Nucleophosmin contributes to vascular inflammation and endothelial dysfunction in atherosclerosis progression

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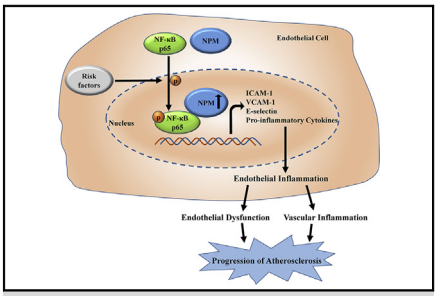
ABSTRACT

Objective: It is unclear whether nucleophosmin (NPM) participates in cardiovascular disease. The present study aimed to investigate the role and underlying mechanisms of NPM in atherosclerosis.

Methods: Levels and location of NPM in human carotid atherosclerotic plaques and healthy controls were detected by real-time polymerase chain reaction, immunoblots, and immunofluorescence. Atherosclerotic prone ApoE$^{-/-}$ mice were fed with a Western diet for 16 weeks as an in vivo model. Human primary umbilical vein endothelial cells (HUVECs) were cultured as an in vitro model.

Results: Compared with controls, we found that NPM levels in human carotid atherosclerotic plaques were more than twice as high as in normal arteries, which mainly localized in endothelial cells. In vivo, adenovirus-containing NPM small hairpin RNA attenuated atherosclerotic lesion and promoted plaque stabilization in ApoE$^{-/-}$ mice fed a Western diet by reducing vascular inflammation, maintaining endothelial function, and decreasing macrophage infiltration. Furthermore, NPM knockdown decreased nuclear factor-κB (NF-κB) p65 phosphorylation. In cultured HUVECs, palmitic acid increased the protein levels of NPM and induced the expression of inflammatory cytokines and monocyte adhesion, whereas NPM knockdown attenuated this effect. In HUVECs, NPM protein physically interacted with NF-κB p65 subunit and promoted its nuclear transposition. NPM also increased the transcriptional activity of NF-κB p65 promoter and enhance its binding to target genes, including interleukin-1β, interleukin-6, intercellular adhesion molecule-1, and E-selectin.

Conclusions: These data provide novel evidence that NPM promotes atherosclerosis by inducing vascular inflammation and endothelial dysfunction through the NF-κB signaling pathway and suggest that NPM may be a promising target for atherosclerosis prevention and treatment. (J Thorac Cardiovasc Surg 2021;161:e377-93)



NPM accelerated the progression of atherosclerosis via NF-κB pathway.

CENTRAL MESSAGE

Nucleophosmin was enriched in human atherosclerotic plaque. Nucleophosmin aggravated atherosclerosis through promoting NF-κB pathway–mediated vascular inflammation and endothelial dysfunction.

PERSPECTIVE

Nucleophosmin has been involved in the pathogenesis of many diseases, however, its role in atherosclerosis remains unknown. This study provided novel evidence that nucleophosmin promoted atherosclerosis by inducing vascular inflammation and endothelial dysfunction through the NF-κB signaling pathway and suggested that nucleophosmin may be a promising target for atherosclerosis prevention and treatment.

See Commentaries on pages e395 and e396.

Video clip is available online.

Atherosclerosis is a chronic vascular inflammatory disease that leads to some life-threatening complications, such as myocardial infarction and stroke. The increasing prevalence

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Abbreviations and Acronyms

ChIP = chromatin immunoprecipitation assay
HUVEC = human umbilical vein endothelial cell
ICAM-1 = intercellular adhesion molecule-1
IL = interleukin
IP = immunoprecipitation assay
NF-κB = nuclear factor-κB
NPM = nucleophosmin
PA = palmitic acid
PCR = polymerase chain reaction
shNPM = small hairpin RNA nucleophosmin
VCAM-1 = vascular cell adhesion molecule-1
VEC = vascular endothelial cell

and mortality have resulted in the loss of productive life and death worldwide in recent years.[1-3] Endothelial dysfunction induced by risk factors is referred to as a pathogenic sine qua non for atherosclerotic cardiovascular disease. Following endothelial impairment, circulating monocytes are then recruited to the intima and differentiated to macrophages, which internalize modified lipoproteins to become foam cells (the hallmark of early lesions).[4]

It is noteworthy that nuclear factor-κB (NF-κB) appears to play a pivotal role in the proinflammatory activation of endothelium in atherogenesis.[5,6] NF-κB is a pleiotropic transcription factor that can be activated by a variety of the pathophysiologic stimuli, resulting in the regulation of multiple effector proteins associated with atherosclerosis, such as inflammatory cytokines, chemokines, adhesion molecules and cell death signals.[7]

Nucleophosmin (NPM, B23, numatrin, or NO38) is a widely expressed and critical phosphoprotein in the nucleoplasmin family.[8] NPM mainly exists in the nucleolus, where it plays its major functions, whereas it can also convert back and forth between the nucleus and the cytoplasm.[9,10] NPM has been implicated in a number of pathways, including ribosome biogenesis, chromatin remodeling, apoptosis, and cell differentiation, participating in several human malignancies.[11,12]

More recently, emerging evidence suggests that NPM controls DNA transcription through interaction with different transcription factors. NPM can either contribute to transcriptional activation, as in the report of Myc-interacting zinc finger protein 1,[13] androgen receptor,[14] ribosomal genes,[15] c-Myc and NF-κB,[16] or to repression, as for the activating protein transcription factor 2α.[17] These studies all point to the potentially vital function of NPM in regulating gene expression in the transcription level.

Nevertheless, little is known about the role and mechanisms of NPM in arterial pathophysiology. In this study, we intended to investigate the role and underlying mechanisms of NPM in exacerbating vascular inflammation and endothelial dysfunction in atherosclerosis progression.

MATERIALS AND METHODS

Expanded methods, as well as additional figures and tables, are available in the Appendix E1.

Clinical Sample Collection

All studies involving humans complied with the Declaration of Helsinki and were approved by the Ethics Committee of Huazhong University of Science and Technology. Written informed consent was obtained from all participants before surgery. Carotid atherosclerotic plaque specimens were explanted from patients undergoing carotid endarterectomy. Control specimens came from normal aorta of patients without atherosclerosis during aortic valve replacement.

Animal Studies

All animal studies followed the guidelines of the Animal Care and Use Committee of Huazhong University of Science and Technology. Eight-week-old male ApoE$^{-/-}$ mice were randomly divided into 4 groups: ApoE$^{-/-}$ mice fed with a chow diet or Western diet for 16 weeks treated with Ad-shNC or Ad-shNPM, respectively (n = 18 for each group). Four weeks before being sacrificed, mice were injected with 5×10^8 plaque-forming unites (5×10^8 pfu/mouse) of either Ad-shNC or Ad-shNPM adenovirus via the tail vein every 2 weeks. Mice were housed individually under a 12-hour light/12-hour dark cycle and sacrificed by cervical dislocation.

NPM Knockdown In Vivo and In Vitro

Adenovirus containing specifically designed small hairpin RNA was used to knock down NPM expression in animal experiments. Small interfering RNA (siRNA) was transfected into HUVECs to knock down NPM expression in cellular experiments.

Cell Culture and Treatment

Human primary umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by using 0.1% collagenase I (Sigma-Aldrich, St Louis, Mo) and cultured in specific endothelial cell medium (ScienCell; Carlsbad, Calif) with 10% fetal bovine serum according to the reagent manufacturer's instructions. HUVECs between passages 3 and 8 were used and treated with palmitic acid (PA) as in vitro model.

Adhesion Assay

Adhesion assay was performed using Endothelial Cell Adhesion Assay Kit (ECM645; Bioscience Research Reagents, Temecula, Calif) according to the manufacturer's instructions.

Immunoprecipitation (IP) Assay

An IP assay was carried out as described in previous studies. In total, 500 μg of total proteins was incubated with NF-κB p65 antibody (8242S; Cell Signaling Technology; Danvers, Mass) and NPM antibody (ab10530; Abcam, Cambridge, United Kingdom) at 4°C overnight with protein A+G magnetic beads (Millipore, Burlington, Mass). The immunoprecipitates were washed and centrifuged 4 times with lysis buffer, followed by western blot analysis. Immunoglobulin G from the same species was used as a negative control.

Gene Overexpression

Human NPM plasmid was obtained from (RC203841; OriGene, Rockville, Md); the full length of human NPM coding sequence was constructed in a functional mammalian expression vector pCMV6. pCMV6-Entry vector was used as a negative control (Entry vector). The cultured HEK293 cells and HUVECs were transfected with plasmids using lipofectamine 2000 (Invitrogen, Carlsbad, Calif) for 24 hours before subsequent experiments.

Luciferase Assay

By using the One Step Cloning Kit (C112-02; Vazyme Biotech, Nanjing, China), NF-κB luciferase reporter plasmid, purchased from Tsingke Biotechnology (Tsingke, China), promoters of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin, and P-selectin, were assembled into pGL3.0 vector (Promega, Madison, Wis). The aforementioned primers are listed in Table E3. HEK293 cells were transfected with an internal control plasmid (pRL-TK; Promega) and plasmids containing other promoter regions simultaneously. Luciferase activity were detected with the Dual Luciferase Reporter Assay Kit (Promega).

Chromatin Immunoprecipitation Assay (ChIP)

ChIP was performed by ChIP assay kit (Millipore) according to the manufacturer's instructions. We used polymerase chain reaction (PCR) to detect DNA samples enriched by NF-κB p65 antibody (8242S; Cell Signaling Technology) to test whether the downstream target genes had changed. Rabbit immunoglobulin G antibody was performed as negative control. Primers used in the ChIP assay are shown in Table E4.

Statistical Analysis

For the data of human characteristics, continuous variables are shown as mean $\pm$ standard deviation when it was normally distributed or median (interquartile range) when it was not and evaluated by the Student *t* test or nonparametric test when appropriate. Categorical variables are presented as percentages and were compared between groups with χ^2 tests.[18]

For the results of animal and cellular experiments, data are presented as the mean $\pm$ standard deviation; statistical differences were evaluated by unpaired Student *t* test between 3 groups or one-way analysis of variance for multiple-group comparisons. Bonferroni corrections were made for the multiple testing. All analysis was performed with SPSS 23.0 (IBM Corp, Armonk, NY), and $P < .05$ was characterized as statistically significant.

RESULTS

NPM Was Enriched in Human Atherosclerotic Plaques

To explore the involvement of NPM in atherosclerosis, we first examined the level of NPM in 12 carotid atherosclerotic plaques and 12 human healthy arteries. The demographic characteristics of patients are summarized in Table E1. The level of NPM mRNA was more than twice as high as that in normal arteries; immunoblots also indicated that the protein level of NPM was elevated in plaque tissues (Figure 1, *A* and *B*). To determine the cellular localization of NPM, we performed dual immunofluorescent staining of human carotid atherosclerotic plaques using antibodies against NPM and CD31 (marker of endothelial cells), CD68 (marker of macrophages), or α-SMA (marker of smooth muscle cells). As shown in Figure 1, *C*, NPM predominantly colocalized with CD31-positive cells, suggesting that NPM was mainly expressed in vascular endothelial cells (VECs).

Inhibition of NPM Attenuated Endothelial Dysfunction and Atherosclerosis in ApoE$^{-/-}$ Mice

To evaluate the effect of NPM on atherosclerosis in vivo, we generated recombinant adenovirus containing small hairpin RNA nucleophosmin (shNPM) and injected it into ApoE$^{-/-}$ mice via the tail vein. As confirmed in Figure 2, *A* and *B*, Ad-shNPM inhibited the transcriptional and translational expression of NPM by 65.2% in the murine aorta. Oil Red O staining showed that Ad-shNPM decreased lesion area at aortic sinus by 45.1% compared with the control treated with Ad-shNC in ApoE$^{-/-}$ mice fed the Western diet, whereas no significant beneficial effect was observed in mice fed with chow diet (Figure 2, *C*). Likewise, NPM knockdown markedly reduced atherosclerotic plaque formation (decreased by 51.3% compared with Ad-shNC-treated mice, Figure 2, *D*). Knockdown of NPM also improved atherosclerotic plaque stability. As shown in Figure 2, *E*, Ad-shNPM-treated mice exhibited smaller necrotic core area, thicker fibrous caps, fewer elastic fiber breaks, as well as decreased macrophage infiltration. Furthermore, Ad-shNPM suppressed NF-κB p65 phosphorylation and decreased the protein level of VCAM-1, ICAM-1, and E-selectin, indicating that knockdown of NPM attenuated endothelial dysfunction and inflammation in ApoE$^{-/-}$ mice fed the Western diet (Figure 2, *F*).

Knockdown of NPM Suppressed the Inflammatory Responses in HUVECs

To investigate the role of NPM in endothelial function, we knocked down NPM in HUVECs by siRNA. Real-time PCR and western blot showed that NPM siRNA efficiently decreased the expression of NPM (Figure E3, *A* and *B*). To explore whether NPM was involved in the regulation of endothelial cell inflammatory reactions, we tested mRNA levels of cytokines and adhesion molecules in HUVECs stimulated by PA[19] with or without NPM knockdown (Figure 3, *A*). Depletion of NPM with siRNA notably reduced the levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor-α in the supernatants of HUVECs incubated with PA (Figure 3, *B*). We further characterized the effect of NPM on the expression adhesion molecules. We measured the expression of ICAM-1, VCAM-1, and E-selectin by western blot analysis and showed that knockdown of NPM suppressed the expression of all these molecules in varying degrees (Figure 3, *C*). After that, we generated endothelial cell monolayer adhesion assay using immunofluorescence technique. Consistent with the aforementioned results, NPM knockdown could significantly

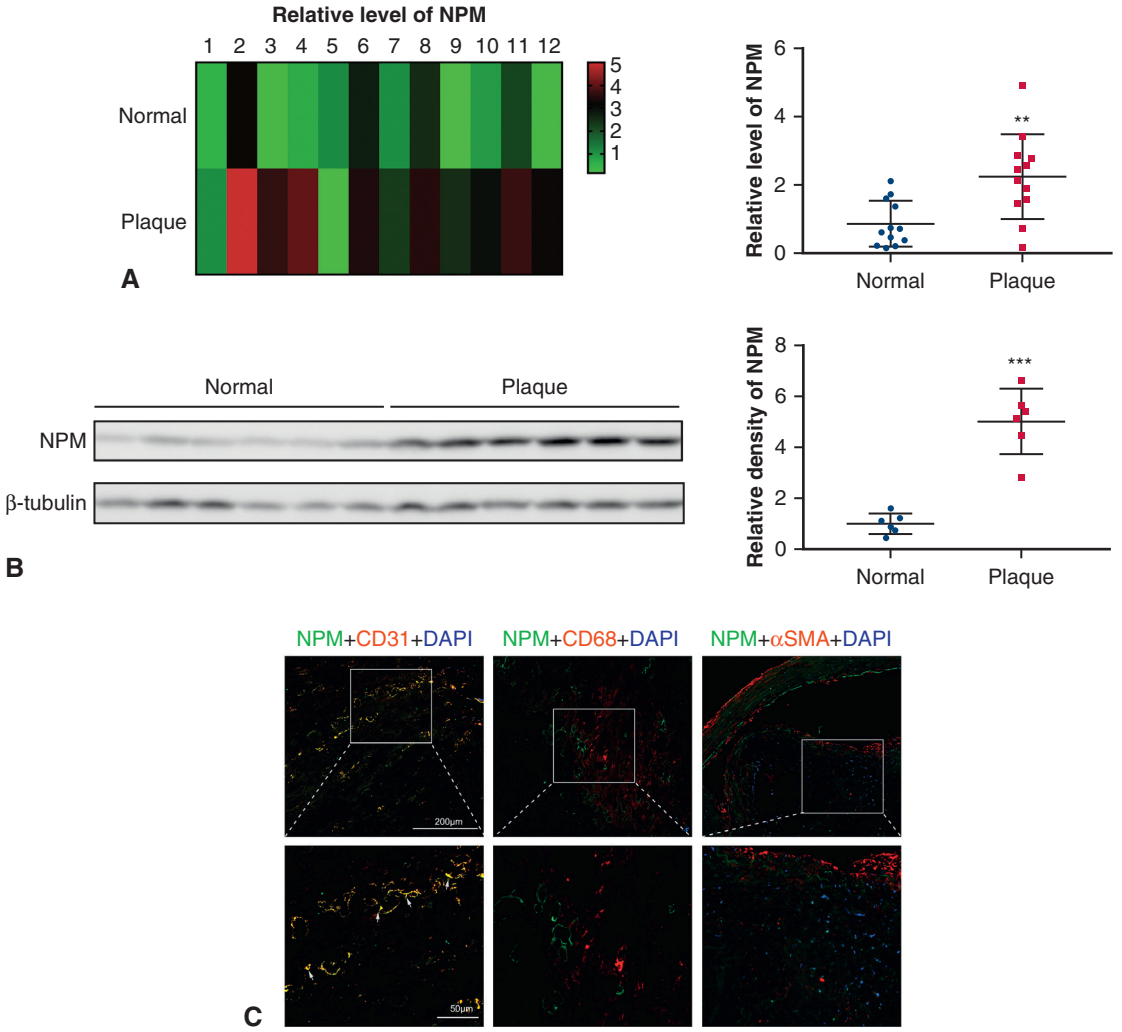


FIGURE 1. NPM was enriched in human atherosclerotic plaques. A, Quantitative real-time polymerase chain reaction showed that NPM mRNA expression was markedly increased in human carotid atherosclerotic plaques compared with healthy arteries, n = 12, $^{**}P < .01$ versus normal. B, Immunoblots showed that NPM protein level in atherosclerotic plaques was significantly greater than that in control, n = 6, $^{***}P < .001$ versus normal. C, Representative images of dual immunofluorescent staining showed that NPM was predominantly expressed in endothelial cells. For co-localization analysis, sections were dual-stained for NPM (*green*) and CD31 (*red*, endothelial marker), α-SMA (*red*, smooth muscle cell marker), or CD68 (*red*, macrophage marker). DAPI was used for nucleus staining (*blue*). NPM and endothelial cell-specific marker double-positive cells are indicated by *arrows*. *NPM*, Nucleophosmin; *DAPI*, 4,6-diamidino-2-phenylindole; *α-SMA*, α-smooth muscle actin.

reduce the number of peripheral blood monocytes attached to PA-activated endothelial cell monolayers (Figure 3, *D*).

NPM Physically Interacted With NF-κB p65 Subunit and Promoted Its Activation

To uncover the underlying mechanism of the aforementioned observations, we explored whether NF-κB pathway, a crucial proinflammatory transcription factor, was involved in the process. The phosphorylation and nuclear translocation of NF-κB p65 subunit is indispensable for its transcriptional activation, we measured the expression level of phospho-p65 (p-p65) to evaluate the activation status of NF-κB not only in total protein but also in nuclear extract and cytoplasmic extract, respectively (Figure 4, *A* and *B*). Immunofluorescent staining showed that PA promoted nucleus translocation of p65, which could be prevented by NPM siRNA (Figure 4, *C*). To investigate how NPM modulated NF-κB transcriptional activity, we hypothesized that NPM could function as a transcriptional co-activator that interacted with NF-κB via physical binding. We immunoprecipitated NF-κB p65 in untreated cells and detected the bound form of NPM with western blot. Conversely, immunoprecipitation of NPM resulted in the co-precipitation of NF-κB p65. Furthermore, treatment with PA strengthened their interaction with each other in HUVECs (Figure 4, *D*). PA treatment and NPM

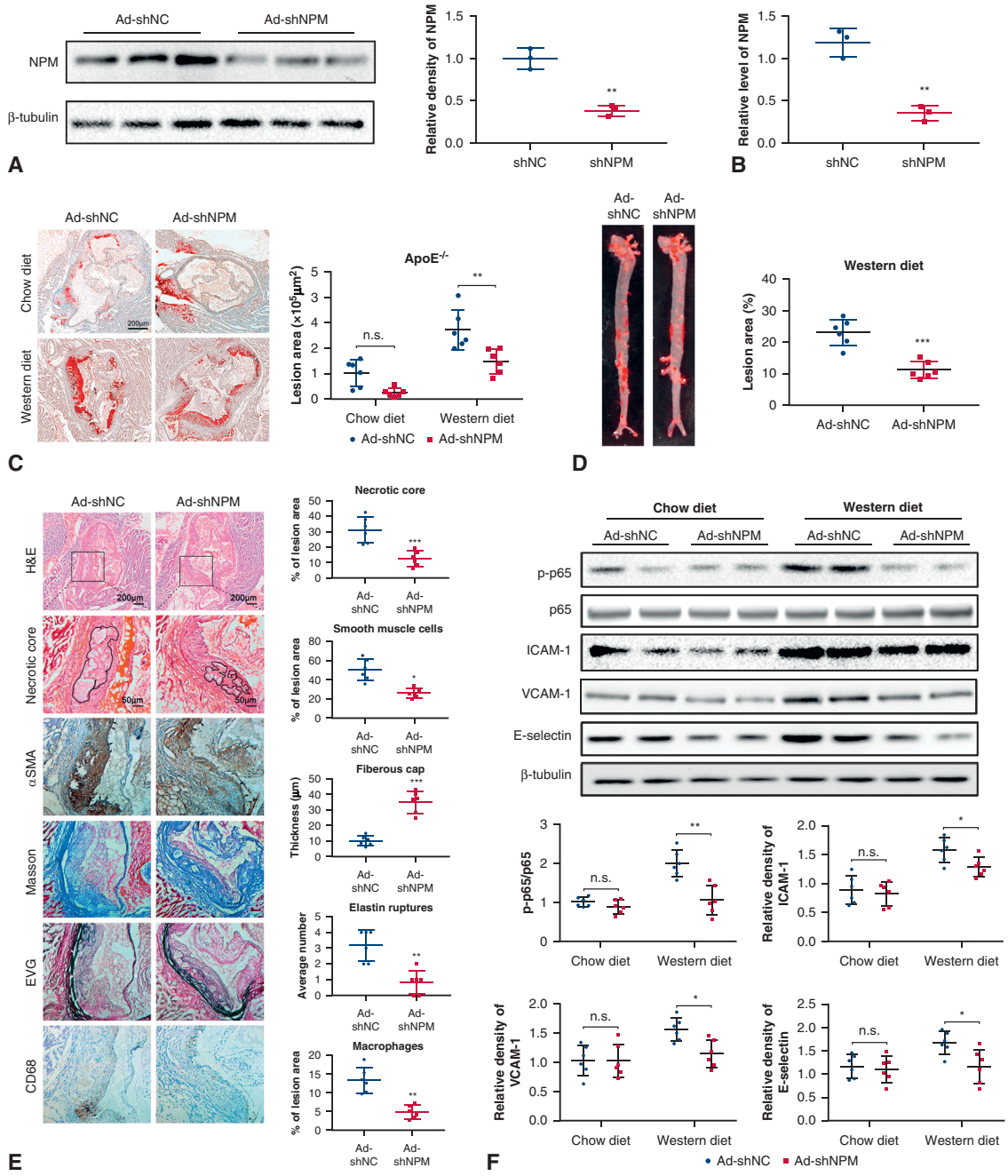


FIGURE 2. Inhibition of NPM attenuated endothelial dysfunction and atherosclerosis in ApoE$^{-/-}$ mice fed the Western diet. A and B, Recombinant adenovirus (Ad-shNPM) significantly decreased the protein and mRNA level of NPM in the murine vessel, n = 3, $^{**}P < .01$ versus shNC. C, ApoE$^{-/-}$ mice fed a chow diet (*upper*) or a Western diet (*lower*) were injected with Ad-shNC or Ad-shNPM. Oil Red O staining showed that Ad-shNPM decreased lesion area at aortic sinus in the ApoE$^{-/-}$ mice fed the Western diet, n = 6, $^{**}P < .01$ versus shNC. D, Representative images of Oil Red O staining in the whole aorta. Quantification analysis showed that Ad-shNPM markedly reduced atherosclerotic plaque formation in ApoE$^{-/-}$ mice fed the Western diet, n = 6, $^{***}P < .001$ versus shNC. E, Representative images of aortic sinus sections stained with Hematoxylin-eosin (*H&E*), Masson's trichrome (Masson), elastica van Gieson (*EVG*), and immunohistochemical staining for α-SMA and CD68. *Circular regions* mark the necrotic core area. The *black arrows* indicate α-SMA staining panels where fibrous caps were formed, which can be used for calculating the thickness of the fibrous caps. The *white arrows* in EVG staining panels indicate the rupture of elastic fibers of tunica media. Histologic quantification analysis showed that Ad-shNPM changed the pathologic features of atherosclerotic lesions compared with Ad-shNC, n = 6, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus shNC. F, Western blot was performed to detect the protein levels of ICAM-1, VCAM-1, E-selectin, and total and phosphorylated NF-κB p65 subunit in the vessel tissues of ApoE$^{-/-}$ mice. Densitometric analysis showed that Ad-shNPM suppressed NF-κB p65 phosphorylation and decreased the protein level of VCAM-1, ICAM-1, E-selectin, n = 6, $^{*}P < .05$, $^{**}P < .01$ versus shNC. Data are expressed as mean ± standard deviation. *Ad-shNC*, Adenovirus expressing normal control small hairpin RNA; *NPM*, nucleophosmin; *shNC*, normal control small hairpin RNA; *shNPM*, nucleophosmin small hairpin RNA; *α-SMA*, α-smooth muscle actin; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1; *n.s.*, no statistical significance.

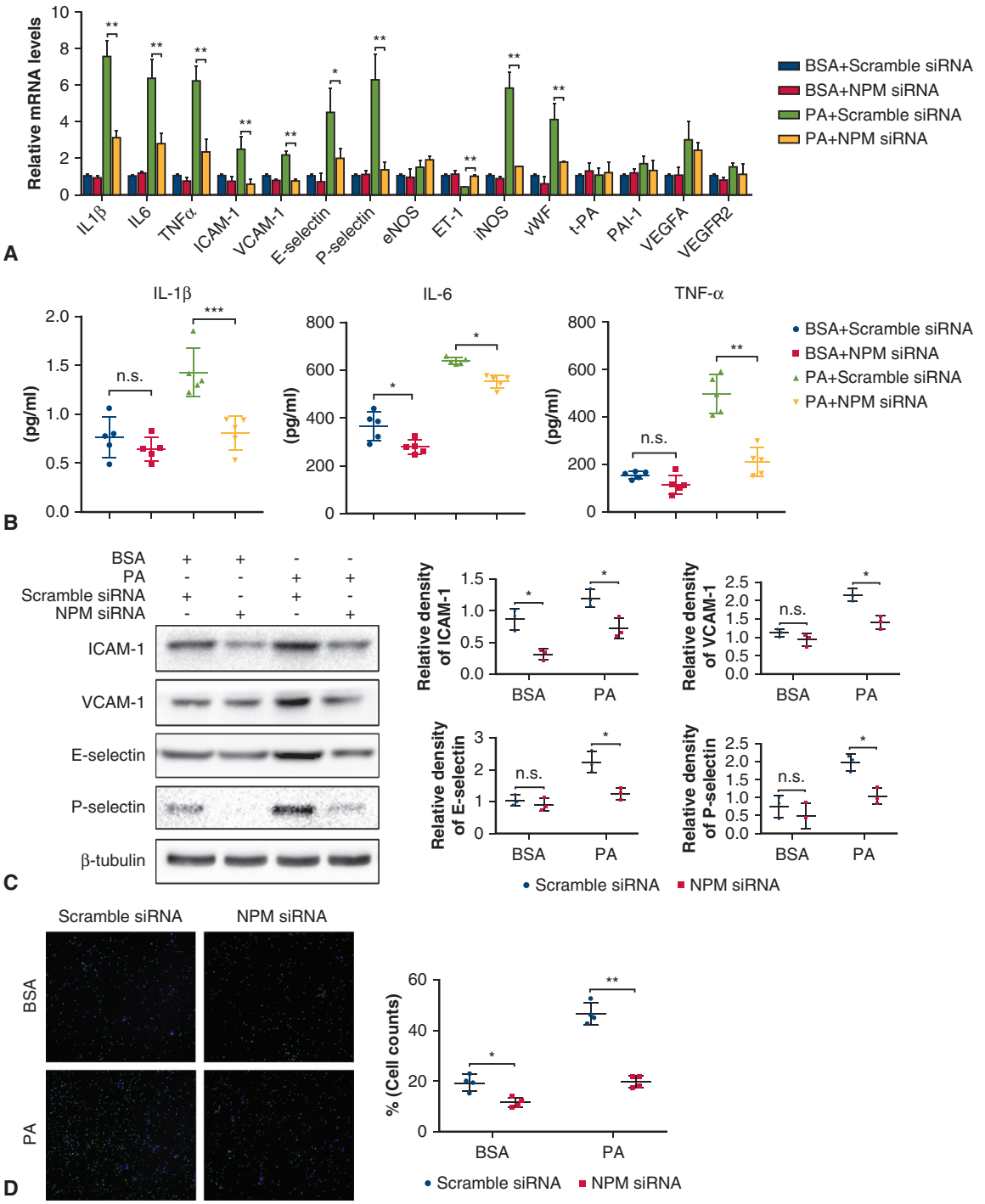


FIGURE 3. Knockdown of NPM suppressed the inflammatory responses in HUVECs. A, Real-time polymerase chain reaction showed that knockdown of NPM by siRNA decreased mRNA expression levels of inflammatory chemokines, adhesion molecules, and procoagulant factors in HUVECs treated with 500 μmol/L PA for 24 hours, n = 3, *$P < .05$, **$P < .01$ versus scramble siRNA. B, Knockdown of NPM with siRNA notably reduced the levels of IL-1β, IL-6, and TNF-α in the supernatants of HUVECs treated with 500 μmol/L PA for 24 hours, n = 5, *$P < .05$, **$P < .01$, ***$P < .001$ versus scramble siRNA. C, Immunoblot for ICAM-1, VCAM-1, E-selectin, and P-selectin in HUVECs showed that NPM knockdown suppressed the expression of all these adhesion molecules, n = 3, *$P < .05$ versus scramble siRNA. D, NPM knockdown significantly reduced the number of peripheral blood monocytes attached to PA-activated endothelial cell monolayers in the adhesion assay, n = 3, *$P < .05$, **$P < .01$ versus scramble siRNA. Data are expressed as mean ± standard deviation. *IL*, Interleukin; *TNF-α*, tumor necrosis factor-α; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1; *eNOS*, endothelial nitric oxide synthase; *ET-1*, endothelin-1; *iNOS*, inducible nitric oxide synthase; *vWF*, von Willebrand factor; *t-PA*, tissue plasminogen activator; *PAI-1*, plasminogen activator inhibitor-1; *VEGFA*, vascular endothelial growth factor A; *VEGFR2*, vascular endothelial growth factor receptor 2; *BSA*, bovine serum albumin; *siRNA*, small interfering RNA; *NPM*, nucleophosmin; *PA*, palmitic acid; *n.s.*, no statistical significance.

overexpression by transfecting NPM plasmid significantly augmented the promoter activity of NF-κB p65, which could also be inhibited by NPM siRNA or NF-κB inhibitor PDTC[20-22] (Figure 4, *E*).

NPM Enhanced the Transcriptional Activity of NF-κB to Its Downstream Target Genes

NF-κB regulates gene expression through binding to specific sites in the promoter of target genes. We examined the promoter activities of the adhesion molecules, including ICAM-1, VCAM-1, E-selectin, and P-selectin. PA treatment and NPM overexpression significantly up-regulated the luciferase activities of all promoters except for ICAM-1, whereas the increased luciferase activity could be reduced by NF-κB inhibitor PDTC (Figure 5, *A*). To further determine the influence of NPM on the DNA binding activity of NF-κB, ChIP experiments was performed. NPM overexpression could enhance more NF-κB binding to its downstream gene promoters, including IL-1β, IL-6, ICAM-1, and E-selectin (Figure 5, *B*).

DISCUSSION

Currently, little is known about NPM involvement in the progression of atherosclerosis. The present study showed that NPM was enriched in human atherosclerotic plaque and depletion of NPM attenuated the atherosclerotic lesions in ApoE$^{-/-}$ mice fed with a Western diet. This effect was accompanied by reduced NF-κB activation as well as macrophage infiltration and adhesion molecules expression. In vitro studies showed that NPM knockdown significantly inhibited inflammatory cytokines production (including IL-1, IL-6, and tumor necrosis factor-α) and adhesion molecules (including ICAM-1, VCAM-1, and E-selectin) as well as NF-κB p65 intranuclear translocation in cultured HUVECs stimulated by PA. Furthermore, NPM modulated NF-κB transcriptional activity via physical interaction with p65 subunit. To our knowledge, the novel findings provided the first evidence that proinflammatory effects induced by NPM/NF-κB interaction in VECs contributed to the development and progression of atherosclerosis.

NPM is an important nucleocytoplasmic shuttling protein that has been reported to participate in metabolic pathways, DNA repair pathways, and regulating apoptosis. NPM malfunction such as overexpressed, mutated, rearranged, or deleted has been described in a varieties of cancers, including lymphoma, acute myeloid leukemia, breast cancer, and gastric cancers.[23] However, emerging evidence suggests that NPM-mediated nucleolar stress is involved in cardiovascular disease such as myocardial infarction and hypertension.[24,25] Studies also have linked NPM to the onset of atherosclerosis. NPM mRNA expression was increased in carotid arteries of aged rats.[26] Oxidized low-density lipoprotein decreased human VEC proliferation through NPM dephosphorylation.[27] Consistent with previous studies, our findings further provided more robust evidence that NPM mRNA and protein levels were increased in human atherosclerotic plaques and mainly localized in VECs. In addition, knockdown of NPM in vivo attenuated atherosclerotic lesions in ApoE$^{-/-}$ mice fed a Western diet.

Endothelial cells not only function as a barrier but also can undergo a dramatic transition in their phenotype in response to injury or other biological stimuli, including pathogen-associated molecular patterns and other damage-associated molecular patterns. Endothelial proinflammatory activation resulted in the expression of leukocyte adhesion molecules, such as ICAM-1, VCAM-1, and E-selectin, which selectively recruited circulating monocytes into intima, where they differentiated into macrophages and internalized modified lipoproteins to become foam cells. In our study, NPM knockdown inhibited the production of inflammatory cytokines and adhesion molecules in HUVECs, thus significantly reducing attachment of monocytes to endothelial cells. In addition, knockdown of NPM in vivo also resulted in decreased expression of ICAM-1, VCAM-1, and E-selectin as well as reduced macrophage infiltration.

Highly unstable plaques are thought to be fast-growing and accompanied by superimposed thrombosis, eventually leading to acute adverse cardiovascular events.[28] Typical features of the vulnerable plaques include massive infiltration of inflammatory cells and a large necrotic core with thin fibrous cap.[29] Compared with control, Ad-shNPM–treated mice turned out to have thicker fibrous caps, smaller central necrotic areas, and fewer elastic fiber breaks, suggesting that in addition to the reduction in lesion size, the plaque stability was also greatly improved. From those results, NPM overexpression probably aggravated atherosclerotic lesion and plaque instability through inducing endothelial proinflammation change. However, these indicators have their limitations and can only indirectly reflect plaque stability.[30-32] Further studies are needed to investigate the effect of NPM on plaque stability more properly.

The NF-κB–mediated proinflammatory signaling pathway plays a crucial role in endothelial dysfunction. Our findings demonstrated knockdown of NPM inhibited PA-induced NF-κB p65 subunit phosphorylation and intranuclear translocation. Furthermore, we found physical interaction between NPM and the NF-κB p65 subunit. NPM also enhanced p65 promoter activity. Pretreatment with NF-κB inhibitor decreased adhesion molecule promoter activity induced by NPM overexpression. All these results demonstrated that NF-κB activation might be responsible for the detrimental role of NPM in atherosclerosis. Interestingly, recent studies suggested that NPM levels tended to be upregulated in aging heart, and SIRT1 may prevent NPM-induced NF-κB activation.[26] Further studies

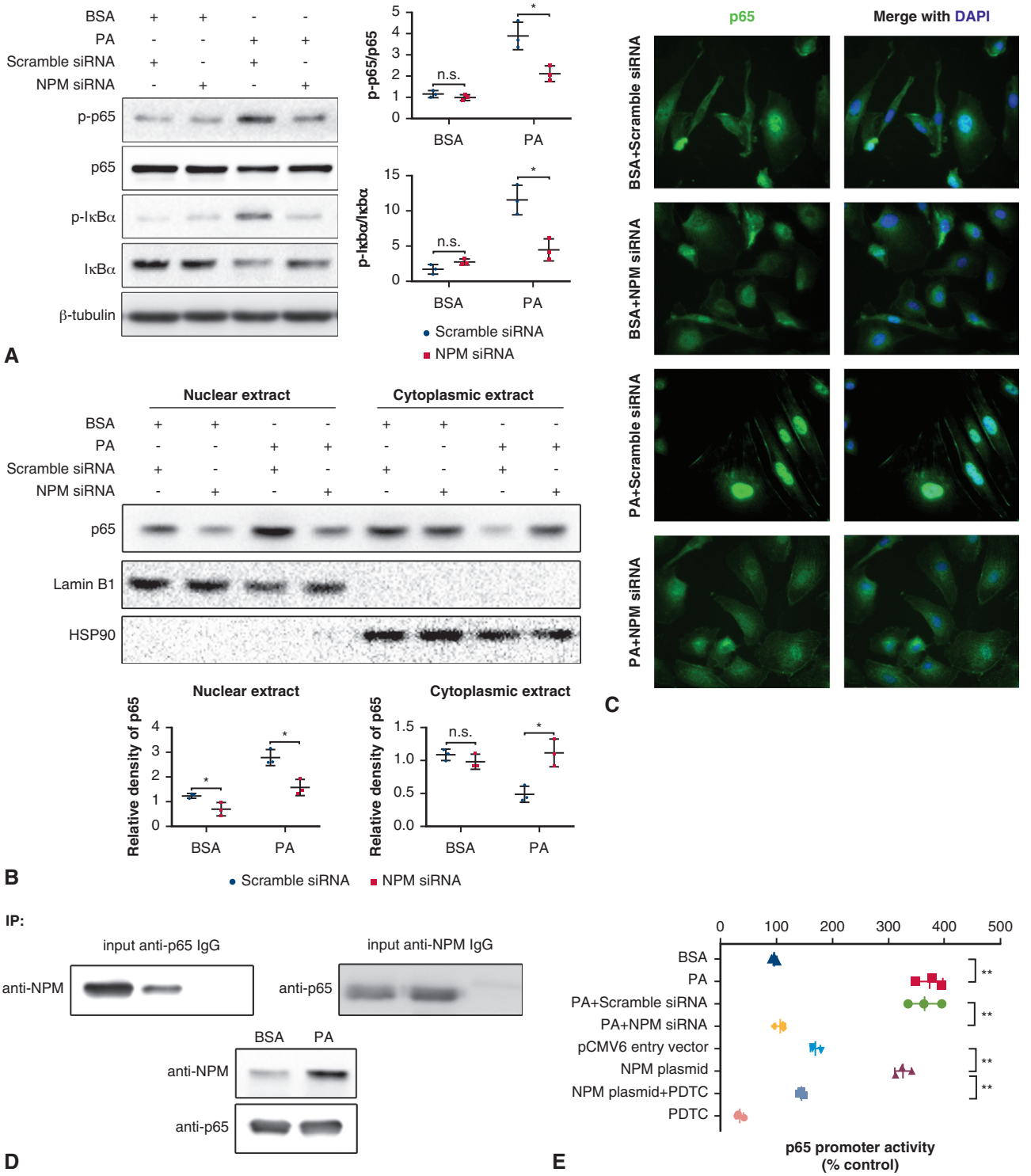


FIGURE 4. NPM physically interacted with NF-κB p65 subunit and promoted its activation. A, Immunoblots analysis of p65, Iκbα, and their phosphorylated protein level in total HUVEC protein after stimulated with 500 μmol/L PA for 1 hour, and NPM siRNA significantly decreased the ratio of p-p65/p65 and p-Iκbα/Iκbα, n = 3, *$P < .05$ versus scramble siRNA. B, Immunoblots of p65 protein level in nuclear extract and cytoplasmic extract showed that PA increased and decreased the expression of p65 in nucleus as well as in cytoplasm respectively, which were both inhibited by NPM siRNA. n = 3, *$P < .05$ versus scramble siRNA. C, Representative immunofluorescent staining against p65 (*green*) showed that NPM knockdown inhibited p65 intranuclear translocation induced by PA. The nucleus was stained with DAPI (*blue*). D, Immunoblots for NPM and NF-κB p65 after immunoprecipitation

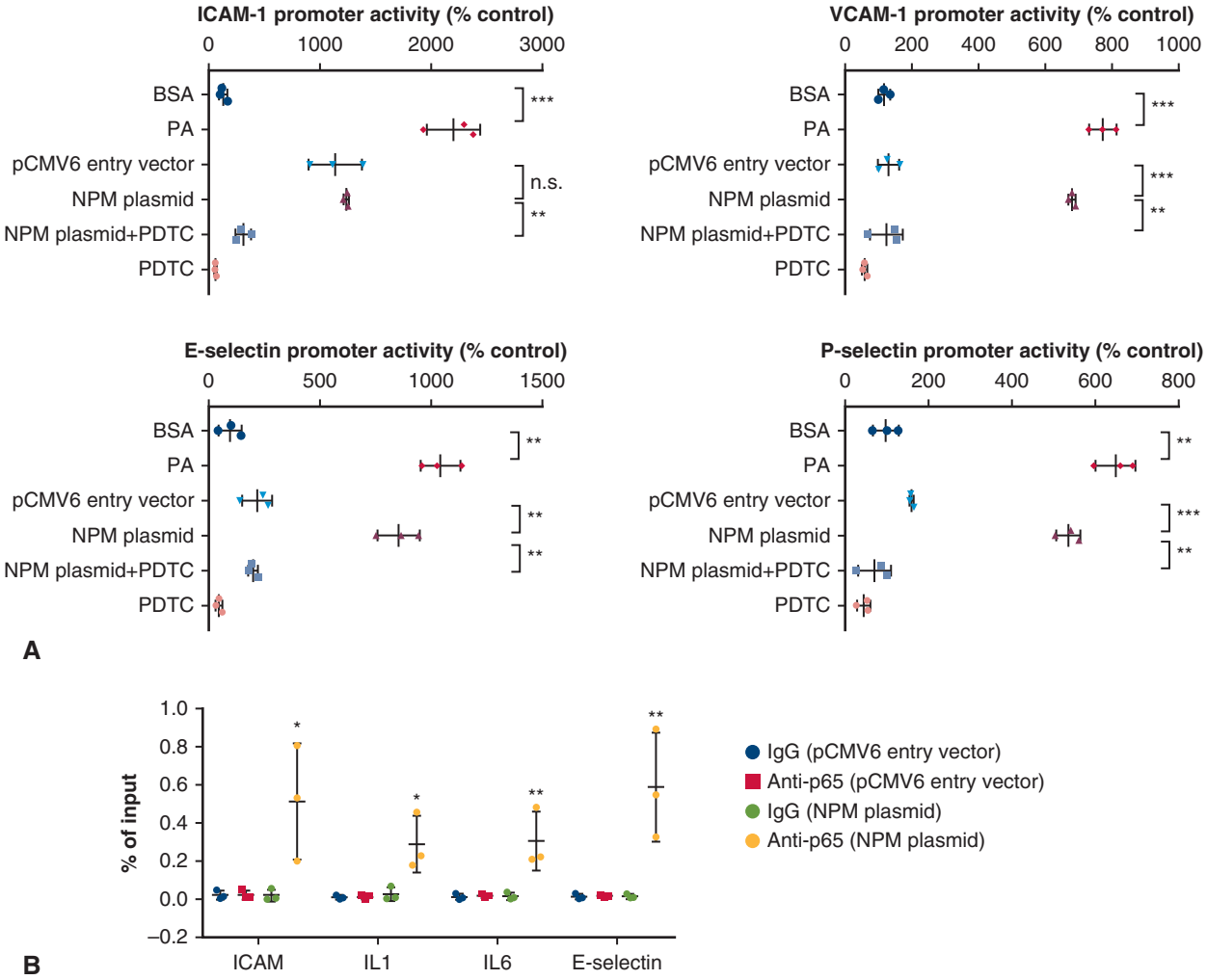


FIGURE 5. NPM enhanced the transcriptional activity of NF-κB to its downstream target genes. A, Luciferase reporter constructs containing promoters of ICAM-1, VCAM-1, E-selectin, and P-selectin were cotransfected with an internal control plasmid pRL-TK into HEK293 cells. PA treatment and NPM overexpression significantly augmented the promoter activity of these adhesion molecules (excluding ICAM-1), which could also be inhibited by NPM siRNA or NF-κB inhibitor PDTC, n = 3, $^{**}P < .01$, $^{***}P < .001$. B, ChIP assay was performed in cultured HUVECs using ChIP standard p65 antibody. IgG acted as negative control. Percentage of amplified signals from IP chromatin to amplified input signals obtained from the same sample indicated that NPM overexpression could significantly enhance NF-κB p65 binding to its downstream gene promoters, including IL-1β, IL-6, ICAM-1, and E-selectin, n = 3, $^{*}P < .05$, $^{**}P < .01$ versus IgG group. Data are expressed as mean ± standard deviation. *ICAM-1*, Intercellular adhesion molecule-1; *BSA*, bovine serum albumin; *PA*, palmitic acid; *pCMV6*, pCMV6 entry vector; *NPM*, nucleophosmin; *PDTC*, pyrrolidine dithiocarbamate, a nuclear factor–kB pathway inhibitor; *n.s.*, no statistical significance; *VCAM-1*, vascular cell adhesion molecule-1; *IL*, interleukin; *IgG*, immunoglobulin G.

are warranted to clarify the role of NPM in regulation of pathophysiologic conditions associated with arterial aging.

In summary, the present study uncovered a previously unrecognized role of NPM in exacerbating endothelial inflammation during the progression of atherosclerosis in ApoE$^{-/-}$ mice. NPM knockdown abrogated PA-induced expression of inflammatory cytokines as well as adhesion molecules in cultured HUVECs and reduced atherosclerosis

with anti-p65 antibody, anti-NPM antibody demonstrated physical binding of NPM and NF-κB. PA stimulation strengthened their interaction with each other in HUVECs. E, Luciferase assay was used to measure NF-κB promoter activity. Luciferase reporter construct containing the promoter of p65 was transfected with an internal control plasmid pRL-TK into HEK293 cells. PA treatment and NPM overexpression significantly augmented the promoter activity of NF-κB p65, which could be inhibited by either NPM siRNA or NF-κB inhibitor PDTC, n = 3, $^{**}P < .01$. Data are expressed as mean ± standard deviation. *BSA*, Bovine serum albumin; *PA*, palmitic acid; *siRNA*, small interfering RNA; *NPM*, nucleophosmin; *HSP90*, heat shock protein 90; *n.s.*, no statistical significance; *DAPI*, 4,6-diamidino-2-phenylindole; *NF-κB*, nuclear factor-κB; *HUVECs*, human primary umbilical vein endothelial cells; *IP*, interleukin; *IgG*, immunoglobulin G.

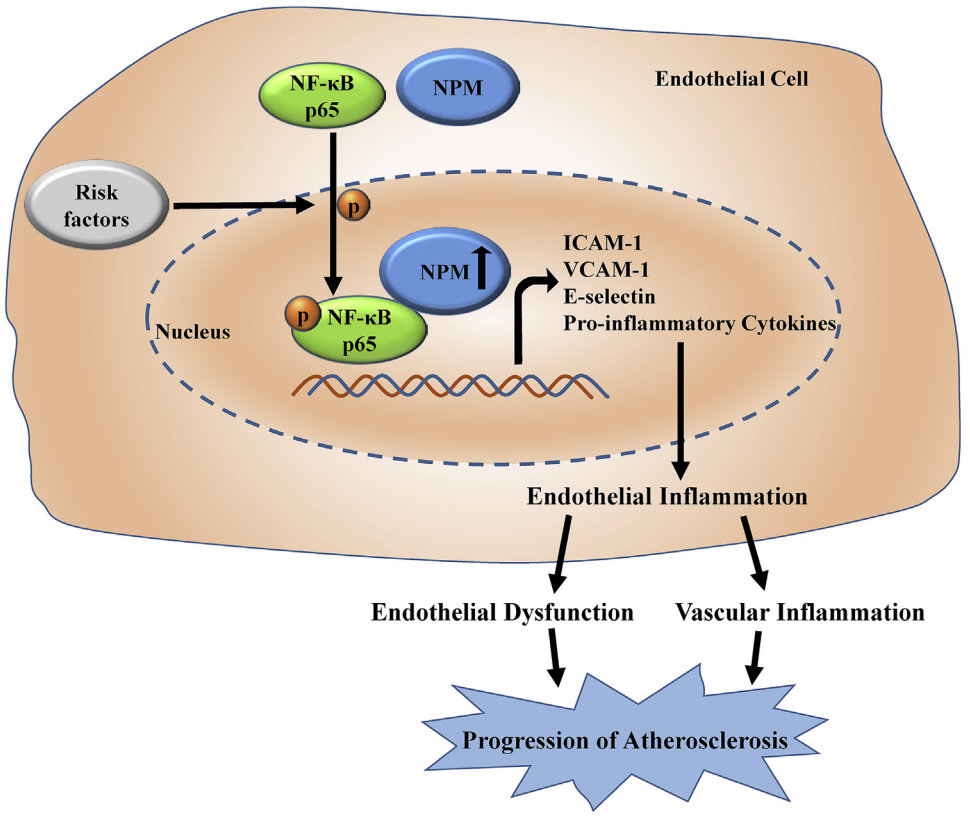
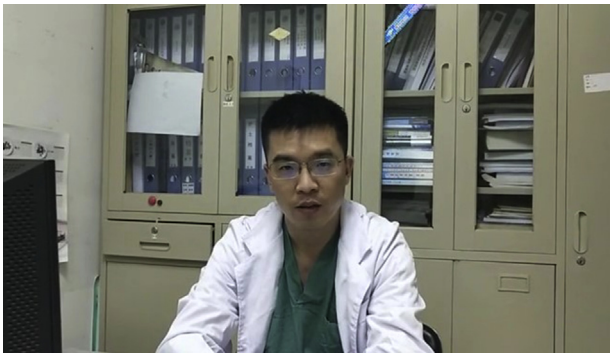


FIGURE 6. How nucleophospmin (*NPM*) contributes to the progression of atherosclerosis. Risk factors induced the expression of nucleophosmin, which subsequently increased the phosphorylation of NF-κB p65 subunit and promoted its translocation to nucleus, resulting production of inflammatory cytokines and adhesion molecules. Vascular inflammation and endothelial dysfunction aggravated atherosclerosis progression. *NF-κB*, nuclear factor-κB; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1.



VIDEO 1. This study uncovered a novel role of nucleophosmin in the progression of atherosclerosis. We found the level of nucleophosmin was greater in human atherosclerotic plaques than that in normal artery. Knockdown of nucleophosmin in vivo decreased macrophage infiltration, suppressed plaque formation, and improved plaque stability in ApoE$^{-/-}$ mice. In vascular endothelial cells, nucleophosmin could bind with NF-κB p65, promote its intra-nuclear translocation, and enhance the expression of downstream target genes. Although this study has some limitations, we still believe that our findings are of general interest to the readers. Video available at: https://www.jtcvs.org/article/S0022-5223(19)32776-X/fulltext.

plaque formation in ApoE$^{-/-}$ mice fed a Western diet. These protective effects were probably mediated via inhibition of NF-κB signaling pathway (Figure 6 and Video 1). All the aforementioned results suggest NPM may be a promising target for slowing atherosclerosis, stabilizing plaque, and reducing associated adverse events.

Study Limitations

Some limitations existed in our work. One was that we did not examine the effect of NPM knockdown on the metabolism of serum lipids in ApoE$^{-/-}$ mice. The other was that we performed all work on male mice; whether NPM made sense in female animals remains unknown, for males and females differ in the development of atherosclerosis.[33,34] In addition, vascular smooth muscle cells can also express ICAM-1 and VCAM-1 in atherosclerosis[35]; whether NPM can directly regulate SMC function is also unknown. Furthermore, although the specimens were ample enough to study, the result would be more robust if we increased the sample size for some comparison.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

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Key Words: nucleophosmin, NF-κB, vascular inflammation, endothelial dysfunction, atherosclerosis

ADULT

APPENDIX E1. MATERIALS AND METHODS

Immunofluorescence

Immunofluorescent staining of human carotid artery sections and primary human primary umbilical vein endothelial cells (HUVECs) was performed as previously described.[E1,E2] To summarize, carotid plaque sections were first incubated with mouse anti-nucleophospmin (NPM) antibody (ab10530; Abcam, Cambridge, United Kingdom), together with rabbit anti-CD31 antibody (ab28464; Abcam), anti-α-smooth muscle actin antibody (ab124964; Abcam), or anti-CD68 antibody (ab125212; Abcam) at 4°C overnight, followed by 30 minutes' incubation with anti-mouse immunoglobulin G (IgG) (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) (4408S; Cell Signaling Technology, Danvers, Mass) and anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor 555 Conjugate) (4413S; Cell Signaling Technology). Cell slides was incubated overnight with anti-nuclear factor-κB (NF-κB) p65 antibody (8242S; Cell Signaling Technology), followed with anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) (4412S; Cell Signaling Technology) for 30 minutes. Signals of individual and merged images for antigen detection were performed using a fluorescence microscope (Olympus, Tokyo, Japan) and AxioVision (version 4.8; Carl Zeiss GmbH, Jena, Germany) software.

NPM Knockdown In Vivo and In Vitro

Adenovirus containing specifically designed small hairpin RNA was used to knock down NPM expression in animal experiments. The target sequence was as follows: GCAGAGTCTGAAGATGAAGAT. Small interfering RNA (siRNA, 50 nM; RiBoBio, Guangzhou, China) was transfected into primary HUVECs by using lipofectamine 2000 (Invitrogen, Carlsbad, Calif) to knock down NPM expression. We designed 3 siRNAs for human NPM and selected the most stable and effective one for the following study. The target sequence was GGAGGAAGATGCAGAGTCA. Adenovirus express non-targeting small hairpin RNA (Ad-shNC) and "universal control" siRNA (siNC) served as negative controls.

Histologic Analysis and Quantification of Atherosclerotic Lesions

Mice were euthanized after fasting for 6 hours. Aorta Oil Red O staining was performed once the mice was sacrificed as previously described.[E3] Aortic root sections were performed simultaneously with hematoxylin-eosin, Oil Red O staining, Masson's trichrome staining, and elastic fibers with Elastica van Gieson staining. For macrophages and vascular smooth muscle cell content, cryosections of aortic root were stained with anti-CD68 antibody or anti-α-smooth muscle actin antibody, respectively. Quantification was performed by using Image-Pro Plus 6.0 (Media Cybernetics, Inc, Rockville, Md) and Image J software (National Institutes of Health, Bethesda, Md).

Cell Culture and Treatment

Cells were used for experiments at passages 3 to 8. For induced endothelial dysfunction model, HUVECs was stimulated with 500 μmol/L Palmitic acid (PA, P0500; Sigma-Aldrich, St Louis, Mo) for 24 hours. PA was first saponified with sodium hydroxide, then completely dissolved by phosphate-buffered saline in combination with fatty acid free bovine serum albumin (BAH66; Equitech-Bio, Kerrville, Tex) in a molar ratio of 1:6. Peripheral blood mononuclear cells (PBMCs) were prepared with Ficoll-Hypaque density gradient centrifugation. CD14+ cells were obtained through positive selection by CD14+ micromagnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). CD14+ monocytes were differentiated in complete RPMI-1640 medium supplemented with recombinant human macrophage colony-stimulating factor (50 ng/mL; R&D Systems, Minneapolis, Minn) for an additional 6 days before adhesion assay.

Quantitative Real-Time PCR

Total RNA was extracted with Trizol (D9108A; Takara Bio, Mountain View, Calif) and reversely transcribed using the RNA PCR kit (RR036A; Takara Bio). Quantitative PCR was performed by the ABI PRISM 7900 Sequence Detector system (Applied Biosystems, Beverly, Mass) according to the manufacturer's instructions. Relative gene expression was calculated by $2^{-\Delta\Delta CT}$ method after normalization to β-actin levels. The primers used in PCR were detailed in Table E2.

Western Blot

Cells or tissues extracts were prepared in ice-cold RIPA buffer mixed with protease inhibitors (Roche, Santa Clara, Calif), nuclear and cytoplasmic proteins were extracted by using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, Mass). Protein concentrations were detected by BCA protein assay kit (Thermo Fisher Scientific). Equal amount of protein was loaded to sodium dodecyl sulfate polyacrylamide gel for electrophoresis and transferred onto nitrocellulose membrane, then incubated with the following primary antibodies: ICAM-1 (ab7815; Abcam); VCAM-1 (ab134047; Abcam), E-selectin (ab18981; Abcam), P-selectin (ab178424; Abcam); NF-κB p65 antibody (8242S; Cell Signaling Technology); phospho-NF-κB p65 (3033S; Cell Signaling Technology); IκBα (ab32518; Abcam), and phospho-IκBα (sc-8404; Santa Cruz Biotechnology, Santa Cruz, Calif). β-tubulin (ab6046; Abcam) was used as total protein control, HSP90 (4877S; Cell Signaling Technology) and Lamin B1 (ab16048; Abcam) were used as cytoplasmic and nuclear protein control respectively.[E4] Then, the nitrocellulose membranes were incubated with a secondary antibody containing horseradish peroxidase for 2 hours in room temperature, and specific protein bands were recorded with Bio-Rad (Hercules, Calif) imaging system.

Enzyme-linked Immunosorbent Assay (ELISA)

ELISA was performed to measure interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) levels in supernatants of cell cultures by using commercial kits according to the manufacturer's instructions. ELISA kits were IL-1β (ELH-IL1b-1; RayBiotech, Peachtree Corners, Ga), IL-6 (ELH-IL6-1; RayBiotech), and TNF-α (ELH-TNFa-1; RayBiotech). The absorbance was read at 450 nm immediately after adding the stop solution. Quantitative results were calculated by standard curves.

Adhesion Assay

Pretreatment of PBMCs consisted of centrifugation in 1000 rpm for 5 minutes, incubation with calcein acetomethoxy dye in a 37°C cell incubator (5% CO_2) for 30 minutes, and washed 3 times with phosphate-buffered saline before incubation with HUVECs. HUVECs were transfected with scramble siRNA or NPM siRNA for 24 hours, followed by treatment with PA (500 μmol/L; Sigma-Aldrich) for another 24 hours and then incubated with the pretreated human PBMCs for 30 minutes. Unattached PBMCs were washed away by phosphate-buffered saline. Cells were then fixed in 4% paraformaldehyde and permeabilized with 0.1% TritonX-100 at room temperature for 10 minutes, and finally the cell nucleus was stained with 2.5 μg/mL 4,6-diamidino-2-phenylindole (DAPI; DNA stain; Invitrogen). PBMCs were identified and counted based on calcein AM green fluorescence (495 nm excitation, 516 nm emission), and total number of cells was counted based on DAPI fluorescence (350 nm excitation, 470 nm emission).

E-References

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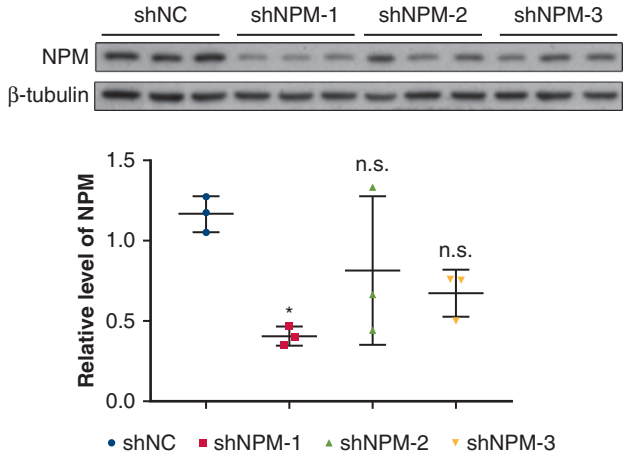

FIGURE E1. To generate adenovirus containing shRNA against NPM (Ad-shNPM), 3 shRNAs for mouse NPM were designed and the most efficient one (decreased by 65.2% compared with Ad-shNC-treated mice) was selected to be recombined into adenoviral vectors. The negative control adenovirus was designed to express non-targeting "universal control" shRNA (Ad-shNC). n = 3, $*P < .05$. Data are expressed as mean ± standard deviation. *shNC*, normal control small hairpin RNA; *shRNA*, small hairpin RNA; *NPM*, nucleophospmin; *n.s.*, no statistical significance.

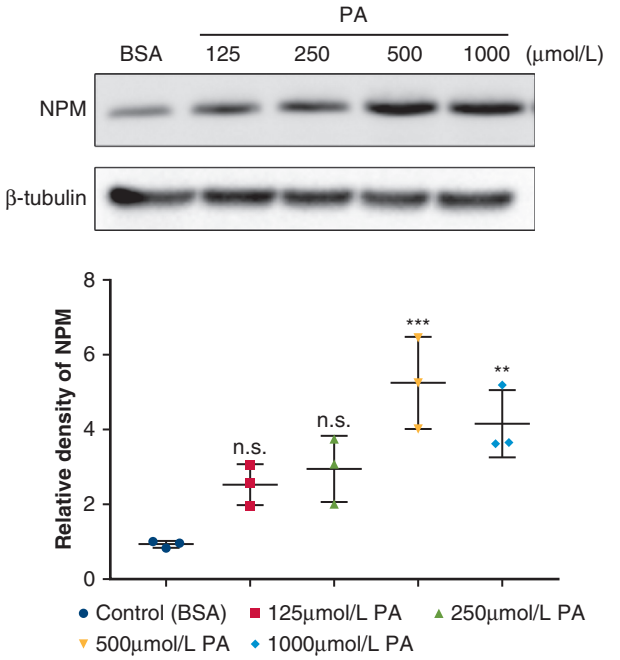

FIGURE E2. We stimulated primary human umbilical vein endothelial cells with a concentration gradient of PA for 24 hours and chose the most optimal dose (500 μmol/L) for subsequent study. BSA was used as vehicle control. n = 3, $**P < .01$, $***P < .001$. Data are expressed as mean ± standard deviation. *PA*, Palmitic acid; *BSA*, bovine serum albumin; *NPM*, nucleophospmin; *n.s.*, no statistical significance.

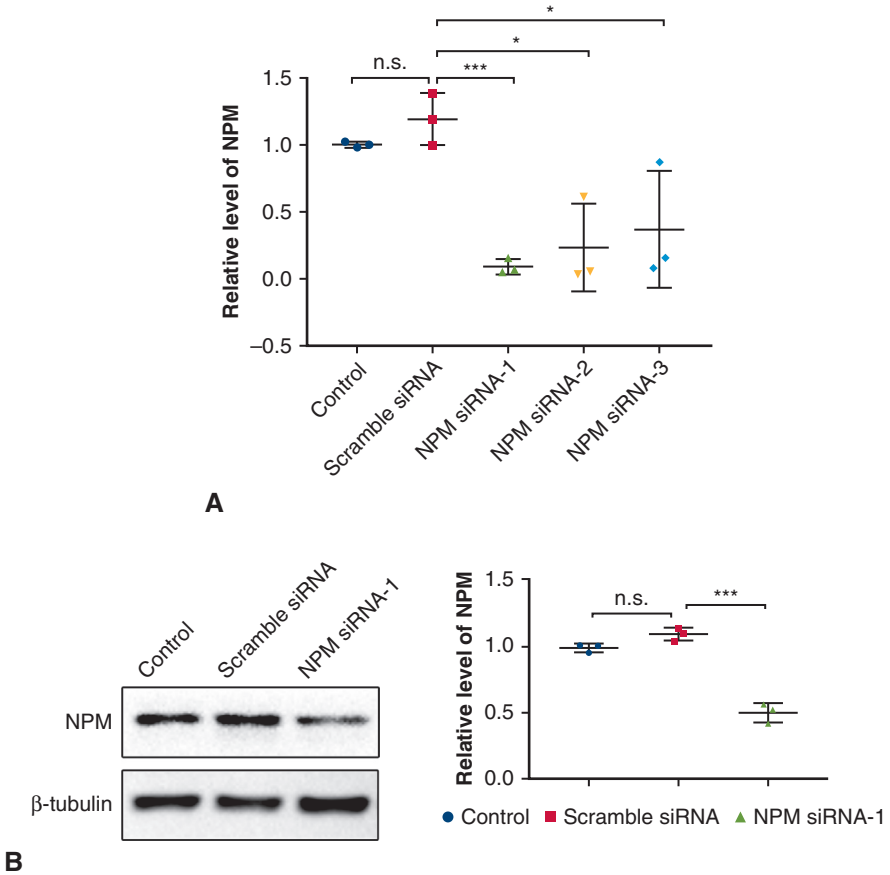

FIGURE E3. Three siRNAs for human NPM were designed and transfected to HUVECs. NPM siRNA-1 turned out to be the most efficient one (decreased by 86.8% compared with shNC-treated HUVECs by using real-time polymerase chain reaction to determine NPM mRNA level) (A) and western blot analysis for protein level (B), n = 3, $*P < .05$, $***P < .001$. Data are expressed as mean ± standard deviation. *NPM*, Nucleophospmin; *siRNA*, small hairpin RNA; *n.s.*, no statistical significance.

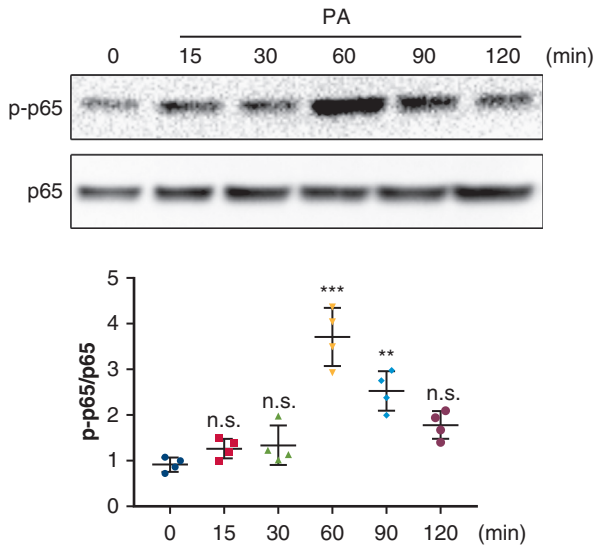

FIGURE E4. Human umbilical vein endothelial cells were stimulated with PA (500 μmol/L) for 15, 30, 60, 90, and 120 minutes. Western blot showed that PA induced the phosphorylation of nuclear factor-kappa B in a time-dependent manner, n = 4, $**P < .01$, $***P < .001$. Data are expressed as mean ± standard deviation. *PA*, Palmitic acid; *n.s.*, no statistical significance.

TABLE E1. Baseline characteristics of patients

Characteristics	Total (n = 24)	Normal (n = 12)	Plaque (n = 12)	*P* value
Age, y	58.96 ± 3.93	58.50 ± 3.92	59.42 ± 4.06	.579
Body mass index, kg/m^2	27.73 ± 2.95	23.56 ± 3.44	25.90 ± 1.823	.050
Smokers, %	58.33	20.83	37.50	.214
Drinkers, %	33.33	12.50	20.83	.667
Diabetes, %	4.17	0.00	4.17	.500
Systolic blood pressure, mm Hg	134 (120-152)	124 (119-139)	141 (131-162)	.240
Diastolic blood pressure, mm Hg	78.58 ± 14.93	72.33 ± 13.77	84.83 ± 13.82	.037
Fasting blood glucose, mmol/L	4.98 ± 0.35	4.81 ± 0.31	5.16 ± 0.30	.011
Total cholesterol, mmol/L	3.77 ± 0.92	3.02 ± 0.42	4.52 ± 0.62	<.0001
Triglycerides, mmol/L	1.25 (0.86-1.97)	0.97 (0.71-1.57)	1.47 (1.10-2.78)	.330
HDL cholesterol, mmol/L	1.20 (0.99-1.52)	1.10 (0.88-1.30)	1.41 (1.02-1.92)	.101
LDL cholesterol, mmol/L	1.83 ± 0.77	1.47 ± 0.32	2.18 ± 0.92	.024
hs-CRP, mg/L	1.70 (0.70-9.16)	0.80 (0.61-1.18)	8.97 (3.36-16.42)	<.0001

HDL, High-density lipoprotein; *LDL*, low-density lipoprotein; *hs-CRP*, high-sensitivity C-reactive protein.

TABLE E2. Real-time PCR primers

Gene	Forward primer	Reverse primer
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
TNF-α	GTGACAAGCCTGTAGCCCATGTT	TTATCTCTCAGCTCCACGCCATT
ICAM-1	ATGCCCAGACATCTGTGTCC	GGGGTCTCTATGCCCAACAA
VCAM-1	GGGAAGATGGTCGTGATCCTT	TCTGGGGTGGTCTCGATTTTA
E-selectin	GCACAGCCTTGTCCAACC	ACCTCACCAAACCCTTCG
P-selectin	ATGGGTGGGAACCAAAAAGG	GGCTGACGGACTCTTGATGTAT
eNOS	TGATGGCGAAGCGAGTGAAG	ACTCATCCATACACAGGACCC
ET-1	AGAGTGTGTCTACTTCTGCCA	CTTCCAAGTCCATACGGAACAA
iNOS	TTCAGTATCACAACCTCAGCAAG	TGGACCTGCAAGTTAAAATCCC
vWF	CCGATGCAGCCTTTTCGGA	TCCCCAAGATACACGGAGAGG
t-PA	AGCGAGCCAAGGTGTTTCAA	CTTCCCAGCAAATCCTTCGGG
PAI-1	ACCGCAACGTGGTTTTCTCA	TTGAATCCCATAGCTGCTTGAAT
VEGFA	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA
VEGFR2	GGCCCAATAATCAGAGTGGCA	CCAGTGTCATTTCCGATCACTTT
h-NPM	GGAGGTGGTAGCAAGGTTCC	TTCACTGGCGCTTTTTCTTCA
m-NPM	ATGGAAGACTCGATGGATATGGA	ACCGTTCTTAATGACAACTGGTG

IL, Interleukin; *TNF-α*, tumor necrosis factor-α; *ICAM-1*, intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1; *eNOS*, endothelial nitric oxide synthase; *ET-1*, endothelin-1; *iNOS*, inducible nitric oxide synthase; *vWF*, von Willebrand factor; *t-PA*, tissue plasminogen activator; *PAI-1*, plasminogen activator inhibitor-1; *VEGFA*, vascular endothelial growth factor A; *VEGFR2*, vascular endothelial growth factor receptor 2; *NPM*, nucleophospmin.

TABLE E3. Primers designed for promoter reporter gene construction

Gene	Primers	Promoter region, bp	Promoter length, bp
ICAM-1	F CTGCCCTGTCATCTCCCT R TCCATTTCACAAAGCGGTA	−1753∼−86	1668
VCAM-1	F CTGGGAGGAGCAGGTAGGA R TTGTTGCAGAGGCGGAGG	−1718∼+66	1784
E-selectin	F TTTGGGTCTTGACATCTT R GGTATCACTGCTGCCTCT	−1903∼+67	1970
P-selectin	F AGCGTGATAGGTATTGTT R TCTGTGACTCTGCTGGTT	−1822∼+61	1883

ICAM-1, Intercellular adhesion molecule-1; *VCAM-1*, vascular cell adhesion molecule-1.

ADULT

TABLE E4. Specific primers used in chromatin immunoprecipitation assay

Gene	Forward primer	Reverse primer
IL-1β	CTTCCACTTTGTCCCACAT	GTGCAGTTGATGTCCACAT
IL-6	GGCAAAGAGAATCTACACT	TCTGTTCTGTGCCCTCATT
ICAM-1	CACTCCCACGGTTAGCGG	CCATTTCACAAAGCGGTAAAC
E-selectin	GGGAAAGTTTTTGGATGCCATT	TGTCCACATCCAGTAAAGAGGAAAT

IL, Interleukin; *ICAM-1*, intercellular adhesion molecule-1.