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Original Research

HSPB1 Orchestrates the Inflammation-Associated Transcriptome Profile of Atherosclerosis in HUVECs

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Abstract

Background: Atherosclerosis (AS), with a profound inflammatory response, is the basis of cardiovascular diseases. Previous reports showed that heat shock protein family B member 1 (HSPB1) has a protective effect against AS, but the specific mechanism is still unclear. In this study, we aim to explore the functions and downstream targets of HSPB1 in human umbilical vein endothelial cells (HUVECs). **Methods**: Expression of the *HSPB1* gene was knocked down in HUVECs. Cellular phenotype was then assessed and transcriptome data (RNA-seq) was analyzed to identify the potential targets regulated by HSPB1. Moreover, RNA-seq data for human fibroatheroma (GSE104140) from the gene expression omnibus (GEO) database was re-analyzed to verify the targets of HSPB1 in AS. **Results**: Silencing of HSPB1 significantly reduced apoptosis ($p < 0.0001$) and increased the proliferation ($p < 0.05$) of HUVECs. The 608 differentially expressed genes (DEGs) were identified after HSPB1 knockdown, including 423 upregulated genes. DEGs, including *CXCL1*, *CXCL8*, *CXCL2*, *TRIB3*, *GAS5*, *SELE*, and *TNIP1*, were enriched in inflammatory and immune response pathways. HSPB1 was also shown to affect alternative splicing patterns of hundreds of genes, especially those enriched in apoptotic processes, including *ACIN1*, *IFI27*, *PAK4*, *UBE2D3*, and *FIS1*. An overlapping gene set was found between the HSPB1-regulated and AS-induced transcriptome. This included 171 DEGs and 250 alternatively spliced genes that were also enriched in inflammatory/immune response- and apoptosis-associated pathways, respectively. **Conclusion**: In summary, HSPB1 knockdown modulates the proliferation and apoptosis of HUVECs by regulating RNA levels and alternative splicing patterns. HSPB1 plays an important role in AS pathogenesis by modulating the inflammatory and immune response. This study provides novel insights for the investigation of future AS therapeutic strategies.

Keywords: HSPB1; atherosclerosis; alternative splicing; DEGs; inflammation and immune response

1. Introduction

Atherosclerosis (AS) is a chronic inflammatory disease caused by atherosclerotic plaques on the inner vascular wall. The AS process involves vascular endothelial cell injury, chronic inflammation, immune dysfunction, and altered lipid metabolism and epigenetic regulation [1–3]. AS is the common pathogenic basis for cardiovascular diseases [4]. In particular, initial injury to vascular endothelial cells promotes the formation of atherosclerotic plaques in the early stage of AS and is an important factor in the pathogenesis of this disease [5]. Venous endothelial cells are also used as model system to investigate the molecular mechanism and vascular inflammation in AS [6,7]. A recent study identified five novel targets of the inflammatory process during AS [8]. However, it is important to identify additional novel regulatory factors to better understand the pathogenesis of AS.

During alternative splicing, the exons of premature message RNAs (mRNAs) can be spliced into different arrangements to produce functionally distinct mRNAs that have crucial roles in various normal or pathological biological processes [9,10]. Studies have reported that aberrant splicing plays a major role in the development of AS [11,12], although the potential regulatory mechanism in this pathogenesis is still unclear. RNA-binding proteins (RBPs) can interact with double-stranded or single-stranded RNAs to form ribonucleoprotein complexes to regulate the fate of RNAs [13]. It is widely accepted that RBPs regulate many post-transcriptional biological processes, such as RNA splicing, translocation, editing of sequences, intracellular localization, and regulation of translation [14]. RBPs interact with RNAs by their specific RNA binding domains to regulate the following biological processes [15]. This process plays an essential role in post-transcriptional regulation in eukaryotes.

RBPs have been associated with the occurrence and progression of various diseases, including AS [16,17]. In an atherosclerotic rabbit model induced by a high-fat diet, PJ34 promoted endothelial repair by inhibiting the function of poly (ADP-ribose) polymerase 1 (PARP1), which is an RBP [18]. Alternative splicing is an important post-transcriptional process regulated by RBPs that increases the

complexity of gene expression, promotes the diversity of encoded proteins, and has important roles in various cellular processes and diseases [19]. However, the underlying mechanism by which RBPs regulate alternative splicing in AS has not been fully elucidated.

Heat shock protein family B member 1 (HSPB1), also referred to as Hsp27, is one of the small heat shock proteins. It can function as a chaperone and interact with other proteins to promote their correct functional folding. HSPB1 can also regulate the differentiation process of multiple cell types [20]. Other studies have reported that HSPB1 is an RBP involved in DNA repair and mRNA splicing [21,22]. Currently, most research on HSPB1 has focused on vascular smooth muscle cells, while its role in AS and the underlying molecular mechanism are still unclear.

To address the above questions, in this study, we knocked down HSPB1 in human umbilical vein endothelial cells (HUVECs) to decipher its functions. The transcriptome profile (RNA-seq) following HSPB1 knockdown was obtained using next-generation sequencing technology, and its potential targets at the transcription and splicing levels were analyzed. Additionally, RNA-seq data for human fibroatheroma (GSE104140) was downloaded from the gene expression omnibus (GEO) database to investigate the molecular targets and regulatory mechanism of HSPB1 in AS. This work lays the foundation for further in-depth research on the molecular pathogenesis of AS and on potential therapeutic strategies for this disease.

2. Materials and Methods

2.1 Small Interference RNA (siRNA)

The siRNA for *HSPB1* (siHSPB1) and non-targeting control siRNA (siNC) were purchased from Genepharma (Suzhou, Jiangsu, China). The sequence of siNC was: 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense). The sequence of siHSPB1 was: 5′-CGGACGAGCUGACGGUCAATT-3′ (sense). We also used the blank cell as background to assess the knockdown level of HSPB1.

2.2 The siRNA Transfection and Cell Culture

The transfection reagent Lipofectamine™ RNAiMAX (13778030, Invitrogen, Carlsbad, CA, USA) was used for siRNA transfection into HUVECs (DFSC-EC-01, Zhongqiaoxinzhou Biotech, Shanghai, China). The cells were cultured at 37 °C with 5% CO_2 in ECSM (ZQ-1304, Zhongqiaoxinzhou Biotech, Shanghai, China) with 10% fetal bovine serum (FBS) (10099-141, Gibco, Shanghai, China). The HUVECs were tested by short tandem repeat (STR) profiling for validation, and tested negative for mycoplasma. Transfected cells were harvested after 48 h for the subsequent experiments.

2.3 Reverse Transcription and Quantitative Polymerase Chain Reaction (RT-qPCR) and Western Blot

RT-qPCR was performed to validate the knockdown efficiency of *HSPB1*. Complete DNA (cDNA) was synthesized according to standard procedures using the Bio-Rad S1000 instrument (Hercules, CA, USA) and Hieff™ qPCR SYBR® Green Master Mix (Low Rox Plus, YEASEN, Shanghai, China). We used the $2^{-\Delta\Delta CT}$ method [23] to calculate the concentration of each transcript, with the level of *HSPB1* mRNA normalized to that of *GAPDH* mRNA.

To evaluate the protein level of HSPB1 in HUVEC cells, the experimental procedure for Western blot was conducted as previously described [24]. The membranes were incubated with the primary antibodies to HSPB1 (1:1000, A0240, Abclonal, Wuhan, Hubei, China) and ACTIN (1:1000, 20536-1-AP, Proteintech, Wuhan, Hubei, China), and then with HRP-conjugated secondary antibody. The secondary antibody (anti-rabbit, 1:5000, SA00001-2, Proteintech, China; or anti-mouse, 1:5000, AS003, ABclonal, China) was detected using enhanced chemiluminescence (ECL) reagent (Bio-Rad, 170506, Hercules, CA, USA).

2.4 CCK-8 Assay for Cell Proliferation Activity

The Cell Counting Kit-8 (CCK-8) assay kit (HY-K0301, MedChemExpress, Monmouth Junction, NJ, USA) was used to evaluate the proliferation of HUVECs. These were seeded into a plate with 96-wells at a density of 10^4 cells per well (6 replicates) and cultured in an incubator for 0, 24, 48, and 72 h. The 10 µL of CCK-8 solution was added to the wells at each time point and then incubated for a further 3 h. The absorbance of each well at a wave length of 450 nm was then measured using a microplate reader (ELX800, Biotek, Winooski, VT, USA). OD450 values were used to evaluate cell proliferation.

2.5 Flow Cytometry for the Detection of HUVEC Apoptosis

The Annexin V-APC/7-ADD kit (KGA1026, KeyGEN BioTECH, Nanjing, Jiangsu, China) was used to detect apoptosis according to the user instructions. We seeded 10^6 HUVECs into plates, cultured for 24 h, and then transfected with siHSPB1 or siNC for 48 h. The cells were subsequently mixed with 5 µL of Annexin V-allophycocyanin (V-APC) and incubated at room temperature for 5 min, followed by incubation with 5 µL of 7-aminoactinomycin D (7-AAD) reagent for 5 min. The two cell groups were then analysed by flow cytometry (FACSCanto, BD, Franklin Lake, NJ, USA) to assess apoptosis.

2.6 Total RNA Extraction and RNA-seq Experiments

The total RNAs were extracted from the siNC and siHSPB1 HUVEC groups (3 replicates) using the TRIzol method. RNA concentration and purity were measured using Smartspec Plus (A260/A280; BioRad, Hercules, CA, USA). After passing the quality inspection, 1 µg of total

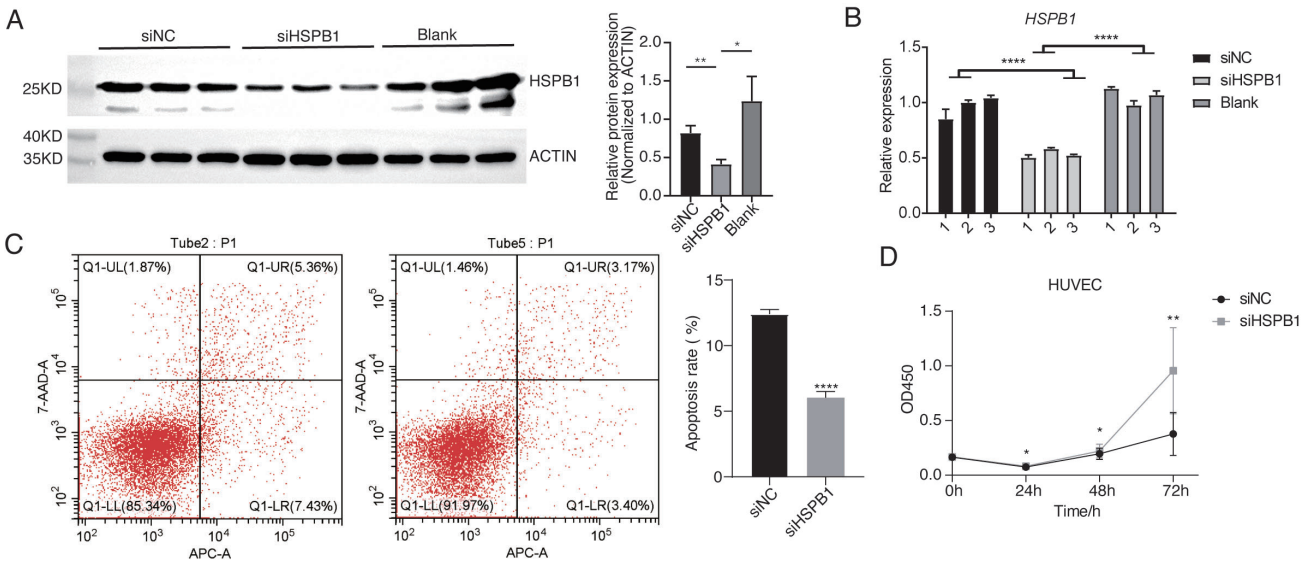

Fig. 1. SiHSPB1 significantly affects the proliferation and apoptosis of HUVECs. (A)Western blot results after HSPB1 knockdown; the right panel was the quantitative results (n = 3). * $p < 0.05$, ** $p < 0.01$. (B) RT-qPCR results after HSPB1 knockdown (n = 3). **** $p < 0.0001$. (C) Flow cytometry and bar plot showing the level of apoptosis after HSPB1 knockdown. The right panel was used for statistical analysis (n = 3). **** $p < 0.0001$. (D) Bar plot showing the rate of cell proliferation after HSPB1 knockdown (n = 6). * $p < 0.05$, ** $p < 0.01$. siNC, non-targeting control siRNA; siHSPB1, The siRNA for HSPB1; HUVECs, human umbilical vein endothelial cells; HSPB1, heat shock protein family B member 1; RT-qPCR, reverse transcription and quantitative polymerase chain reaction; APC-A, allophycocyanin-A.

RNA was used to construct an RNA-seq library using the VAHTS Universal V8 RNA-seq Library Prep Kit for Illumina (NR605, Vazyme, Nanjing, Jiangsu, China). The ligation product was then purified and the 300–500 bp size fraction was selected. The products were then amplified, purified, and stored at –80 °C. High-quality RNA sequences were obtained using the NovaSeq 6000 sequencing platform (Illumina, San Diego, CA, USA) for paired-end 150 nt sequencing.

2.7 Retrieval and Processing of Published Data

"Atherosclerosis" was used as the keyword to retrieve and select the appropriate dataset from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). The GSE104140 dataset was downloaded for further analysis, and has not been used in any previous published study.

2.8 Identification of Differentially Expressed Genes, and GO Pathway Analysis

The FASTX-Toolkit (Version 0.0.13, The Hannon Lab, New York, NY, USA) was used to discard low-quality reads from the two RNA-seq datasets. HISAT2 2.2.1 (https://daehwankimlab.github.io/hisat2/) [25] was used to align filtered reads onto the human GRCh38 genome. Differentially expressed genes (DEGs) were identified using DESeq2 https://bioconductor.org/packages/release/bioc/html/DESeq2.html [26], with criteria of (fold-change) FC $\geq$3/2 or $\leq$2/3, and a p-value < 0.01 used to define a significant difference.

Gene Ontology (GO, http://geneontology.org/) was used to describe the functions of gene sets. GO enrichment analysis was conducted to explore the functions of DEGs. These were first mapped to various terms in the GO database and the number of genes associated with each term was counted. Hypergeometric distribution testing was then used to obtain significantly enriched GO terms (p-value $<$ 0.01) associated with DEGs based on the GO annotation of the entire genome.

2.9 Alternative Splicing Events Analysis

We used ABLas pipeline (ABLife, Wuhan, Hubei, China), as previously described [27], to compare the differences in alternative splicing events (ASEs) and define and quantify the regulated alternative splicing events (RASEs) between groups. To identify RASEs that were different after silencing the *HSPB1* gene, Student's t-test method was used to calculate changes in alternative splicing of the same splicing type for each gene between *siHSPB1* and *siNC*. The Events with significance at a p-value cutoff of 5% were considered to be RASEs.

2.10 Statistical Analysis

The Gene Ontology (GO) enriched pathways were identified using the KOBAS 2.0 server [28] (version 2.0, Peking University, Beijing, China). Experimentally quantitative data were presented by mean $\pm$ standard deviation (SD). Student's t-test was used to calculate the difference between siHSPB1 and siNC groups (Prism version 8.0,

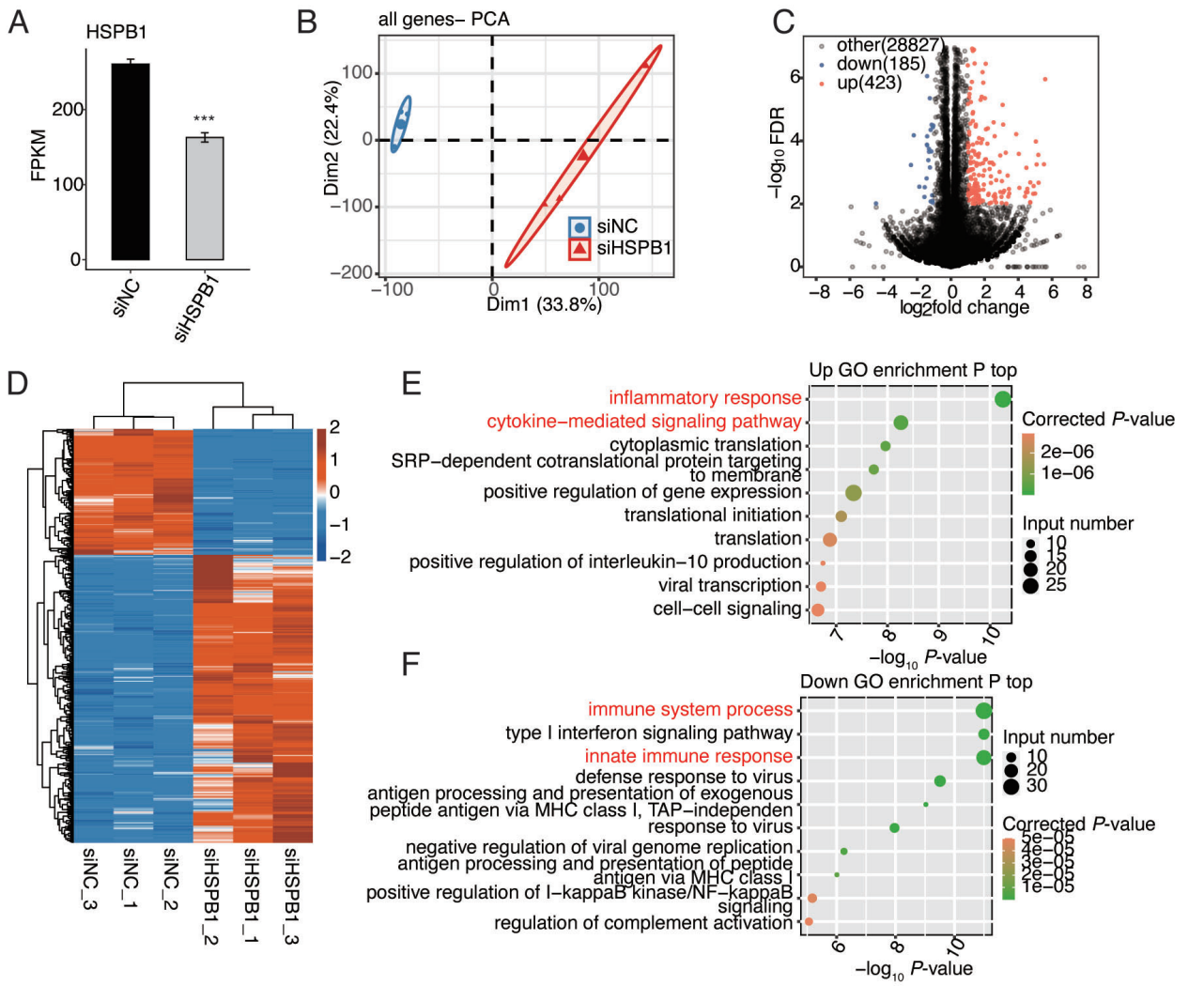

Fig. 2. HSPB1 regulates gene expression in HUVECs. (A) Bar plot demonstrating the changed expression level of HSPB1 from RNA-seq (n = 3). *** $p < 0.001$. (B) PCA result based on the FPKM values of all expressed genes after HSPB1 knockdown, with confidence ellipse drawn for each group. (C) Volcano plot showing the pattern and number of DEGs between siHSPB1 and siNC groups. (D) Heat map showing the expression pattern of all detected DEGs by hierarchical clustering. (E) Bubble plot showing the top 10 enriched GO biological processes amongst the up-regulated DEGs. The two most enriched pathways are highlighted with red font. (F) The same analysis as in (E), but for the down-regulated DEGs. PCA, principal component analysis; DEGs, differentially expressed genes; GO, Gene Ontology; FPKM, fragments per kilobase per million.

GraphPad, Boston, MA, USA) with p-value < 0.05 as significant difference. Sequencing data was analyzed using R software (version 4.3.1, The R Foundation, Vienna, Austria) and plotted using GraphPad Prism software (Version 9.5.0, GraphPad, Boston, MA, USA) and R software.

3. Results

3.1 Knockdown of HSPB1 Significantly Altered the Apoptosis and Proliferation of HUVECs

To examine its role in HUVECs, the *HSPB1* gene was silenced using the siRNA transfection method (siHSPB1). The mRNA and protein expression levels of HSPB1 were then evaluated to determine the silencing efficiency. The HSPB1 protein (Fig. 1A, **Supplementary Fig. 1**) and mRNA (Fig. 1B) in HUVECs showed significantly lower levels in the siHSPB1 group compared to the siNC and blank groups ($p < 0.0001$). Then we used the siNC group as the background in following experiments. We next investigated the changes in phenotype induced by HSPB1 knockdown in HUVECs. Flow cytometry analysis revealed that the apoptosis ratio in the siHSPB1 group was significantly lower than in the siNC group (Fig. 1C, $p < 0.0001$). Moreover, CCK-8 analysis showed that the cell proliferation rate in the siHSPB1 group was significantly higher than that in the siNC group (Fig. 1D, $p < 0.05$). These results indicate that HSPB1 can obviously modulate the cellular phenotype of HUVECs.

3.2 HSPB1 Regulates the Gene Expression Pattern in HUVECs

To clarify the potential mechanism by which HSPB1 regulates HUVECs, RNA-seq and bioinformatics analysis was performed on the siNC and siHSPB1 groups of HUVECs. The sequencing results showed a significant decrease (p-value < 0.001) in *HSPB1* gene expression in the siHSPB1 group (Fig. 2A), indicating successful knockdown. Then we performed principal component analysis (PCA) on all of the expressed genes from the siNC and siHSPB1 groups. PCA result demonstrated a clear separation between the two groups (Fig. 2B), indicating that HSPB1 knockdown altered the pattern of global transcription. DEGs between these two groups were identified using DESeq2. The fold-change (FC) and p-value were used to determine whether a gene was differentially expressed (assessment criteria: FC $\geq$3/2 or $\leq$2/3, and p-value $<$ 0.01). A total of 608 DEGs, including 423 upregulated genes and 185 downregulated genes, were finally identified (Fig. 2C,D).

GO analysis of the upregulated genes revealed enrichment of the following pathways: inflammatory response, cytokine-mediated signalling pathway, cytoplasmic translation, signal recognition particle (SRP)-dependent cotranslational proteins targeting membranes, positive regulation of gene expression, translation initiation, translation, positive regulation of interleukin-10 production, viral transcription, and intercellular signalling processes (Fig. 2E). This indicates that HSPB1 participates in inflammatory responses and in cytokine-mediated signalling pathways. GO analysis of the 185 downregulated genes revealed enrichment for immune system processes, innate immune response, type I interferon signalling pathway, defence response to viruses, antigen processing, exogenous peptide antigen through major histocompatibility complex (MHC) class I, viral response, transporter associated with antigen processing (TAP)-independent presentation, negative regulation of viral genome replication, antigen processing, peptide antigen presentation through MHC class I, I-$\kappa\beta$ kinase/nuclear factor kappa β (NF-$\kappa\beta$), positive regulation of signalling pathways, and regulation of complement activation in biological pathways (Fig. 2F). In summary, the above results indicate that HSPB1 modulates the inflammatory and immune response of HUVECs, both of which are closely associated with the pathogenesis of AS [29].

3.3 HSPB1 Modulated the Alternative Splicing Pattern of Genes Associated with Apoptosis in HUVECs

By analysing the alternative splicing events regulated by HSPB1 (Fig. 3A), HSPB1 knockdown was found to promote exon hopping and suppress cassette exons. These events may be characteristic of the regulation of AS by HSPB1. To investigate the potential influence of HSPB1-regulated AS events (RASEs) and genes (RASGs), we carried out functional enrichment analysis for RASGs. This revealed that RASGs were mainly enriched in the processes of apoptosis, the I-$\kappa\beta$ kinase/NF-$\kappa\beta$ biological functional pathway, mRNA processing, RNA splicing, protein transport, mRNA splicing during glycosphingolipid metabolism, type I interferon signalling pathway, and the regulation of cell morphology (Fig. 3B). siHSPB1 was found to induce the splicing pattern of genes that participate in the apoptotic process, consistent with the phenotype alteration observed for HUVECs. A total of 26 overlapping genes were found between RASGs and DEGs (Fig. 3C), of which *ACIN1*, *IFI27*, *PAK4*, *UBE2D3*, and *FIS1* were enriched in the apoptotic pathway. Therefore, we further analysed the RASEs in *FIS1* and *UBE2D3* by siHSPB1. The mutually exclusive 5′ untranslated region (5pMXE) of *UBE2D3* was included by siHSPB1 (Fig. 3D), whereas the 5pMXE of *FIS1* was excluded by siHSPB1 (Fig. 3E).

3.4 HSPB1 Regulates the Expression Pattern of Genes in the Vascular Tissue of Patients with Atherosclerosis

To further decipher the regulatory targets of HSPB1 in AS, we downloaded the RNA-seq data (GSE104140) of human fibroatheroma patients from the GEO database. This study analysed and compared the RNA-seq transcriptome data from early vascular disease (diffuse intimal thickening, SAMP_DIT) and two late AS states (calcification, SAMP_cal and non-calcified fibrous AS, SAMP_no cal). Data for early vascular disease (SAMP_DIT) and late calcified fibrous AS (SAMP_cal) were used for analysis. The results indicated that HSPB1 expression was significantly lower (p-value < 0.001) in the SAMP_cal group than in the SAMP_DIT group (**Supplementary Fig. 2A**), implying that it may have an important role in AS. Moreover, thousands of DEGs were identified between the SAMP_cal *vs.* SAMP_DIT groups, including 2740 up-regulated and 987 down-regulated genes (**Supplementary Fig. 2B,C**). An overlap analysis was performed between 608 HSPB1-regulated DEGs and 3716 genes that were differentially expressed according to the GSE104140 RNA-seq data. This identified 171 overlapping DEGs (Fig. 4A, $p = 8.87 \times 10^{-28}$, hypergeometric test). GO pathway enrichment analysis for these overlapped DEGs revealed that inflammatory response showed the strongest enrichment (Fig. 4B). Then we performed a heatmap analysis showing the expression pattern of the 22 DEGs from the inflammatory response pathway, including 6 down-regulated DEGs and 16 up-regulated DEGs (Fig. 4C). Finally, we selected 8 DEGs that showed significant differences in expression and were related to inflammatory response pathways. Their expression pattern according to HSPB1 (HUVEC) KD RNA-seq (Fig. 4D) and GSE104140 RNA-seq (Fig. 4E) data is shown using bar graphs.

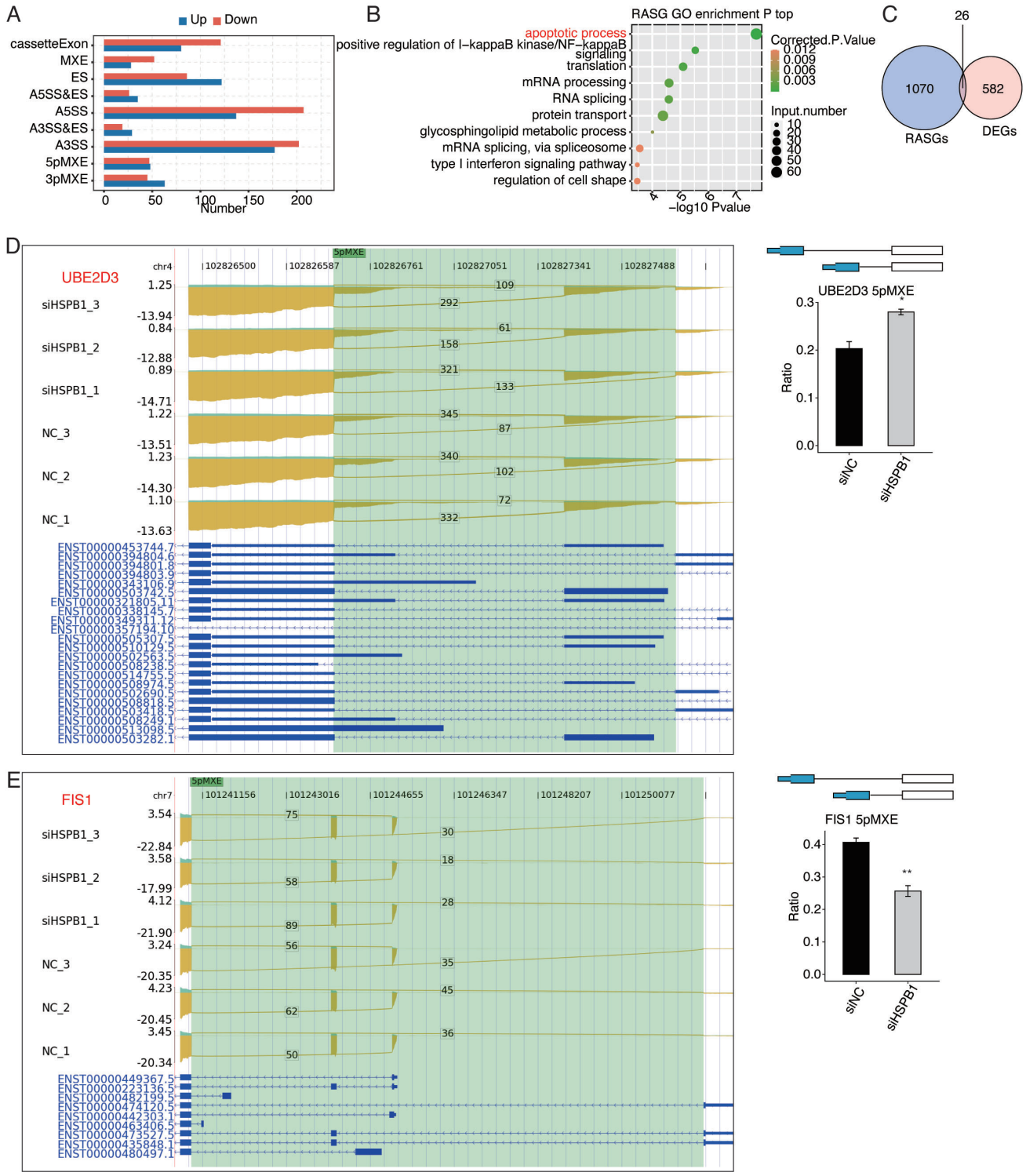

Fig. 3. HSPB1 regulates alternative splicing pattern of genes in HUVECs. (A) Bar plot demonstrating the HSPB1-regulated alternative splicing events (RASEs). (B) Bubble plot showing the top 10 enriched biological process pathways for regulated alternative splicing genes (RASGs). The apoptotic process is highlighted in red. (C) Venn diagram demonstrating the overlapped genes between RASGs and DEGs. (D) HSPB1 regulates the 5pMXE splicing event for UBE2D3. The left panel shows the IGV-sashimi plot with the sequencing density on transcripts, while the right panel shows the RNA-seq validation of ASEs. Error bars represent the mean ± SEM (n = 3). * $p < 0.05$. (E) The same analysis as in (D), but showing the 5pMXE for FIS1. Error bars represent the mean ± SEM (n = 3). ** $p < 0.01$. SEM, standard error of the mean; ASEs, alternative splicing events; 5pMXE, mutually exclusive 5′ untranslated region; IGV, integrative genomic viewer.

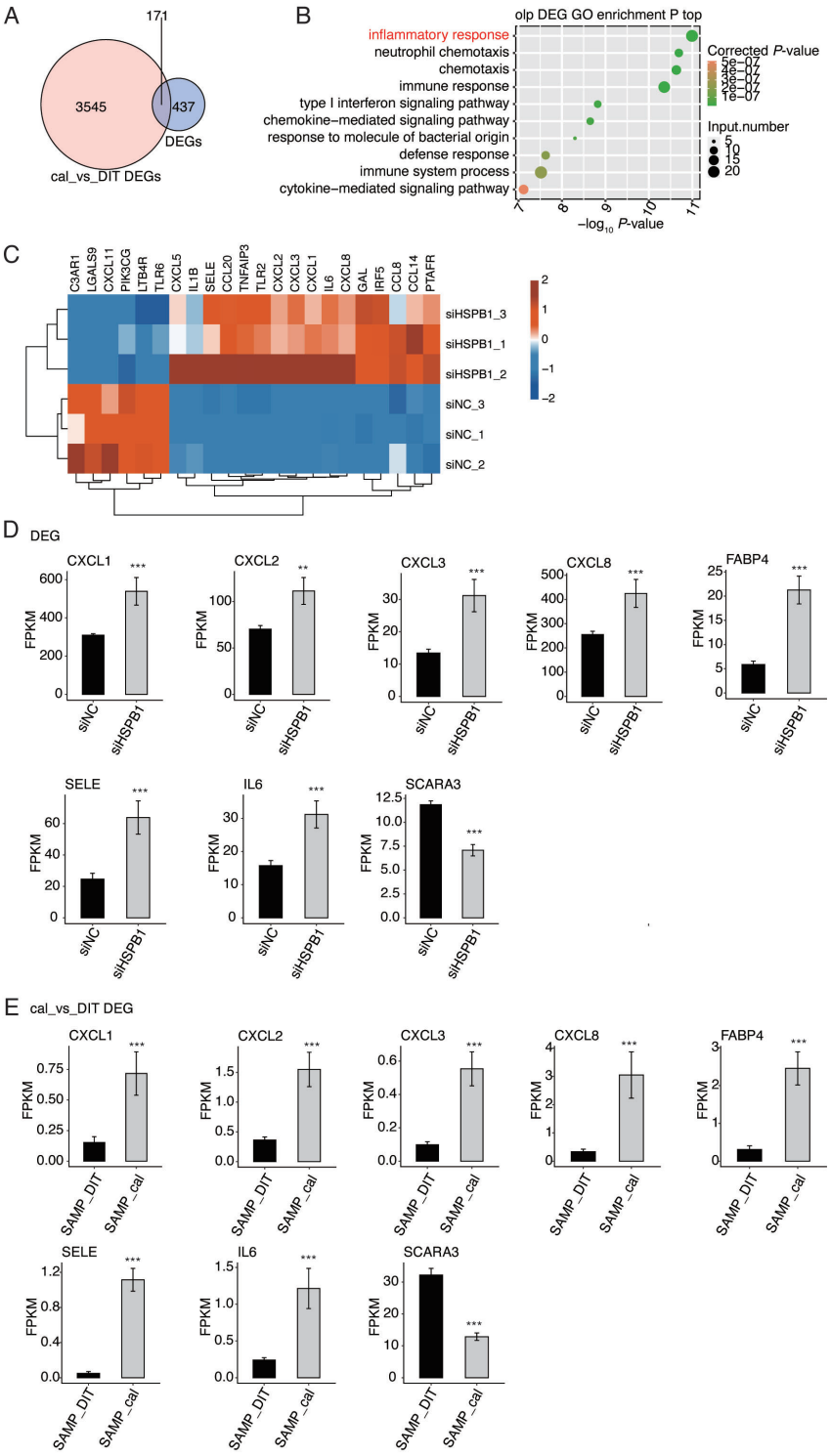

Fig. 4. HSPB1 regulates the expression of genes that were changed in the vascular tissue of AS patients. (A) Venn diagram demonstrating overlapped genes between the GSE104140 dataset and post-HSPB1 knockdown of HUVECs. (B) Scatter plot demonstrating the top 10 enriched GO biological processes for the overlapping DEGs. The inflammatory response is highlighted. (C) Heat map of the overlapped genes for the inflammatory response by hierarchical clustering. (D) Bar plot showing the changed expression pattern of overlapped DEGs between the siNC and siHSPB1 groups. Error bars represent the mean ± SEM (n = 3). ***$p < 0.001$, ** $p < 0.01$. (E) The same analysis as in (D), but for the GSE104140 RNA-seq dataset. Error bars represent the mean ± SEM (n = 9). *** $p < 0.001$. AS, atherosclerosis.

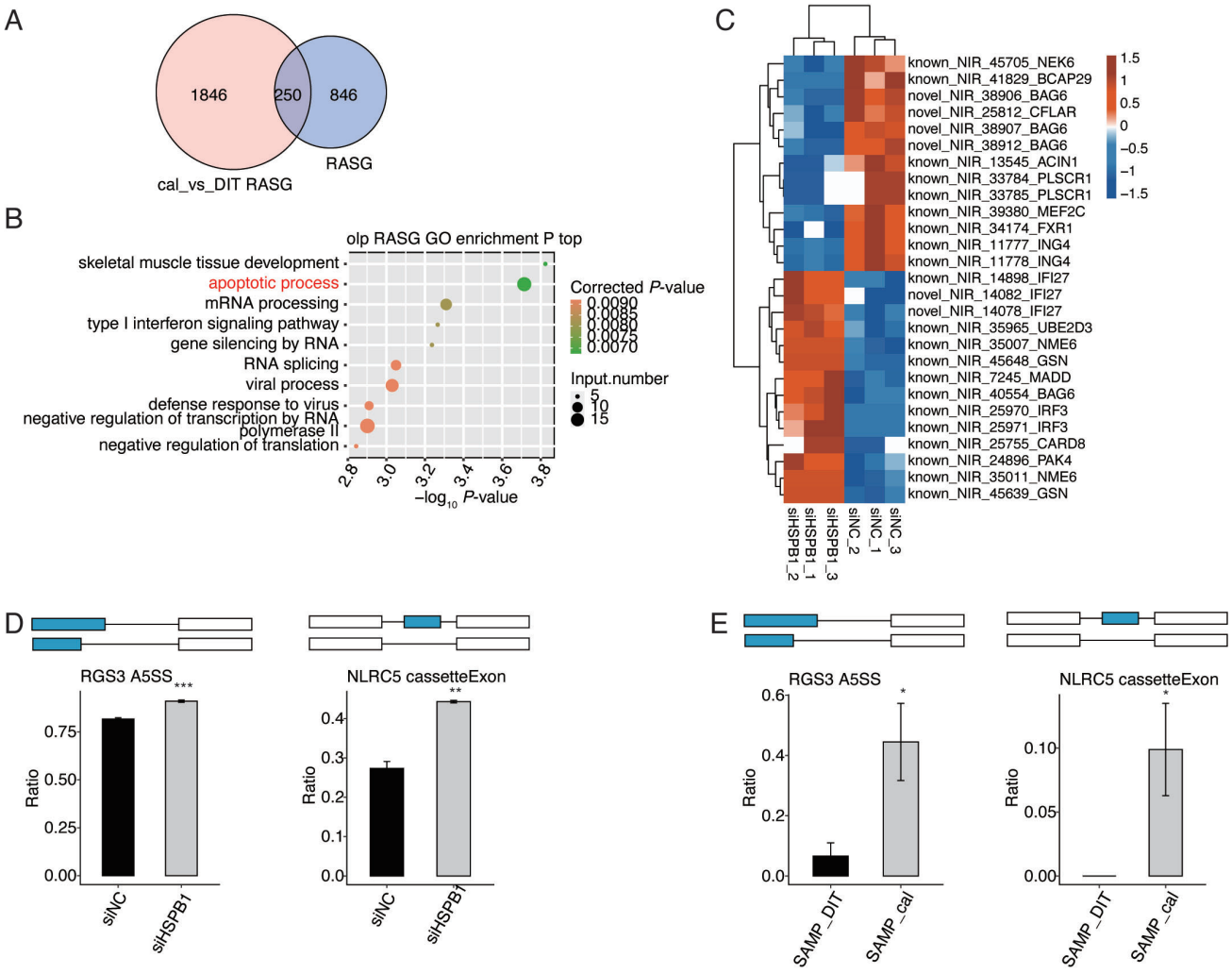

Fig. 5. HSPB1 modulates the splicing pattern of genes in the vascular tissue of AS patients. (A) Venn diagram demonstrating the overlapped RASGs between the GSE104140 dataset and the HSPB1 knockdown RNA-seq of HUVECs. (B) Scatter plot showing the top 10 enriched GO biological processes for the overlapping RASGs. The apoptotic process is highlighted. (C) Hierarchical clustering heat map of overlapping alternative splicing events and genes in the apoptotic process. (D,E) HSPB1 regulates alternative splicing for RGS3 and NLRC5. Schematic diagrams and RNA-seq validation for the RASEs. Error bars represent the mean ± SEM (n = 3 and n = 9 for D and E, respectively). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

3.5 HSPB1 Regulates the Alternative Splicing of Genes in the Vascular Tissue of AS Patients

To further study the role of HSPB1 in regulating alternative splicing in AS, we overlapped 1096 RASGs identified by RNA-seq of HSPB1 (HUVEC) KD genes with 2096 RASGs identified by RNA-seq of the GSE104140 dataset. This revealed a total of 250 overlapping RASGs (Fig. 5A, $p = 4.35 \times 10^{-38}$, hypergeometric test), many of which were enriched in the apoptotic process, as shown by GO pathway enrichment analysis (Fig. 5B). Overlapping RASGs from the apoptotic process were displayed through heatmaps, revealing obvious differences between the siHSPB1 and siNC groups (Fig. 5C). Finally, the distribution of the splicing model in siHSPB1 HUVECs and in AS patients, as well as bar plots showing the dysregulated splicing pattern of regulator of G-protein signaling 3 (*RGS3*) and NOD-like receptor family CARD domain containing 5 (*NLRC5*), are presented in Fig. 5D,E. A previous study reported that NLRC5 can regulate vascular remodelling by directly inhibiting smooth muscle cell dysfunction via its interaction with peroxisome proliferators-activated receptors γ (PPARγ) [30]. In summary, interaction analysis of the splicing pattern suggests that HSPB1 participates in the progression of AS by modulating the splicing of important genes.

4. Discussion

Cardiovascular diseases are a major high-risk disease in humans and are associated with an elevated rate of mortality. AS is the dominant pathological basis for cardiovas-

cular diseases [31,32], and their occurrence and development are associated with lipid infiltration, platelet activation, thrombosis, endometrial damage, inflammatory reactions, oxidative stress, activation of vascular smooth muscle cells, and changes in vascular endothelial cell (EC) apoptosis [1,33,34]. In this study, we explored the function of HSPB1 in HUVECs and linked its targets to the dysregulated genes observed in the vascular tissue of AS patients. HSPB1 was found to be an important regulator that affects essential biological processes in the pathogenesis and development of AS. Our study provides another avenue for the discovery of novel molecular therapeutic targets for AS.

Several studies have validated that the proliferation of endothelial cells, including venous endothelial cells may promote the development of AS [35,36]. Moreover, increased expression of proapoptotic proteins in endothelial cells covering vascular lesions was reported [37]. Extracellular vesicles derived from endothelial cells can promote intercellular communication and prevent or promote disease progression through the transfer of cellular contents such as proteins and microRNAs [34]. Therefore, investigating the regulation of endothelial cell function is crucial for the development of strategies that impede the progression of AS. HSPB1 is one of the family of small heat shock proteins. A previous study on coronary heart disease found that HSPB1 reduces the level reactive oxygen species in the aorta and the progression of AS by inhibiting mitochondrial apoptosis, thereby playing a protective role [38]. However, the specific regulatory mechanism involved is still unclear. To date, most of the research reports on HSPB1 have focused on vascular smooth muscle cells (VSMCs). HSPB1 was found to regulate the autophagy and apoptosis of VSMCs in arteriosclerosis obliterans, thus exerting an anti-atherosclerotic effect [39,40]. Here, we focused on the functional mechanism of HSPB1 in endothelial cells (HUVECs) by first knocking down HSPB1 and then analysing the transcriptome changes using high-throughput sequencing technology. Potential targets for HSPB1-mediated regulation of transcription and alternative splicing in HUVECs were identified.

Furthermore, we studied the DEGs induced by knocking down HSPB1 protein in HUVECs. The most important discovery was that many inflammatory factors were upregulated following HSPB1 knockdown. Of note, chemokine ligand 1 (CXCL1), CXCL8, CXCL2, selectin E (SELE), and TNFAIP3 interacting protein 1 (TNIP1) were enriched in the inflammatory response pathway, which is closely related to the pathogenesis of AS [1,41]. These findings suggest that HSPB1 induces the dysfunction of endothelial cells by activating inflammatory response pathways [42]. Several recent studies have shown that CXCL1, CXCL8, CXCL2, tribbles pseudokinase 3 (TRIB3), growth arrest specific 5 (GAS5), SELE, and TNIP1 are implicated in the pathogenesis of AS [43,44]. CXCL1 is a small molecular weight cytokine belonging to the CXC chemokine family, and is mainly involved in processes such as vascular growth and inflammation [43]. The long noncoding RNA (lncRNA) *GAS5* can also modulate inflammation and oxidative stress in macrophages [44]. Interestingly, the upregulated DEGs by siHSPB1 were also enriched in cytokine-mediated signalling pathways. Cytokines and inflammatory responses are inter-related and can occur simultaneously in many inflammation-associated diseases, including AS [45]. This indicates that knockdown of HSPB1 can induce changes in immune-related genes.

Alternative splicing is unique to eukaryotes [46]. Many cells show aberrant regulation of alternative splicing, resulting in the production of spliceosomes that are different to those seen in normal cells, and leading to abnormal protein expression. Such abnormalities in protein expression are tightly associated with the pathogenesis and development of various diseases. Previous studies have reported abnormal alternative splicing in cardiovascular diseases, suggesting it has an important role in AS [47,48]. Vascular endothelial growth factor A (VEGF-A) can increase proliferation of endothelial cells, promote macrophage infiltration and foam cell formation [49], thereby having a key role in the pathogenesis of AS. The alternative splicing subtype of *VEGF-A*, referred to as VEGF165, is more conducive to the development of AS [50]. Further research on RBPs that regulate pre-mRNA alternative splicing can provide a theoretical basis for the clinical targeted treatment of AS. Following knockdown of the HSPB1 protein in HUVECs, a large number of genes were observed to undergo alternative splicing events. Functional enrichment analysis of DEGs revealed that pathways related to apoptosis, translation, mRNA processing, and RNA splicing were enriched and were also related to AS disease. Among them, the *ACIN1*, *IFI27*, *PAK4*, *UBE2D3*, and *FIS1* genes were associated with the apoptosis pathway. The apoptosis of endothelial cells was previously reported to influence the pathogenesis of AS [51]. P21 (RAC1) activated kinase 4 (PAK4) is one of the P21-activated kinases that regulates inflammation and is involved in the initiation of AS and other cardiovascular diseases [52,53]. Our flow cytometry results showed a significant reduction in the percentage of apoptotic cells in the siHSPB1 group (Fig. 1C). Dysregulated alternative splicing of such genes may lead to altered apoptosis and inflammation, and subsequently to AS. In summary, HSPB1 knockdown affects genes involved in cell apoptosis and extracellular matrix decomposition at both the levels of expression and alternative splicing, thereby inhibiting the apoptosis of HUVECs and having important influence in the pathogenesis of AS [39,40]. However, further research is necessary to investigate the specific regulatory mechanism how these genes contribute to the occurrence and development of AS.

By integrating the transcriptome data from AS patients, we also found an obvious overlap between the HSPB1-regulated transcriptome in HUVECs and the dys-

regulated transcriptome from AS tissues. The overlapping DEGs were enriched in inflammatory and immune response pathways, and the overlapped RASGs were enriched in apoptotic process pathways. This highlights the profound impact of HSPB1 on the dysregulated transcriptome profile induced by AS.

Although the present research found that HSPB1 participates in the occurrence and development of AS, our findings are limited to cellular experiments and bioinformatics analysis. The specific regulatory molecular mechanism involved therefore requires additional clarification. Further in-depth research using animal models and other technologies should be conducted to fully explore the molecular mechanism by which HSPB1 regulates AS.

5. Conclusion

In summary, by integrating the HSPB1-regulated and AS-induced transcriptome profiles, our research found that HSPB1 is closely involved in the pathogenesis and progression of AS. This study demonstrates a theoretical basis for the use of HSPB1 as a new biomarker for the diagnosis of AS. Moreover, it provides another strategy for the discovery of novel molecular therapeutic targets for AS.

Availability of Data and Materials

The data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors made substantial contributions to this study. ZH conceptualized, designed and supervised the study. GZ, LZ performed experiments, interpreted the data, and drafted the manuscript. YM, LY, LH collected the samples. ZH revised and approved the manuscript. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

All authors declare no conflicts of interest. Despite receiving sponsorship from Wuhan Nissi Biotech, the judgments in data interpretation and writing were not influenced by this relationship.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL36306.

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