# *Original Research*

# *Highlight article*

# HnRNP K regulates inflammatory gene expression by mediating splicing pattern of transcriptional factors

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## Impact Statement

We for the first time used RNA-seq methodology to reveal how hnRNP K functions in the transcription and alternative splicing regulation in HeLa cells. The results showed that hnRNP K influenced the proliferation of HeLa cells. On the molecular level, the results suggested that hnRNP K positively modulates the splicing of genes involved in transcriptional process of and the RNA level of genes implicated in inflammation. More importantly, analysis of transcription factors (TFs) and binding motifs indicated that several TFs regulated by hnRNP K at splicing level bind to the promoter of differentially expressed genes (DEGs) associated with inflammatory response. Therefore, we speculate that hnRNP K regulates alternative splicing of TFs, which then bind to inflammatory genes and change its expression. The hnRNP K-TFs-inflammatory genes axis provides novel molecular mechanisms of hnRNP K involved in HeLa cells and offers new treatment targets in the future.

## Abstract

HnRNP K is a heterogeneous nuclear ribonucleoprotein and has been identified as an oncogene in most solid tumors via regulating gene expression or alternative splicing of genes by binding both DNA and pre-mRNA. However, how hnRNP K affects tumorigenesis and regulates the gene expression in cervical cancer (CESC) remains to be elucidated. In these data, higher expression of hnRNP K was observed in CESC and was negatively correlated with the patient survival time. We then overexpressed hnRNP K (hnRNP K-OE) and found that its overexpression promoted cell proliferation in HeLa cells ($P=0.0052$). Next, global transcriptome sequencing (RNA-seq) experiments were conducted to explore gene expression and alternative splicing profiles regulated by hnRNP K. It is shown that upregulated genes by hnRNP K-OE were associated with inflammatory response and an apoptotic process of neuron cells, which involves in cancer. In addition, the alternative splicing of those genes regulated by hnRNP K-OE was associated with transcriptional regulation. Analysis of the binding features of dysregulated transcription factors (TFs) in the promoter region of the inflammatory response genes regulated by hnRNP K revealed that hnRNP K may modulate the expression level of genes related to inflammatory response by influencing the alternative splicing of TFs. Among these hnRNP K-TFs-inflammatory gene regulatory networks, quantitative reverse transcription polymerase chain reaction (RT-qPCR) experiments and gene silencing were conducted to verify the hnRNP K-IRF1-CCL5 axis. In conclusion, the hnRNP K-TFs-inflammatory gene regulatory axis provides a novel molecular mechanism for hnRNP K in promoting CESC and offers a new therapeutic target.

## Introduction

Cancer remains a global major public health issue and effective treatments for cancer are still being investigated. Cervical cancer (CESC) ranks third in cancer incidence worldwide and is of the top four leading risk factors for mortality among women.[1] In spite of advances in disease detection and the application of vaccines against human papillomavirus (HPV) that can effectively prevent the disease,[2–5] there has been no significant improvement in the therapeutic effect for CESC during the past few decades.[6,7] Currently, chemotherapy,[8] surgery,[9] and radiotherapy[10] are the most therapeutic regimen for CESC. However, resistance to chemotherapy

of CESC cells results in poor treatment efforts. Hence, it is of great importance to provide a novel approach to cancer treatment through disclosing the mechanism of CESC development and singling out certain targets.

Dysregulation of many RNA-binding proteins (RBPs) is discovered during the progression of various tumors,[11] since RBPs play important role in mRNA processing, including mRNA stability,[12] transport,[13] alternative splicing,[14] and translation,[15] they are commonly regarded as therapeutic targets and diagnostic indicators.[16] Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is an RBP and belongs to the subfamily of hnRNPs with a size of 65 kDa. Most of hnRNPs are present in nucleus, while hnRNP K seems to

shuttle between cytosol and nucleus;[17] therefore, hnRNP K participates in multiple biological and cellular processes, especially in transcriptional and post-transcriptional regulation. For example, hnRNP K was known as a transcription factor with an ability to bind to a particular DNA sequence and to interact with other transcription regulators.[18] Meanwhile, hnRNP K can regulate pathways in carcinogenesis and tumor suppression.[19] Most interestingly, sumoylation of hnRNP K induces p53 transcriptional activation.[20] In addition to transcription regulation, hnRNP K can also function as gene splicing mediator. HnRNP K includes the third exon of MRPL33 in colorectal cancer tissue and is involved in tumor progression.[21] Peng *et al.*[22] suggested that upregulation of hnRNP K in gastric cancer (GC) activates the expression of SRSF1, which is a famous splicing factor. Then, SRSF1 induces the expression of the isoform of oncogene CD44, and ultimately promotes the production, migration, and invasion of GC cell. As an oncogene, hnRNP K has been widely investigated in multiple solid cancers, with cancers of lung,[19] prostate,[23–25] colon,[26] renal cell,[27] and the nasopharynx included,[28] which indicates the close connection between hnRNP K and tumor formation and development. However, the function of hnRNP K in CESC was rarely reported. Zhang *et al.*[29] found that nujiangexathone, a compound from *Garcinia nujiangensis*, can inhibit the progression of CESC by downregulating hnRNP K, eventually causing cell-cycle arrest. One recent study demonstrated hnRNP K promotes malignant phenotypes of HeLa cells by regulating lncRNA-LINC00263,[30] while the precise mechanism of hnRNP K implicated in CESC remained elusive. As an RBP, whether hnRNP K also has considerable roles in the CESC development by modulating transcriptome profile associated with cancer needs to be further investigated.

To further explore the mechanisms and functions of hnRNP K in CESC, it was overexpressed in HeLa cells, which could significantly promote HeLa cell proliferation. RNA sequencing was performed to discover hnRNP K–modulated splicing and gene expression pattern in HeLa cells. Functional enrichment analysis revealed that differentially expressed genes (DEGs) were significantly enriched in inflammation pathways. On the contrary, alternative splicing genes regulated by hnRNP K (RASGs) were significantly enriched in the pathways of transcriptional regulation. Combined with analyzing the target genes of transcription factors (TFs) regulated by hnRNP K, the motifs of those TFs were observed in the promoter regions of inflammatory response DEGs. The results suggest that hnRNP K mediates the transcription of inflammatory-response-related genes and this regulation may be mediated by the splicing of TFs. The hnRNP K-TFs-inflammatory genes axis potentially sheds light on the mechanisms of hnRNP K implicated in CESC and offers new therapy target in the future.

## Materials and methods

### Construction of plasmid and small interfering RNA

Pairs of primers were prepared by CE Design V1.04. Every primer included a set of sequences specific to each gene and the vector pIRES-hrGFP-1a. GFP, which is a fluorescence label contained in plasmid, is widely used as genetically encoded fluorescent fusion tags. The primers are listed below.

F-primer: agcccgggcggatccgaattcATGGAAACTGAACAGCCAG

R-primer: gtcatccttgtagtcctcgagGAAAAACTTTCCAGAATACTGC

The pIRES-hrGFP-1a vector was digested in EcoRI (NEB, 3101S, England) and XhoI (NEB, R0146V, England) at 37°C for 2–3 h. A 1.0% agarose gel was used to run the enzyme-digested vector. And Qiagen column kit was used to purify the vector. With TRIzol reagent, total RNA was separated from HeLa cells. After purification, cDNA was generated by the reverse transcription of RNA. The fragment inserted was synthesized via amplification of polymerase chain reaction (PCR). By the ClonExpress® II One Step Cloning Kit (Vazyme, C112, China), both the PCR-mediated insertion and a linearized vector digested via EcoRI and XhoI (NEB) were ligated in a PCR microtube. Chemical transformation was used to introduce plasmids into the *Escherichia coli* strain. Placed on the LB agar plates with 1 μL/mL ampicillin, the cells were incubated for one night. To screen out the colonies, colony PCR was performed with universal primers (on the backbone vector) by 28 cycles. For IRF1 silencing, small interfering RNA (siIRF1) GCUACACAGUUCCAGGCUAUT (sense) and non-targeting control siRNA (siNegative): 5'-UUCUCCGAACGUGUCACGUTT-3' (sense) were obtained from Gemma (Suzhou, China).

### Cell culture and transfection

Both HeLa and A549 cells were obtained from the Institute of Biochemistry and Cell Biology (Shanghai, China). Cell culture conditions and transfection methods were followed from a previously published paper.[31]

The efficiency of overexpressed hnRNP K (hnRNP K-OE) and IRF1 knockdown was assessed using quantitative reverse transcription polymerase chain reaction (RT-qPCR) method with an internal control gene GAPDH. First, RT-qPCR was performed to determine hnRNP K expression and GAPDH. The density of hnRNP K and IRF1 was standardized to the GAPDH level according to the $2^{-\Delta\Delta CT}$ calculation method, which was reported by Livak and Schmittgen in 2001.[32] The primers were presented in Supplemental Table S1. What's more, Western blot (WB) was also performed, the purpose of which was to evaluate the efficiency of hnRNP K overexpression; anti-hnRNP K (11426-1-AP, 1:1000; Proteintech, China) was used against hnRNP K and GADPH was used as internal control.

### Cell proliferation and apoptosis experiments

To assess how hnRNP K overexpression or IRF1 silencing impacts cell proliferation, an MTT assay experiment was conducted as described previously,[33] in which MTT-produced crystals were melted in dimethyl sulfoxide (DMSO). Then, the optical density (OD) values were determined at an optical wavelength of 570 nm. For cell apoptosis, $10^5$ cells were seeded into 24-well culture plates and incubated at 37°C and

5% $CO_2$ for 24 h. Then, the apoptosis level was assessed by flow cytometry (CytoFLEX; Beckman, USA) following one published paper.[34]

### RNA-seq experiment

Total RNA from HeLa cell was extracted using TRIzol (15596-018, Ambion, USA). The total RNA purification, genomic DNA removal, quantity and quality determination of purified RNA, and RNA integrity were conducted or assessed following the published methods.[31]

Before sequencing, 1 µg total RNA was utilized to construct RNA-seq library of each sample. First, conjugated magnetic beads with oligo (dT) (61005, Invitrogen; Thermo Fisher Scientific, Inc., USA) were used to purify and concentrate the polyA mRNAs. The fragmented RNAs were end-repaired, followed by ligating at the 5′ end at 95°C. Then, purified RNAs were reverse-transcribed (RT) to cDNAs and stored at −80°C after amplifying. The libraries were constructed to perform paired-end high-throughput sequencing with 150 nt on the HiSeq X Ten system (Illumina, USA).

### RNA-seq data and DEGs analysis

First, raw reads with unknown bases were trimmed off/eliminated. Then, sequencing adaptors and short reads not more than 16 nt were also discarded with FASTX-Toolkit (version 0.0.13). Clean reads were aligned onto the human genome (GRCh38) using TopHat2[35] software. We counted uniquely mapped reads to calculate FPKM (Fragments Per Kilobase of exon per Million reads) values for detected genes as their expression level.[36] The DEGs were screened out using the R Bioconductor package edgeR[37] with false discovery rate (FDR) < 0.05 and fold change (FC) ⩾ 2 or ⩽ 0.5 as criteria.

### Alternative splicing analysis

To identify differential alternative splicing events (ASEs), regulated ASEs (RASEs) were determined with ABLas program.[38] According to the uniquely mapped reads and splice junction reads, ASEs were commonly divided into eight types, which contains exon skipping (ES), mutually exclusive exons (MXES), the MXE combined with alternative 5′promoter (5pMXE), the MXE combined with alterative 3′promoter (3pMXE), intron retention (IR), mutually exclusive 3′ untranslated regions (UTRs), alternative 5′ splice site (A5SS) and alternative 3′ splice site (A3SS). To identify hnRNP K-RASEs, Fisher's exact test was utilized to count the statistical *P* value. The RASE difference was defined as ratio between alternatively spliced reads and constitutively spliced reads. Ratio > 0.2 and *P* value < 0.05 were the thresholds to identify RASEs.

### DEGs and RASEs verification

To verify identified DEGs and RASEs from the RNA-seq data, RT-qPCR experiment was conducted. We designed specific primer sequences for RASEs, following one published study.[39] The primer sequences utilized in qPCR are shown in Supplemental Table S1. The detailed experimental procedures had been described in the above part.

### WB experiment

Except for RT-qPCR, WB experiment was conducted to assess the protein level of CCL5 in A549 cells and HeLa cells; total protein was obtained from hnRNP K-OE and control cells with radioimmunoprecipitation assay (RIPA) buffer. The western assay was performed according to the description of previous study.[40] The information of primary antibodies used in experiment was as follows: CCL5 (1:500; Sigma, USA) and GAPDH (A19056, 1:1000; ABclonal, China). Bound secondary antibody (HRP Goat Anti-Rabbit IgG [H + L], AS014 1:10000; ABclonal, China) was identified with enhanced chemiluminescence (ECL) reagent (32106; Thermo Scientific, USA). We used GAPDH or β-actin as internal controls.

### Other statistical analysis

Functional enrichment statistics of selected gene sets were predicted using GO and KEGG pathway annotation with the KOBAS 2.0 server.[41] *P*-value was calculated by hypergeometric test and was then corrected by Benjamini–Hochberg FDR controlling procedure to identify the enrichment. Unpaired Student's *t*-test was conducted to make comparisons between two groups. *P*-value < 0.05 was regarded as significant difference. The statistical values were presented as the mean ± standard deviation with at least three biological replicates, except for the RNA-seq.

## Results

### Expression of hnRNP K and prognostic value in CESC

HnRNP K is an extensively expressed gene in multiple cancers; its expression between tumor and normal tissue in different cancer types from TCGA is shown in Figure 1(A) using GEPLA2, a web-based tool. HnRNP K had a higher expression level in tumor than in normal tissues in 12 of 16 cancer types, indicating that hnRNP K was inclined to act as an oncogene. Furthermore, we assessed the prognostic value of hnRNP K in CESC based on TCGA samples and found that elevated hnRNP K expression was correlated with poor prognosis of patients with CESC (Figure 1(B)).

### HnRNP K overexpression promotes HeLa cell proliferation

To uncover the mechanisms of hnRNP K functioning in CESC, an hnRNP K–overexpression HeLa cell model was constructed. The hnRNP K expression level was examined by conducting RT-qPCR and WB experiments (Figure 1(C) and (D)), indicating that hnRNP K was successfully overexpressed in HeLa cells. In conformity with the results of MTT assay (Figure 1(E)), the proliferation level was increased in hnRNP K–overexpressed HeLa cells in comparison with control (*P* = 0.0052), demonstrating that highly expressed hnRNP K promotes proliferation in HeLa cells.

### HnRNP K–regulated HeLa transcriptomes

In order to comprehensively study hnRNP K–mediated regulation in HeLa cell, cDNA libraries were constructed with
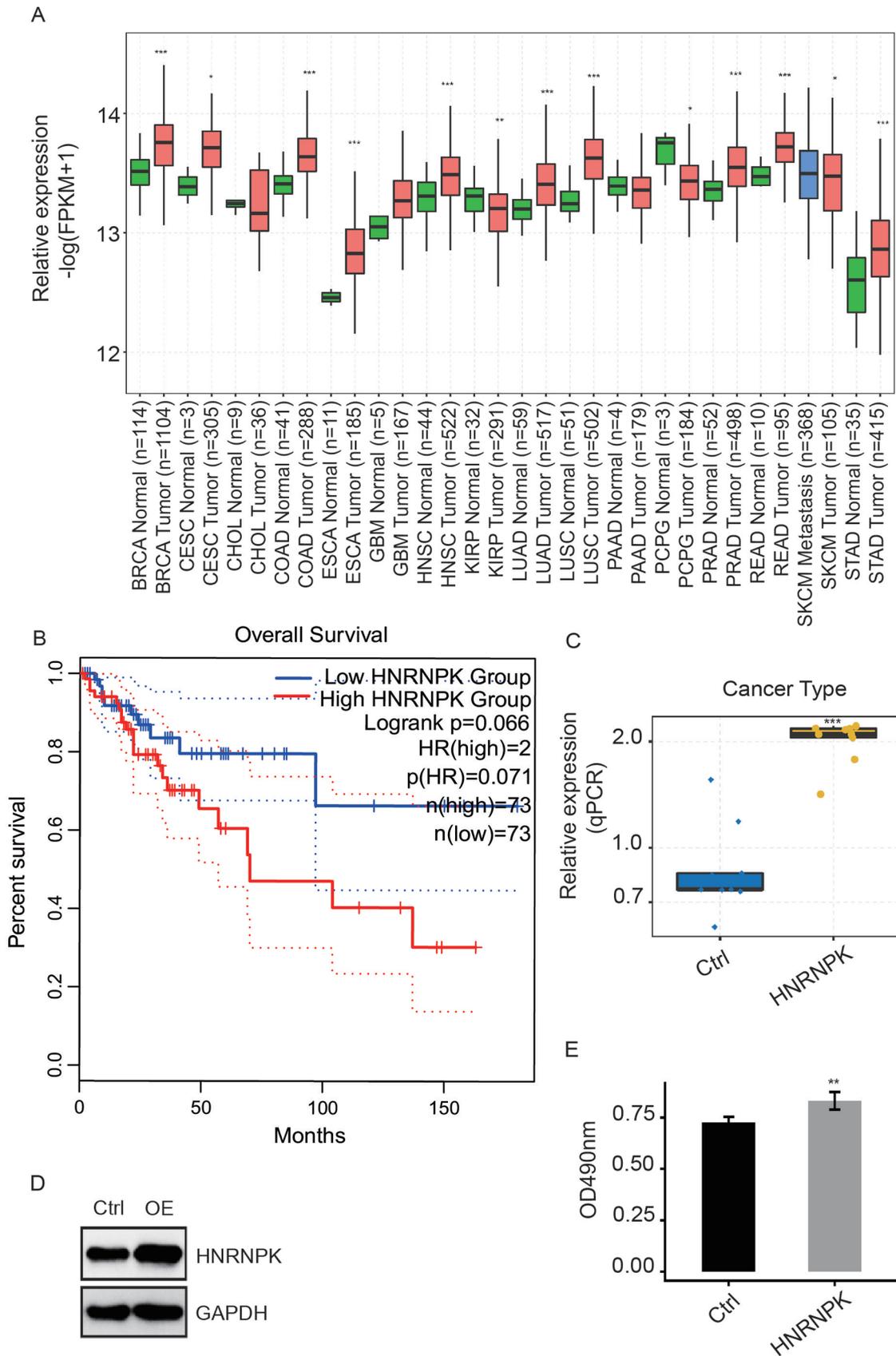
**Figure 1.** HnRNP K overexpression promotes the proliferation of HeLa cells. (A) Box plot showing the relative expression of hnRNP K in tumor and normal samples from TCGA. (B) Line plot showing the Kaplan–Meier analysis of the connection between hnRNP K expression and clinical prognosis based on TCGA data. (C-D) HnRNP K expression was validated by RT-qPCR (C) and WB (D) after treatment with hnRNP K-OE or control vector. (E) MTT assay demonstrated that HeLa cells being treated with hnRNP K-overexpression vector increased significantly. A comparison between control and hnRNP K-OE HeLa cells was made using Student's *t*-test. **$P < 0.01$; ***$P < 0.001$. (A color version of this figure is available in the online journal.)
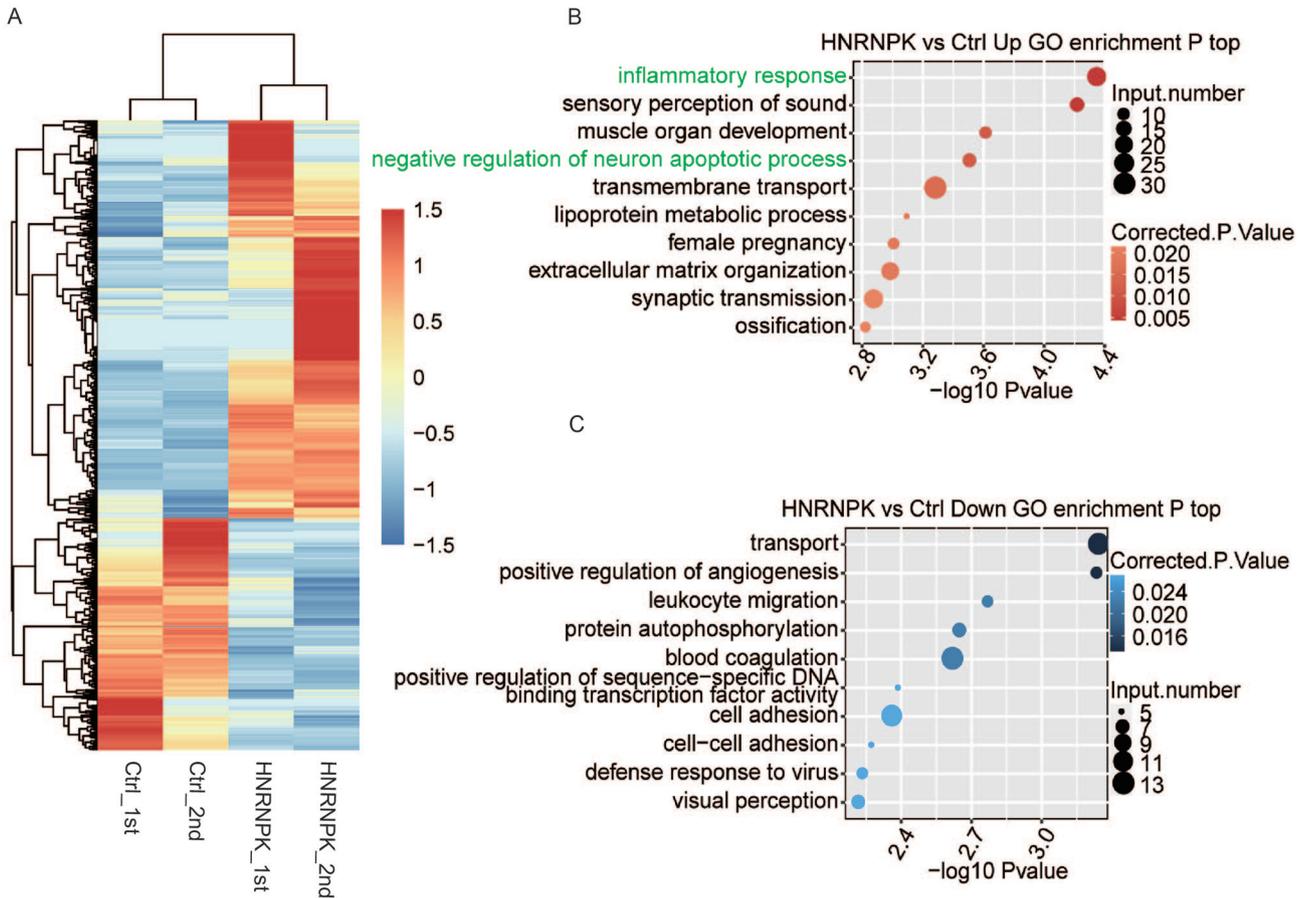
**Figure 2.** Changes of gene expression analysis responding to hnRNP K-OE. (A) Heat map of all 2405 DEGs identified in this study. FPKM values are log2-transformed and then median-centered by each gene. (B-C) The top 10 GO biological process terms of genes upregulated (B) and downregulated (C) by hnRNP K-OE. (A color version of this figure is available in the online journal.)

hnRNP K-OE cells and control cells (2 biological replicates). We then obtained four RNA-seq samples by high-through-put sequencing method.

The mean and standard deviation of raw data was $94.2 \pm 4.4$ million per sample. A total of $90.1 \pm 4.9$ million reads per sample, which were prepared for downstream bioinformatics analysis, were retained after discarding low-quality sequences and adaptor sequences. Then, we used TopHat2 to map the filtered reads onto the human GRCh38 genome, which revealed that 74.80–82.19% were mapped and 94.94–95.70% were uniquely mapped.

To show the expression values of the genes detected from the uniquely mapped reads, we calculated the FPKM values. In total, 26,247 genes were attained with FPKM > 0, among which 11,810 genes had FPKM > 1 in at least one sample. The principal component analysis (PCA) result revealed that the top two components account for 79% of the variation, and hnRNP K-OE and control samples were clearly separated from each other, indicating that hnRNP K-OE can extensively affect expression in HeLa cells (Supplemental Figure S1(A)).

### HnRNP K selectively regulates DEGs associated with inflammatory response

Based on gene expression values, we further studied genes that were significantly regulated by hnRNP K. To identify the DEGs, we used edgeR program[37] with criteria of absolute FC ≥ 2 or ≤ 0.5 and FDR < 0.05. A total of 2405 DEGs with 1519 upregulated and 886 downregulated genes were identified between hnRNP K-OE and control cells, which indicated that hnRNP K extensively regulates gene transcription (Supplemental Figure S1(B)). The difference between the number of upregulated and downregulated genes indicates that hnRNP K may have a preferential regulation in upregulating gene expression. Detailed expression levels of the DEGs were presented in Supplemental Table S2. The hierarchical clustering of all DEGs was constructed and revealed the obvious expression change between the two datasets (Figure 2(A)).

To investigate the enriched functions of these DEGs, 2405 DEGs were analyzed based on GO and KEGG pathway enrichment analysis. As a result, 632 up DEGs and 311 down DEGs were classified into 35 GO terms and 30 GO terms, respectively. Upregulated DEGs in hnRNP K-OE cells were chiefly enriched in cancer-associated pathways, including inflammatory response, transmembrane transport, lipoprotein metabolic process, and extracellular matrix organization (Figure 2(B)). The inflammatory response (corrected $P$ value = 0.0043), which was the most enriched upregulated term in biological process, contains 23 inflammation-related genes, including *CCL5, AOC3, NOS2, BCL6, FOS, IL17F, ADAM8, UCN,* and *CD14/180.* The downregulated genes
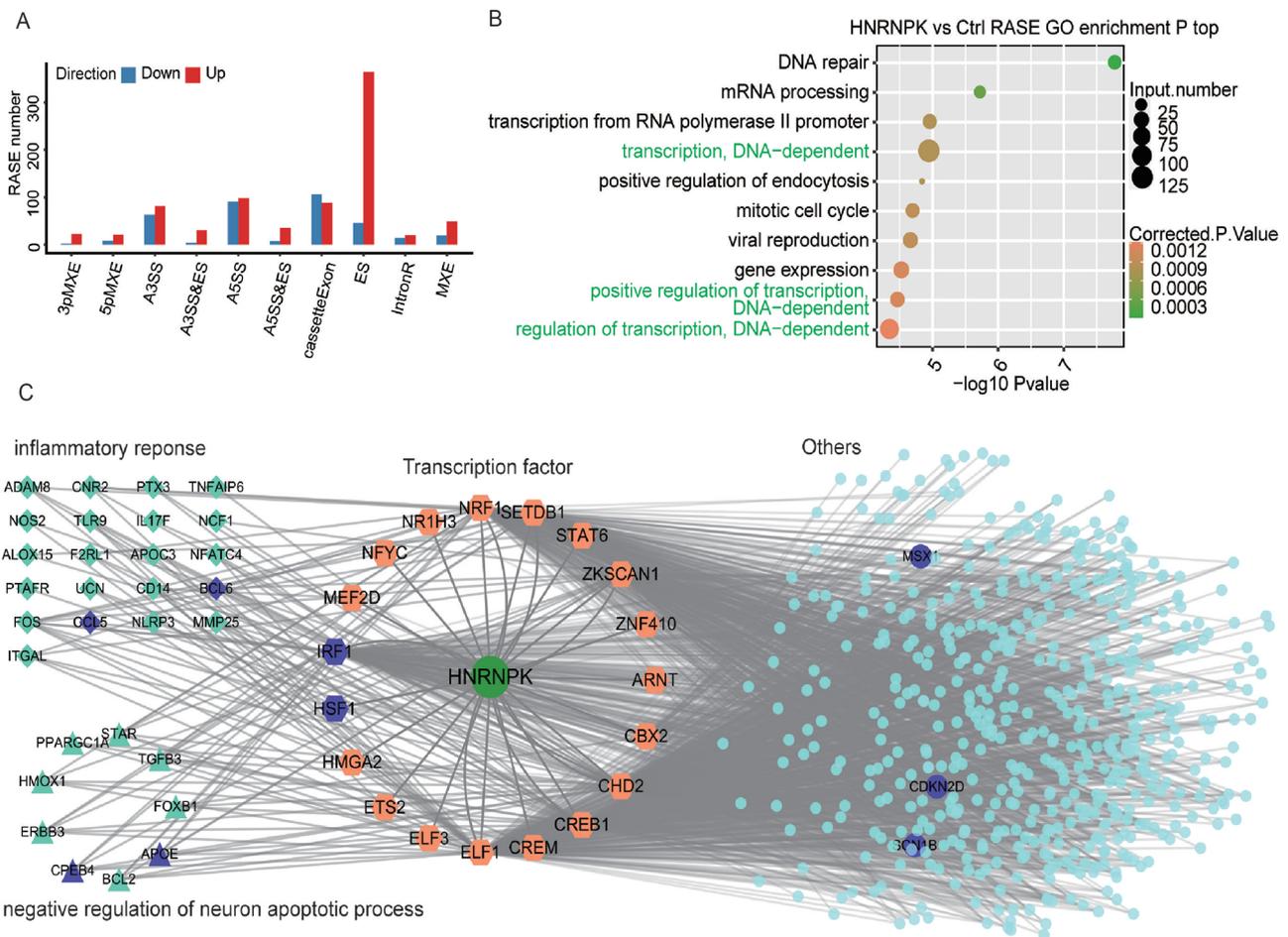
**Figure 3.** HnRNP K extensively changed ASEs in HeLa cells. (A) Categories of alternative splicing events regulated by hnRNP K. (B) The top 10 GO biological process pathways of regulated alternative splicing genes. (C) HnRNP K-TFs-targets regulation network in HeLa cells. Hexagons represent transcription factors. Squares represent inflammatory response genes and triangles represent apoptotic-related genes. (A color version of this figure is available in the online journal.)

were mostly related to transport, positive regulation of angiogenesis, positive regulation of sequence-specific DNA binding transcription factors, cell adhesion, and cell–cell adhesion (Figure 2(C)). According to the results of KEGG analysis, DEGs were enriched in pathways, including rheumatoid arthritis, amoebiasis, ATP-binding cassette (ABC) transporters, peroxisome proliferator-activated receptor (PPAR) signaling pathway, transcriptional misregulation in cancer and legionellosis (Supplemental Figure S1(C) and (D)). It is notable that the most enriched upregulated annotations in both GO and KEGG analyses were associated with inflammation. The findings above indicate that hnRNP K can extensively affect the expression levels of inflammation-associated genes.

**HnRNP K extensively regulated alternative splicing profile in HeLa cells**

The impact of hnRNP K on alternative splicing (AS) in HeLa cells was explored according to the transcriptome data. From the aligning result, nearly 51% are junction reads, including 162,918 known junction reads and 221,547 novel junction reads. A stringent threshold of $P$ value $\leqslant 0.05$ and changed $T$-value $\geqslant 0.2$ was set as the criteria to identify RASEs with a high confidence; A5SS, A3SS, and ES were the three most

prevalent AS events induced by hnRNP K; and exon skipping (ES) events were extremely induced in hnRNP K-OE samples (Figure 3(A)). The results showed that hnRNP K extensively modulated ASEs in HeLa cells.

**HnRNP K modulates gene expression by regulating alternative splicing of transcription factors**

To discover the functions of alternative splicing genes regulated by hnRNP K, GO (Figure 3(B)) and KEGG (Supplemental Figure S2(A)) functional clustering analyses were conducted. The results demonstrated that these genes were highly enriched in transcription-associated pathways, in which large number of transcription factors (TFs) were included. We hypothesized that overexpression of hnRNP K might modulate gene expression by altering the AS of TFs.

To prove the abovementioned hypothesis, we extracted 68 hnRNP K–regulated TFs enriched in regulation of transcription, transcription, or DNA-dependent pathway. Based on TRRUST and ENCODE Transcription Factor Targets dataset, the DNA binding motifs of 19 out of 68 TFs were presented in the promoter regions of 573 DEGs induced by hnRNP K. GO analysis showed that these 573 DEGs were mainly involved in inflammatory response, extracellular matrix organization pathways and negative regulation of neuron
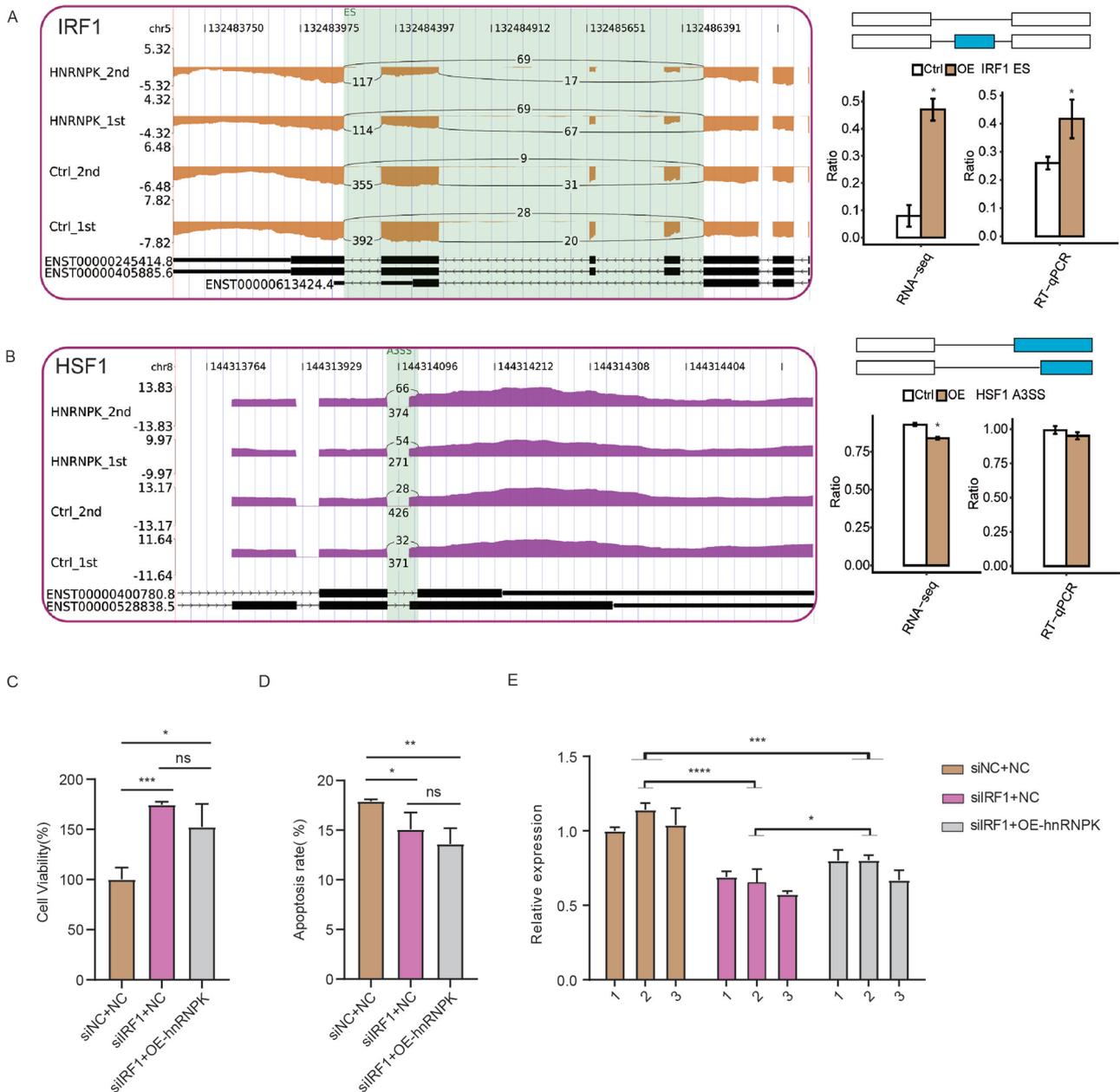
**Figure 4.** Validation of hnRNP K-IRF1-CCL5 axis in HeLa cells. IGV-sashimi plots (left panel) and RT-qPCR validation (right panel) showed ASEs and the changed ratio of transcription factors, including (A) IRF1 and (B) HSF1. The schematic diagrams present the structures of AS (right panel, top) and Model (right panel, middle). Boxes denote the exon sequences. The horizontal line denotes the intron sequences. (C) Bar plot showing the cell proliferation rate in three groups. (D) Bar plot showing the cell apoptosis rate in three groups. (E) Bar plot showing the expression level of CCL5. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; Student's *t*-test for A-D; two-way ANOVA test for E. (A color version of this figure is available in the online journal.)

apoptotic process (Figure 3(B)), which was highly similar to that in Figure 2(B). The results confirmed the hypothesis that the hnRNP K–regulated TF alternative splicing plays an important role in establishing the regulation network of hnRNP K (Figure 3(C)).

## Validation of hnRNP K-IRF1-CCL5 axis in HeLa cells

Among the hnRNP K-TF-inflammatory gene regulatory network, we were particularly interested in the hnRNP K-IRF1-CCL5 axis. Inflammatory mediator CCL5 was found to play key roles in cancer progression, while IRF1 encodes

an activator of immune response genes.[42,43] Previous study revealed that IRF1 binds to a TTTTC motif-containing mouse CCL5 promoter.[44] To confirm our finding that hnRNP K may regulate inflammatory response and tumorigenesis via IRF1-CCL5 pathway, we performed RT-qPCR to further study their expression relationship. The ASEs regulated by hnRNP K in IRF1, HSF1, HDAC10 and BRD4 were verified by RT-qPCR experiment, which confirmed the RNA-seq results (Figure 4(A), (B) and S2(C) and (D)), except that HSF1 showed no significant difference (Figure 4(B)). We then silenced IRF1 expression by small interfering RNA (siIRF1) in HeLa cells to explore its influence on cellular phenotypes and the expression level of CCL5. SiIRF1 significantly

increased proliferation rate and decreased apoptotic level of HeLa cells (Figure 4(C) and (D)), consistent with its known functions in cancer.[45] Under hnRNP K-OE condition, siIRF1 could also reverse maintain the increased proliferation rate and decreased apoptotic level (Figure 4(C) and (D)). Furthermore, we checked the expression level of CCL5 and found siIRF1 significantly reduced CCL5 expression level under hnRNP K-OE and normal conditions (Figure 4(E)). These results indicate that IRF1 regulates cellular phenotype of HeLa cells and could rescue CCL5 expression in hnRNP K-OE cells.

Other than CCL5, six other hnRNP K–upregulated DEGs (*CPEB4*, *MSX1*, *BCL6*, *APOE*, *SCN1B*, and *CDKN2D*) from inflammatory response and negative regulation of neuron apoptosis pathways, which are important biological process during tumorigenesis, were chosen for the purpose of qPCR validation. The results were shown in Figure 5(A), in which all seven genes were significantly increased along with hnRNP K overexpression. Please be noted that the total RNA template used for this validation experiments was the same as in RNA-seq experiments. HnRNP K–induced expression of CCL5 was also validated in WB experiments (Figure 5(B)).

To further validate the above results, we performed a new set experiment from cell culture and plasmid transfection in triplicates. Using the total RNA from this new set of experiments, we conducted RT-qPCR experiment to assess the expression levels of the same seven genes, which demonstrated highly consistent results with the previous experiments (Figure 6(A)).

### HnRNP K regulates expression of CCL5 in A549 cells

To confirm our findings that CCL5 is an important target of hnRNP K, a plasmid with hnRNP K overexpression was transfected into A549 cells. The expression of CCL5 and hnRNP K was validated by RT-qPCR and WB (Figure 6(B)). Our results demonstrated the successful overexpression of hnRNP K in A549 cells. In addition, overexpression of hnRNP K significantly induced the expression of CCL5, conforming to the results from HeLa cells.

### Discussion

HnRNP K is a multifunctional protein implicated in RNA metabolism, RNA stability and translation, RNA splicing, and DNA transcription. Various lines of evidences have confirmed that aberrant expression of hnRNP K is contributing to tumor proliferation. For example, hnRNP K promotes cell proliferation in lung cancer,[19] bladder cancer,[46] and colon cancer.[26] In accordance with previous studies, results in our study revealed that hnRNP K overexpression extensively increased the proliferation of HeLa cells.

According to previous studies, hnRNP K was elevated in bladder cancer, which regulates expression of several genes to promote bladder cell proliferation, chemoresistance to cisplatin, and apoptosis inhibition.[46] Gao *et al.* reported that hnRNP K has the ability to increase tumor metastasis by regulating the expression of genes with cell motility, angiogenesis, and extracellular matrix functions. HnRNP K is also overexpressed and correlated with mutant p53 in pancreatic

carcinoma.[47] Similarly, hnRNP K promotes the progression of prostate cancer by regulating the androgen receptor (AR) translational apparatus and its altered pattern of expression has potential values for diagnosis and prognosis in prostate cancer.[23] Hence, we adopted a high-throughput transcriptomic RNA-seq approach to reveal how hnRNP K affects tumor cells in transcriptome. Hybridization-based approaches can only be used to detect transcripts corresponding to sequences that already exist, whereas RNA-seq approaches have no upper limit on quantification.[48] In our study, transcriptome analysis of hnRNP K overexpression and control cells was performed, and a total of 2405 DEGs and 1052 RASGs were detected, suggesting that the activation of hnRNP K has a wide range of effects.

DEGs regulated by hnRNP K were unbiasedly analyzed and were shown to be enriched in multiple KEGG pathways and GO functional pathways. Previous study demonstrated that inflammation is involved in cancer progression, involving aberrant tissue repair, genotoxicity, proliferation, invasion, and migration.[49] In this study, hnRNP K upregulates expression of CCL5 and BCL6 among inflammatory response. As one of the chemokine superfamily members, CCL5 is well recognized in immune environment, in which it induces leukocyte-directed motility.[50] The expression of CCL5 is induced by inflammatory insult, whereas blocked when inflammation subsides. CCL5 acts mainly in inflammatory reactions, which are confirmed to be highly expressed by numerous tumor cells and exert certain pathogenic influences on malignant tumors and metastases.[51–53] According to previous studies, the level of CCL5 was found markedly increased in tissue and plasma of patients with CESC.[54] Besides, our hypothesis that hnRNP K regulates the expression of CCL5 was confirmed in A549 cells (Figure 6(B)). BCL6 can interact with large numbers of genes directly or indirectly and it is mainly required for germinal centers formation, which are necessary to produce an effective humoral immune response.[55] BCL6 also plays an important role in breast cancer,[56] ovarian cancer,[57] and non-small-cell lung cancer progression.[58] Above all, this study indicates that hnRNP K might contribute greatly to cancer progression by activating the inflammatory oncogenes. Meanwhile, it is of necessity to validate the functions of hnRNP K in CESC as well as other cancers by testing its expression level in tumor tissues, analyzing its influence on cell condition, and exploring the underlying mechanisms.

The extensive prevalence of AS suggests the importance of genomic complexity-regulated biological process. As an important RBP, hnRNP K is found to mediate the AS pattern of many genes. Tyson-Capper and Gautrey[59] described hnRNP K as a splicing enhancer which promotes inclusion exon 2 Mcl-1 and subsequently produces the antiapoptotic Mcl-1$_L$. HnRNP K could regulate alternative splicing of MRPL33 and then promote production of splice variant with the third exon inclusion in colorectal cancer cells.[21] Other than mediating splicing via direct binding with pre-mRNA, hnRNP K also activated the expression of the splicing regulator SRSF1 and then induced splicing activity.[22] We found that hnRNP K overexpression significantly changes the AS of numbers of genes in HeLa cells according to previous studies. A panel of those genes is enriched in transcription
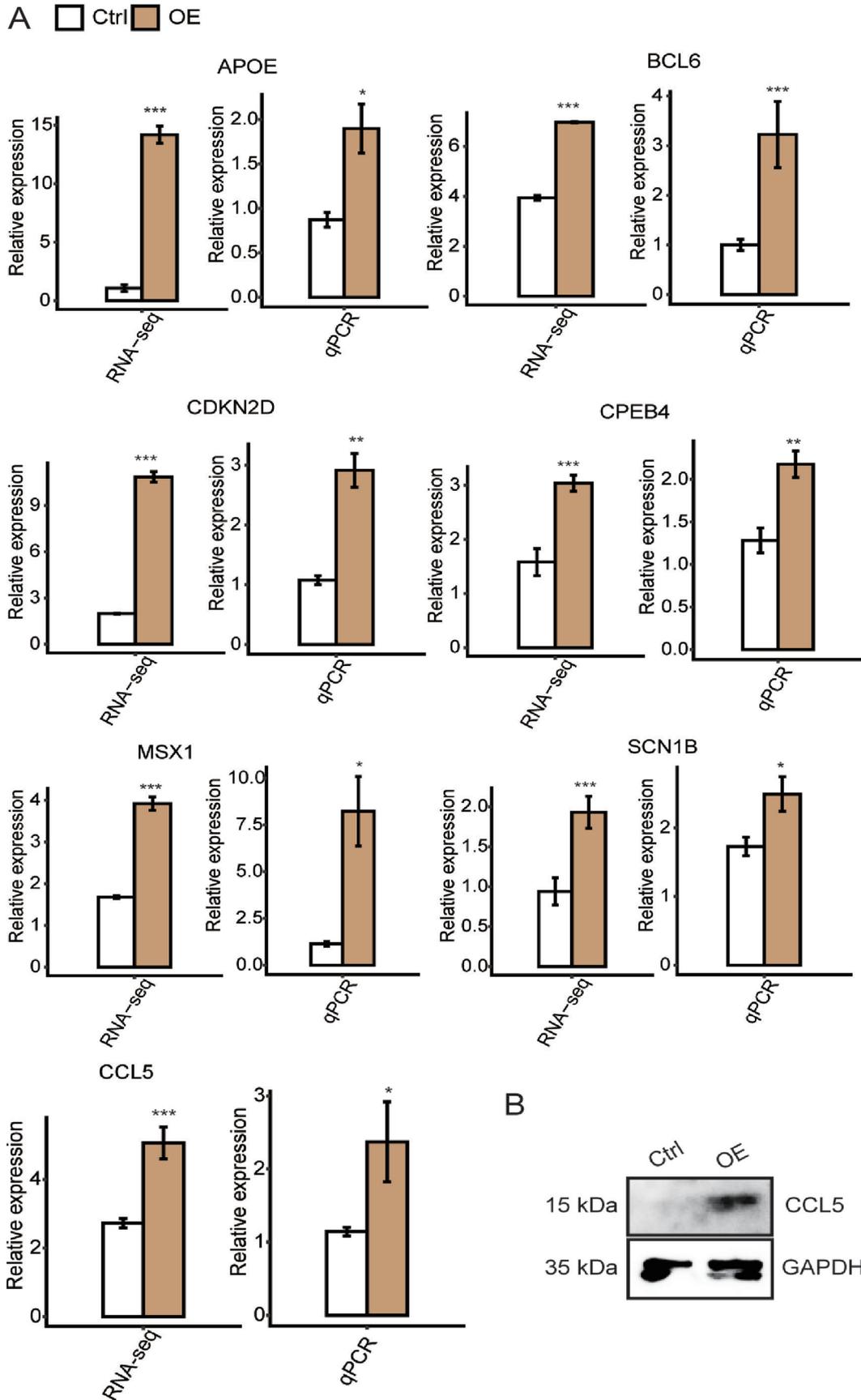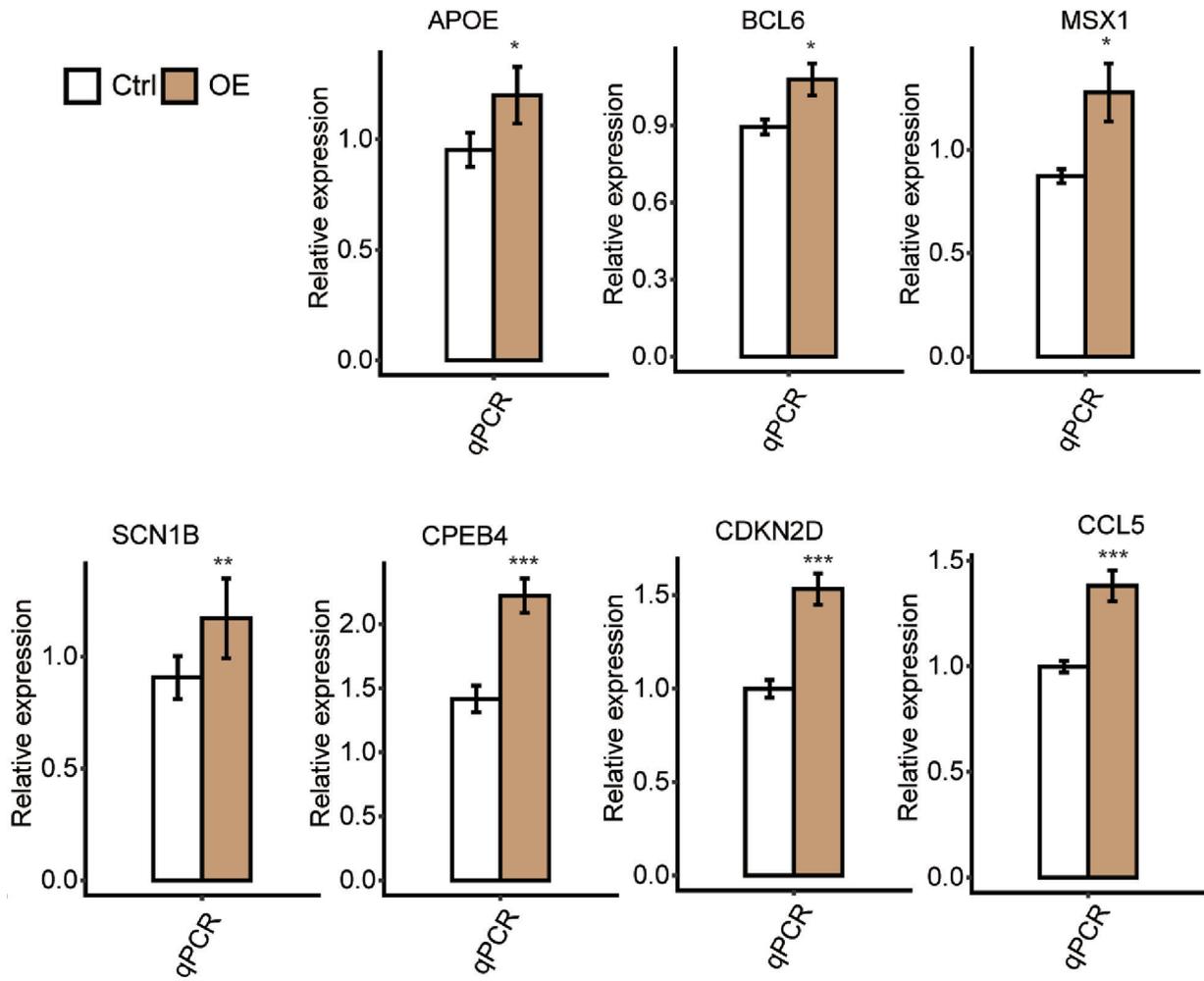
**Figure 5.** Validation of hnRNP K–regulated genes by qPCR and Western blot. (A) Bar plot showing the expression levels of seven genes with hnRNP K-OE and control vector in HeLa cells. (B) Validating the effect of hnRNP K–regulated gene CCL5 by conducting Western blot experiment in HeLa cells. (A color version of this figure is available in the online journal.)
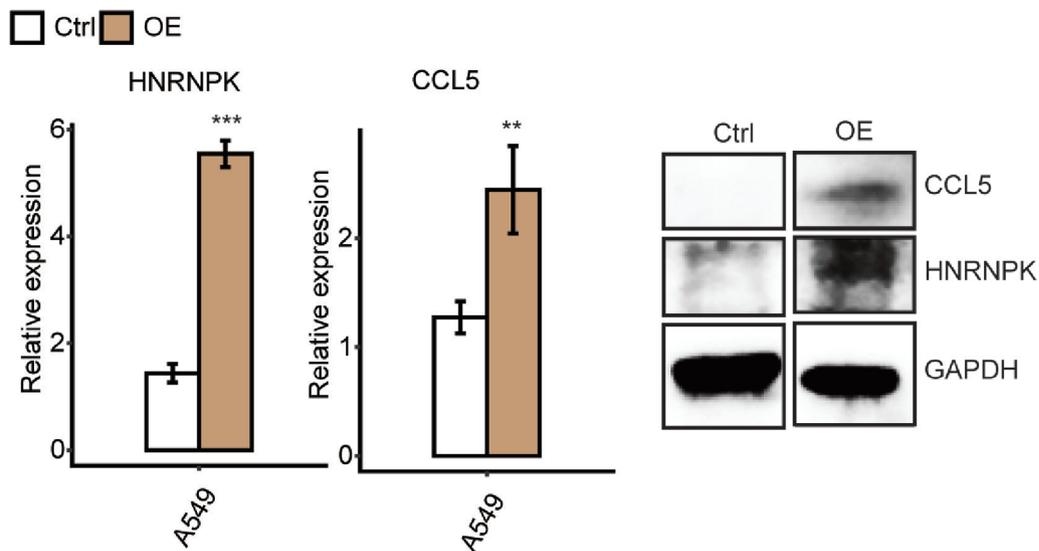
**Figure 6.** (A) A new set of experiment validating hnRNP K–regulated DEGs shown in Figure 4(A) in triplicate. (B) Validating the effect of hnRNP K–regulated gene CCL5 by qPCR and Western blot in A549 cells. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (A color version of this figure is available in the online journal.)

or regulation of transcription and in DNA-dependent pathways, including IRF1 and HSF1, which are well-known transcription factors. Belluti suggested that alternative splicing of cancer-related transcription factors can generate isoforms that promote cell proliferation, differentiation block, and apoptosis resistance.[60] IRF1 encodes a protein serving as an activator of genes implicated in both innate and acquired immune response. It was reported that IRF1 was implicated in CESC through STAT3/IRF1 pathway.[61] Lee *et al.*[62] discovered IRF1 variants with the exclusion of exons 7, 8, or 9, a crucial mechanism for negatively regulating IRF1 in CESC. Based on transcription factor and binding motif analysis, IRF1 was found to bind to the promoter of several genes, including CCL5 and BCL6, which are important in inflammatory response (Figure 3(C)). This result is consistent with the finding of Liu *et al.*[44] who have identified IRF1 bound to mouse CCL5 promoter directly with a TTTTC motif at –147 to –143 and provided a novel defense mechanism against tumor development. We validated IRF1 could affect cellular phenotypes and rescue CCL5 expression under hnRNP K-OE condition in HeLa cells; we speculate that hnRNP K indirectly modulates the expression level of genes implicated in inflammatory responses via regulating AS of TFs.

Except for IRF1, the other three transcription factors also play essential roles in inflammation process via modulating gene expression. For example, HSF1 activates the inhibition of NF-kB cytokine production mediated by hypercapnia[63] or exhibits anti-inflammatory functions in hepatocytes induced by zerumbone.[64] IL-6 was induced by oxidant stress. CXCL8 was remarkably inhibited by BRD4 restriction, indicating its essential function in regulating inflammatory genes.[65] HDAC10 was reported to alleviate inflammation after intracerebral hemorrhage[66] or promote IL-1β-mediated inflammatory activation of mesenchymal stem cells derived from synovium.[67] In addition, previous studies have shown that HSF1, BRD4, and HDAC10 were implicated in CESC,[68–70] supporting our hypothesis that hnRNP K might regulate expression of genes involved in inflammation via modulating alternative splicing of transcription factors. Nevertheless, the splicing isoforms of these genes and their exact functions on CESC remain to be disclosed. Another interesting point that needs to be further investigated is whether human papillomavirus (HPV) regulates hnRNP K expression. It has been reported hnRNP K inhibited the protein level of HPV-16 L2 mRNA *in vitro*,[71] while no other study reported the relationship between HPV and hnRNP K in cervical cancer.

## AUTHORS' CONTRIBUTIONS

JT and SL devised the study. YD and RY conducted the experiments. SL and DC collected and analyzed the dada. SL prepared this manuscript. JT oversaw and revised the final manuscript. All authors have read and approved the final manuscript.

## DECLARATION OF CONFLICTING INTERESTS

## FUNDING

## DATA AVAILABILITY

The raw reads of RNA-seq data have been deposited in NCBIs Gene Expression Omnibus (GEO) with accession number GSE148385.

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## SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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