NSun2 Deficiency Protects Endothelium From Inflammation via mRNA Methylation of ICAM-1

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Rationale: Vascular endothelial inflammation, including the expression of intercellular adhesion molecule 1 (ICAM-1), is a key event in vascular diseases. However, the mechanisms underlying the regulation of ICAM-1 are largely unknown.

Objective: To investigate the mechanisms on the regulation of ICAM-1 by NOP2/Sun domain family, member 2 (NSun2)-mediated mRNA methylation and the impact of NSun2–ICAM-1 regulatory process in vascular inflammation and allograft arteriosclerosis.

Methods and Results: By using in vitro, in cells, and in vivo methylation assays, we showed that the tRNA methyltransferase NSun2 methylated the ICAM-1 mRNA. Methylation by NSun2 promoted the translation of ICAM-1, thereby increasing the adhesion of leukocytes to endothelial cells. Tumor necrosis factor-α or homocysteine activated the methyltransferase activity of NSun2 by repressing the phosphorylation of NSun2 by Aurora-B. The levels of ICAM-1 induction and of leukocyte adhesion to vascular endothelium observed with homocysteine treatment in wild-type rats were markedly decreased in NSun2−/− rats. In a rat model of aortic allograft, the lack of donor NSun2 impaired the formation of allograft arteriosclerosis.

Conclusions: NSun2 upregulates the expression of ICAM-1 by methylating ICAM-1 mRNA. This regulatory process impacts on vascular inflammation and allograft arteriosclerosis. (Circ Res. 2016;118:944-956. DOI: 10.1161/CIRCRESAHA.115.307674.)

Key Words: arteriosclerosis ■ inflammation ■ intercellular adhesion molecule-1 ■ methylation ■ NSun2

Vascular inflammation is an early and pivotal event in the process of atherosclerosis.1 The production of adhesion molecules and their shedding onto the endothelial and leukocytic surfaces play an indispensable role in mediating the interaction between endothelial cells and blood constituents or the extracellular matrix.2,3 As an important member of the interaction between endothelial cells and blood constituents or the extracellular matrix, the lack of donor NSun2 impaired the formation of allograft arteriosclerosis.

Although ICAM-1 is constitutively expressed, its up-regulation on cytokine-activated vascular endothelial cells has been linked with the strength of leukocytes’ adhesion to endothelial cells at the early stage of atherosclerosis. Indeed, elevation of ICAM-1 expression by proinflammatory factors (eg, tumor necrosis factor-α [TNF-α], lipopolysaccharide, homocysteine) has been described as a typical feature in the inflammatory response of the endothelial cells.5–9 Although the mechanisms controlling ICAM-1 expression are not fully elucidated, regulation at the transcriptional level has been intensively studied. For example, transcriptional factors including nuclear factor-κB, cAMP response element-binding protein, Ets, and SP1 control the transcription of ICAM-1 by binding to the ICAM-1 promoter; histone modification has also been implicated in the transcriptional regulation of ICAM-1.10–13 In addition to transcriptional regulation, the contribution of posttranscriptional gene regulatory events to the expression of ICAM-1 is becoming apparent.14 RNA binding protein HuR has been found to regulate the expression of ICAM-1 in a nuclear factor-κB–dependent manner.15 In addition, microRNAs miR-221, miR-222, miR-223, miR-339, and miR-296 are also repressors for the expression of ICAM-1.16–19

Methylation is a prevalent modification for noncoding RNAs such as tRNA, rRNA, piwi RNA, Drosophila small
Nonstandard Abbreviations and Acronyms

BCECF  bis-carboxyethyl-carboxyfluorescein
CDS  coding sequence
EEL  external elastic lamina
HUVEC  human umbilical vein endothelial cell
ICAM-1  intercellular adhesion molecule-1
NSun2  NOP2/Sun domain family, member 2
PCR  polymerase chain reaction
qPCR  quantitative polymerase chain reaction
TNF-α  tumor necrosis factor-α
UTR  untranslated region
VCAM-1  vascular cell adhesion molecule-1
WT  wild type

Interfering RNA, microRNAs, and lncRNAs,20–27 as well as for the 5’ cap and 3’ untranslated region (UTR) of mRNAs.20,29,31
Thus far, methylation of these RNAs has been linked to the efficiency and accuracy of translation,32,33 RNA stability,24,27 and to the biogenesis of small RNAs.24,34,35 NOP2/Sun domain family, member 2 (NSun2) Myc-induced SUN domain-containing protein is a tRNA methyltransferase and is localized predominantly in the nucleus.36,37 Apart from tRNA, NSun2 may have a wide range of substrates. For example, NSun2 has been shown to methylate vault-noncoding RNA, miR-125b, and the 3’UTRs of p16, p53, Bak1, E2F3, and ErbB2 mRNAs.30,31,34 However, whether NSun2-mediated RNA methylation is involved in the process of vascular inflammation remains to be studied.

In the present study, we report that NSun2 methylates ICAM-1 mRNA in 5’UTR and 3’UTR and in the coding sequence (CDS). Methylation by NSun2 promotes the expression of ICAM-1 at the translational level. This regulatory process impacts on vascular endothelial inflammation and arteriosclerosis.

Methods

An expanded Materials and Methods are available in the Online Data Supplement.

Ethics Statement

All rat husbandry and experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Health Science Center of Peking University, and all efforts were made to minimize the animals’ suffering.

Rats

NSun2−/− and NSun2+/− rats were generated from Sprague Dawley rats using the Rat TALEN method (Rat NSun2 Gene ID: 361191) in Kangweida Technology Co. Ltd. (Wuhan, China). The constructs used are described in their previous article.38 Deletion of NSun2 was confirmed by polymerase chain reaction (PCR) and sequence analysis. Primers used for PCR were as follows: CACCTGTCCCGATCACTGAC and ACAGCCTGGCCCTACACTCA. Some 3’ primers contained the poly-T sequence [poly-T] TTTTTTTTTTTTTT TT. Primers used for preparing the templates are listed in Online Table I. In vitro transcription were performed by using an in vitro transcription kit (Promega, Madison, WI) following the manufacturer’s instructions.

In Vitro Methylation Assays

For in vitro methylation assays, His-tagged NSun2 was expressed in E. coli and purified as previously described.39 Reaction mixtures (50 μL) containing 0.2 mmol/L His-NSun2, 0.01 mmol/L RNA, and 1 μCi of [3H]-labeled 5'-adenosyl-L-methionine (Amersham Bioscience, USA) in reaction buffer (5 mmol/L Tris-HCl [pH 7.5], 5 mmol/L EDTA, 10% glycerol, 1.5 mmol/L dithiothreitol, and 5 mmol/L MgCl2) supplemented with inhibitors (leupeptin [1 μg/mL], aprotinin [1 μg/mL], 0.5 mmol/L phenylmethylsulfonyl fluoride, and RNasin [5 U/μL]) were incubated for 30 minutes at 37°C. E. coli trNA (0.01 mmol/L; Sigma, St. Louis, MO) and ICAM-1 cDNA (0.01 mmol/L) were used as a positive control and a negative control, respectively. Unincorporated [3H]-adenosyl-L-methionine was removed by using QiAQuick Spin Columns (Qiagen, Germany), and incorporated radioactivity was measured by liquid scintillation counting.

Measurement of Methylation in Cells or In Vivo

For methylation assays in cells or in vivo, 1 μg of anti-met-C antibody (Abcam, Cambridge, MA), 20 μg of cellular RNA or rat vascular RNA, and 20 μL (in 50% slurry) protein-G Sepharose were incubated in immunoprecipitation (IP) buffer (150 mmol/L NaCl, 0.1% NP-40, 10 mmol/L Tris-HCl [pH 7.4]) and 1 U/μL RNasin in 500 μL at 4°C for 2 hours. The IP beads then were washed 5 times with IP buffer. RNA isolated from the IP beads was subjected to real-time qPCR analysis.

HPLC-MS Analysis

In vitro methylated RNA fragments (1 μg) were digested with nuclelease P1 (Sigma, St. Louis, MO) and alkaline phosphatase (New England Biolabs, Beverly, MA). The formation of m5C or m6A was analyzed by high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis at Tsinghua University Mass Spectrometry Center (Beijing, China).

In Vitro Translation Assays

For in vitro translation assays, a cell-free translation system (Promega, Madison, WI) in rabbit reticulocyte lysate was used. Luc-5’UTR, Luc-CDS, or Luc-3’UTR chimeric transcript (0.01 mmol) was in vitro transcribed from pGL3-5’UTR, pGL3-CDS, or pGL3-3’UTR reporter and methylated by NSun2 or kept untreated. The methylated and nonmethylated transcripts were used for in vitro translation assays. The translation efficiency was determined by measuring the activity of firefly luciferase.

Leukocyte Adhesion in Rat Mesenteric Venules

The rats were anesthetized with sodium pentobarbital (30 mg/kg body weight), and the femoral vein was cannulated for the administration of the various reagents. For observation of mesenteric microcirculation, rat mesentery was drawn out of the abdominal cavity carefully and gently. The local microcirculation was recorded in real time using an upright microscope (DMiLS; Leica, Mannheim, Germany) with a supersensitive CCD camera (US-301; UNIQ Vision, Santa Clara, CA). Single, unbranched venules without an obvious bend and with diameters ranging from 30 to 50 μm and lengths of ≥200 μm were selected. The image was projected onto a monitor (J2118A; TCL, Seoul, Korea) and recorded with a DVD recorder (DVRR25; Malata, Seoul, Korea) and recorded with a DVD recorder (DVRR25; Malata, Seoul, Korea).
Shenzhen, China). To examine leukocyte adhesion in venules, the dynamic behavior of leukocytes was examined by replaying the recorded movie. Adherent leukocytes were defined as cells that attached to the venule for >30 seconds, and the number of adherent leukocytes was counted along venules (200 μm in length).

**Immunohistochemical Analysis**

Thoracic aorta were fixed and sectioned at 7 μm. Immunohistochemistry was used to document the expression of NSun2 and ICAM-1 on vascular endothelium. After overnight incubation with anti-NSun2 antibody (1:200, Santa Cruz, CA) or an anti-ICAM-1 antibody (1:50, Santa Cruz, CA), slides were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibodies (1:200). The intensities of NSun2 and ICAM-1 were detected using the SABC method. Images were collected using a BX60 microscope (Olympus Optical Co Ltd, Japan) equipped with a Sony 3 CCD camera and television monitor.

**Immunofluorescence Staining**

For immunofluorescence assay, frozen sections were incubated with an anti-NSun2 (1:200) or anti-ICAM-1 (1:50) antibody, or anti-CD45 (1:200; BD Biosciences, San Jose, CA), or with a negative control antibody (IgG). After staining with secondary Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) or Alexa Fluor 633-conjugated antigoat IgG (Invitrogen, Carlsbad, CA), the fluorescent signal was monitored by confocal laser-scanning microscopy (Leica, Germany). Nuclei were counterstained with Hoechst 33342 (Sigma, St. Louis, MO).

**Adhesion of Leukocytes to HUVECs and the Rat Vascular Endothelium**

For adhesion of leukocytes to human umbilical vein endothelial cells (HUVECs), THP-1 mononuclear cells were cultured in RPMI 1640 medium containing 3 mmol/L bis-carboxyethyl-carboxyfluorescein (BCECF; Sigma, St. Louis, MO). The BCECF-labeled THP-1 cells (5×10^5 cells/mL) were incubated with HUVECs for 30 minutes. The adherent monocytes from 9 random selected vision areas were counted using fluorescence microscopy (Leica, Germany).

For adhesion of leukocytes to the rat vascular endothelium, the rat leukocytes were isolated from the spleen of WT rat and cultured in RPMI 1640 medium containing 3 mmol/L BCECF. The thoracic aorta (30 mm) were isolated from rats and opened longitudinally. The BCECF-labeled spleen leukocytes (5×10^5 cells/mL) were incubated with the aortic segments for 30 minutes. The adherent leukocytes from 9 random selected vision areas were counted using fluorescence microscopy.

**Aorta Transplantation**

Donor Sprague Dawley rats were anesthetized with pentobarbital sodium (30 mg/kg body weight). The thoracic aorta was severed between the diaphragm and the subclavian artery. The arterial stumps of the intercostal artery were electrocoagulated to stop bleeding. The lumens of the blood vessels was washed with heparinized normal saline. The thoracic aortic graft was cut into a 1-cm section. The recipient Wistar rats were anesthetized, a midline incision was made, and the peritoneum was opened. The operation was performed under a dissecting stereomicroscope (Olympus SZH 10, Japan). Microhemostat clamps were placed below the renal arteries and above the aortic bifurcation, and a segment of this section was dissected. The thoracic aortic grafts were end-to-end anastomosed using a 9-0 suture. After transplantation, all recipients received gentamicin to prevent acute infection. Four weeks after transplantation, the rats were anesthetized, and the grafts were harvested. The grafts were frozen in liquid N₂ and histological sectioning (7 μm) began at the middle of the grafts to avoid effects of the sutures.

**Morphometric Analyses**

The frozen sections were stained with hematoxylin and eosin for histological evaluation. The intima was defined as the region between the lumen and the internal elastic lamina. The media was defined as the region between the internal and external elastic lamina (EEL). Using a BX60 microscope equipped with a Sony 3 CCD camera and television monitor and interfaced to SPOT analysis software, images were first scanned, saved, and then overlaid with different lines to trace the lumen, the internal elastic lamina, and the EEL. The neointimal area was determined by subtracting the area enclosed by the internal elastic lamina from the area of the EEL. Eight cross-sections were obtained by selecting the first of every 10 sections from each graft.

**Results**

NSun2 Promotes the Adhesion of THP-1 Cells to HUVECs by Upregulating ICAM-1

The present study was prompted by our finding that the protein levels of ICAM-1 were greatly decreased in HUVECs with NSun2 silenced. As shown in Figure 1A and Online Figure I, transfection of HUVECs by 3 different small interfering RNAs targeting NSun2 markedly reduced the protein level of ICAM-1 (by >65%; P<0.001), but not those of vascular adhesion molecule-1 (VCAM-1), P-selectin, and E-selectin. It is widely accepted that proinflammatory factors (eg, TNF-α, lipopolysaccharide, homocysteine) induce the expression of ICAM-1. We therefore tested whether NSun2 mediated the effect of TNF-α or homocysteine in inducing the expression of ICAM-1. As shown in Figure 1B, the protein level of ICAM-1, but not that of NSun2, increased dramatically when HUVECs were stimulated with TNF-α or homocysteine. In cells with NSun2 silencing, the effect of TNF-α or homocysteine on the protein expression of ICAM-1 was remarkably diminished (Figure 1B). Therefore, TNF-α or homocysteine induces ICAM-1 expression, at least in part, in an NSun2-dependent manner. To further investigate the mechanisms underlying NSun2-regulated expression of ICAM-1, the levels of ICAM-1 mRNA and pre-mRNA in the cells represented in Figure 1B were analyzed by reverse transcription followed by real-time qPCR. As shown in Figure 1C, the pre-mRNA and mRNA levels of ICAM-1 in cells with NSun2 silencing and in control cells were comparable, indicating that the regulation of ICAM-1 by NSun2 did not involve regulation at the levels of transcription. Also, NSun2 might not regulate the mRNA turnover of ICAM-1 because knockdown of NSun2 did not alter the levels and the half-life of ICAM-1 mRNA (Figure 1C; Online Figure IIA). Instead, NSun2 might regulate the translation of ICAM-1 because knockdown decreased the de novo synthesized ICAM-1 protein and the presence of ICAM-1 mRNA in the polysomes (Online Figure IIB and IIC). However, TNF-α or homocysteine might induce the expression of ICAM-1 at the levels of transcription or mRNA turnover because the pre-mRNA and mRNA levels of ICAM-1 shown in cells exposed to TNF-α or homocysteine were significantly higher than those shown in the untreated cells (Figure 1C).

To test the impact of the NSun2–ICAM-1 regulatory process on the adhesion of leukocytes to endothelial cells, the HUVECs represented in Figure 1B were incubated with BCECF-labeled THP-1 cells, whereas the cells adhering to the HUVEC monolayers were assessed. As shown in Figure 1D, there were much fewer adherent THP-1 cells observed on HUVECs with NSun2 knockdown than on the control cells.
NSun2 regulates ICAM-1

Stimulation with TNF-α or homocysteine greatly increased the adhesion of THP-1 cells, and this effect was less pronounced in cells silenced with NSun2 (P<0.001). Furthermore, incubation of HUVECs with an anti–ICAM-1 antibody greatly diminished the effect of TNF-α or homocysteine in inducing the adhesion of THP-1 cells to the HUVECs (Online Figure III). Together, by upregulating ICAM-1, NSun2 is able to promote the adhesion of leukocytes to HUVECs.

NSun2 Is a Positive Regulator of Vascular Endothelial Inflammation

To further analyze the role of NSun2 in vivo, we generated an NSun2-knockout rat model by using the TALEN method (Online Figure IV A, Schematic). Western blot analysis showed that NSun2 was ubiquitously expressed in rat tissues (Online Figure IVB). The deletion of NSun2 was confirmed by sequence analysis. As shown, deletion of rat NSun2 resulted in a loss of 14 nucleotides (positions 7966–7979) in exon 4 of NSun2 gene (Online Figure IVC), which resulted in a frame shift from full-length NSun2. A substantial reduction in NSun2 and loss of NSun2 expression were observed in various tissues of heterozygous rats (NSun2+/−) and homozygous rats (NSun2−/−), respectively (Online Figure IVD). Consistent with the observations from NSun2-deficient mice, NSun2−/− rats, but not NSun2+/− rats, were nearly sterile (data not shown) and exhibited weight loss and small body size (Online Figure IV E and IVF). In addition, deletion of NSun2 did not influence the morphology and ratio of the blood cells (Online Figure V; Online Table III and IV).

To address the role of NSun2 on vascular inflammation, NSun2−/− rats and their wild-type littermates (WT) were intravenously injected with homocysteine (50 mg/kg) or with saline for 1 hour. Injection of homocysteine greatly increased the concentration of homocysteine in the plasma (Online Table...
and no difference was observed between the WT rats and the NSun2−/− rats (Online Table V). The adherent leukocytes in the endothelium of mesenteric venules were analyzed. As shown in Figure 2A and the Online Videos I–VIII, stimulation with hyperhomocysteinemia increased the adhesion of leukocytes to the endothelium of WT rats, with the greatest increase occurring 60 minutes after treatment (P<0.05); this effect was markedly diminished in NSun2−/− rats (P<0.05). To further test the effect of NSun2 deletion in vascular inflammation, thoracic aortas removed from the homozygous (−/−) rats, heterozygous (+/−) rats, and their WT littermates were stimulated with TNF-α (1 ng/mL), homocysteine (100 μmol/L), or saline (PBS). At 24 hours later, the thoracic aortas were cocultured with BCECF-labeled rat spleen leukocytes for additional 30 minutes. The adherent leukocytes on the vascular endothelium were visualized by fluorescence microscopy. As shown in Figure 2B, stimulation of WT rats with TNF-α or homocysteine induced significantly the adherent leukocytes. This effect was markedly attenuated in NSun2+/− rats (TNF-α, P<0.001; homocysteine, P<0.001) and further attenuated in NSun2−/− rats (TNF-α, P<0.001; homocysteine, P<0.001). These results extend the findings in cultured HUVECs (Figure 1D) to an in vivo setting.

**NSun2 Is Required for the Upregulation of ICAM-1 in Vascular Endothelial Inflammation**

To test whether NSun2 regulates ICAM-1 in vivo, NSun2−/− rats and their WT littermates were intravenously injected with homocysteine (50 mg/kg) or with saline. One hour later, RNA and protein lysates prepared from the thoracic aortas were subjected to Western blot and real-time qPCR analyses, respectively. As shown in Figure 3A, NSun2 protein was undetectable in NSun2−/− rats. In WT rats, hyperhomocysteinemia increased the protein levels of ICAM-1 (by ≈3.7-fold; P<0.01) but did not change that of NSun2. Deletion of NSun2 diminished the effect of hyperhomocysteinemia in inducing the protein levels of ICAM-1 (≈3.7-fold versus 1.9-fold, P<0.01). However, the constitutive levels of ICAM-1 protein in WT rats and NSun2−/− rats were comparable. As shown in Figure 3B, deletion of NSun2 didn’t influence the protein levels of ICAM-1.

![Figure 2](http://circres.ahajournals.org/)

**Figure 2. Deletion of NOP2/Sun domain family, member 2 (NSun2) attenuates vascular inflammation.** A, NSun2-deficient (NSun2−/−) rats and their wild-type (WT) littermates were intravenously injected with homocysteine (Hcy, 50 mg/kg) or with saline. At the times indicated, the adherent leukocytes in the endothelium of mesenteric venules were analyzed. The data are shown as the mean±SEM (n=5 or 6); significance was analyzed by using 1-way ANOVA followed by the Student–Newman–Keuls test (*P<0.05 vs WT control; #P<0.05 vs WT hyperhomocysteinemia [HHcy]; Bars: 20 μm). Series videos of the above results are included in the Online Data Supplement. B, Thoracic aortas isolated from the NSun2−/− and NSun2+/− rats and from their WT littermates were treated with tumor necrosis factor-α (TNF-α, 1 ng/mL), Hcy (100 μmol/L), or PBS for 24 h and then cocultured with bis-carboxyethyl-carboxyfluorescein (BCECF)-labeled rat spleen leukocytes for additional 30 minutes. The adherent leukocytes were visualized by fluorescence microscopy. The number of the adherent leukocytes in 9 random fields was counted. The data are shown as the mean±SEM (n=3); significance was analyzed by using 1-way ANOVA followed by the Student–Newman–Keuls test (**P<0.001; Bars: 100 μm).
levels of VCAM-1. Consistent with the results shown in Figure 3A, NSun2 mRNA was also undetectable in NSun2−/− rats (Figure 3C, left). The constitutive levels of ICAM-1 and VCAM-1 mRNAs in NSun2−/− rats and WT rats were comparable (Figure 3C, middle and right). Stimulation of WT rats with hyperhomocysteinemia induced the mRNA levels of ICAM-1 and VCAM-1. The data shown as the means±SEM (n=3); significance was determined by using 1-way ANOVA followed by the Student–Newman–Keuls test (**P<0.01). B, Protein lysates from tissues described in (A) were subjected to Western blot analysis to assess the protein levels of NSun2, vascular adhesion molecule-1 (VCAM-1), and GAPDH. The data are shown as the means±SEM (n=3); significance was determined by using 1-way ANOVA followed by the Student–Newman–Keuls test (*P<0.05). C, RNA isolated from tissues described in (A) was subjected to real-time quantitative polymerase chain reaction to assess the mRNA levels of NSun2, ICAM-1, and VCAM-1. The data are shown as the means±SEM (n=3); significance was determined by using 1-way ANOVA followed by the Student–Newman–Keuls test (*P<0.05; **P<0.01; ***P<0.001). HHcy indicates hyperhomocysteinemia; and ns, no significance.

Figure 3. NOP2/Sun domain family, member 2 (NSun2) mediates the homocysteine (Hcy)-induced expression of intercellular adhesion molecule-1 (ICAM-1) in the thoracic aortas. A, NSun2−/− rats and their wild-type (WT) littermates were intravenously injected with Hcy (50 mg/kg) or with saline. One hour later, lysates prepared from the thoracic aortas were subjected to Western blot analysis to assess the protein levels of NSun2, ICAM-1, and GAPDH (left). The data are shown as the means±SEM (n=3); significance was determined by using 1-way ANOVA followed by the Student–Newman–Keuls test (right; **P<0.01). B, Protein lysates from tissues described in (A) were subjected to Western blot analysis to assess the protein levels of NSun2, vascular adhesion molecule-1 (VCAM-1), and GAPDH. The data are shown as the means±SEM (n=3); significance was determined by using 1-way ANOVA followed by the Student–Newman–Keuls test (right; *P<0.05). C, RNA isolated from tissues described in (A) was subjected to real-time quantitative polymerase chain reaction to assess the mRNA levels of NSun2, ICAM-1, and VCAM-1. The data are shown as the means±SEM (n=3); significance was determined by using 1-way ANOVA followed by the Student–Newman–Keuls test (*P<0.05; **P<0.01; ***P<0.001). HHcy indicates hyperhomocysteinemia; and ns, no significance.

NSun2–ICAM-1 Axis Affects the Process of Allograft Arteriosclerosis

In previous studies, it has been described that ICAM-1 is critical for the development of allograft arteriosclerosis.41,42 We therefore tested whether the NSun2-ICAM-1 axis impacts on the process of arteriosclerosis. To this end, the thoracic aortic allografts of WT or NSun2−/− rats (Sprague Dawley rats) were end-to-end transplanted into the abdominal aortas of Wistar rats. Four weeks after the surgery, the formation of allograft arteriosclerosis was assessed. As shown, the allografts from NSun2−/− donors developed much smaller neointima than those from WT donors (Figure 5A, left). The neointimal area, media area, the ratio of neointima/media area, and EEL length in the allografts of the NSun2−/− donors were mitigated compared with that observed for the WT donors (P<0.01 or P<0.05; Figure 5A, right). Given that the body size of NSun2−/− rats was smaller than that of their WT littermates (Online Figure IVE), it was not surprising that a lack of the local NSun2 lowered the media area and EEL length (Figure 5A, right). Consistent with
the observations shown in Figure 5A, the intensity of ICAM-1 protein and the number of CD45+ leukocytes in the allografts of the NSun2−/− donors were reduced compared with those of the WT donors (Figure 5B and 5C). These results indicate that the local expression of NSun2 is necessary for the modification of neointima formation.

NSun2 Methylates ICAM-1 mRNA In Vitro, in Cells, and In Vivo

To test whether NSun2 methylates ICAM-1 mRNA, in vitro transcribed mRNA fragments of ICAM-1 (Figure 6A, Schematic) were subjected to in vitro methylation assays by using 3H-labeled S-adenosyl-L-methionine and purified His-NSun2. As shown in Figure 6B (left), 3H incorporation into tRNA as well as the 5ʹUTR, CDS, and 3ʹUTR fragments of ICAM-1 was significantly higher than what was seen with a negative control substrate (cDNA). Further study showed that NSun2 methylated fragments 5ʹUTR-A, CDS-A, CDS-B, CDS-C, 3ʹUTR-A, 3ʹUTR-B, and 3ʹUTR-C but not fragments 5ʹUTR-B and 3ʹUTR-Ab, indicating that NSun2 methylated ICAM-1 mRNA at multiple sites (Figure 6B, middle). The methylation of ICAM-1 mRNA by NSun2 was specific because NSun2 was unable to methylate the 3ʹUTR fragment of VCAM-1 (Figure 6B, right). Knockdown of NSun2 diminished the effectiveness of TNF-α or homocysteine in elevating the levels of methylated ICAM-1 mRNA (1.81±0.139 versus 0.271±0.019 for TNF-α, P<0.001; 1.55±0.240 versus 0.361±0.071 for homocysteine, P<0.001; Figure 6D, right).

Because the NSun2−/− rats showed reduced expression of ICAM-1 (Figure 3A), we tested whether NSun2 methylates ICAM-1 mRNA in cells. RNA isolated from the HUVECs described in Figure 1C was immunoprecipitated using an anti-m5C antibody. The presence of ICAM-1 mRNA in the IP materials was analyzed using real-time qPCR. As shown in Figure 6D (left), the m5C antibody effectively immunoprecipitated ICAM-1 mRNA. As a negative control, IP using IgG failed to immunoprecipitate ICAM-1 mRNA. The level of methylated ICAM-1 mRNA was decreased in cells silenced with NSun2 (1.01±0.043 versus 0.158±0.034; P<0.001), but it increased in cells stimulated with TNF-α (1.01±0.043 versus 1.81±0.139; P<0.001) or homocysteine (1.01±0.043 versus 1.55±0.240; P<0.001; Figure 6D, right). Knockdown of NSun2 diminished the effectiveness of TNF-α or homocysteine in elevating the levels of methylated ICAM-1 mRNA (1.81±0.139 versus 0.271±0.019 for TNF-α, P<0.001; 1.55±0.240 versus 0.361±0.071 for homocysteine, P<0.001; Figure 6D, right).

Because the NSun2−/− rats showed reduced expression of ICAM-1 (Figure 3A), we tested whether the methylation of ICAM-1 mRNA by NSun2 occurs also in rat. As shown in Figure 6E, the rat ICAM-1 fragments 5ʹUTR+CDS and 3ʹUTR were methylated by NSun2 in vitro. Furthermore, the RNA described in Figure 3C was used for the measurement of the in vivo methylated ICAM-1. As shown in Figure 6F, the level of methylated ICAM-1 mRNA observed in NSun2−/− rats...
was lower than that observed in WT rats (0.992±0.072 versus 0.200±0.061; *P<0.05). Stimulation of the WT rats with hyperhomocysteinemia significantly induced the levels of methylated ICAM-1 mRNA (0.992±0.072 versus 2.387±0.471; **P<0.001); this effect was significantly diminished in similarly stimulated NSun2−/− rats (2.387±0.471 versus 0.279±0.102; ***P<0.001). Therefore, NSun2 is able to methylate human and rat ICAM-1 mRNA in vitro, in cells, and in vivo.

**Methylation of ICAM-1 by NSun2 Mediates NSun2-Dependent Expression From Heterologous Reporters at Translational Level**

Next, we analyzed the activity of pGL3-derived reporters bearing the ICAM-1 mRNA fragments (Figure 7A, Schematic). As shown in Figure 7B, knockdown of NSun2 reduced the reporter activity of pGL3-5′UTR (by 41%; *P<0.001) and of pGL3-3′UTR (by 42%; *P<0.001), but it did not change those of pGL3-CDS, pGL3-5′UTR-B, and pGL3-3′UTR-Ab. Likewise, the protein levels of firefly luciferase expressed by pGL3-5′UTR and pGL3-3′UTR, but not those expressed by pGL3-CDS, pGL3-5′UTR-B, or pGL3-3′UTR-Ab, were reduced in cells with NSun2 silencing (Figure 7C). However, knockdown of NSun2 did not alter the levels of pGL3-5′UTR and pGL3-3′UTR chimeric transcripts (Figure 7D), suggesting that methylation by NSun2 may influence the expression of ICAM-1 at the level of translation. To further confirm this view, luciferase (Luc), luc-5′UTR, luc-CDS, or luc-3′UTR reporter transcript (Figure 7E, left, Schematic) was in vitro transcribed from pGL3, pGL3-5′UTR, pGL3-CDS, or pGL3-3′UTR reporter. These transcripts then were in vitro methylated by NSun2 and subjected to in vitro translation assays. As shown in Figure 7E, the expression of Luc in NSun2−/− was lower than that observed in WT rats (0.992±0.072 versus 0.200±0.061; *P<0.05). Stimulation of the WT rats with hyperhomocysteinemia significantly induced the levels of methylated ICAM-1 mRNA (0.992±0.072 versus 2.387±0.471; **P<0.001); this effect was significantly diminished in similarly stimulated NSun2−/− rats (2.387±0.471 versus 0.279±0.102; ***P<0.001). Therefore, NSun2 is able to methylate human and rat ICAM-1 mRNA in vitro, in cells, and in vivo.

TNF-α and Homocysteine Activate NSun2 via Repression of Aurora-B

The protein kinase Aurora-B, which is essential for the segregation of eukaryotic chromosomes, represses the
methyltransferase activity of NSun2 by phosphorylating NSun2 at Ser139. On the basis of the finding that TNF-α or homocysteine elevated the levels of methylated ICAM-1 mRNA without influencing the protein levels of NSun2 (Figure 1B; Figure 6D), we investigated whether TNF-α or homocysteine could activate NSun2 via repression of Aurora-B. As shown, stimulation with TNF-α (1 ng/mL) or homocysteine (100 μmol/L) reduced the mRNA levels (Figure 8A) and protein levels (Figure 8B) of Aurora-B, suggesting that TNF-α or homocysteine might repress the expression of Aurora-B at the levels of transcription or mRNA turnover. As a result, treatment of HUVEC cells with TNF-α or homocysteine reduced the Serine10 phosphorylation of histone H3, a typical substrate of Aurora-B (Figure 8C). To test the effect of Aurora-B–mediated NSun2 phosphorylation in methylating ICAM-1 mRNA, purified NSun2 was in vitro phosphorylated by immunocipitated Aurora-B or kept untreated. The phosphorylated or unphosphorylated NSun2 was used for in vitro methylation assays. As shown in Figure 8D, Aurora-B increased the Serine phosphorylation level of NSun2 (left), and 3H incorporation to the 3ʹUTR of ICAM-1 mRNA by Aurora-B–treated NSun2 was decreased (right). Therefore, phosphorylation by Aurora-B represses the activity of NSun2 in methylating ICAM-1 mRNA. Importantly, TNF-α or
homocysteine reduced the Serine phosphorylation levels of NSun2 (Figure 8E). Inhibition of Aurora-B by hesperadin (250 nmol/L) reduced the Serine phosphorylation of NSun2, thereby increasing the levels of methylated ICAM-1 mRNA (Figure 8F and 8G) and ICAM-1 protein (Figure 8H, left and right, lanes 1 and 3). Furthermore, inhibition of Aurora-B by hesperadin enhanced the effect of TNF-α or homocysteine in increasing the protein levels of ICAM-1 (Figure 8H, left and right, lanes 2 and 4). Moreover, mutation of Ser139 of NSun2 (S139A) enhances the ability of NSun2 in methylating reporter transcripts of pGL3-5′ UTR and pGL3-3′ UTR and in elevating the luciferase activities of these reporters (Online Figure VII). These results indicate that TNF-α and homocysteine may activate NSun2 via repression of Aurora-B.

Discussion

NSun2 has been implicated in the regulation of cell proliferation, stem cell differentiation, testis differentiation, and human cancers. The present study suggests that NSun2 is also a positive determinant for vascular endothelial inflammation and arteriosclerosis (Figures 1–5). By methylating ICAM-1 mRNA, NSun2 elevates the expression of ICAM-1 at the translational level (Figures 1, 6, and 7; Online Figure II). This regulation is specific because knockdown or deletion of NSun2 has no effect on the expression of VCAM-1, P-selectin, and E-selectin (Figures 1A and 3B; Online Figure I). The NSun2–ICAM-1 regulatory process can partly mediate the effect of TNF-α or homocysteine on inducing the endothelial inflammatory response because knockdown or knockout of NSun2 partly diminishes the effect of TNF-α or homocysteine in inducing the expression of ICAM-1 and thereby reduces the adhesion of leukocytes to the endothelial cells (Figure 1B and 1D; Figures 2–5). Unlike methylation at the 5′ UTR and the 3′ UTR, methylation at the CDS of ICAM-1 mRNA does not significantly influence the expression of ICAM-1 (Figure 7B, 7C, and 7E). Therefore, whether mRNA methylation is functional in gene regulation may depend on the location of the methylation or on other unknown factors. The intensity of endothelial ICAM-1 shown in NSun2−/− rats is lower than that shown in WT rats (Figure 4). However,
Western blot analysis showed that the constitutive levels of ICAM-1 protein in the thoracic aorta tissue of NSun2−/− rats are comparable to those of WT rats (Figure 3A). This inconformity is probably because ICAM-1 is expressed predominantly in the endothelial cells, which account only for a small proportion of the thoracic aorta tissue.

In previous studies, we have shown that the induction of NSun2 is responsible for oxidative stress–induced expression of p16, p53, Bak1, and ErbB2.30,31 The fluctuation of NSun2 in the cell cycle and in stem cell differentiation is also linked to the function of NSun2 in these processes.39,44 Regulation at the transcriptional level is important for the expression of NSun2 during these processes.36,39 In addition, posttranslational modification influences the methyltransferase activity of NSun2. For instance, Aurora-B has been shown to repress the methyltransferase activity of NSun2 by phosphorylating NSun2 at Ser139.43 This is further confirmed by our findings that mutation of Ser139 enhances the ability of NSun2 in methylating ICAM-1 and in regulating ICAM-1 expression (Online Figure VII). Notably, stimulation with TNF-α or homocysteine does not alter the protein levels of NSun2 (Figures 1B, 3A, and 8E). Instead, TNF-α or homocysteine reduces the levels of Aurora-B, thereby activating NSun2 by decreasing the serine phosphorylation of NSun2 (Figures 8B and 8E). Given that NSun2 may be modified posttranslationally (NCBI web site: http://www.phosphosite.org/proteinAction.do?id=2901&showAllSites=true), it is possible that signaling pathways other than that of Aurora-B may also contribute to the regulation of NSun2 during vascular endothelial inflammation.

Conclusions

In addition to arteriosclerosis, vascular endothelial inflammation is a critical event in other human diseases, including hypertension, restenosis, septic shock, autoimmune diseases, and ischemia/reperfusion damage.46,47 It is plausible that the NSun2-ICAM-1 regulatory axis may also modulate these diseases. Apart from ICAM-1 mRNA, NSun2 has been shown
to catalyze the methylation of other mRNAs (eg, p53 mRNA, p16 mRNA) and noncoding RNAs. Therefore, whether NSun2 methylates mRNAs other than ICAM-1 mRNA or noncoding RNAs involved in the process of vascular inflammation or atherosclerosis should be carefully studied.

Acknowledgments

We are grateful to Zhihong Wang, Lina Wang, and Prof Deling Kong (Nankai University, Tianjin, China) for their help in performing the aorta transplantation experiments.

Sources of Funding

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Disclosures

None.

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Novelty and Significance

What Is Known?

• The intercellular adhesion molecule-1 (ICAM-1), which mediates leukocytes adhesion to endothelium, is a key mediator of vascular inflammation and initiator of early stage atherosclerosis.

• NOP2/Sun domain family, member 2 (NSun2) is a tRNA methyltransferase.

What New Information Does This Article Contribute?

• By methylating ICAM-1 mRNA, NSun2 promotes ICAM-1 translation and thereby leukocyte adhesion to vascular endothelium.

• NSun2−/− rats are resistant to allograft arteriosclerosis.

Apart from transcriptional regulation, the contribution of posttranscriptional gene regulatory events to the expression of ICAM-1 is an emerging area of research. In the present study, we report that the translation of ICAM-1 is enhanced by NSun2-mediated mRNA methylation. The NSun2–ICAM-1 axis impacts on tumor necrosis factor-α and homocysteine-induced leukocyte adhesion to endothelium as well as to the process of allograft arteriosclerosis. These findings may provide a promising intervention target for the prevention and treatment of inflammatory vascular diseases.
NSun2 Deficiency Protects Endothelium From Inflammation via mRNA Methylation of ICAM-1
Yuhong Luo, Juan Feng, Qingbo Xu, Wengong Wang and Xian Wang

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SUPPLEMENTAL MATERIAL

Detailed Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from segments of human umbilical cord vein by collagenase digestion and cultured in medium 199 (Gibco, USA) supplemented with 10% FBS as previously described. For treatment of HUVECs with TNFα or Hcy, unless otherwise indicated, cells at approximately 80% confluence were exposed to 1 ng/ml TNFα (R&D Systems, Minneapolis, MN) or 100 µmol/L DL-homocysteine (Hcy) (Sigma, St. Louis, MO) or 10 µg/ml ICAM-1 blocking antibody (R&D Systems, Minneapolis, MN) for 24 h before collecting protein or running adhesion experiments, and for 12 h for collection of RNA. THP-1 cells were grown in RPMI 1640 medium (Gibco, USA) containing 10% FBS. HeLa cells were cultured in Dulbecco’s modified Eagle’s medium (Gibco, USA) supplemented with 10% FBS.

Antibodies and Western blot analysis

Whole-cell and rat tissue lysates were prepared, and extractions containing equal amounts of protein were resolved by 10% SDS-PAGE for Western blot analysis. The blots were incubated with primary antibodies against NSun2 (Santa Cruz, CA), ICAM-1 (Santa Cruz, CA), VCAM-1 (Santa Cruz, CA), E-selectin (Abcam, Cambridge, MA), P-selectin (Santa Cruz, CA), Aurora-B (Abcam, Cambridge, MA), GAPDH (Cell Signal Technology, Danvers, MA), eIF-5 (Santa Cruz, CA), p-Ser10H3 (Cell Signal Technology, Danvers, MA), phospho-Serine (Abcam, Cambridge, MA), firefly luciferase (Promega, Madison, WI), Renilla luciferase (MBL, Japan), and IRDye 800DX- or 700DX-conjugated secondary antibody (Rockland, Gilbertsville, PA). The immunofluorescence signal was detected by Odyssey infrared imaging (LI-COR Biosciences, Lincoln, NE).

Plasmid construction and transfection

For constructing pGL3-promoter reporter plasmids, the ICAM-1 fragments were amplified by PCR and inserted into the pGL3-promoter vector (Clontech, USA). To amplify the 5’UTR, CDS, 3’UTR, 5’UTR-B and 3’UTR-Ab of ICAM-1 mRNA, we used the following primer pairs: CCCAAGCTTTTAGCCTCGGCGAGGAAACG and CATGCCATGGCTGTGGAGGAAGCATAGCGA for 5’UTR, GCTCTAGAGCAACCTACGCTCGTAT and GCTCTAGATTCAAGGAGGCTTGCCTTGT for CDS, GCTCTAGAAACCCGACGCTTCCCTGAA and GCTCTAGATGGCAGTTGAGAAAGCTTTA TTA for 3’UTR, CCAAGCTTTAGGAGGAAAGGCCGAGGTGT and CATGCCATGGCTGTGGAGGAAGCATAGCGA for 5’UTR-B, GCTCTAGAAACCTACGCTTACCTAAACACTA and GCTCTAGACCCAGCAGACGTTTCAG for 3’UTR-Ab.

All plasmid transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Knockdown of NSun2

To silence NSun2, cells were transfected with siRNA① (10 nmol/L) targeting NSun2 (GAGATCCTCTTCTATGATC), or with siRNA ② (10 nmol/L) targeting NSun2 (TGGAATATTAGTCGTCA), or with siRNA ③ (10 nmol/L) targeting NSun2 (CGAATGATGTGAGAACAACAA) or with a control siRNA (UUGUUGAAGCGUG UCACGUTT) using RNAiMAX (Invitrogen, Carlsbad, CA). Unless otherwise indicated,
cells were collected for analysis 48 h after transfection with siRNA or control siRNA. All knockdown interventions caused less than 1% cell death (by FACS analysis, data not shown).

**Analysis of nascent protein**

One million cells were incubated with 1 mCi (1 Ci=37 GBq) L-[35S]methionine and L-[35S]cysteine (Easy Tag EXPRESS, NEN/PerkinElmer) per 60-mm plate for 20 min, whereupon cells were lysed using TSD lysis buffer (50 mmol/L Tris, pH 7.5, 1% SDS, 5 mmol/L DTT), and lysates were immunoprecipitated using either anti-ICAM-1 antibody (Santa Cruz, CA) or Immunoglobulin G (IgG) for 1 h at 4°C. After extensive washes in TNN buffer (50 mmol/L Tris, pH 7.5, 250 mmol/L NaCl, 5 mmol/L EDTA and 0.5% NP-40), the immunoprecipitated material was resolved by 10% SDS-PAGE, transferred onto PVDF membranes and visualized using a PhosphorImager (Molecular Dynamics).

**Preparation of polysomal fractions**

In total, 20 million cells were incubated for 15 min with 100 mg/ml cycloheximide, and total lysates (500 μl) were layered onto a cushion of 30% sucrose in ice-cold buffer containing 20 mmol/L HEPES (pH 7.4), 50 mmol/L potassium acetate, 5 mmol/L magnesium acetate, 1 mmol/L dithiothreitol, 1 U/μl of RNasin, 1 μg/ml of leupeptin, 1 μg/ml of aprotinin, and 0.5 mmol/L phenylmethylsulfonyl fluoride. After centrifugation (Beckman SW40; 100,000 × g for 2 h, 4°C), RNA from the pellet (polysomal fraction) was prepared and used for RT-qPCR analysis.

**Luciferase reporter assays**

HeLa cells were transfected with an siNSun2 or a control siRNA. At 48 h later, cells were further transfected with 0.1 μg pGL3-derived reporter plasmid, together with 0.01 μg a vector expressing *Renilla* luciferase and cultured for additional 24 h. The ratio of firefly luciferase activity to *Renilla* luciferase activity was measured using a luciferase assay system (Promega, Madison, WI).

**Plasma Hcy level**

For analysis of the plasma Hcy levels, rats were treated with Hcy for 60 minutes. The anti-coagulated (50 U/ml heparin) blood samples (50 μl) from the rat femoral vein were sent to Peking University Third Hospital Clinic Laboratory for the determination of total Hcy concentration.

**Measurement of mRNA half-life**

To measure the half-life of endogenous ICAM-1 mRNA, actinomycin D (2 μg/ml, Sigma, St. Louis, MO) was added into the cell culture medium and total RNA was prepared at the times indicated and subjected to RT-qPCR analysis using ICAM-1-specific primers.

**In Vitro Phosphorylation**

Purified NSun2 proteins were used as substrates for *in vitro* phosphorylation by anti-Aurora-B antibody-immunoprecipitated Aurora-B (from HUVEC cells). The reaction was performed for 30 min at 25°C in 100 μl of a reaction mixture containing anti-Aurora-B antibody-immunoprecipitated Aurora-B, 0.05 nmol NSun2, 25 mmol/L Tris (pH 7.5), 2 mmol/L MgCl2, 200 μmol/L ATP and 0.1 μmol/L calyculin A, as previously reported. The reaction mixtures were processed for SDS-PAGE and were analyzed for the level of phosphorylated NSun2.

**Routine blood tests and blood smear tests**

For routine blood tests, 20 μl anti-coagulated blood samples from rat caudal vein were sent to Peking University Laboratory Animal Center for the determination of basic hematological characterizations including WBC, Platelet, RBC counts and so on.
For blood smear tests, a drop (about 50 µl) of blood samples from rat caudal vein were used for the preparation of blood smears. After Swiss dyeing, the morphology of WBC and differential WBC count were detected under a BX60 microscope (Olympus Optical Co, Ltd, Japan) equipped with a Sony 3CCD camera and television monitor.

Statistical analysis

All data are shown as the mean ± SEM unless otherwise stated. The data were analyzed using GraphPad Prism software. Statistical analysis was performed with one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons and with Student’s unpaired t-test for comparisons between two groups. A value of $p<0.05$ was considered statistically significant.
Supplemental Figures and Figure Legends

Online Figure I. Knockdown of NSun2 decreases the expression of ICAM-1. **Upper**, HUVECs were transfected with a siRNA targeting NSun2 (siN①, siN②, or siN③) or with a control siRNA. After 48 h, cell lysates were prepared and subjected to Western blot analysis to assess the protein levels of NSun2, ICAM-1, VCAM-1, P-selectin, E-selectin, and GAPDH. **Bottom**, The density of the Western blot analysis of NSun2 expression in HUVECs silenced with siN①, siN②, or siN③ was presented as the mean ± SEM from 3 independent experiments; significance was analyzed by using Student’s t-test (***, p<0.001).
Online Figure II. Knockdown of NSun2 decreases the nascent protein levels of ICAM-1 and the presence of ICAM-1 mRNA in the polysome. A, HUVECs were transfected with a siRNA targeting NSun2. 48 h later, Actinomycin D (2 μg/ml) was added into the cell culture medium and total RNA was prepared at 0 h, 2 h, 4 h, 6 h, 9 h, 12 h, and subjected to RT-qPCR analysis using ICAM-1-specific primers. B, HUVECs were transfected with a siRNA targeting NSun2. 48 h later, cells were incubated with 35S-labeled methionine and cysteine for 20 minutes. The nascent protein levels of ICAM-1 were analyzed, as described in “Detailed Methods”. C, HUVECs were transfected with a siRNA targeting NSun2. 48 h later, polysomes were isolated as described in “Detailed Methods” and subjected to real-time qPCR analysis to assess the levels of ICAM-1 mRNA in the polysomes. The data are shown as the mean ± SEM from 3 independent experiments; significance was analyzed by using Student’s t-test (***, p<0.001).
Online Figure III. Blocking ICAM-1 reduces endothelial adhesivity. At 48h after transfection with NSun2 siRNA or control siRNA, HUVEC cells were treated with TNFα (1 ng/ml), Hcy (100 µmol/L), or PBS, along with or without ICAM-1 blocking antibody (10 µg/ml) for 24 h, and then cocultured with BCECF-labeled THP-1 cells for additional 30 minutes. The adherent THP-1 cells were visualized by fluorescence microscopy. The number of the adherent THP-1 cells in 9 random fields was counted. The data are shown as the mean ± SEM (n=3); significance was analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test (***, p<0.001; Bars: 100 µm).
Online Figure IV. Validation of NSun2 -/- rats. A, Schematic representation depicting the rat NSun2 gene. B, The protein levels of NSun2 in various tissues of rat were determined by Western blot analysis. The signal density of NSun2 was normalized to that of GAPDH. C, DNA sequence analysis was performed to confirm the deletion of NSun2 in the NSun2 -/- rats. The deleted nucleotides are indicated. D, Protein lysates were prepared from the tissues of wild type, NSun2 +/-, and NSun2 -/- rats and subjected to Western blot analysis to assess the protein levels of NSun2 and GAPDH. E and F, The body size (E) and weight (F) of wild type, NSun2 +/-, and NSun2 -/- rats were shown. The body weights are shown as the mean ± SEM (n=8) and significance was analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test (***, p<0.001).
Online Figure V. The morphology of WBC of NSun2 knockout rats. The morphology of WBC of NSun2 null rats and control rats was determined as described in “Detailed Methods”.
Online Figure VI. NSun2 methylates the N$^5$-cytosine of ICAM-1 mRNA. Full-length 5'UTR, CDS, and 3'UTR fragments of ICAM-1 (1 µg) were incubated with non-isotopic SAM in the presence (+NSun2) or in the absence of His-NSun2. Fragments then were digested with P1 nuclease and dephosphorylated by calf intestinal alkaline phosphatase. The presence of m$^5$C was analyzed by HPLC-MS analysis. N$^5$-methylated cytidine (m$^5$C, standard, 25 ng) was served as a positive control. The respond value for each sample was indicated.
Online Figure VII. Phosphorylation of NSun2 by Aurora-B represses the effect of NSun2 in methylating ICAM-1 mRNA and in regulating the expression of ICAM-1. A, HeLa cells were transfected with a vector expressing NSun2 (pNSun2) or with a vector expressing a variant of NSun2 with mutated Ser139 (pNSun2-S139A), at 48 h later, the cells were further transfected with a pGL3-derived vector bearing the 5′UTR (pGL3-5′UTR) or 3′UTR (pGL3-3′UTR) of ICAM-1 together with a pRL-CMV control reporter and cultured for another 24 h. Firefly luciferase activity against Renilla luciferase activity was analyzed. The data are shown as the mean ± SEM from 3 independent experiments; significance was determined by using Student’s t-test (*, p<0.05; **, p<0.01). B, RNA prepared from cells described in A was subjected to IP assays by using an antibody recognizing mC. RNA isolated from IP materials was subjected to real-time qPCR to assess the levels of methylated reporter transcripts (Luci-5′UTR and Luci-3′UTR). Data represent mean ± SEM from 3 independent experiments; significance was analyzed by Student’s t-test (**, p<0.01).
Supplemental Tables and supporting information

### Primers for real-time qPCR

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primer sequence</th>
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</thead>
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<td>Human NSun2</td>
<td>TQAGTAGATCAATAAAGCCGTAG and ACACAAAATAAGGACAGGAAGCAAT</td>
</tr>
<tr>
<td>Rat NSun2</td>
<td>TTTGCTAACCACCGAACCAC and CAAAAGAAACGACAG</td>
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<td>Human ICAM-1</td>
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<td>Human VCAM-1</td>
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<td>Firefly luciferase</td>
<td>GATTACCAAGGATTTTGA and GACACCTTTTATGGGAC</td>
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<td>Renilla luciferase</td>
<td>CACATATTGAGCCAGTTGCG and GACACGAGAATTTTCCTTC</td>
</tr>
<tr>
<td>Human Aurora-B</td>
<td>CAGTGGGACACCCGACAT and CTTGAGCCCTAAGGAGCAG</td>
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</table>

### Primers used for real-time qPCR.

#### Online Table I. Primers used for real-time qPCR.

<table>
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<tr>
<th>mRNA fragments</th>
<th>Primer sequence</th>
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<tr>
<td>Human ICAM-1 5’UTR</td>
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<td>Human ICAM-1 5’UTR-B</td>
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<td>Human ICAM-1 CDS-C</td>
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<td>Human ICAM-1 3’UTR</td>
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<td>Luc + 3’UTR</td>
<td>(T7)TTAGGCTGGCGCCGGAAGCG and (poly-T)TTTCTGCCAAGGAGCTTCCCTGC</td>
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#### Online Table II. Primers used for preparing the templates for in vitro transcription.


Online Table III. Routine blood test of NSun2 knockout rats. The basic hematological characterizations were determined as described in “Detailed Methods”. Data represented the means ± SEM (n=3); significance was analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test. WBC, White Blood Cell; RBC, Red Blood Cell; PLT, Blood Platelet; HGB, Hemoglobin; LYM, Lymphocyte; GRN, Neutrophile Granulocyte.

|                  | NSun2 +/- | NSun2 +/- | NSun2 +/-  
<table>
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<tr>
<td><strong>WBC (×10^9/L)</strong></td>
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<td><strong>RBC (×10^12/L)</strong></td>
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<tr>
<td><strong>PLT (×10^9/L)</strong></td>
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<td>186.00 ± 20.55</td>
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<tr>
<td><strong>HGB (g/L)</strong></td>
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<td><strong>LYM (×10^9/L)</strong></td>
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<tr>
<td><strong>GRN (×10^9/L)</strong></td>
<td>7.30 ± 2.48</td>
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<tr>
<td><strong>LYM%</strong></td>
<td>46.77 ± 7.75</td>
<td>52.00 ± 7.71</td>
<td>55.33 ± 8.42</td>
</tr>
<tr>
<td><strong>GRN%</strong></td>
<td>46.03 ± 7.09</td>
<td>40.73 ± 7.26</td>
<td>34.80 ± 7.07</td>
</tr>
</tbody>
</table>

Online Table IV. Blood smear count of NSun2 knockout rats. The differential WBC count was determined as described in “Detailed Methods”. Data represented counts of each rat (n=2); significance was analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test. BN, Stab form Neutrophil; SN, Segmented Neutrophil; LYM, Lymphocyte; E, Eosinophil; M, Monocyte.

<table>
<thead>
<tr>
<th></th>
<th>BN %</th>
<th>SN %</th>
<th>LYM %</th>
<th>E %</th>
<th>M %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NSun2 +/-</strong></td>
<td>1</td>
<td>30</td>
<td>57</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td><strong>NSun2 +/-</strong></td>
<td>0</td>
<td>21</td>
<td>74</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td><strong>NSun2 +/-</strong></td>
<td>1</td>
<td>26</td>
<td>61</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td><strong>NSun2 +/-</strong></td>
<td>0</td>
<td>23</td>
<td>71</td>
<td>3</td>
<td>3</td>
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<tr>
<td><strong>NSun2 +/-</strong></td>
<td>1</td>
<td>26</td>
<td>72</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>NSun2 +/-</strong></td>
<td>0</td>
<td>23</td>
<td>70</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

Online Table V. Measurement of plasma Hcy concentration. NSun2 +/- (n=5) and their wild type littermates (n=6) were intravenous injected with Hcy (50 mg/kg) or with saline for 60 minutes. The plasma Hcy concentration was determined as described in “Detailed Methods”. Data represented the means ± SEM; significance was analyzed by using one-way ANOVA followed by the Student-Newman-Keuls test (*, p<0.05 compared with WT rats; †, p<0.05 compared with NSun2 +/- rats).
Supplemental References


Legends for Video files

Supplemental videos have been uploaded.

**Online Video I-II:** Wild type rats were intravenously injected with saline. The videos showed the adherent leukocytes in the endothelium of mesenteric venules at 0 and 60 min. Related to Figure 2A.

**Online Video III-IV:** Wild type rats were intravenously injected with Hcy (50 mg/kg). The videos showed the adherent leukocytes in the endothelium of mesenteric venules at 0 and 60 min. Related to Figure 2A.

**Online Video V-VI:** NSun2-deficient (NSun2 -/-) rats were intravenously injected with saline. The videos showed the adherent leukocytes in the endothelium of mesenteric venules at 0 and 60 min. Related to Figure 2A.

**Online Video VII-VIII:** NSun2-deficient (NSun2 -/-) rats were intravenously injected with Hcy (50 mg/kg). The videos showed the adherent leukocytes in the endothelium of mesenteric venules at 0 and 60 min. Related to Figure 2A.