Article

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Blocking *CCR10* Expression Activates m6A Methylation and Alleviates Vascular Endothelial Cell Injury

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Background: Cardiovascular disease, one of the most common types of disease in clinical practice today, carries a very high risk of disability and death. This research aims to examine genome-wide changes in injured human dermal microvascular endothelial cells (HDMECs) using the Ribonucleic Acid sequencing (RNA-Seq) technique, and to search for key genes influencing N6-methyladenosine (m6A) methylation, thus gaining new insights into future clinical diagnosis and treatment of cardiovascular diseases (CVDs) and laying a foundation for follow-up research.

Methods: Impaired HDMECs (injury group), established by endotoxin intervention, were analyzed by RNA-Seq for differentially expressed genes (DEGs) relative to normal HDMECs (control group). Then, DEGs that might be associated with m6A methylation were selected for expression blocking to observe m6A methylation alterations. The migration, angiogenesis, and inflammatory response of damaged HDMECs were detected by cell scratch assay, western blotting, and Enzyme-linked Immunosorbent Assay (ELISA) experiments, respectively.

Results: In this study, 20 DEGs were screened out from the two groups by RNA-Seq, of which 17 were up-regulated and 3 were down-regulated. The C-C motif chemokine receptor 10 (*CCR10*) was selected for subsequent analysis. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) identified elevated *CCR10* expression and reduced m6A methylation levels in the injury group ($p < 0.05$). After blocking *CCR10* expression in damaged HDMECs by BI6901 (a *CCR10* specific blocker) m6A methylation, cell activity, vascular endothelial growth factor A (VEGFA) and CD31 protein expression, as well as relative length and branches of tube formation were found to be increased compared with the injury group, while the levels of inflammatory factors interleukin-1 (IL-1), interleukin-1 (IL-6) and tumor necrosis factor-α (TNF-α) were decreased ($p < 0.05$).

Conclusions: Blocking *CCR10* expression can activate m6A methylation, promote cell activity, inhibit inflammatory reactions and alleviate HDMEC injury, which may become a breakthrough in future diagnosis and treatment of cardiovascular diseases.

Keywords: *CCR10*; m6A methylation; endothelial cell injury; RNA-Seq; cell migration

Introduction

Coronary heart disease, stroke, arrhythmia and heart failure are collectively referred to as cardiovascular diseases (CVDs), which belong to the most common types of diseases in clinics at present with the largest cumulative cases worldwide [1]. CVDs have a predilection for middle-aged and elderly people, with an incidence of about 20–30% in people over the age of 60 [2]. With the increasingly serious global population aging, the incidence of CVDs has shown an expanding trend, with a rise of about three times that of 2010 [3]. Moreover, as a progressive disease, the potential threat of CVDs cannot be ignored. Statistics from the World Health Organization (WHO) show that of the 56 million deaths worldwide in 2015, approximately 18 million are attributed to CVDs, and the number of deaths from CVDs is expected to reach 24 million in the next 20 years [4]. Therefore, for CVDs with an increasing incidence and potential threats, finding an effective and safe cure is the key to ensuring patients' life safety. Researchers believe that an in-depth understanding of the nosogenesis of CVDs is the basis for finding new therapeutic schemes, but the exact pathogenesis of many CVDs is not yet completely clear. Referring to related research, we found a commonality of CVDs, that is, they are all accompanied by varying degrees of vascular endothelial cell (VEC) damage. For example, the inflammatory reaction and necrosis of VECs can promote the occurrence of myocardial infarction [5], while blocking the oxidative stress reaction of VECs can inhibit atherosclerosis [6], and enhancing VEC circulation is a new direction to treat rheumatic heart disease [7]. Therefore, how to protect VECs from injury may be a breakthrough in the future prevention and treatment of CVDs.

Epitranscriptomics is an emerging biological research field, which has received extensive attention in the regulation of VEC damage in recent years [8,9]. RNA methylation is the most common type of RNA modification in eukaryotes, accounting for 60% of the total Ribonu-

cleic Acid (RNA) modifications [10]. Among them, N6-methyladenosine (m6A) modification is considered to be the most common, reversible and dynamic eukaryotic messenger RNA (mRNA) transcription modification among all RNA modification types, accounting for about half of all methylated ribonucleotides [11]. In recent years, increasing studies have shown that m6A methylation is strongly linked to the occurrence and development of CVDs such as myocardial hypertrophy, heart failure, ischemic cardiomyopathy, aortic aneurysm and vascular calcification [12,13]. As a newly discovered type of post-transcriptional regulation, dynamic and reversible m6A modification is the most common type of internal modification of RNA methylation [14]. m6A modification not only plays a critical part in various cell biological processes but is also closely related to CVDs [15]. However, it is still unclear how it is involved in CVDs. Therefore, this study examined genome-wide changes in injured human dermal microvascular endothelial cells (HDMECs) by Ribonucleic Acid sequencing (RNA-Seq) technology to further explore the pathogenesis of CVDs and search for key genes that affect m6A methylation. Through RNA-Seq, we found 20 differentially expressed genes (DEGs) in injured HDMECs, among which the C-C motif chemokine receptor 10 (*CCR10*) attracted our attention. *CCR10*, or orphan chemokine receptor G Protein-Coupled Receptor (GPR2), is one of the members of CC chemokine receptors and a 7-transmembrane G protein-coupled receptor [16]. In humans, it is selectively expressed by Immunoglobulin A (IgA) antibody-secreting cells in salivary glands, small intestine, large intestine, appendix and tonsil, and was initially found to be related to inflammatory reactions such as psoriasis and allergic contact dermatitis [17,18]. Recent evidence has shown the important potential significance of *CCR10* in multiple neoplastic diseases such as non-small-cell lung carcinoma and liver cancer [19,20]. As for CVDs, although no studies have confirmed the significance of *CCR10* in the disease, we have found the important influence of *CCR10* on modulating cell biological behaviors such as cell fibrosis, angiogenesis and wound healing [21,22], as well as the close correlation of its specific ligands C-C motif chemokine ligand 27 (CCL27) and C-C motif chemokine ligand 28 (CCL28) with human immune regulation [23]. All of these pathological processes are common manifestations of CVDs. Moreover, *CCR10* was found to share 51% homology with the interleukin-8 (IL-8) receptor [24], while m6A and IL-8 have a synergistic reaction in mediating inflammatory infiltration of neutrophils [25].

Therefore, we speculate that *CCR10* may affect the occurrence of VEC injury through m6A. Therefore, this research will carry out experimental analysis on this, thus gaining new insights into future clinical diagnosis and treatment of CVDs and laying a foundation for follow-up research.

Materials and Methods

Cell Culture

HDMECs, ordered from Hunan Fenghui Biotechnology Co., Ltd. (FH08530046; Changsha, China), were incubated in a medium comprising 5.55 mM glucose (56499C, Sigma-Aldrich, St. Louis, MO, USA), 5% FBS (fetal bovine serum) (12107C, Sigma-Aldrich, St. Louis, MO, USA) and 1% endothelial cell growth supplement (8728, ScienCell, Santiago, CA, USA) at 5% CO_2 and 37 °C. The culture medium was changed once every 3 days, and the passage was carried out when the cell confluency reached 70–80%. The fourth to ninth-generation cells were used for follow-up experiments. The cells used in this experiment were Short Tandem Repeat (STR)-identified and tested for mycoplasma.

HDMEC Injury Treatment

After passaging the cells, HDMECs were assigned to either the injury group or the control group, in which the latter was routinely cultured in high glucose DMEM medium + 10% fetal bovine serum + penicillin/streptomycin double antibody (12103C, Sigma-Aldrich, St. Louis, MO, USA), while the former was subjected to damage treatment (24 h) by adding endotoxin to the medium at the concentration of 5 μg/mL (A5159, Sigma-Aldrich, St. Louis, MO, USA).

RNA-Seq

RNAiso Plus (EN0602, ThermoFisher Scientific, Waltham, MA, USA) was used for cell RNA isolation, and RNA purity was verified on a Fragment Analyzer Bioanalyzer using a standard sensitivity RNA analysis kit (A54984, ThermoFisher Scientific, Waltham, MA, USA), followed by RNA-Seq. Briefly, the mRNA was fragmented after poly (A)-based mRNA enrichment with oligo (dT) magnetic beads using 18 total RNA samples as the input material. Following the first and second strands, complementary DNA (cDNA) synthesis, end repair, 3'-adenylation and ligation of fork-tail linkers were performed. After a Polymerase Chain Reaction (PCR) amplification, the library quality was verified on the Fragment Analyzer Bioanalyzer, with a fragment size of 150 bp, and the paired-end library was sequenced using BGISEQ-2000 (read length: 2 × 150).

qRT-PCR

After total RNA separation from cells by Trizol (15596018, ThermoFisher Scientific, Waltham, MA, USA) and Ultraviolet (UV) spectrophotometer (Prove 600, Sigma-Aldrich, St. Louis, MO, USA) verification for its purity, reverse transcription of RNA into cDNA was carried out by referring to the kit (18064014, ThermoFisher Scientific, Waltham, MA, USA) manuals. The reaction system used was the following: 10× amplification buffer 10 μL, dNTP mixture 200 μmol/L, primers 10 pmol, cDNA

Table 1. Primer sequences.

	Forward (5′-3′)	Reverse (5′-3′)
CCR10	GAG GCC ACA GAG CAG GTT TC	CCT GGA CAT CGG CCT TGT A
GAPDH	GAC AGT CAG CCG CAT CTT CT	AAA TGA GCC CCA GCC TTC TC

0.1 μg, Taq DNA polymerase 2.5 μL, followed by amplification reaction under the conditions of 95 °C for 10 min, 94 °C for 1 s, 60 °C for 6 s, and 72 °C for 10 s, for a total of 45 cycles (see Table 1 for sequences of primers). The $2^{-\Delta\Delta Ct}$ method was employed to calculate the *CCR10* mRNA expression relative to *GAPDH*. In addition, the m6A methylation expression was quantified using the Magna MeRIPTMm6A kit. The isolated RNA was fragmented by an RNA fragmentation buffer. After saving one-tenth of the total RNA as input, the remaining RNAs were used for immunoprecipitation with m6A antibody (Millipore Sigma, Darmstadt, Germany, ABE572) coated on magnetic beads A/G (Millipore Sigma, Darmstadt, Germany, 16–663). After being washed with the immunoprecipitation buffer three times, the m6A-modified RNAs were eluted with an elution buffer. Finally, the eluted RNAs were recovered by ethanol precipitation and then subjected to Real-Time Quantitative Reverse Transcription PCR (qRT-PCR) (Q960 Fluorescence Quantitative PCR Instrument, Di Nai Bio, Hangzhou, China).

CCR10-Blocking Treatment

HDMECs were divided into the injury, control and BI6901 groups. The treatment of the injury group and control group was the same as above. The BI6901 group was first treated with endotoxin to establish the injury model and then intervened by the *CCR10*-specific blocker BI6901 (HY-116835, MCE, Trenton, NJ, USA, 50 ng/mL). qRT-PCR verified the blocking effect, and changes in m6A methylation were determined.

Wound-Healing Assay

When the confluency of cells (4×10^5/mL) seeded in the wells of 6-well plates reached 80–90%, a 10 μL pipette tip was utilized to vertically scratch the culture plate. After 12 h, the scratched area was observed, and the cell migration was calculated (0 h scratch distance – 24 h scratch distance)/0 h scratch distance $\times$ 100%.

Western Blotting

Radio immunoprecipitation assay (RIPA) (89901, ThermoFisher Scientific, Waltham, MA, USA) buffer was utilized to lyse the cells and extract the total protein. After bicinchoninic acid (BCA) quantification, sodium dodecyl sulfate (SDS) electrophoresis was performed and the protein was transferred to a Polyvinylidene Difluoride (PVDF) membrane (ThermoFisher Scientific, Waltham, MA, USA), followed by 10 min of blocking with 5% defatted milk and the subsequent addition of Vascular endothelial growth factor A (VEGFA) (1:1000) and CD31 (1:1000) primary antibody proteins (SAB1306008, SAB1306529, Sigma-Aldrich, St. Louis, MO, USA) for overnight incubation at 4 °C. The membrane was rinsed the next day and a secondary antibody (1:2000) (04-1571, Sigma-Aldrich, St. Louis, MO, USA) was added and incubated. ECL (WBULS0100, Sigma-Aldrich, St. Louis, MO, USA) development was performed 4 h later, and the protein bands were scanned to analyze relative protein expression by Image J software (version 1.8.0.112, National Institutes of Health, Bethesda, MD, USA).

Tube Forming Ability Test

After the overnight dissolution of Matrigel (LV-6828-2-10ml, Liver Biotechnology, Shenzhen, China) at 4 °C, the gel was spread on an ice box the next day and placed in a 37 °C incubator for half an hour. When the cells were about 80% confluent, they were digested with trypsin and resuspended with DMEM/F12 containing 10% fetal bovine serum (FBS), after which 1 mL of the resuspended solution was added into the wells of a 12-well plate. After 24 h, the cells were observed and imaged under a biological inverted microscope (200$\times$), and the length of tube formation was calculated.

Measurement of Inflammatory Factors (IFs)

The cells were digested by trypsin and the serum was collected by centrifugation to determine interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α levels by Enzyme-linked Immunosorbent Assay (ELISA). All the kits were supplied by China TransGen Biotech (NE102, NE101, NE104, Beijing, China), and the operation process strictly followed the kit manuals.

Statistical Analysis

Statistical analysis was performed with the use of SPSS23.0 (IBM, Armonk, NY, USA). In this study, each experiment was done in triplicate, and the results were recorded in the form of mean $\pm$ standard deviation ($\bar{x} \pm$ s). The independent sample *t*-test was used for inter-group comparisons, and one-way Analysis of Variance (ANOVA) and LSD *post hoc* tests were applied for multi-group comparisons, with significance determined at $p < 0.05$.

Results

RNA-Seq

First, we tried to screen the DEGs between damaged HDMECs and normal HDMECs after endotoxin treat-

ment, to explore the scheme of regulating m6A methylation. Twenty differentially expressed genes (DEGs), namely Nitric Oxide Synthase 2, Epstein-Barr Virus-Induced 3, Phosphoinositide-3-Kinase Regulatory Subunit 2, Unc-5 Netrin Receptor A, Xg Glycoprotein (Xg Blood Group), 2'-5'-Oligoadenylate Synthetase Like, Thy-1 Cell Surface Antigen, Zinc Finger Protein 281, MX Dynamin Like GTPase 2, *CCR10*, Small Cajal Body-Specific RNA 12, Fibronectin Type-III Domain-Containing Protein, Small Nucleolar RNA, H/ACA Box 31B, ISY1-RAB43 Readthrough, NHR Domain-Containing Protein, Growth Differentiation Factor 5, Zinc Finger Protein 8, Histone H2A, Rhomboid Domain-Containing Protein, and ENSG00000285943 were screened from the injured and control groups by RNA-Seq, including 17 up-regulated DEGs and 3 down-regulated ones (Fig. 1). Hierarchical cluster analysis was performed based on normalized read counts reflecting gene expression, following log z-transformation in the row direction (Fig. 2). *CCR10* was selected for subsequent analysis.

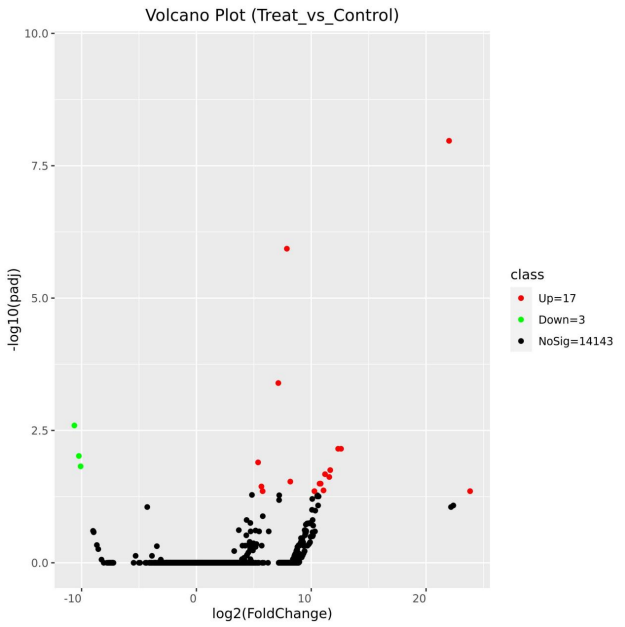

Fig. 1. Volcano plot of DEGs after human dermal microvascular endothelial cells damage, with red indicating upregulated genes and green indicating downregulated genes.

Alterations of CCR10 Expression and m6A Methylation after HDMEC Injury

The PCR detection revealed notably higher *CCR10* gene expression in the injury group (3.28 ± 0.25) compared with the control group ($p < 0.05$) (Fig. 3A). However, the m6A methylation degree was lower in the injury group (0.82 ± 0.04) than in the control group ($p < 0.05$) (Fig. 3B).

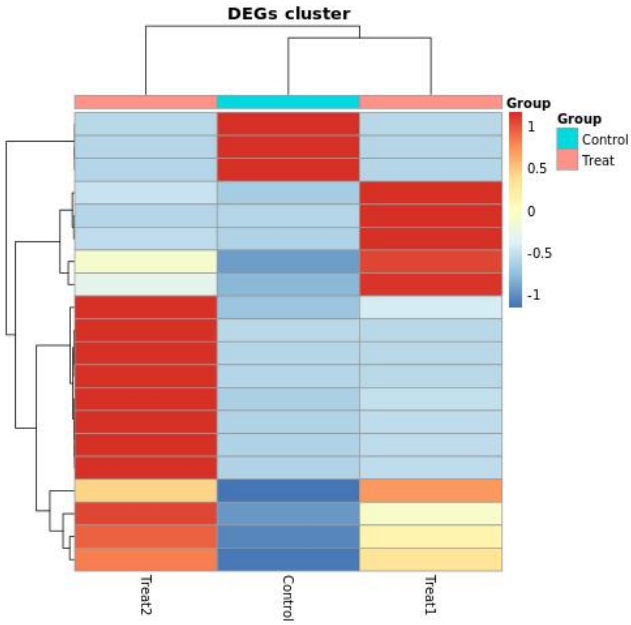

Fig. 2. Heat map of THE expression of 20 DEGs after human dermal microvascular endothelial cells injury. Treatment 1 and 2: Cells damaged by endotoxin.

Impact of CCR10 on m6A Methylation

1.88 times compared with the control group ($p < 0.05$) (Fig. 4A), indicating that the blocking by BI6901 was successful. Subsequently, m6A methylation was found to be the highest in the control group among the three groups, and m6A methylation in the BI6901 group was higher compared with the injury group ($p < 0.05$) (Fig. 4B).

Influence of CCR10 on Cell Migration Capacity

The scratch assay was used to detect the effect of *CCR10* on cell migration ability. As shown in Fig. 5A, the cell migration rate of the BI6901 group was (49.29% ± 4.04), lower than that of the control group and higher than that of the injury group ($p < 0.05$) (Fig. 5B).

Impact of *CCR10* on Angiogenesis

The western blot showed lower VEGFA and CD31 protein levels in the injury group compared with the control group ($p < 0.05$) (Fig. 6A); While the VEGFA and CD31 protein levels in the BI6901 group were lower than those in the control group but higher than those in the injury group ($p < 0.05$) (Fig. 6B). The tube formation ability test (Fig. 6C) showed that the relative length of tubule formation and the relative number of branches formed were the highest in the control group among the three groups, while those in the BI6901 group were higher compared with the injury group ($p < 0.05$) (Fig. 6D,E).

Influence of *CCR10* on IFs

Finally, the ELISA results (Fig. 7A–C) revealed that IL-1, IL-6 and TNF-α levels in the BI6901 group

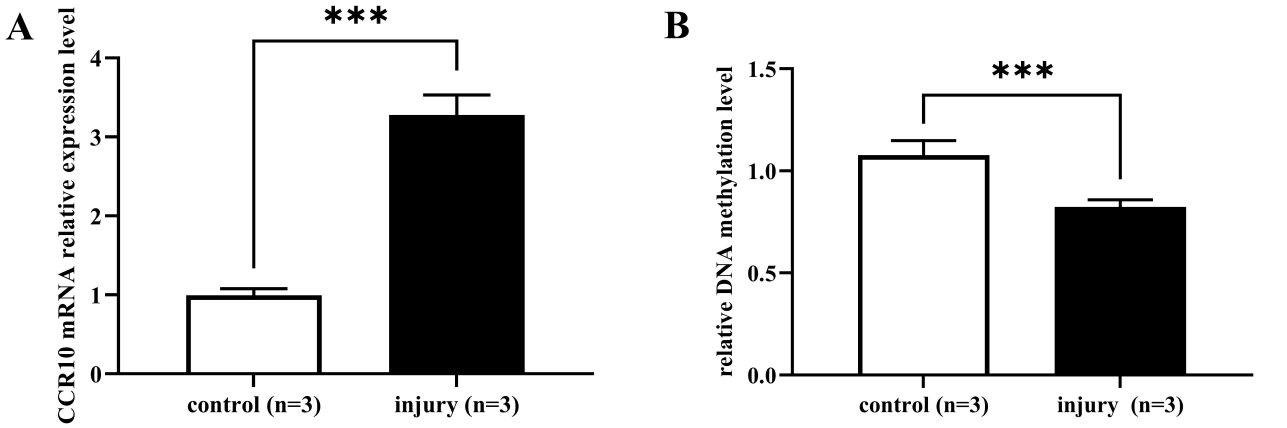

Fig. 3. PCR of *CCR10* gene expression and m6A methylation degree after HDMEC injury. (A) Comparison of *CCR10* gene expression between the injury group and the control group (n = 3). (B) Comparison of m6A methylation degree between the injury group and the control group (n = 3). $^{***}p < 0.001$.

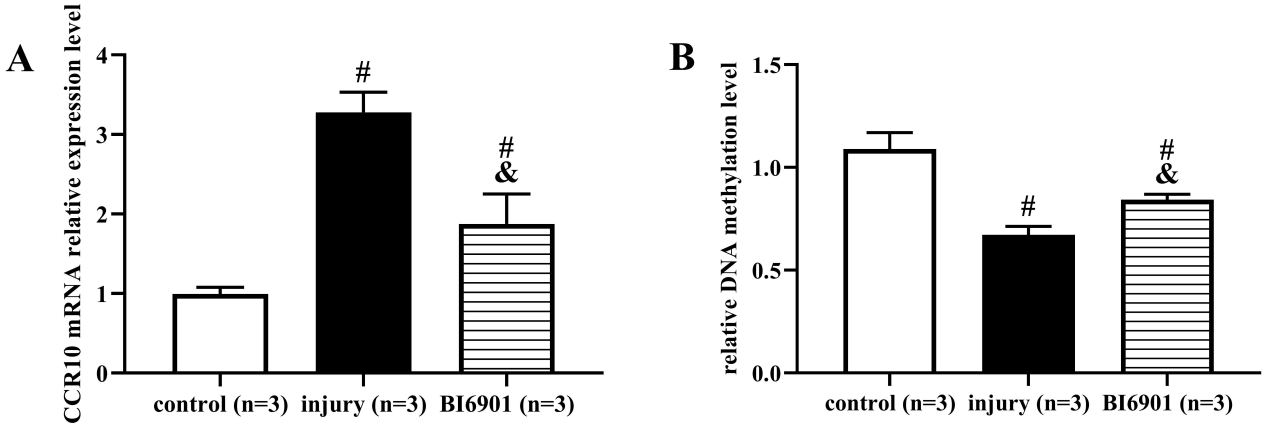

Fig. 4. Impact of *CCR10* on m6A methylation. (A) Comparison of *CCR10* expression among the BI6901 group, injury group and control groups (n = 3). (B) Comparison of m6A methylation degree among the BI6901 group, injury group and control groups (n = 3). Compared with the control group, $^{\#}p < 0.05$; Compared with the injury group, $^{\&}p < 0.05$.

were (15171.17 ± 2260.06 pg/mL), (19501.94 ± 3531.65 pg/mL), and (12038.22 ± 1994.64 pg/mL), respectively, higher than those in the control group but lower than those in the injury group ($p < 0.05$).

Discussion

Endotoxin-induced damage of HDMECs, a very classical treatment in clinical practice, has been proven to simulate the pathological manifestations of various CVDs such as calcification of inlet and outlet vessels and heart failure [26,27]. Through RNA-Seq, we found that *CCR10* was abnormally expressed in HDMECs under endotoxin injury. As we previously described, *CCR10* is closely related to cardiovascular disease and may have a synergistic reaction with m6A.

Therefore, to confirm the relationship between *CCR10* and m6A, we detected the *CCR10* expression by qRT-PCR and found elevated *CCR10* expression levels in damaged HDMECs, confirming the above RNA-Seq results. m6A methylation was significantly reduced, which is consistent with our expectations and the results of previous studies [28]. It also suggests that both of them may participate in the onset and development of HDMEC injury. Subsequently, we blocked *CCR10* expression in cells to observe changes in m6A methylation and cell biological behavior. Blocking *CCR10* led to increased m6A methylation degree, cell migration and angiogenesis capacity in damaged HDMECs, and decreased IFs. This shows that blocking *CCR10* expression in damaged HDMECs can reactivate m6A methylation, enhance cell activity and inhibit inflammatory reactions, suggesting that *CCR10* is expected to become a new therapeutic target for CVDs. Reviewing the

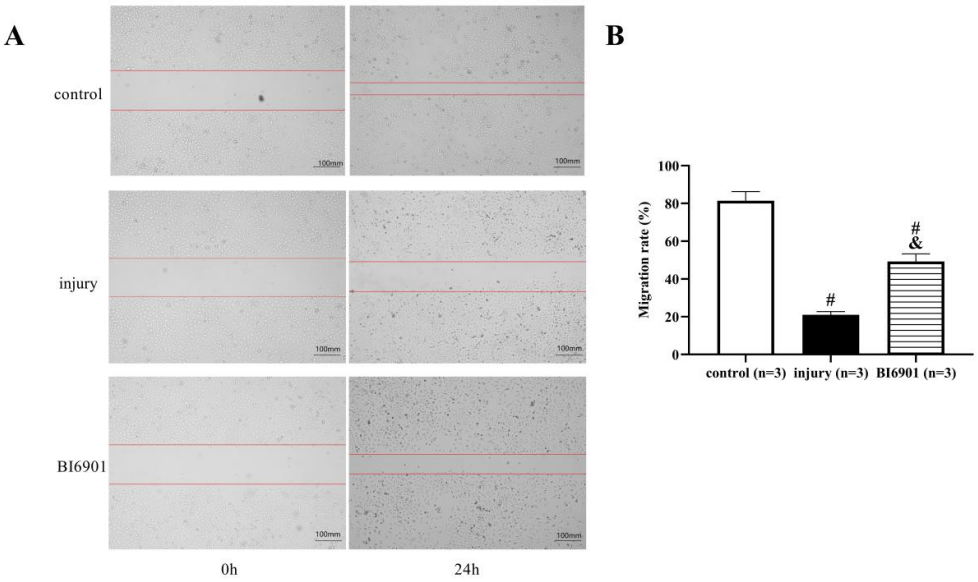

Fig. 5. Influence of *CCR10* on cell migration capacity. (A) Cell scratch test results. (B) Cell migration rate. Scale bar: 100 mm. Compared with the control group, $^{\#}p < 0.05$; Compared with the injury group, $^{\&}p < 0.05$ (n = 3).

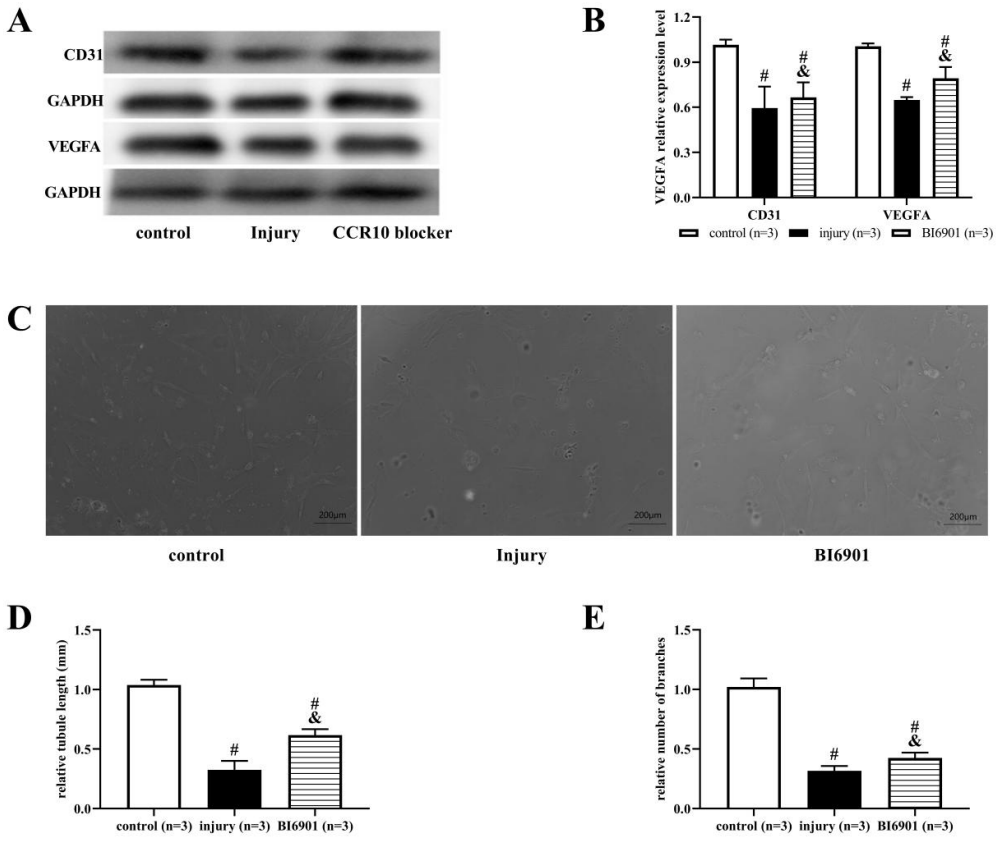

Fig. 6. Impact of *CCR10* on angiogenesis. (A) Western blotting. (B) Comparison of VEGFA and CD31 protein expression levels. Comparison with the control group, $^{\#}p < 0.05$; Comparison with the injury group, $^{\&}p < 0.05$. (C) Tubule formation experiment. Scale bar: 200 μm. (D) Comparison of the relative length of tube formation. (E) Comparison of relative branches of tube formation. Compared with the control group, $^{\#}p < 0.05$; Compared with the injury group, $^{\&}p < 0.05$ (n = 3).

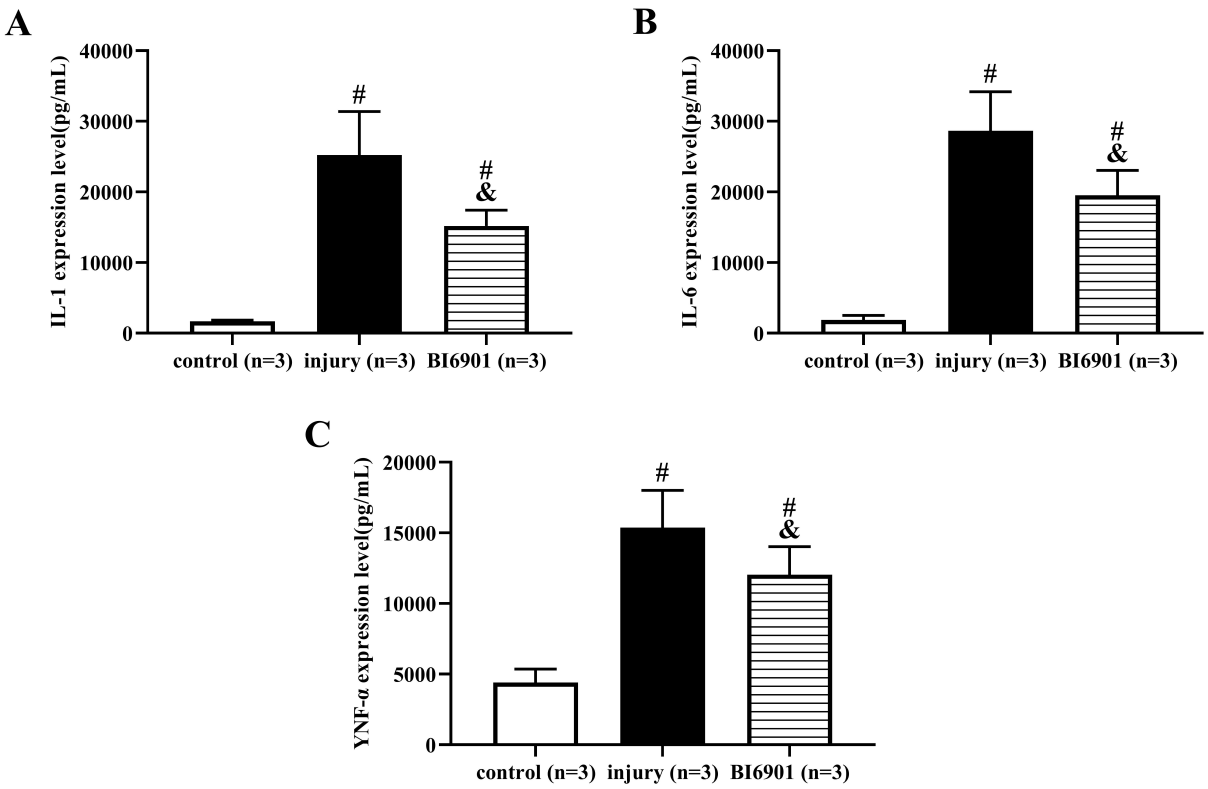

Fig. 7. Impact of *CCR10* on inflammatory factors. (A) Comparison of IL-1 expression. (B) Comparison of IL-6 expression. (C) Comparison of TNF-α expression. Comparison with the control group, $^{\#}p < 0.05$; Comparison with the injury group, $^{\&}p < 0.05$ (n = 3).

past literature, we also found that the *CCR10* antibody has the ability to regulate the synergy between intestinal B cells and T cells, maintain intestinal stability and mediate lymphatic endothelial cell migration [29,30], which can also verify our experimental results, indicating that *CCR10* has an important potential influence on the integrity and activity of cells in the body. Similarly, *CCR10* is reported to activate inflammatory reactions in keratinocytes and promote the occurrence of inflammatory skin lesions [31], confirming the synergistic relationship between *CCR10* and cellular inflammatory reactions. The above-mentioned has important potential therapeutic significance for inflammatory diseases such as coronary heart disease and myocardial fibrosis in the future.

However, the specific mechanism through which *CCR10* regulates m6A methylation has not been discussed, which is still the focus of our further research. In addition, since this experiment is conducted based on endotoxin-treated HDMECs, the role played by *CCR10* in human samples with specific CVDs is still unclear, requiring our supplementary experiments for verification. In the follow-up study, we will conduct a more in-depth and comprehensive experimental analysis of the effects of *CCR10* on m6A and the specific mechanisms of its effects on a variety of CVDs, so as to provide more convincing clinical evidence.

Conclusions

Conclusively, blocking *CCR10* expression can activate m6A methylation, promote cell activity, inhibit inflammatory reactions and alleviate HDMEC injury, which may become a breakthrough in future diagnosis and treatment of CVDs.

Availability of Data and Materials

The datas used and/or analyzed during the current study are available from the corresponding author.

Author Contributions

ZZ and HY—are the co first author, they are mainly responsible for collecting experimental data and ensuring the integrity of the paper; ZZ and HY—also carried out data analysis and participated in writing papers; XW and LY—contributed to manuscript revision and project management. All authors contributed to the article and approved the submitted version.

Ethics Approval and Consent to Participate

This study was approved by the ethics committee of our hospital (TC20201185).

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Conflict of Interest

The authors declare no conflict of interest.

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