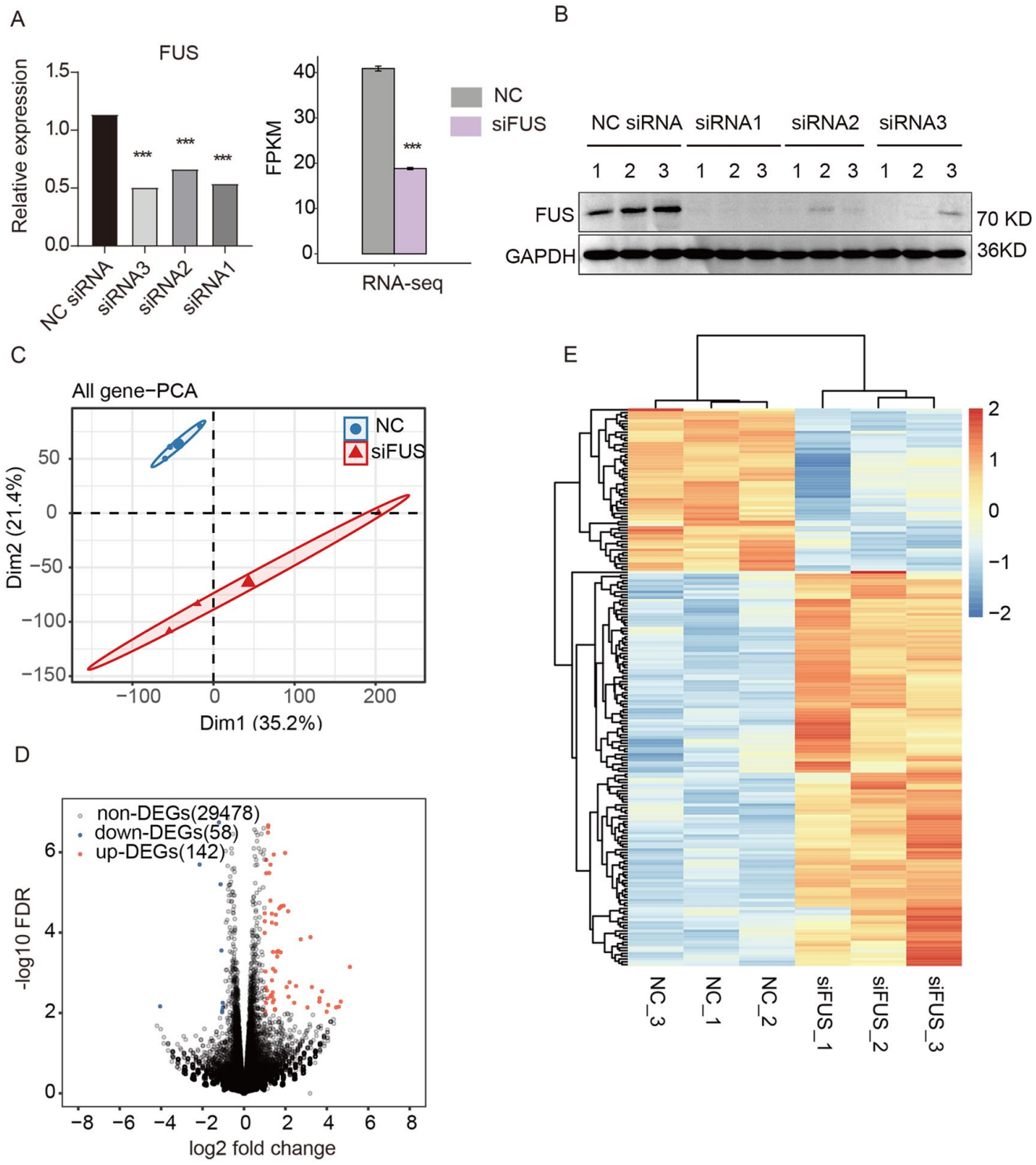

Figure 1. Differential gene expression responding to FUS knockdown in HUVECs. (A) Bar plot showing the knockdown efficiency for three siRNAs of FUS from RT-qPCR result (left panel) and RNA-seq data (right panel). (B) Western blot result showing the decreased level of FUS in HUVECs. (C) PCA result showing the global expression pattern of RNA-seq data. (D) Volcano plot showing the upregulated (red) and down-regulated (blue) genes. (E) Heatmap showing the high expression (red) and low expression (blue) of DEGs.

stimulus," "G2/M transition of mitotic cell cycle," "cell population proliferation" and other pathways. The top 10 terms of GO analyses are shown in Figure 3(B). Then, we overlapped the FUS- RASGs and DEGs and found that 12 genes were significant differences in the expression level and AS level (Figure 3(C)). This study evaluated potential differential splicing events identified by RNA-seq results using qRT-PCR. The results of RASEs validated through

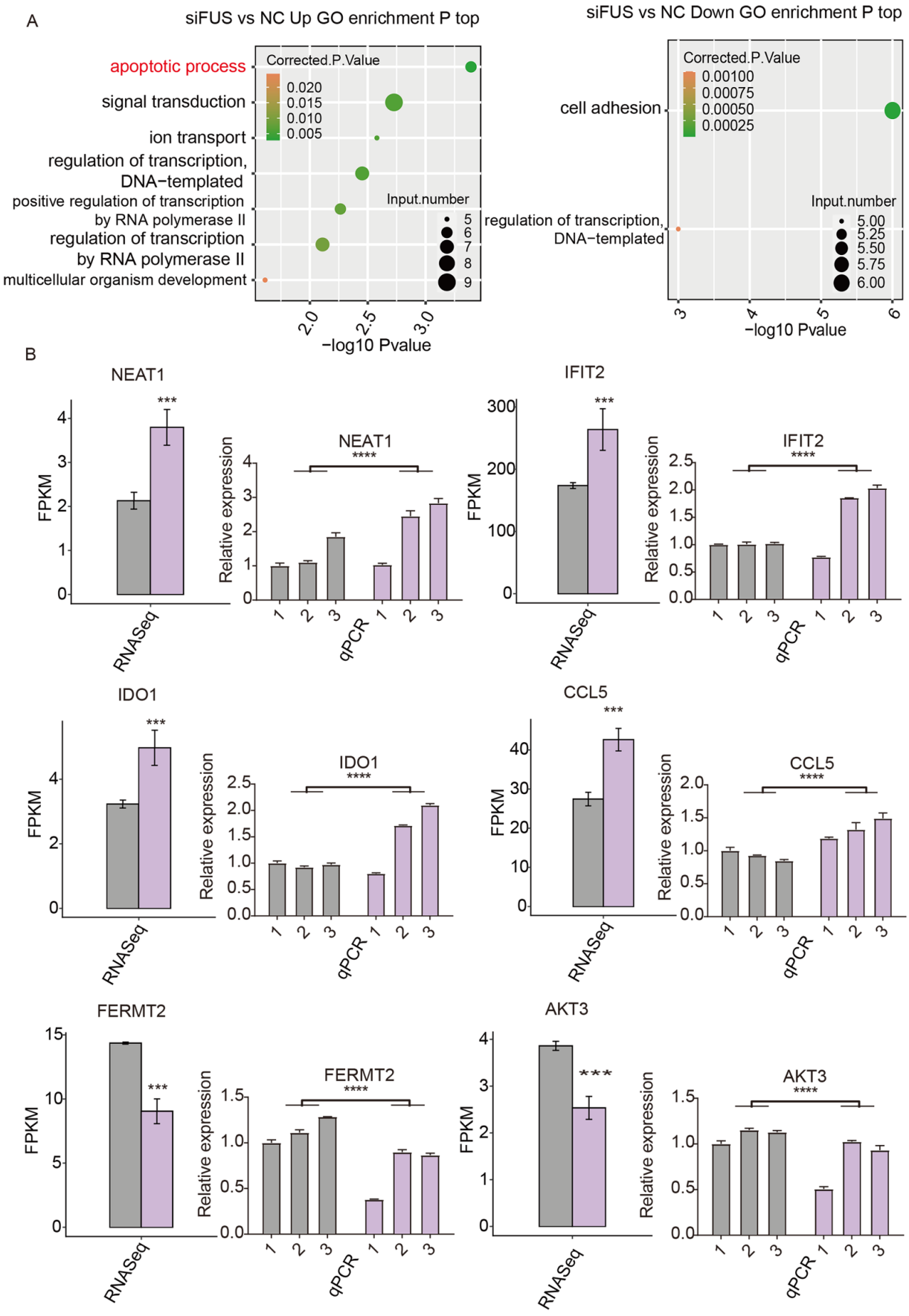

Figure 2. FUS regulates the expression of apoptosis-related genes. (A) The top 10 GO terms of up or down-regulated genes. (B) Genes expression of FUS regulated from RNA-seq data and qRT-PCR validation.

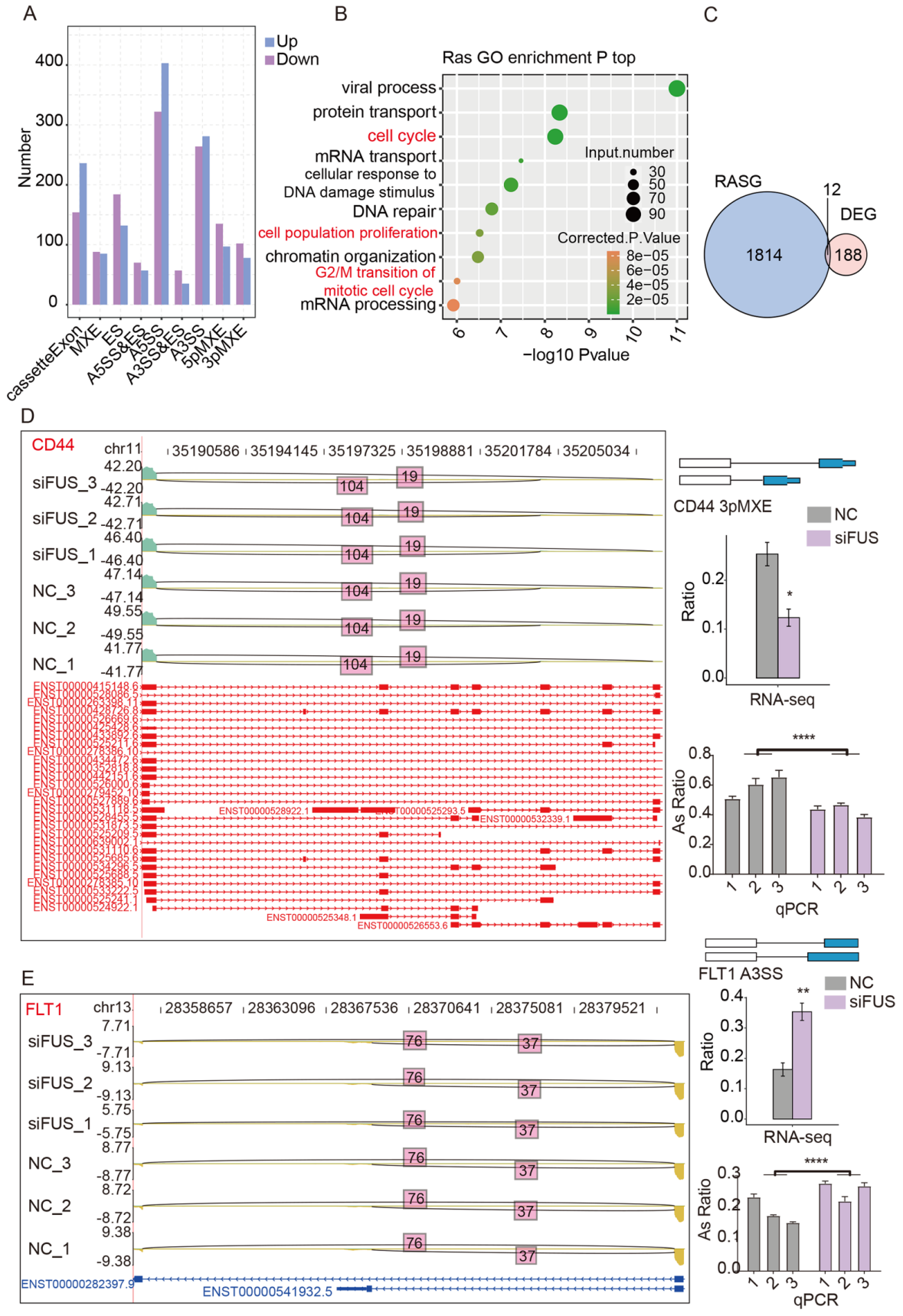

Figure 3. FUS regulates gene alternative splicing in HUVECs. (A) Frequency distribution of different alternative splicing events types regulated by FUS. (B) The top 10 GO biological processes of RASGs. (C) Venn diagram illustrating the overlap genes of RASGs and DEGs. (D and E) FUS regulates the alternative splicing of CD44 and FLT1. IGV-sashimi diagrams illustrate AS changes in FUS knockdown cells and control cells (left panel). The schematic plots describe the structures of ASEs (right panel, top). The constitutive exon sequences are represented by white boxes, and the intron sequences are represented by the horizontal line, while the alternative exons are represented by blue boxes. The bottom of the right panel shows the RNA-seq quantification and qRT-PCR validation of ASEs.

the qPCR strategy were in accordance with the RNA-seq data, which showed that FLT1 and LRRFIP1 were considerably increased, while CD44 was distinctly decreased in the siFUS samples (Figures 3(D), (E) and S1). These genes are involved in vascular inflammation and proliferation, and neointima formation directly or indirectly engaged in atherosclerosis.[26–28]

Discussion

The regulatory mechanism of the RNA-binding protein FUS-mediated transcription in the process of arteriosclerosis is still unclear. To study the underlying molecular functions of FUS in regulating gene expression that may be associated with atherosclerosis, we attained FUS-regulated transcriptome data in HUVECs by RNA-seq strategy. Transcriptome sequencing analysis showed that the knockdown of FUS resulted in an extensive change of gene expression profiles in HUVECs. FUS knockdown caused upregulation or downregulation of several genes in HUVECs, including IDO1, CCL5, NEAT1, IFIT2, FERMT2, and AKT3. In addition, these genes were mainly enriched in "apoptotic process," "signal transduction," "ion transport," "multicellular organism development" and other terms. In addition, FUS regulates the AS of multiple genes, including FLT1, CD44, and LRRFIP1. Changes in the expression levels of these genes are mainly involved in biological processes associated with cell proliferation.

Apoptosis affects the morphology and structure of atherosclerotic arteries, as well as plaque stability. There are many kinds of cells under the apoptosis or necrosis process in atherosclerotic plaque, including endothelial cells, VSMCs, and inflammatory cells. In addition, excessive cell apoptosis leads to plaque instability. Our findings revealed that the expression of genes involved in the apoptotic process, including IFIT2, was specifically overexpressed after the knockdown of FUS expression. Stawowczyk *et al.* reported that IFIT2 expression activates caspase-3 to promote apoptosis via a mitochondrial pathway in various human cell lines. The apoptotic effect of IFIT2 is independent of the P53 signaling pathway but depends on pro-apoptotic proteins including Bax and Bak.[29] A recent study showed that IFIT1, IFIT2, and IFIT3 were significantly overexpressed in M1-polarized macrophages, and immunohistochemical staining of ApoE−/− mice tissues also confirmed the expression level of IFIT, suggesting that IFITs may be potential markers of atherosclerosis.[30]

Significant upregulation of IDO1, CCL5, and NEAT1 genes was consistently revealed by RNA-seq and qPCR of HUVECs. As a rate-limiting enzyme, IDO1 catalyzed tryptophan degradation through the kynurenine pathway, which has been investigated in the pathogenesis of atherosclerosis. IDO1 is expressed in various cells of the artery wall, including endothelial cells, VSMCs, and immune cells.[31–34] Proinflammatory modulators could induce IDO1 to mediate vasodilation and immune regulation in endothelial cells.[35,36] The role of IDO1 in atherogenesis is complicated, which can not only promote the progress of atherosclerosis but also against established atherogenesis. Liang *et al.*[37] found that IDO1 expression and activity can accelerate the progression of atherosclerosis in atherosclerotic blood samples and foam cells. In a cohort study of young adults (aged 24–39 years), Pertovaara *et al.*[38] found that IDO1 activity was significantly related to carotid intima-media thickness (IMT), an early atherosclerosis biomarker, in young women but not in men. The most likely mechanism is IDO1 participated in the immune activation of early atherosclerosis. In addition, another cohort study of 921 older populations (age range 46–76 years) confirmed that IDO1 activity positively correlated with carotid IMT in both males and females.[39] In contrast, Polyzos *et al.*[40] reported that inhibition of IDO1 with 1-MT in *Apoe*$^{-/-}$ mice accelerates atherosclerosis, vascular inflammation, and protein distribution and promotes an unfavorable lipoprotein profile. Krohn *et al.*[41] found that CCL5 upregulated in neointimal versus medial smooth muscle cells compared with medial cells in atherosclerosis-prone ApoE$^{-/-}$ mice, which mediated monocyte adhesion and promoted neointima formation. Braunersreuther *et al.*[42] reported that CCL5 could recruit leukocytes to infiltrate the arterial wall; the enhanced infiltration of immune cells accelerated the progression of atherosclerosis. The recruitment of macrophages and T cells to plaque area was found to be inhibited using CCL5 antagonists, but it was not confirmed which immune cells CCL5 acted on.[43] Using q-PCR analysis and immunofluorescent staining, Li *et al.* reported that CCL5 was mainly expressed in CD45 + leukocytes of the aorta and colocalized with MAC3, a macrophage marker, which indicated that the immune cells mainly expressing CCL5 in atherosclerotic plaque are macrophages. Moreover, IFN-γ was found to promote CCL5 expression in macrophages.[44] A recent study showed that NEAT1 was elevated in the blood serum of patients with atherosclerosis. Low-density lipoprotein (ox-LDL) could induce atherosclerosis by promoting endothelial cell dysfunction and activating foam cell formation, regulating VSMCs and other mechanisms. Downregulation of NEAT1 expression reduces ox-LDL-induced HAECs cell proliferation and triggers apoptosis, suggesting that NEAT1 might be involved in the prevention of atherosclerosis.[45] Similarly, Vlachogiannis *et al.*[46] indicated that the expression of lncRNA NEAT1 was substantially elevated in carotid atherosclerotic arterial tissues than normal tissues, which was consistent with the results in serum. Wang *et al.*[47] demonstrated that lncRNA NEAT1 inhibited ox-LDL-induced lipid deposition and inflammatory reaction in THP-1 cells through sponge miR-342-3p, suggesting that NEAT1 is a potential marker of atherosclerosis.

FUS knockdown caused the downregulation of several genes in HUVECs, including FERMT2 and AKT3. FERMT2 is a cytoplasmic protein necessary for platelet aggregation and thrombosis, which is involved in the process of atherosclerosis.[48] Compared to control samples, the expression of FERMT2 was down-regulated in arterial plaque and positively correlated with the DEGs of smooth muscle-rich atherosclerotic plaque. FERMT2 might regulate neovascularization and vascular permeability in plaque.[49] A recent study demonstrated that the expression level of FERM2 decreased substantially in patients with atherosclerosis. FERM2 could interact with serum response factor to

transcriptionally inhibits smooth muscle cell-specific gene expression and regulate its cell location during endothelial-to-mesenchymal transition.[50] AKT3 is one of three closely related serine-threonine protein kinases that regulate a variety of biological pathways, such as cell proliferation, growth, metabolism, and angiogenesis. Evidence gathered in the review indicates that AKT3 is highly expressed in atherosclerosis, and its deletion inhibits the development of atherosclerosis.[51] Ding *et al.*[52] found that knockout of Akt3 enhanced LDL receptor-independent uptake and elevated ACAT1 expression level in Akt3$^{-/-}$ mice, which in turn promoted cholesterol accumulation, an early pivotal process in the development of atherosclerosis. However, the authors did not elucidate the mechanism of Akt3 deficiency in promoting macrophage lipoprotein uptake. Fortunately, in the following study, they found Akt3 inhibits phagocytosis of macrophages through WNK1/SGK1/Cdc42 pathway and protects against atherosclerosis.[53]

In this study, a total of 2780 RASEs with significant differences were identified through the analysis of RNA-seq data after the knockdown of FUS, which confirms that FUS broadly regulates differential splicing of genes in HUVECs. GO terms in which the differential RASGs were mainly enriched were associated with cell proliferation. For the AS results, we also confirmed that FLT1 and LRRFIP1 were upregulated, while CD44 was decreased in siFUS samples using qRT-PCR. This study provides new viewpoints on the differential splicing regulation of FUS in atherosclerosis. FLT1 is expressed in vascular endothelial cells, SMCs, and immune cells, which is essential in regulating angiogenesis, the development process of the embryonic vascular system, cell migration, cell survival, macrophage function, and chemotaxis. Thus, FLT1 may participate in the development of atherosclerotic plaques and angiogenesis.[26] To investigate the role of FLT1 in different stages of plaque formation, Aernout *et al.* chose different time points (5, 10, or 20 weeks of age) to treat ApoE$^{-/-}$ mice with anti-Flt1 for 5 weeks. Anti-Flt1 administration significantly inhibits atherosclerotic plaques, with a 50% reduction in aortic root plaques in early and intermediate lesions and a 50–25% reduction in the growth of advanced atherosclerotic plaques. Further study found that anti-Flt1 reduced macrophage infiltration by 40% in early and advanced atherosclerosis and inhibited the inflammation caused by macrophages in the adventitia, which may exert atherosclerosis protection by affecting the function of macrophages.[54] Platelets are the main cells in the process of atherosclerotic thrombosis. Through the study of 500 subjects, Goodall *et al.* found that LRRFIP1 protein is abundant in the cytoplasm of megakaryocytes and platelets, which is an important part of the platelet cytoskeleton. Based on the protein-protein interaction network analysis, the study reported that LRRFIP1 interacts with FLI1 and DBN1 proteins, all of which are involved in the construction of actin-binding and remodeling functional cassettes of cytoskeletal proteins. To further verify the role of LRRFIP1 protein in thrombogenesis, a significant reduction in thrombogenesis was observed after silencing LRRFIP1 by morpholino injection.[27] On the other hand, the proliferation and migration of VSMCs could cause arterial intima thickening, which is a key pathological process of atherosclerosis. Khachigian *et al.*[55] found that inhibition of LRRFIP1 expression blocked VSMC proliferation. Interestingly, Choe *et al.* observed the number of VSMCs at 1, 3, and 5 days after overexpression of LRRFIP1, and the results indicated that the number of cells decreased slightly at 1 day and increased at 3 or 5 days. In addition, Choe concluded that LRRFIP1 induced VSMC proliferation by the ERK1/2 signaling pathway.[56] Our results indicated that changes in LRRFIP1 expression possibly affect signaling pathways, such as "cell cycle" and "cell population proliferation," which are closely associated with VSMCs proliferation and intimal hyperplasia. Local inflammation is an important pathological process of atherosclerosis and plaque rupture.[57] Several recent studies have demonstrated that CD44 was highly expressed in vascular endothelial cells, inflammatory cells, and VSMCs under inflammatory conditions.[28] An early study on mice reported that CD44-deficient ApoE$^{-/-}$ mice reduced the development of atherosclerosis, and macrophages in the lesions were decreased by 90% compared with CD44 wild-type mice.[58] The conclusion was consistent with a study in humans; Krettek *et al.*[59] found that CD44 was present in macrophages of atherosclerotic plaques, and its level is more than ten times higher than that in healthy tissue. FUS may mediate gene regulation indirectly by affecting the function of its protein partners and directly through its RNA and DNA binding activity. Further research is needed to thoroughly investigate the potential molecular mechanisms, especially how FUS regulates gene expression level and AS by playing as an RBP.

Our study is the first to investigate that silencing FUS had regulatory effects on gene splicing and transcription in HUVECs. FUS positively regulates the transcription of genes related to the apoptotic process. Moreover, AS of genes regulated by FUS were mainly enriched in cell proliferation-related pathways. Our findings support a hypothesis that FUS might act as a vital role in atherosclerosis by regulating the expression and AS of proliferation-related genes, which provides new insights for guiding the signal network of atherosclerosis and potentially FUS-targeted therapy.

AUTHORS' CONTRIBUTIONS

JH designed the work. SY interpreted data. YG and NY drafted the manuscript. SJ approved the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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SUPPLEMENTAL MATERIAL

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REFERENCES

1. Falk E. Pathogenesis of atherosclerosis. *J Am Coll Cardiol* 2006;**47**: C7–12
2. Pieczynska MD, Yang Y, Petrykowski S, Horbanczuk OK, Atanasov AG, Horbanczuk JO. Gut microbiota and its metabolites in atherosclerosis development. *Molecules* 2020;**25**:594
3. Peng J, Xiao X, Hu M, Zhang X. Interaction between gut microbiome and cardiovascular disease. *Life Sci* 2018;**214**:153–7
4. Ghosh S, Zhao B, Bie J, Song J. Macrophage cholesteryl ester mobilization and atherosclerosis. *Vascul Pharmacol* 2010;**52**:1–10
5. Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardeña G. Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann N Y Acad Sci* 2000;**902**:230–9239
6. Frostegård J. Immunity, atherosclerosis and cardiovascular disease. *Bmc Med* 2013;**11**:117
7. Lunde BM, Moore C, Varani G. RNA-binding proteins: modular design for efficient function. *Nat Rev Mol Cell Biol* 2007;**8**:479–90
8. Ramírez CM, Lin CS, Abdelmohsen K, Goedeke L, Yoon JH, Madrigal-Matute J, Martin-Ventura JL, Vo DT, Uren PJ, Penalva LO, Gorospe M, Fernández-Hernando C. RNA binding protein HuR regulates the expression of ABCA1. *J Lipid Res* 2014;**55**:1066–76
9. Tan AY, Manley JL. The TET family of proteins: functions and roles in disease. *J Mol Cell Biol* 2009;**1**:82–92
10. Yamaguchi A, Takanashi K. FUS interacts with nuclear matrix-associated protein SAFB1 as well as Matrin3 to regulate splicing and ligand-mediated transcription. *Sci Rep* 2016;**6**:35195
11. Antonescu CR, Elahi A, Humphrey M, Lui MY, Healey JH, Brennan MF, Woodruff JM, Jhanwar SC, Ladanyi M. Specificity of TLS-CHOP rearrangement for classic myxoid/round cell liposarcoma: absence in predominantly myxoid well-differentiated liposarcomas. *J Mol Diagn* 2000;**2**:132–8
12. Shing DC, McMullan DJ, Roberts P, Smith K, Chin S-F, Nicholson J, Tillman RM, Ramani P, Cullinane C, Coleman N. FUS/ERG gene fusions in Ewing's tumors. *Cancer Res* 2003;**63**:4568–76
13. Ng TL, O'Sullivan MJ, Pallen CJ, Hayes M, Clarkson PW, Winstanley M, Sorensen PH, Nielsen TO, Horsman DE. Ewing sarcoma with novel translocation t(2;16) producing an in-frame fusion of FUS and FEV. *J Mol Diagn* 2007;**9**:459–63
14. Higelin J, Demestre M, Putz S, Delling JP, Jacob C, Lutz AK, Bausinger J, Huber AK, Klingenstein M, Barbi G, Speit G, Huebers A, Weishaupt JH, Hermann A, Liebau S, Ludolph AC, Boeckers TM. FUS mislocalization and vulnerability to DNA damage in ALS patients derived hiPSCs and aging motoneurons. *Front Cell Neurosci* 2016;**10**:290
15. Wu H, Zhao ZA, Liu J, Hao K, Yu Y, Han X, Li J, Wang Y, Lei W, Dong N, Shen Z, Hu S. Long noncoding RNA Meg3 regulates cardiomyocyte apoptosis in myocardial infarction. *Gene Ther* 2018;**25**: 511–23
16. Wang Q, Yang Y, Fu X, Wang Z, Liu Y, Li M, Zhang Y, Li Y, Li PF, Yu T, Chu XM. Long noncoding RNA XXYLT1-AS2 regulates proliferation and adhesion by targeting the RNA binding protein FUS in HUVEC. *Atherosclerosis* 2020;**298**:58–69
17. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* 2001;**25**:402–8
18. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;**162**:156–9
19. Kim D, Langmead B, Salzberg SL. HISAT: a fast spliced aligner with low memory requirements. *Nat Methods* 2015;**12**:357–60
20. Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol* 2010;**28**:511–5
21. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;**15**:550
22. Jin L, Li G, Yu D, Huang W, Cheng C, Liao S, Wu Q, Zhang Y. Transcriptome analysis reveals the complexity of alternative splicing regulation in the fungus Verticillium dahliae. *BMC Genomics* 2017;**18**:130
23. Xia H, Chen D, Wu Q, Wu G, Zhou Y, Zhang Y, Zhang L. CELF1 preferentially binds to exon-intron boundary and regulates alternative splicing in HeLa cells. *Biochim Biophys Acta Gene Regul Mech* 2017;**1860**:911–21
24. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li CY, Wei L. KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* 2011;**39**:W316–22
25. Kavurma MM, Bhindi R, Lowe HC, Chesterman C, Khachigian LM. Vessel wall apoptosis and atherosclerotic plaque instability. *J Thromb Haemost* 2005;**3**:465–72
26. Saito Y. The role of the PlGF/Flt-1 signaling pathway in the cardiorenal connection. *J Mol Cell Cardiol* 2021;**151**:106–12
27. Goodall AH, Burns P, Salles I, Macaulay IC, Jones CI, Ardissino D, de Bono B, Bray SL, Deckmyn H, Dudbridge F, Fitzgerald DJ, Garner SF, Gusnanto A, Koch K, Langford C, O'Connor MN, Rice CM, Stemple D, Stephens J, Trip MD, Zwaginga J-J, Samani NJ, Watkins NA, Maguire PB, Ouwehand WH. Transcription profiling in human platelets reveals LRRFIP1 as a novel protein regulating platelet function. *Blood* 2010;**116**:4646–56
28. Johnson P, Ruffell B. CD44 and its role in inflammation and inflammatory diseases. *Inflamm Allergy Drug Targets* 2009;**8**:208–20
29. Stawowczyk M, Van Scoy S, Kumar KP, Reich NC. The interferon stimulated gene 54 promotes apoptosis. *J Biol Chem* 2011;**286**:7257–66
30. Huang C, Lewis C, Borg NA, Canals M, Diep H, Drummond GR, Goode RJ, Schittenhelm RB, Vinh A, Zhu M, Kemp-Harper B, Kleifeld O, Stone MJ. Proteomic identification of interferon-induced proteins with tetratricopeptide repeats as markers of M1 macrophage polarization. *J Proteome Res* 2018;**17**:1485–99
31. Wang Q, Zhang M, Ding Y, Wang Q, Zhang W, Song P, Zou M-H. Activation of NAD(P)H oxidase by tryptophan-derived 3-hydroxykynurenine accelerates endothelial apoptosis and dysfunction in vivo. *Circ Res* 2014;**114**:480–92
32. Niinisalo P, Oksala N, Levula M, Pelto-Huikko M, Järvinen O, Salenius JP, Kytömäki L, Soini JT, Kähönen M, Laaksonen R, Hurme M, Lehtimäki T. Activation of indoleamine 2,3-dioxygenase-induced tryptophan degradation in advanced atherosclerotic plaques: Tampere vascular study. *Ann Med* 2010;**42**:55–63
33. von Rango U, Krusche CA, Beier HM, Classen-Linke I. Indoleamine-dioxygenase is expressed in human decidua at the time maternal tolerance is established. *J Reprod Immunol* 2007;**74**:34–45
34. Yun TJ, Lee JS, Machmach K, Shim D, Choi J, Wi YJ, Jang HS, Jung I-H, Kim K, Yoon WK, Miah MA, Li B, Chang J, Bego MG, Pham TNQ, Loschko J, Fritz JH, Krug AB, Lee S-P, Keler T, Guimond JV, Haddad E, Cohen EA, Sirois MG, El-Hamamsy I, Colonna M, Oh GT, Choi J-H, Cheong C. Indoleamine 2,3-dioxygenase-expressing aortic plasmacytoid dendritic cells protect against atherosclerosis by induction of regulatory T cells. *Cell Metab* 2016;**24**:886
35. Furset G, Fløisand Y, Sioud M. Impaired expression of indoleamine 2, 3-dioxygenase in monocyte-derived dendritic cells in response to Toll-like receptor-7/8 ligands. *Immunology* 2008;**123**:263–71
36. Wang Y, Liu H, McKenzie G, Witting PK, Stasch JP, Hahn M, Changsirivathanathamrong D, Wu BJ, Ball HJ, Thomas SR, Kapoor V, Celermajer DS, Mellor AL, Keaney JF Jr, Hunt NH, Stocker R. Kynurenine is an endothelium-derived relaxing factor produced during inflammation. *Nat Med* 2010;**16**:279–85
37. Liang H, Chen M, Qi F, Shi L, Duan Z, Yang R, He J, Lou B, Li Y, Yang Q. The proatherosclerotic function of indoleamine 2, 3-dioxygenase 1 in the developmental stage of atherosclerosis. *Signal Transduct Target Ther* 2019;**4**:23
38. Pertovaara M, Raitala A, Juonala M, Lehtimäki T, Huhtala H, Oja SS, Jokinen E, Viikari JS, Raitakari OT, Hurme M. Indoleamine 2,3-dioxygenase enzyme activity correlates with risk factors for atherosclerosis:

the cardiovascular risk in young Finns study. *Clin Exp Immunol* 2007;**148**:106–11
39. Niinisalo P, Raitala A, Pertovaara M, Oja SS, Lehtimäki T, Kähönen M, Reunanen A, Jula A, Moilanen L, Kesäniemi YA, Nieminen MS, Hurme M. Indoleamine 2,3-dioxygenase activity associates with cardiovascular risk factors: the health 2000 study. *Scand J Clin Lab Invest* 2008;**68**:767–70
40. Polyzos KA, Ovchinnikova O, Berg M, Baumgartner R, Agardh H, Pirault J, Gisterå A, Assinger A, Laguna-Fernandez A, Bäck M, Hansson GK, Ketelhuth DFJ. Inhibition of indoleamine 2,3-dioxygenase promotes vascular inflammation and increases atherosclerosis in Apoe-/- mice. *Cardiovasc Res* 2015;**106**:295–302
41. Krohn R, Raffetseder U, Bot I, Zernecke A, Shagdarsuren E, Liehn EA, van Santbrink PJ, Nelson PJ, Biessen EA, Mertens PR, Weber C. Y-box binding protein-1 controls CC chemokine ligand-5 (CCL5) expression in smooth muscle cells and contributes to neointima formation in atherosclerosis-prone mice. *Circulation* 2007;**116**:1812–20
42. Braunersreuther V, Steffens S, Arnaud C, Pelli G, Burger F, Proudfoot A, Mach F. A novel RANTES antagonist prevents progression of established atherosclerotic lesions in mice. *Arterioscler Thromb Vasc Biol* 2008;**28**:1090–6
43. Veillard NR, Kwak B, Pelli G, Mulhaupt F, James RW, Proudfoot AEI, Mach F. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* 2004;**94**:253–61
44. Li Y, Liu X, Duan W, Tian H, Zhu G, He H, Yao S, Yi S, Song W, Tang H. Batf3-dependent CD8α dendritic cells aggravates atherosclerosis via Th1 cell induction and enhanced CCL5 expression in plaque macrophages. *EBioMedicine* 2017;**18**:188–98
45. Zhang X, Guan MX, Jiang QH, Li S, Zhang HY, Wu ZG, Cong HL, Qi XH. NEAT1 knockdown suppresses endothelial cell proliferation and induces apoptosis by regulating miR-638/AKT/mTOR signaling in atherosclerosis. *Oncol Rep* 2020;**44**:115–25
46. Vlachogiannis NI, Sachse M, Georgiopoulos G, Zormpas E, Bampatsias D, Delialis D, Bonini F, Galyfos G, Sigala F, Stamatelopoulos K, Gatsiou A, Stellos K. Adenosine-to-inosine Alu RNA editing controls the stability of the pro-inflammatory long noncoding RNA NEAT1 in atherosclerotic cardiovascular disease. *J Mol Cell Cardiol* 2021;**160**:111–20
47. Wang L, Xia JW, Ke ZP, Zhang BH. Blockade of NEAT1 represses inflammation response and lipid uptake via modulating miR-342-3p in human macrophages THP-1 cells. *J Cell Physiol* 2019;**234**:5319–26
48. van Lammeren GW, Pasterkamp G, de Vries JP, Bosch L, de Haan JJ, de Kleijn DP, Moll FL, Vink A. Platelets enter atherosclerotic plaque via intraplaque microvascular leakage and intraplaque hemorrhage: a histopathological study in carotid plaques. *Atherosclerosis* 2012;**222**:355–9
49. Oksala N, Pärssinen J, Seppälä I, Klopp N, Illig T, Laaksonen R, Levula M, Raitoharju E, Kholova I, Sioris T, Kähönen M, Lehtimäki T, Hytönen VP. Kindlin 3 (FERMT3) is associated with unstable atherosclerotic plaques, anti-inflammatory type II macrophages and upregulation of beta-2 integrins in all major arterial beds. *Atherosclerosis* 2015;**242**:145–54
50. Chen D, Zhang C, Chen J, Yang M, Afzal TA, An W, Maguire EM, He S, Luo J, Wang X, Zhao Y, Wu Q, Xiao Q. MiRNA-200c-3p promotes endothelial to mesenchymal transition and neointimal hyperplasia in artery bypass grafts. *J Pathol* 2021;**253**:209–24
51. Yu H, Littlewood T, Bennett M. Akt isoforms in vascular disease. *Vascul Pharmacol* 2015;**71**:57–64
52. Ding L, Biswas S, Morton RE, Smith JD, Hay N, Byzova TV, Febbraio M, Podrez EA. Akt3 deficiency in macrophages promotes foam cell formation and atherosclerosis in mice. *Cell Metab* 2012;**15**:861–72
53. Ding L, Zhang L, Kim M, Byzova T, Podrez E. Akt3 kinase suppresses pinocytosis of low-density lipoprotein by macrophages via a novel WNK/SGK1/Cdc42 protein pathway. *J Biol Chem* 2017;**292**:9283–93
54. Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P. Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 2002;**8**:831–40
55. Khachigian LM, Santiago FS, Rafty LA, Chan OL, Delbridge GJ, Bobik A, Collins T, Johnson AC. GC factor 2 represses platelet-derived growth factor A-chain gene transcription and is itself induced by arterial injury. *Circ Res* 1999;**84**:1258–67
56. Choe N, Kwon JS, Kim JR, Eom GH, Kim Y, Nam KI, Ahn Y, Kee HJ, Kook H. The microRNA miR-132 targets Lrrfip1 to block vascular smooth muscle cell proliferation and neointimal hyperplasia. *Atherosclerosis* 2013;**229**:348–55
57. Taleb S. Inflammation in atherosclerosis. *Arch Cardiovasc Dis* 2016;**109**:708–15
58. Cuff CA, Kothapalli D, Azonobi I, Chun S, Zhang Y, Belkin R, Yeh C, Secreto A, Assoian RK, Rader DJ, Puré E. The adhesion receptor CD44 promotes atherosclerosis by mediating inflammatory cell recruitment and vascular cell activation. *J Clin Invest* 2001;**108**:1031–40
59. Krettek A, Sukhova GK, Schönbeck U, Libby P. Enhanced expression of CD44 variants in human atheroma and abdominal aortic aneurysm: possible role for a feedback loop in endothelial cells. *Am J Pathol* 2004;**165**:1571–81

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