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ORIGINAL RESEARCH

METTL16 Contributes to Coronary Heart Disease by Inducing TET2 m6A Modification

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Background: Coronary heart disease (CHD) ranks as the primary cause of global morbidity and mortality. Despite of the progress in exploring risk factors and developing medications, considerable residual risk persists. In recent years, epigenetic regulation has emerged as a critical regulatory mechanism across various diseases. This study aimed to investigate the effects of methyltransferase-like protein 16 (METTL16) on CHD and the potential molecular mechanisms.

Methods: A CHD mouse model was established and treated with METTL16 depletion treatment. The METTL16 RNA level was measured by qPCR assay. Cardiac function was estimated by using echocardiography. Tissue damage and cardiac fibrosis were analyzed by deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and Masson's trichrome staining. The blood samples were collected to measure lipid levels and cardiac function biomarkers. Vascular smooth muscle cells (VSMCs) were isolated, and cell viability and proliferation were detected by cell counting kit 8 (CCK-8) and colony formation assay. Cell apoptosis was determined by flow cytometry. Expression of apoptosis biomarkers was detected by Western blotting assay. The m6A enrichment on TET2 mRNA was determined by methylated RNA immunoprecipitation (MeRIP) assay and cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR).

Results: We observed elevated expression of METTL16 in cardiac tissues of CHD mice, and knockdown of METTL16 notably recovered the cardiac function, alleviated cardiac fibrosis, and decreased lipid levels. Knockdown of METTL16 suppressed VSMC proliferation and elevated cell apoptosis. METTL16 directly modulated the m6A enrichment on TET2 mRNA, and overexpression of TET2 could reverse the inhibitory effects of siMETTL16 on VSMC proliferation.

Conclusion: METTL16 affects the cardiac damage and function during CHD via epigenetically modulating the m6A modification of TET2.

Keywords: coronary heart disease, epigenetic regulation, N6-methyladenosine, METTL16

Introduction

Coronary heart disease (CHD) ranks as the primary cause of global morbidity and mortality, characterized as a chronic, immune-inflammatory, fibroproliferative ailment propelled by lipids.[1] Despite considerable progress in understanding associated risk factors and developing preventive measures and medications targeting lipoprotein-related risks, a considerable residual risk persists.[1–3] For example, statin medications effectively reduce plasma cholesterol levels and lower the risk of coronary events by 20–30%.[4] However, this underscores the presence of significant residual risk and emphasizes the importance of exploring alternative treatment approaches.[5,6] Vascular smooth muscle cells (VSMCs) have long been associated with phenotypic modulation/plasticity or dedifferentiation.[7,8] Various studies have established that death of VSMCs during CHD development is prevalent and leads to lesion formation and abnormal cardiac function.[9,10] Therefore, improving the VSMC viability is a promising strategy for CHD treatment.

In recent years, epigenetic regulation has emerged as a critical regulatory mechanism across various diseases.[11–14] Among these epigenetic regulation forms, N6-methyladenosine (m6A) stands out as one of the most prevalent epigenetic modifications, exerting a significant impact on gene expression and function.[15,16] This modification significantly impacts the stability of RNAs and the subsequent translation efficiency.[15,16] The m6A modification is a reversible process that regulated by methyltransferases and demethylases.[17,18] METTL16 is one of the two representative methyltransferases that mostly studied.[19,20] It has been reported that METTL16 exhibits dual functionality in gene regulation wither dependent or independent of its methyltransferase

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activity.[21] Within the cell nucleus, METTL16 acts as an m6A writer, depositing m6A into hundreds of specific messenger RNA targets. Simultaneously, in the cytosol, METTL16 promotes translation independently of m6A.[21] Studies have revealed abnormal expression of METTL16 in multiple diseases, especially cancers and cardiovascular diseases.[18,22,23]

In this study, we explored the role of METTL16 in coronary heart disease and investigated the potential molecular mechanisms.

Materials and Methods

Mouse Model

To establish the CHD model, male BALB/c mice that aged 6 weeks-old were bought from Vital River Laboratory (Beijing, China). After acclimation for one week, the mice in model group were fed with high-fat diet for 8 weeks. Standard chow was supplied to mice in control group. The mice in treatment group were intravenously injected with siRNAs (20 nmol in 50 μL PBS) every three days. Each group contains 10 mice. All experiments were performed under the approval of the Animal Ethics Committee of The Third Affiliated Hospital of Wenzhou Medical University. The cardiac function was measured by transthoracic echocardiography (Vevo 2100 ultrasound system, Vevo 2100 ultrasound system) after treatment. Left ventricle anterior wall thickness in diastole (LVAW) and left ventricular posterior wall end diastole (LVPWd) were measured and calculated. The successful establishment of CHD model was verified by ST-segment elevation on the electrocardiography. The procedures were conformed to the Guide for the Care and Use of Laboratory Animal published by the US National Institutes of Health (NIH publication, 8th edition, 2011).

Masson's Trichrome Staining and Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Staining

The cardiac tissues were collected after treatment and made into paraffine embedded samples. The apoptotic cells were stained with TUNEL apoptosis detection kit (Beyotime, China). Tissue fibrosis was measured by Masson's trichrome according to manufacturer's protocol. Images were taken by electron microscope (Leica, Germany).

Evaluation of Arterial Blood

After treatment, the mice were anesthetized by sodium pentobarbital. The abdominal aorta was isolated and inserted with a puncture needle. Then the arterial blood was collected by a syringe. The blood sample was centrifuged at 3000 rpm and 4°C for 5 minutes, and serum was obtained to determine blood lipid levels. The levels of total cholesterol (TC; A111-1-1, Nanjing JianCheng, China), triglyceride (TG; #S0219M, Beyotime, China) and low-density lipoprotein cholesterol (LDL-C; A113-1-1, Nanjing JianCheng, China), cardiac troponin (cTnT; JL27509, Jonlnbio, China), creatine kinase isoenzyme (CK-MB; CSB-E05140h, CUSABIO, China), and creatine kinase (CK; A032-1-1, Nanjing JianCheng, China) were measured by ELISA using commercial kits following manufacturer's protocols.

Cell Culture

VSMCs were isolated from mice according to previous protocols.[24] In short, BALB/c mice were anesthetized, then the heart and aorta were excised and placed in Hanks' balanced salt solution (200 U/mL). After removing the adipose tissues, the blood vessel was cut open, and the inner lining was gently scraped to remove the endothelial cells. Next, scrape off the vascular media layer and transfer it to a petri dish lid for fine mincing. Place the minced tissue into an inverted culture flask and add Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum. Put the flask into an incubator, and after about 2 hours, once the tissue adheres to the flask surface, turn it upright. Change the medium approximately every 5 days. After around 3 weeks, when the cells have grown to confluence, proceed with passaging and subsequent experiments.

Cell Transfection

VSMCs were placed into 6-well plate overnight to growth. The siRNAs target METTL16 (siMETTL16) and the negative control (siNC) were mixed with Lipofectamine 2000 reagent for 20 min. The mixture was then added into the cultured VSMCs and incubated for 48 h. After that, cells were collected and used for subsequent experiments.

Colony Formation

The VSMCs were suspended in completed culture medium and seeded into 6-well plates at a density of 1000 cells per well. After incubation for 10 days, the colonies were stained with 0.5% crystal violet for 20 min. Imaged of colonies were taken by a digital camera.

Cell Apoptosis Detection

To detect cell apoptosis, VSMCs were analyzed with Annexin V-Fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (Beyotime, China). Cells were suspended in binding buffer that added with 5 μL Annexin V-FITC and 5 μL PI reagent and were incubated for 30 min in dark. The samples were then detected using flow cytometry.

Western Blot Analysis

Cells were lysed with radio-immunoprecipitation assay (RIPA) lysis buffer that added with proteinase inhibitors (Beyotime, China). The cell lysates were centrifuged at 12000 g for 10 min, and total proteins were collected. The total proteins (50 μg) were separated in 6% to 12% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% skimmed milk and hatched with anti-Bcl2 (#17071), anti-Bax (#2772), anti-Caspase 3 (#9662), and anti-β-actin (#4967) antibody overnight at 4°C. All antibodies were bought from cell signaling technology (CST, USA) and used at a dilution of 1:1000 in PBST buffer. After that, the protein bands were then hatched with goat anti-rabbit or anti-mouse secondary antibody for 1 h at room temperature and reacted with an enhanced chemiluminescence reagent. The visualized protein bands were then visualized in an imaging system (Tanon, China).

Quantitative PCR (qPCR) Analysis

Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen, USA). Then, 1 μg of total RNA was reverse-transcribed into complementary DNA (cDNA) using HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). The cDNA was then labeled with SYBR Green reagent (Bio-Rad, USA) and detected by qPCR as follows: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C followed by 60°C for 1 min. Relative gene expression was detected using 2-ΔΔCt method. Primers were listed: METTL16: sense, CAAGGACAAACCACCTGACTT, antisense, GTGGGAATT AGTCTCTCCAAAGG; TET2: sense, AAGCAGCCGTCAGCCAAAT, antisense, TTCCGTGTTGGGAAAGCATCT; GAPDH: sense, AGGTCGGTGTGAACGGATTTG, antisense, GGGGTCGTTGATGGCAACA.

Methylated RNA Immunoprecipitation (MeRIP) Assay

MeRIP experiment was performed to analyzed m6A enrichment on mRNA using the Magna MeRIP m6A Kit (Millipore, USA) in accordance with manufacturer's protocol. The precipitants were analyzed using qPCR assay.

Cross-Linking Immunoprecipitation and qPCR (CLIP-qPCR)

Cells were irradiated with UV for crosslink of protein to RNA, followed by homogenization and sonication. Cell lysates were collected after centrifuge with 20000 g for 10 min. Protein A/G beads were mixed with anti-METTL16 or anti-m6A antibody and incubated for 60 min. The beads were then added into cell lysates and hatched for 2 hat 4°C. After that, the samples were centrifuged and labeled with PNK reagent. RNA was extracted for qPCR analysis.

Statistical Analyses

Data were shown as the mean ± standard deviation (SD) and analyzed using SPSS software. For comparison between two groups, data exhibiting a normal distribution were assessed using an unpaired Student's *t*-test. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was employed, followed by Tukey's test. Statistical significance was set at $p < 0.05$.

Results

METTL16 Contributes to Cardiac Injury in CHD Mouse Model

To investigate the function of METTL16 in the regulation of CHD, we constructed the CHD mouse model. We observed that METTL16 was upregulated in myocardial tissues of CHD mouse and depletion of METTL16 by siRNA significantly reduced METTL16 expression in the model (Figure 1A). The depletion of METTL16 repressed the cardiac injury and fibrosis in CHD mouse model (Figure 1B). Cardiac function impaired by CHD was improved after METTL16 knockdown in the mice, indicated by EF, FS, diastolic LVID, systolic LVID, diastolic LVAW, diastolic, and systolic LVAW (Figures 1C and S1A). The levels of TC, TG, and LDL-C induced in CHD mice were inhibited by METTL16 depletion (Figure 1D). The levels of myocardial injury markers, including CK, CK-MB, and cTnT, were suppressed by METTL16 knockdown in CHD mice (Figure 1E). The depletion of METTL16 attenuates myocardial apoptosis induced by CHD in the mice (Figure S1B), suggesting that METTL16 contributes to cardiac injury in CHD mouse model.

METTL16 Promotes Vascular Smooth Muscle Cell Viability in vitro

Next, we investigated the effect of METTL16 on vascular smooth muscle cells (VSMCs). The effectiveness of METTL16 depletion by siRNAs was validated in VSMCs (Figure 2A). We selected siMETTL1602 for subsequent experiments. The viability and proliferation of VSMCs were repressed by METTL16 depletion (Figure 2B and C). The knockdown of METTL16 induced VSMCs apoptosis (Figure 2D). Bcl-2 expression was reduced, and BAX and cleaved caspase 3 expression were enhanced by METTL16 siRNA in VSMCs (Figure 2E). Consistently, the LDH activity was upregulated by METTL16 depletion in VSMCs (Figure 2F), indicating that METTL16 promotes vascular smooth muscle cell viability in vitro.

METTL16 Upregulates TET2 Expression by m6A Modification

Then, we identified that the depletion of METTL16 repressed TET2 expression in VSMCs (Figure 3A). The enrichment of m6A and METTL16 on TET2 RNA was observed in VSMCs (Figure 3B and C), in which METTL16 knockdown repressed the enrichment of m6A on TET2 RNA (Figure 3D). The mRNA stability of TET2 was significantly repressed by METTL16 depletion in VSMCs (Figure 3E), suggesting that METTL16 upregulates TET2 expression by m6A modification.

METTL16 Enhances Vascular Smooth Muscle Cell Viability by Upregulating TET2 in vitro

Interestingly, we found that overexpression of TET2 restored METTL16 depletion-inhibited viability and proliferation of VSMCs (Figure 4A and B). The apoptosis of VSMCs induced by METTL16 knockdown was repressed by TET2 overexpression (Figure 4C). The overexpression of TET2 reversed METTL16 siRNA-regulated expression of Bcl-2, BAX, and cleaved caspase 3 in VSMCs (Figure 4D). In addition, METTL16 depletion-enhanced LDH activity was inhibited by TET2 overexpression in VSMCs (Figure 4E), suggesting that METTL16 enhances vascular smooth muscle cell viability by upregulating TET2 in vitro.

Discussion

Epigenetic regulation, including the DNA and RNA methylation, has been widely reported to participate in the development of CHD.[25–28] For example, the methyltransferases METTL14 was found to be overexpression in both coronary heart disease and in an LPS-stimulated cell model.[29] Depletion of METTL14 stimulated the polarization of macrophages to M2 phenotype, repressed the formation of foam cells and inhibited cell migration through the NF-κB/IL-6 signaling pathway.[29] METTL14 knockdown transgenic mice exhibited suppressed macrophage inflammation and polarization and the accumulation of atherosclerotic plaques.[29,30] A recent analysis on CHD cases basing on Gene Expression Omnibus (GEO) database identified the correlation between epigenetic regulatory work with CHD development and demonstrated that the high m6A modification pattern was correlated with an enhanced infiltration and abundance of immune cells.[31] Another study on clinical samples demonstrated that patients with CHD showed notably higher m6A methyltransferase activity. The expression of METTL14

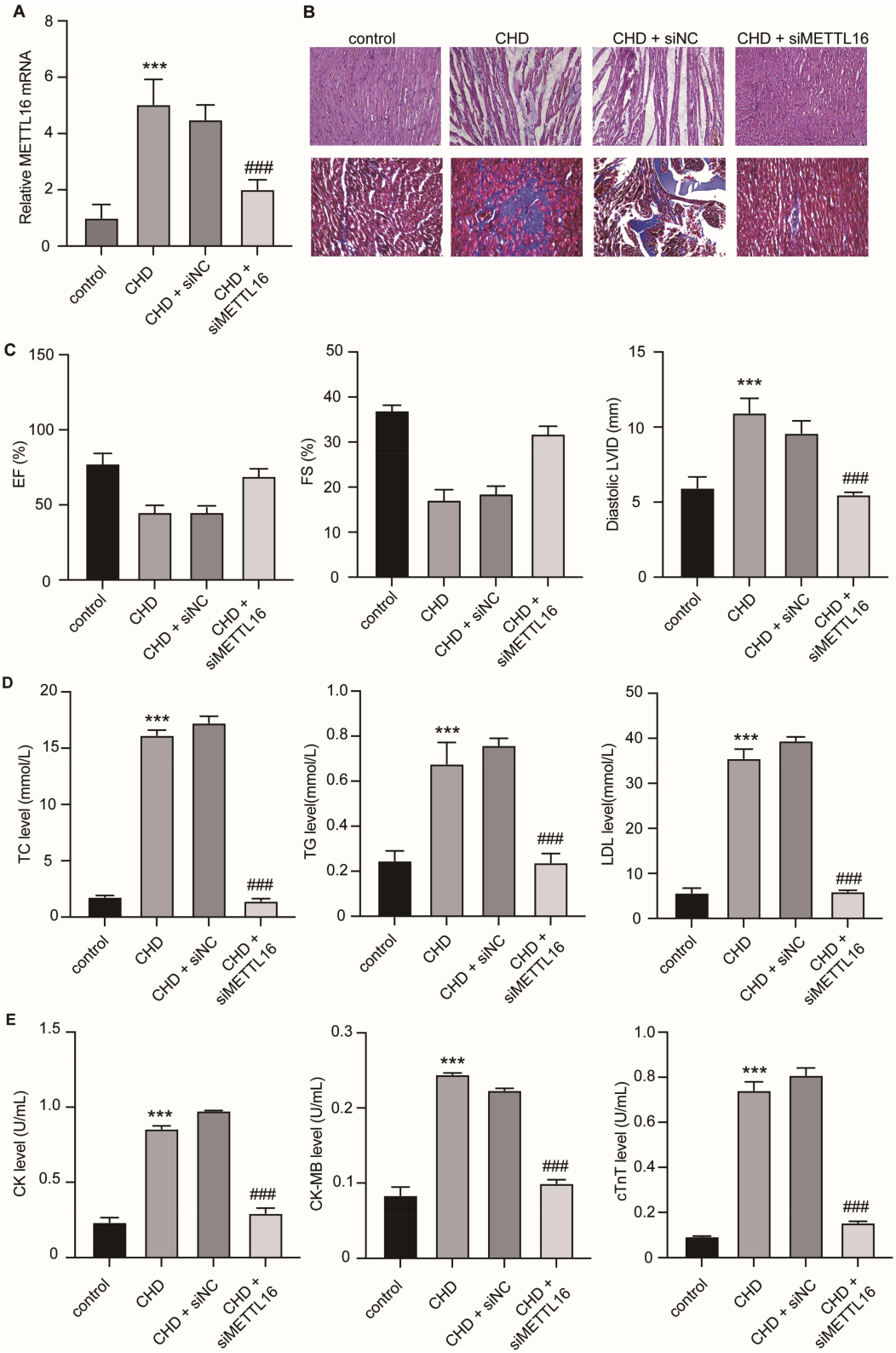

Figure 1 METTL16 contributes to cardiac injury in CHD mouse model. (**A**) The expression of METTL16 was detected by qPCR. (**B**) Cardiac injury was detected by H&E and Masson staining. (**C**) Cardiac function was detected by echocardiography. (**D**) The levels of total cholesterol (TC), triglyceride (TG) and low-density lipoprotein cholesterol (LDL-C) were measured by ELISA. (**E**) The indicated myocardial markers were analyzed. ***$p<0.001$, ###$p<0.001$.

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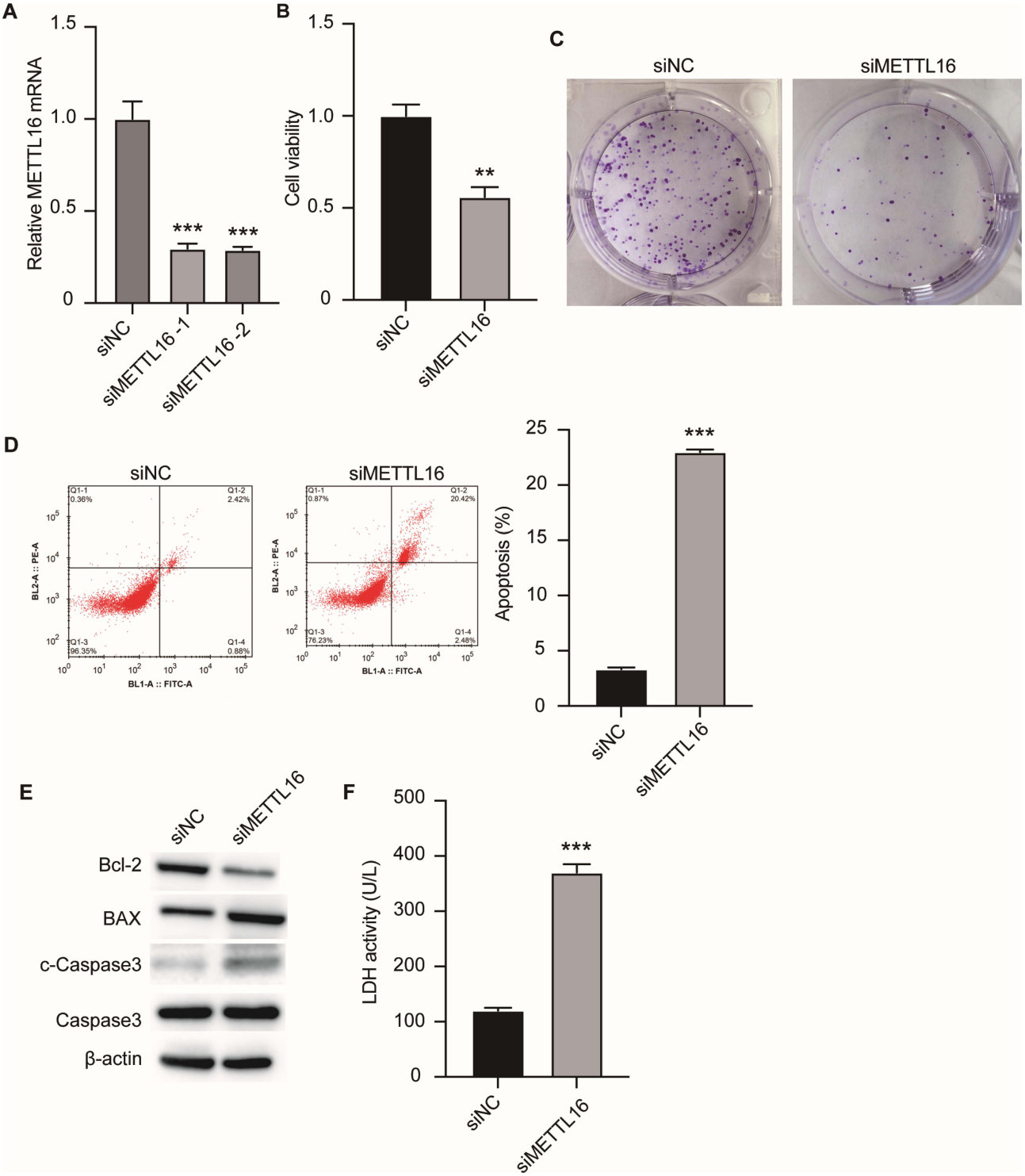

Figure 2 METTL16 promotes vascular smooth muscle cell viability in vitro. (**A–F**) The VSMCs were treated with METTL16 siRNA. (**A**) The expression of METTL16 was detected by qPCR. (**B**) Cell viability was measure by CCK-8. (**C**) Cell proliferation was analyzed by colony formation assay. (**D**) Cell apoptosis was detected by flow cytometry. (**E**) Apoptosis markers were determined by Western blot. (**F**) LDH activity was analyzed. ***p*<0.01, ****p*<0.001.

showed significant differences concerning the number of branches with lesions and were associated with Gensini score and coronary inflammation.[32] Moreover, METTL14 activates FOXO1 expression in a TNF-α-induced endothelial cell model through upregulating its m^{6}A modification, triggering endothelial cell inflammatory and contributing to atherosclerotic plaque formation.[33] Nevertheless, the effects of METTL16 on CHD development is still unclear. Here, we identified elevated

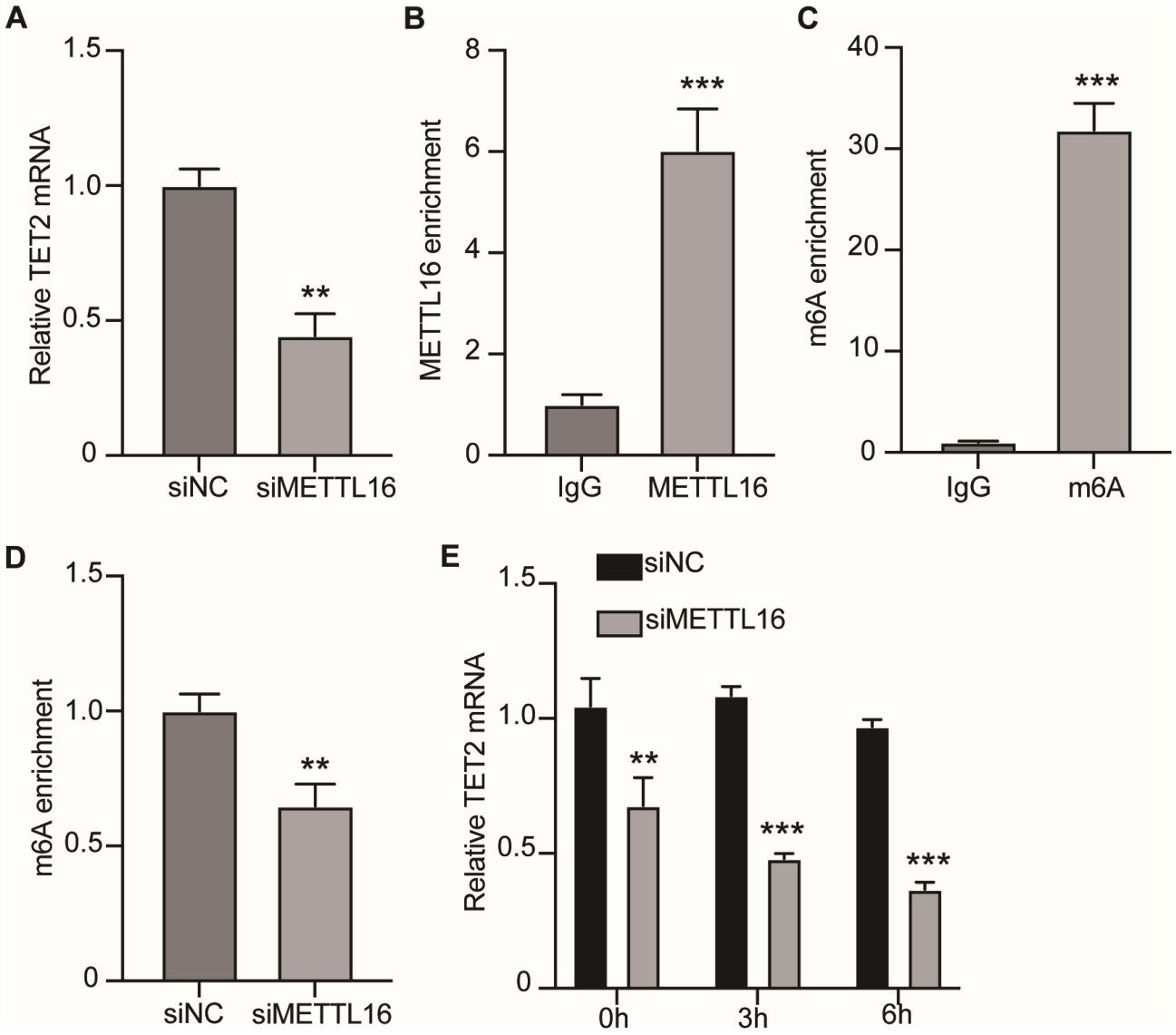

Figure 3 METTL16 upregulates TET2 expression by m6A modification. (**A**) The expression of TET2 was measured by qPCR in VSMCs treated with METTL16 siRNAs. (**B** and **C**) Enrichment of m6A and METTL16 on TET2 mRNA was measured by CLIP-qPCR in VSMCs. (**D**) Enrichment of m6A on TET2 mRNA was detected by MeRIP-qPCR in VSMCs treated with METTL16 siRNAs. (**E**) The stability of TET2 mRNA was analyzed by actinomycin D assay in VSMCs treated with METTL16 siRNAs. $^{**}p<0.01$, $^{***}p<0.001$.

expression of METTL16 in cardiac tissues of CHD mice, and knockdown of METTL16 notably recovered the cardiac function and alleviated tissue damages. Further in vitro studies confirmed that knockdown of METTL16 led to repressed viability of VSMCs and elevated cell apoptosis.

TET methylcytosine dioxygenase 2 (TET2) is an enzyme that catalyze the modification of methylated DNA and the following chromatin remodeling to modulate downstream gene expression.[34] Precious study has identified a negative correlation between TET2 level and severity of atherosclerotic disease in patient samples.[34] Besides, Ostriker et al reported that the expression and activity of TET2 is suppressed in a murine femoral artery denudation model of restenosis, and TET2 upregulated the 5hmC modification on the promoter region of critical VSMC differentiation genes.[35] Moreover, TET2 displays transcriptional upregulation in CHD patients, indicating its potential as a diagnostic tool. Mechanistic studies suggest that TET2 contributes to inflammatory responses and cardiomyocyte apoptosis in rats by demethylating microRNA-126 and subsequently regulate the PI3K-AKT signaling pathway.[36] These reports indicated the critical role of TET2 in CHD progression. We identified that METTL16 directly modulated the m6A enrichment on TET2 mRNA, and overexpression of TET2 could reverse the inhibitory effects of siMETTL16 on VSMC proliferation. Consistently, previous studies indicated that

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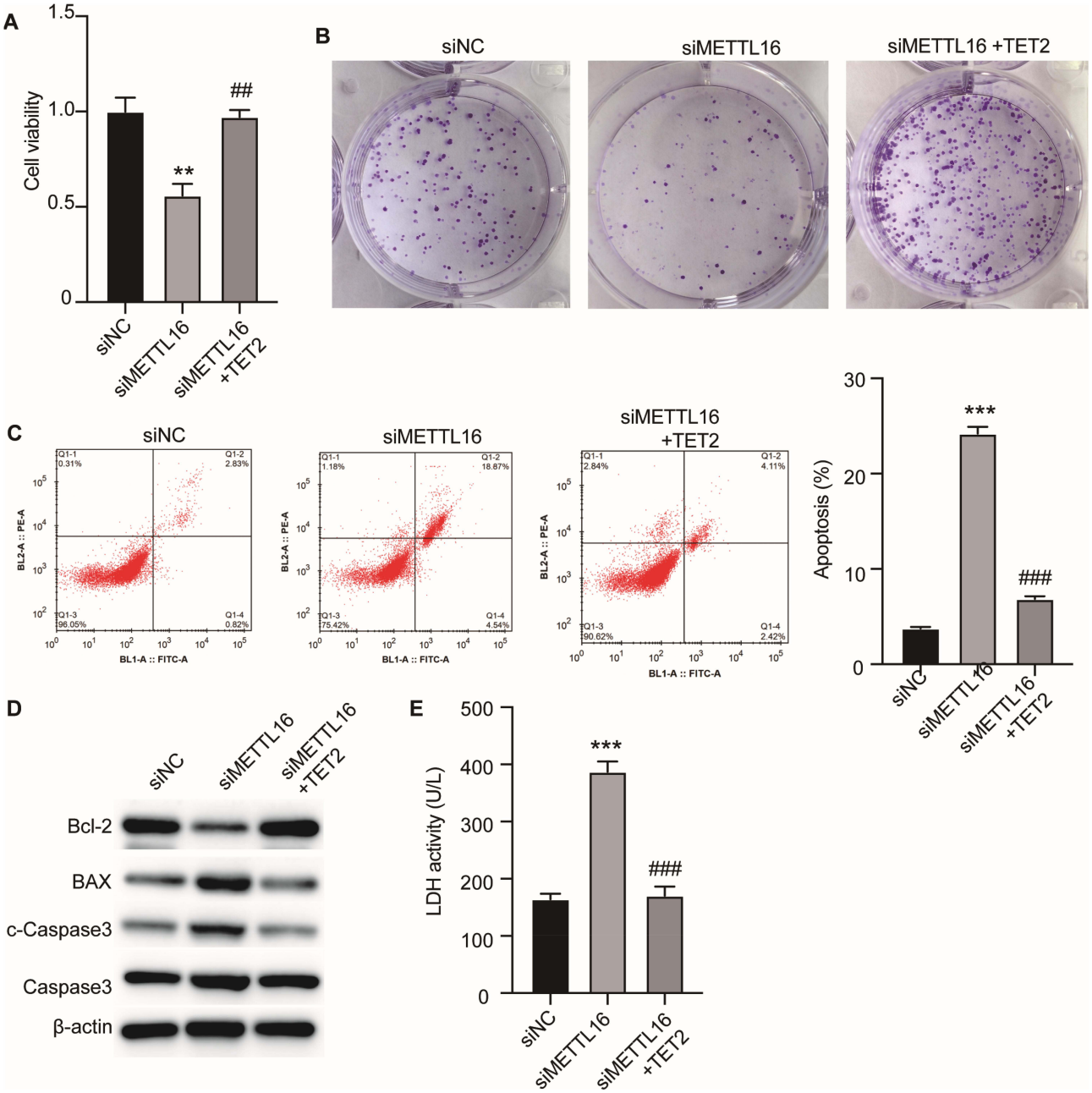
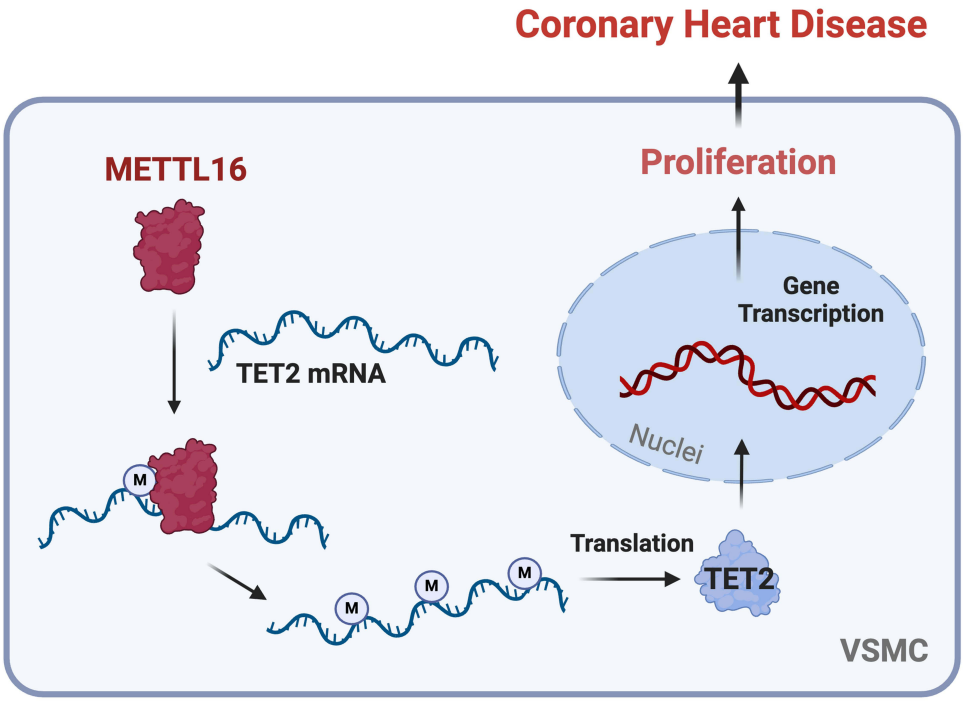


Figure 4 METTL16 enhances vascular smooth muscle cell viability by upregulating TET2 in vitro. (**A–E**) The VSMCs were treated with METTL16 siRNA and TET2 overexpressing plasmids. (**A**) Cell viability was measure by CCK-8. (**B**) Cell proliferation was analyzed by colony formation assay. (**C**) Cell apoptosis was detected by flow cytometry. (**D**) Apoptosis markers were determined by Western blot. (**E**) LDH activity was analyzed. $^{**}p<0.01$, $^{\#\#}p<0.01$, $^{***}p<0.001$, $^{\#\#\#}p<0.001$.

TET2 mediated the differentiation and abnormal proliferation of vascular smooth muscle cell and is a potential therapeutic strategy for diseases associated with intimal hyperplasia, including restenosis and atherosclerosis.[37] Our in vitro results showed that knockdown of METTL16 suppressed VSMC proliferation and TET2 overexpression reversed these effects. These results suggested that knockdown of METTL16 possibly alleviated the abnormal VSMC proliferation during CHD via regulating the TET2 signaling. Hence, targeting METTL16 could notably affect the TET-mediated transcriptional events that correlated with CHD progression and implied a promising clinical application.

Conclusion

To summarize, we identified elevated expression of METTL16 in CHD mouse model, and knockdown of METTL16 significantly alleviated tissues damage and recovered cardiac function via epigenetically modulating the m6A



Scheme 1 METTL16 modulates the m6A modification of TET2 mRNA to promote its translation and expression, which consequently induces the proliferation of VSMCs and chronic heart disease.

modification of TET2 (Scheme 1). Our findings may expose a novel insight into the pathogenesis of CHD and provide a promising target for CHD treatment.

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Disclosure

The authors report no conflicts of interest in this work.

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