DNA-dependent protein kinase (DNA-PK) permits vascular smooth muscle cell proliferation through phosphorylation of the orphan nuclear receptor NOR1

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Aims	Being central part of the DNA repair machinery, DNA-dependent protein kinase (DNA-PK) seems to be involved in other signalling processes, as well. NOR1 is a member of the NR4A subfamily of nuclear receptors, which plays a central role in vascular smooth muscle cell (SMC) proliferation and in vascular proliferative processes. We determined putative phosphorylation sites of NDA-PK in NOR1 and hypothesized that the enzyme is able to modulate NOR1 signalling and, this way, proliferation of SMC.
Methods and results	Cultured human aortic SMC were treated with the specific DNA-PK inhibitor NU7026 (or siRNA), which resulted in a 70% inhibition of FCS-induced proliferation as measured by BrdU incorporation. Furthermore, FCS-stimulated up-regulation of NOR1 protein as well as the cell-cycle promoting proteins proliferating cell nuclear antigen (PCNA), cyclin D1, and hyperphosphorylation of the retinoblastoma protein were prevented by DNA-PK inhibition. Co-immunoprecipitation studies from VSM cell lysates demonstrated that DNA-PK forms a complex with NOR1. Mutational analysis and kinase assays demonstrated that NOR1 is a substrate of DNA-PK and is phosphorylated in the N-terminal domain. Phosphorylation resulted in post-transcriptional stabilization of the protein through prevention of its ubiquitination. Active DNA-PK and NOR1 were found predominantly expressed within the neointima of human atherosclerotic tissue specimens. In mice, inhibition of DNA-PK significantly attenuated neointimal lesion size 3 weeks after wire-injury.
Conclusion	DNA-PK directly phosphorylates NOR-1 and, this way, modulates SMC proliferation. These data add to our understanding of vascular remodelling processes and opens new avenues for treatment of vascular proliferative diseases.
Keywords	Cardiovascular diseases • Atherosclerosis • Molecular biology • Signal transduction

1. Introduction

Proliferation of vascular smooth muscle cells (SMC) plays an important role in the pathogenesis of vascular proliferative diseases such as atherosclerosis and restenosis. Percutaneous coronary intervention (PCI) with stenting is the most widely performed procedure for the treatment of symptomatic coronary disease. Merging evidence suggests in-stent neo-atherosclerosis as an important substrate for both in-stent restenosis (ISR) and late-stent thrombosis. *De novo* atherosclerotic changes of neointima are supposed to be the cause of the restenosis. This neointima mostly consists of proliferative SMC and a proteoglycan-rich extracellular matrix.[1]

Among others, proliferation of vascular SMC is regulated by growth factors and cytokines during the formation of atherosclerotic lesions.[2] However, different pathways involving nuclear receptors are implicated in cell-cycle progression, too. These same pathways often have a part in

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DNA repair, as well. DNA-double strand breaks (DSB) are one of the most potent DNA lesions and can either be repaired by homologous recombination or non-homologous end-joining (NHEJ).[3,4] One key player in this NHEJ-pathway is the serine/threonin kinase DNA-dependent protein kinase (DNA-PK).[5] DNA-PK consists of a catalytic subunit (DNA-PKcs) and the two regulatory subunits Ku70 and Ku80 acting as regulatory elements.[5] The mechanism of DNA-DSB repair includes the assembly of Ku70 and Ku80 with the catalytic subunit DNA-PKcs, an autophosphorylation of DNA-PKcs and the recruitment of additional NHEJ components. It was suggested that DNA-PK is a molecular sensor for DNA damage that enhances DSB repair via phosphorylation of many downstream targets.[6] However, there is growing evidence that DNA-PK is involved in signalling other than needed for DNA repair, as well. Aside from DNA repair, the two important responses to DSBs are cell-cycle arrest that gives the cells enough time to repair damage and apoptosis that eliminates cells with severe damage. DNA-PK also plays an important role in mounting an innate immune response to viral infection or bacterial DNA.[7] Interestingly, DNA-PKcs and Ku are also present at the telomere where they are involved in capping telomeres and maintaining telomere length. Recently, our group was able to show an involvement of DNA-PK in the oestrogen-regulated proliferation of breast cancer cells.[8] We wanted to further investigate this intriguing feature of DNA-PK and to look closer into its participation in cell-cycle progression of SMC.

The neuron-derived orphan receptor (NOR1), a member of the NR4A subfamily of nuclear receptors, which are ligand independent and therefore constitutively active, plays a central role in SMC proliferation and in vascular proliferative processes such as neointima formation.[9,10] Another distinctive feature of the NR4A family of nuclear receptors is that they are rapidly induced by various acute stimuli and are functioning in adaptive and stress-responsive physiological functions.[11] Looking for downstream targets of DNA-PK, we found several putative phosphorylation sites in NOR1. We therefore hypothesized that DNA-PK modulates NOR1 signalling.

2. Methods

2.1 Cell culture

All culture media and supplements were purchased from PAA Laboratories (Germany) unless otherwise specified. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. COS1 cells were purchased from DSMZ (Germany) and maintained in DMEM (high glucose) and RPMI1640, respectively, with 10% FCS and antibiotic/antimycotic solution (PAA Laboratories, Germany). Primary cultures of vascular smooth muscle cells were obtained by explant technique from human aortic sections, derived from coronary artery bypass surgery. Vascular SMC were cultivated in DMEM (low glucose) with 10% FCS and antibiotic/antimycotic solution. The experiments using vascular SMC were repeated three times with independent cell preparations. The vascular SMCs were used at passage 3–8. All procedures involving human materials were been approved by the local Ethics Committee complying with the principles of the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMNCs) were isolated as described.[12] Adherent cells were further cultivated on Costar® ultra-low attachment 6-well plates (Corning, Inc.) for 14 days to obtain monocyte-derived macrophages.

2.2 Plasmids

Constructs of the NOR1 were produced by PCR amplification using plasmid cDNA described in.[11] The resulting fragments from positions 1, Δ39, Δ79, and Δ138 were cloned into the *Nhe*I and *Bam*HI cloning site of the pcDNA3-Flag vector (Invitrogen, Darmstadt, Germany). In addition, the full-length

cDNA of human NOR1 was cloned into pTagGFP-N (Evrogen). Reporter construct containing eight copies of the NBRE (NGFI-B response element) consensus sequence upstream of a minimal prolactin promoter driving expression of the firefly luciferase gene have been described.[11]

2.3 Reagents and antibodies

The following agents and antibodies were used: anti-pRB-Ser729, anti-γH2AX, and anti-cyclin D1 (DC S6) from New England BioLabs (Frankfurt/Main, Germany); Anti-Ku70 (#sc-1487) and anti-DNA-PKcs (#sc-5282) from Santa Cruz Biotechnology (Heidelberg, Germany); Anti-Ku70, anti-Ubiquitin from DAKO (Hamburg, Germany), and anti-α-actin from Abcam (Cambridge, UK); Anti-human NGFI-Bγ/NOR-1/NR4A3 from R&D Systems (Darmstadt, Germany) and mouse anti-nuclear Receptor NOR-1(NR4A3) from Abgent (Heidelberg, Germany). Anti-PCNA, anti-p21, and non-immune IgGs from Upstate Biotechnology (Charlottesville, VA, USA). Mouse anti-Flag M2 was from Sigma. DNA-PK inhibitors NU7026 and DMNB were from Calbiochem (La Jolla, CA, USA).

2.4 Luciferase assay

Human vascular SMC transfected with NBRE or internal control were washed with phosphate-buffered saline (Mg^{2+}- and Ca^{2+}-free) and lysed in 150 μL/well luciferase cell culture lysis reagent. Luciferase assays were performed using the luciferase assay system of Promega according to the manufacturer's instructions (Promega, Mannheim, Germany) and quantified with a luminometer (LB9506, Berthold, Bad Wildbad, Germany).

2.5 Cell proliferation assays

Cell-proliferation assays were performed using the BrdU proliferation assay system of Roche according to the manufacturer's instructions (Roche, Mannheim, Germany). Briefly, human vascular SMC passage three to eight were plated in 96-well tissue-culture microplates and subjected to synchronization by serum-deprivation in 0.1% FBS for at least 24 h followed by stimulation with 10% FBS as indicated

2.6 Reverse transcription and quantitative PCR

Isolation of RNA and quantitative real-time RT−PCR analyses were performed as described previously.[8]

2.7 Immunoprecipitation

Immunoprecipitation was performed as described previously.[8] In addition, we used the GFP Isolation Kit (#130-091-125), according to the manufacturer's instructions (Miltenyi, Bergisch Gladbach, Germany), for isolation of GFP-tagged NOR1.

2.8 Transfection

Transfection of human vascular SMC with siRNA was performed using Lipofectamine (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. The siRNA oligonucleotides with 3′-TT overhangs were purchased from MWG-BIOTECH AG (Ebersberg, Germany). The following siRNA sequences were used: siKu70-1: 5′-GAUGCCCUUUAC UGAAAAAdTdT-3′; siKu70-2: 5′-UUCUCUUGGUAACUUUCCCTTd TdT-3′; siDNA-PK: 5′-GAUCGCACCUUACUCUGU UTTdTdT-3; siGL-3: 5′-CUUACGCUGAGUACUUCGAUUdTdT-3; siNOR1-1: 5′-GUGC GAACCUGCGAGGGCUdTdT-3′; siNOR1-2: 5′-CAAGAGAACAGUGC AGAAAdTdT-3′; siKu80-1: 5′-CGAGUAACCAGCUCAUAAAdTdT-3′; siKu80-2: 5′-CCUUCAAGAGAAAGUGGAAdTdT-3′. The concentration of siRNAs was 20 nmol/L during transfection. In most experiments, siRNA transfection was repeated after 24 h. COS1 cells were transfected using Nanofectin (PAA, Cölbe, Germany). Briefly, cells were grown to ∼60% confluence. Plasmid DNA (3 μg) and Nanofectin (10 μL in phenol red-free DMEM) were added to the COS-7 cells for 4 h at 37°C followed by addition of Dulbecco's modified Eagle's medium and incubation at normal growing conditions.

2.9 *In vitro* phosphorylation assay

Recombinant human NOR1 (Abnova, Heidelberg, Germany) or purified GST-NOR1 fusion protein (wild-type and mutants) were incubated with DNA-PK (Promega, Mannheim, Germany) at 30°C in a total volume of 30 μL of DNA-PK kinase assay buffer containing 10 μCi of [γ-32P]ATP (5000 Ci/mmol). Phosphoprotein products were detected by PAGE (10% gel), Coomassie Blue staining, and autoradiography.

2.10 Histology and immunochemistry

Human carotid endatherectomy specimens were obtained from patients with a carotid stenosis. Tissue was embedded in OCT for immunohisto-chemistry or directly frozen in liquid nitrogen and stored until further use at −80°C. Cryosections (5 μm) were stained using a commercially available DAB kit (Dako, Hamburg, Germany) and digital images of the plaques were captured using an Axiovert II microscope (Zeiss, Oberkochen, Germany). Haemotoxylin/eosin was used for assessment of tissue architecture. Colour Images from the Haematoxilin DAB-staining were separated using the ImageJ colour deconvolution plugin (http://www.mecourse.com/landinig/software/cdeconv/cdeconv.html). Haematoxilin staining was used to determine the total area of the slice, DAB-stain was used to determine the CD68 positive area with the ImageJ particle analyser function with sub-sequent binary images. Subsequent calculations, i.e. Ku70, NOR1, and gamma HA positive area within the CD68 positive area were assessed using the ImageJ image calculator with the masks of the binary images. The study was approved by the Ethical Committee of the University. The patients gave informed consent and the investigation conforms with the principles outlined in the Declaration of Helsinki (Cardiovasc Res 1997;35:2−3)

2.11 Mouse femoral artery injury

All *in vivo* experiments were performed on adult male C57/BL6 mice purchased from Charles River (Sulzfeld, Germany). The mice were anaesthetized by ip injection of 150 mg/kg body weight ketamine hydrochloride (Ketanest, Phar-macia/Pfizer, Mannheim, Germany) and 8 mg/kg body weight xylazine hydro-chloride (Rompun 2%, Bayer, Leverkusen, Germany). Mouse femoral artery injury was performed as described previously.[13] Briefly, after dilatation, the artery was covered with 50 μL of a 25% thermosensitive pluronic F-127 gel containing NU7026[14] or DMNB[15] (50 μg/mL) or vehicle (DMSO) only. Three weeks after dilatation, the femoral artery was carefully excised, fixed in 4% PFA, and embedded in Tissue Tek OCT embedding medium (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands). The starting point of the analysis was 2 mm proximal of the profunda femoris artery, which could be clearly detected while sectioning the mounted artery. Five millimetre slices were cut and every third one was collected until the inguinal ligament for analysis. From these samples, object slides 20, 40, 60, 80, 100, and 120 were used for the morphometric analysis as described previously.[16] All proce-dures involving experimental animals were approved by the local Institutional Committee for Animal Research and complied with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, updated 2011).

2.12 Statistical analysis

Data are given as mean ± SEM. Statistical analysis was performed by ANOVA. Post-test multiple comparison was performed by the method of Bonferroni.

3. Results

3.1 DNA-PK modulates FCS-induced proliferation of human vascular SMC

FCS-induced proliferation of human vascular SMC was significantly reduced when DNA-PK was inhibited as demonstrated by bromodeox-yuridine incorporation (*Figure 1A*). FCS treatment also up-regulated the cell-cycle-promoting protein, proliferating cell-nuclear antigen, and
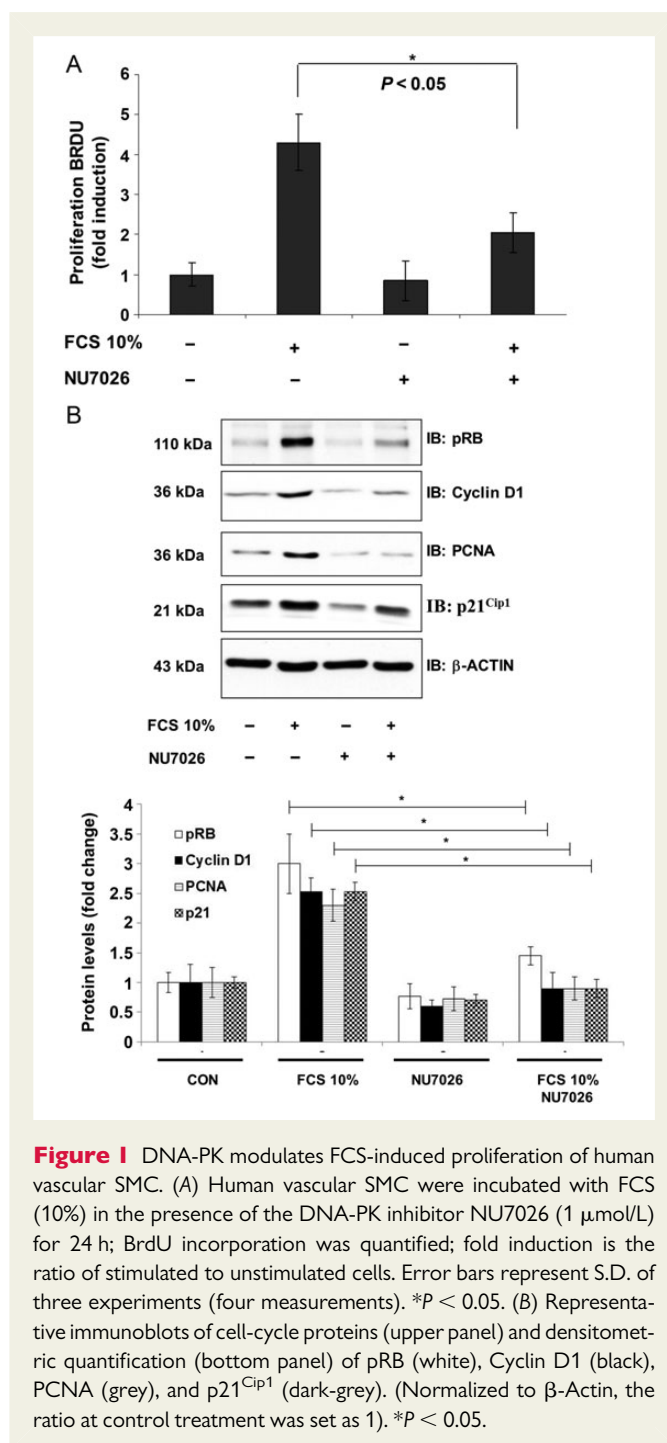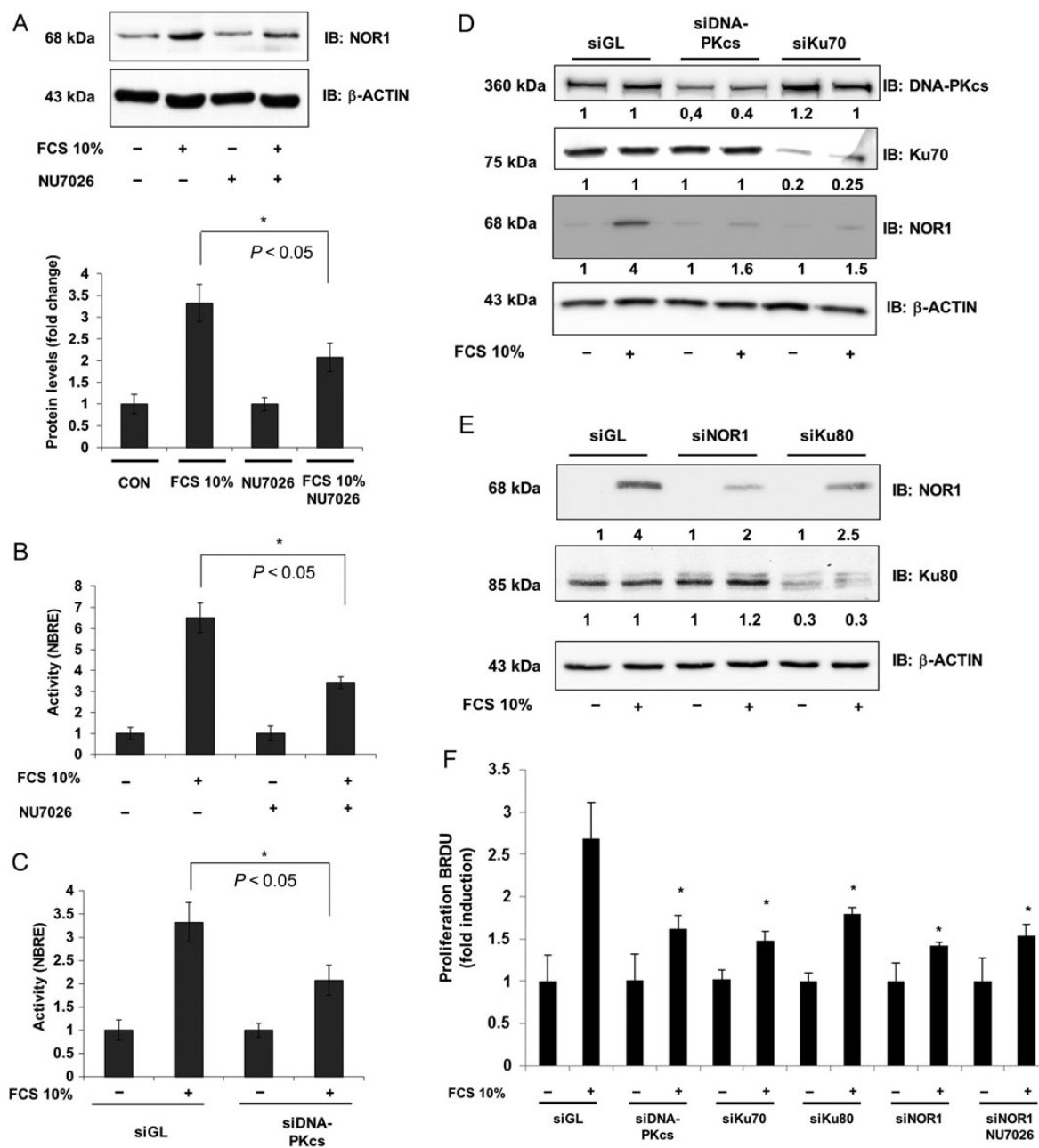

Figure 1 DNA-PK modulates FCS-induced proliferation of human vascular SMC. (*A*) Human vascular SMC were incubated with FCS (10%) in the presence of the DNA-PK inhibitor NU7026 (1 μmol/L) for 24 h; BrdU incorporation was quantified; fold induction is the ratio of stimulated to unstimulated cells. Error bars represent S.D. of three experiments (four measurements). *$P < 0.05$. (*B*) Representa-tive immunoblots of cell-cycle proteins (upper panel) and densitomet-ric quantification (bottom panel) of pRB (white), Cyclin D1 (black), PCNA (grey), and p21^{Cip1} (dark-grey). (Normalized to β-Actin, the ratio at control treatment was set as 1). *$P < 0.05$.

resulted in hyperphosphorylation of the retinoblastoma protein, effects that were all sensitive to DNA-PK inhibition by the specific DNA-PK inhibitor NU7026 (*Figure 1B*).

3.2 DNA-PK modulates FCS-induced NOR1 stability and reporter gene expression

Blocking of DNA-PK with the specific DNA-PK inhibitor NU7026 sig-nificantly reduced FCS-induced NOR1 protein expression (*Figure 2A*). DNA-PK-dependent NOR1 receptor down-regulation resulted in reduced promoter activity as determined by luciferase reporter gene under the control of the consensus site NBRE (*Figure 2B*). Blocking

Figure 2 DNA-PK modulates FCS-induced NOR1 stability and reporter gene expression. (*A*) Immunoblot analysis of NOR1 expression and densito-metric quantification (bottom panel) after FCS treatment for 48 h and DNA-PK inhibition by the specific DNA-PK inhibitor NU7026 (1 μmol/L). β-Actin was used as a loading control. The experiments were independently repeated three times. *$P < 0.05$. (*B*) Human vascular SMC were incubated for 24 h with FCS (10%), NU7026 (1 μmol/L) and combinations thereof. NBRE-dependent gene expression was quantified by measuring luciferase activity. The lucifer-ase activities were normalized to the internal transfection control, and values obtained from cells receiving vehicle (−) were set as 1. Error bars represent S.D. of three experiments (each four measurements). *$P < 0.05$. (*C*) Human vascular SMC were co-transfected either with GL3 control siRNA or with siRNA targeting DNA-PKcs and NBRE reporter plasmid. Where indicated, cells were treated with FCS (10%) for 24 h. NBRE-dependent gene expression was quantified by measuring luciferase activity. Error bars represent S.D. of three experiments (each two measurements). *$P < 0.05$. (*D* and *E*) Human vascular SMC were transfected either with GL3 control siRNA (siGL3) or with siRNA targeting DNA-PK subunits (siKu70, siKu80, siDNA-PKcs) and NOR1 (siNOR1). After 24-h pretreatment with siRNA, cells were treated or not with FCS (10%) for 24 h, and lysates were analysed by immunoblotting (IB) with specific antibodies as indicated. Knock down was significantly reduced compared with controls. $P < 0.05$. (*F*) Human vascular SMC were trans-fected either with GL3 control siRNA (siGL3) or with siRNA targeting DNA-PK subunits (siKu70, siKu80, siDNA-PKcs) and NOR1 (siNOR1). SMC were incubated with FCS (10%) in the presence of the DNA-PK inhibitor NU7026 (1 μmol/L) for 24 h; BrdU incorporation was quantified; fold induction is the ratio of stimulated to unstimulated cells. Error bars represent S.D. of three experiments (four measurements). *$P < 0.05$. FCS treated vs. control. (*F*) Human vascular SMC were co-transfected either with GL3 control siRNA or with siRNA targeting DNA-PKcs and NBRE reporter plasmid. Where indicated, cells were treated with FCS (10%) for 24 h. NBRE-dependent gene expression was quantified by measuring luciferase activity. Error bars represent S.D. of three experiments (each two measurements). (*$P < 0.05$ vs. siGL. $n = 3$).

the DNA-PK subunit DNA-PKcs by specific siRNA resulted in reduced NOR1 activity, as well (*Figure 2C*). To clarify the role of DNA-PK in NOR1 function, we further used siRNA to knock down DNA-PK levels. Reduction of DNA-PK protein levels by specific siRNA caused a pronounced decrease in total NOR1 protein levels (*Figure 2D and E*). Consequently, FCS-induced proliferation of human vascular SMC was significantly reduced (*Figure 2F*). Blocking of NOR1 by specific siRNA in combination with DNA-PK inhibitor yielded no further reduction of proliferation. These results indicate that DNA-PK permits vascular SMC proliferation through regulation of NOR1 (*Figure 2F*).
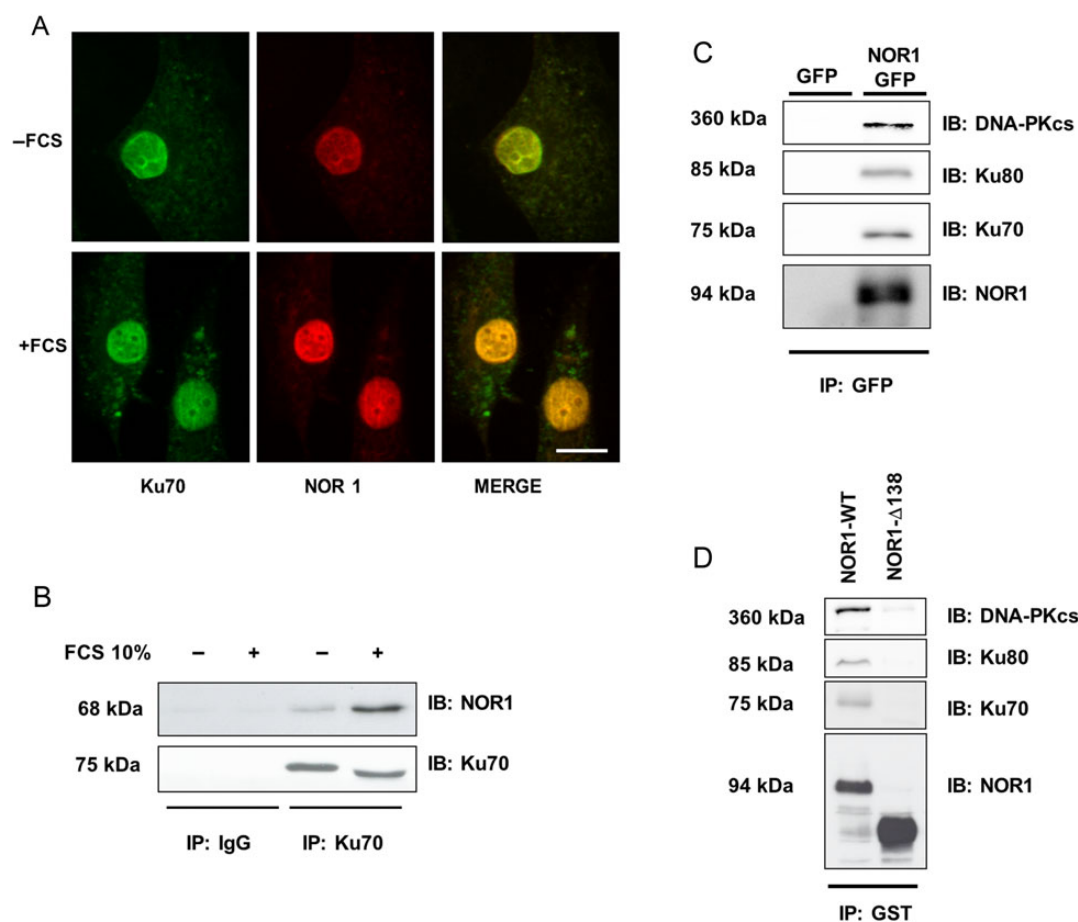
3.3 Interaction of DNA-PK with NOR1

DNA-PK has been reported as a kinase able to form complexes with substrates in different signalling pathways. To clarify if NOR1 is a substrate of DNA-PK, we investigated whether NOR1 and DNA-PK can physically associate *in vitro*. This association was confirmed by immunofluorescence and co-localization assays in human vascular SMC (*Figure 3A*). Co-localization of NOR1 and Ku70 was observed mainly in the nuclei of untreated cells, and increased after FCS stimulation.

The interaction of Ku70 and NOR1 was further confirmed by immunoprecipitation of endogenous Ku70 and subsequent analysis of the immune complexes for the presence of NOR1 or Ku70. We observed an association of Ku70 and NOR1 in untreated cells that was augmented after FCS treatment (*Figure 3B*). Interaction of NOR1 with DNA-PK subunits was further confirmed by immunoprecipitation of GFP-fused NOR1 overexpressed in COS7 cells (*Figure 3C*). We then examined which domain of NOR1 interacts with DNA-PK. For this purpose, a GST pull-down assay was performed. GST-fused NOR1 and a deletion mutant lacking the N-terminal domain of NOR1 were mixed with cell lysates containing DNA-PK. After overnight incubation, GST-NOR1 was eluted from the resin and DNA-PK subunits in the elutant were analysed by immune-blotting (*Figure 3D*). A DNA-PK signal was detected only when full-length NEMO was present, indicating that NOR1 interacts with its N-terminal domain with DNA-PK *in vitro*.

3.4 DNA-PK phosphorylates NOR1

The inhibition of FCS-dependent NOR1 transcriptional activation by NU7026 or using specific siRNA indicated a role of DNA-PK in

Figure 3 DNA-PK interacts with NOR1. (*A*) Distribution of endogenous NOR1 (red) and Ku70 (green) visualized by immunostaining of human vascular SMC after stimulation with FCS (10%) and subsequent imunofluorescence imaging. The experiments were independently repeated three times (size bar 10 μm); (*B*) Lysates from FCS-stimulated cells were immunoprecipitated (IP) with anti-Ku70 followed by immunoblotting (IB) with anti-NOR1 and anti-Ku70. The experiments were independently repeated three times. $P < 0.05$. (*C*) Lysates from COS7 transfected cells were immunoprecipitated (IP) with anti-GFP followed by immunoblotting (IB) with indicated antibodies. (*D*) GST-fused NOR1 and a deletion mutant of NOR1 were mixed with cell lysates containing DNA-PK. After overnight incubation, GST-NOR1 was eluted from the resin and DNA-PK in the elutant was analysed by immune-blotting.

NOR1 activation. Since phosphorylation by DNA-PK is an important step in the regulation of many transcription factors, we investigated whether NOR1 is a target of DNA-PK.

By *in vitro* kinase assay, using recombinant human NOR1 and commercially available DNA-PK, we observed a significant NOR1 phosphorylation by DNA-PK (*Figure 4A*). Phosphorylation of NOR1 by GSK-3 was used as a positive control (S. Medunjanin *et al.*, unpublished data). We then examined the domain of NOR1 phosphorylated by DNA-PK. Therefore, different truncated mutants were generated, and we were able to identify the N-terminal domain as a domain phosphorylated by DNA-PK (*Figure 4B*). Amino acid sequence comparison of the N-terminal domain of NOR1 with known DNA-PK substrates revealed multiple DNA-PK phosphorylation sites within the NOR1 N-terminal domain (*Figure 4C*). To identify the sites phosphorylated by DNA-PK, we generated phosphorylation site-specific NOR1 mutants in which the serine residues were mutated to alanine individually and mutants in which several serine's were replaced by alanine (collectively 12 serine or threonine residues, see *Figure 4C*). We further generated GST-NOR1 fusion proteins of wild-type NOR1 and the different NOR1 mutants described (*Figure 4D*). By means of *in vitro* kinase assays, we tested DNA-PK for its ability to phosphorylate these

NOR1 fusion proteins (*Figure 4D*). Surprisingly, we observed that phosphorylation of all NOR1 mutants was similar to wild-type NOR1. In addition to *in vitro* kinase assay, we analysed phosphorylation of NOR1 by DNA-PK by mass-spectrometric analysis and observed that mutations of NOR1 on one site led to compensatory increased phosphorylation by DNA-PK on other sites (data not shown), because the N-terminal domain of NOR1 is very rich in serine or threonine residues (*Figure 4C*). Furthermore, overexpression of the different NOR1 mutants resulted in the loss of transcriptional activation of NOR1 by FCS (*Figure 4E*).

3.5 DNA-PK prevents NOR1 ubiquitination and degradation

We further evaluated the involvement of DNA-PK in NOR1 stabilization. We generated different NOR1 truncated mutants with deletion of the N-terminal part (*Figure 5A*). In comparison to wild-type NOR1, we observed reduced expression of NOR1 mutant proteins after incubation of cells for up to 48 h after transfection (*Figure 5B*). Next, we studied the impact of the N-terminal domain of NOR1 on its transcriptional activity. Co-transfection of full-length NOR1 and NBRE reporter
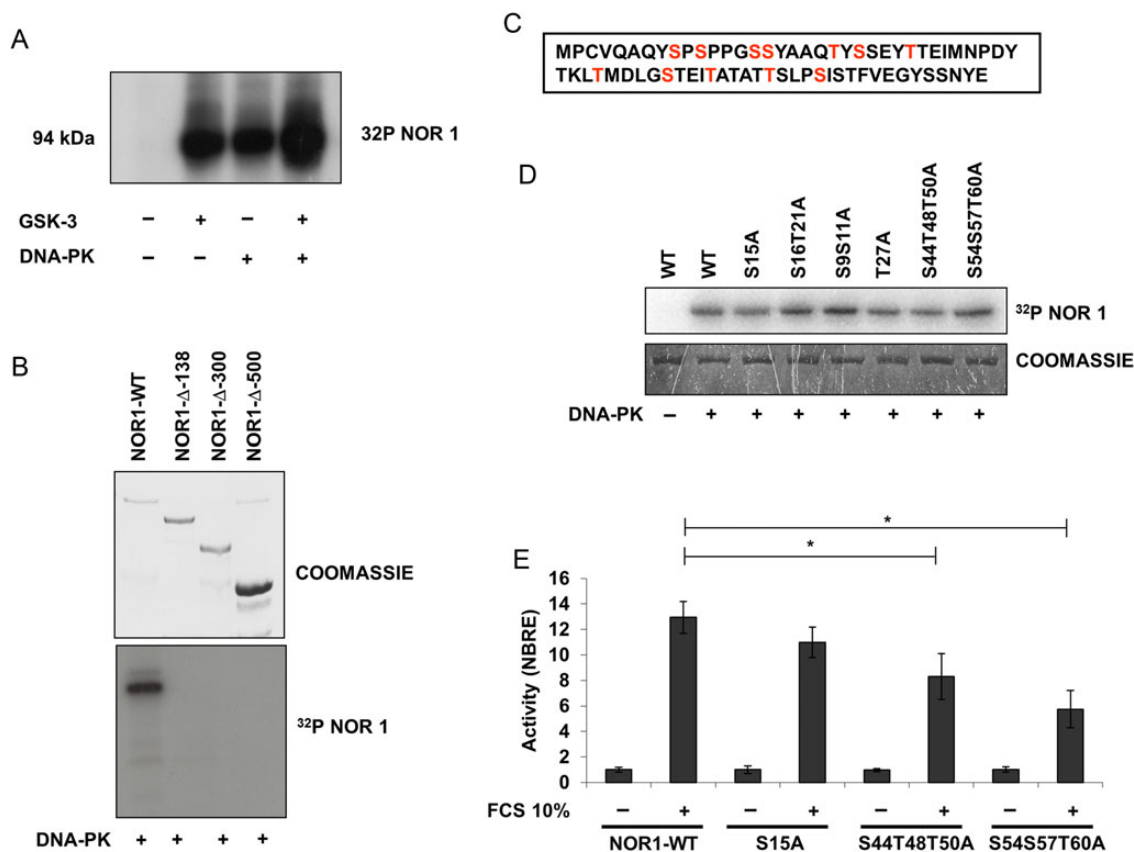
Figure 4 DNA-PK phosphorylates NOR1. (*A*) *In vitro* kinase assay using recombinant human 1 μg NOR1 as substrate for DNA-PK (50 ng). DNA-PK was incubated with 1 μg recombinant human NOR1 and 10 μCi of [γ-32P]ATP at 30°C for 30 min. The experiments were repeated three times. $P < 0.05$. (*B*) NOR1 is a substrate of DNA-PK and is phosphorylated at the N-terminus. (*C*) DNA-PK putative phosphorylation sites are labelled in red. (*D*) *In vitro* kinase assay using wild-type and mutant GST-NOR1 fusion proteins (1 μg) as substrates for DNA-PK (10 ng). The experiments were repeated three times. (*E*) COS1 cells were co-transfected with NBRE reporter plasmid and either wild-type NOR1 or NOR1 mutants. After stimulation with FCS (10%), NBRE-dependent gene expression was quantified by measuring luciferase activity. Data are expressed as -fold luciferase activity measured in untreated cells. Data represent the mean of three independent experiments performed in triplicate. *$P < 0.05$.
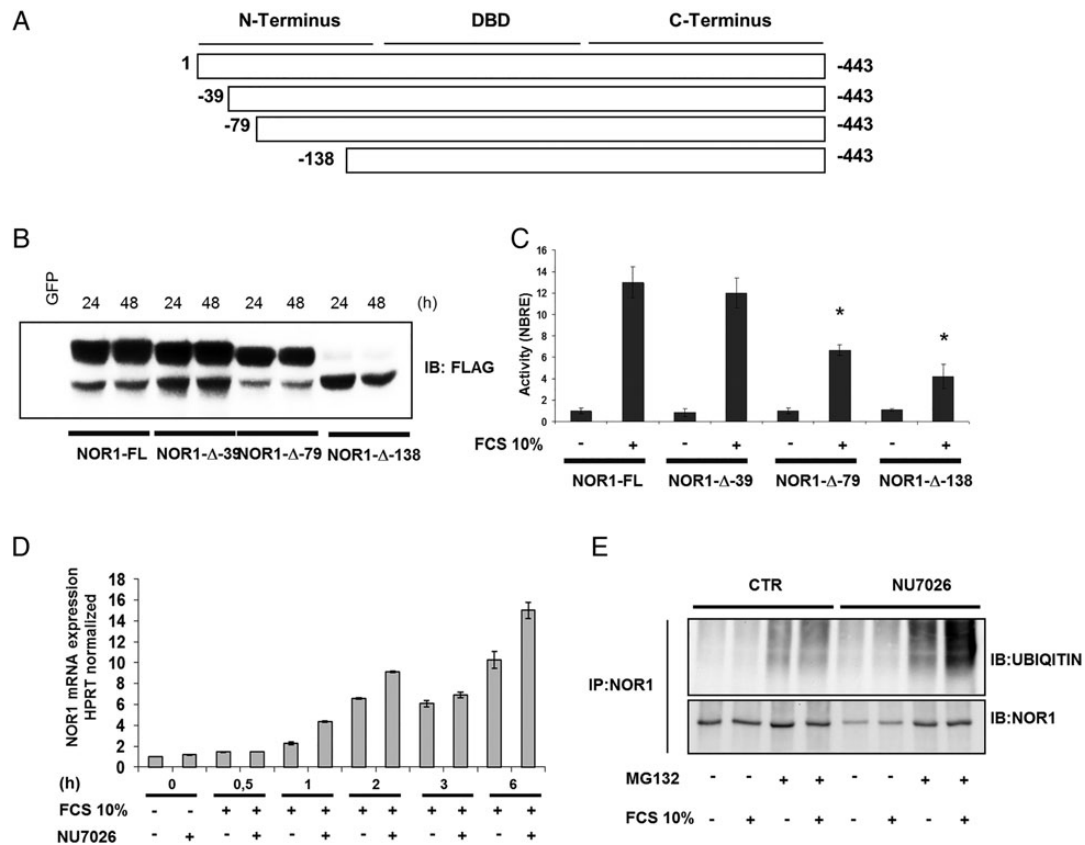
Figure 5 DNA-PK prevents NOR1 ubiquitinylation and degradation. (*A*) Schematic representation of NOR1 mutants generated by PCR. (*B*) N-terminal part of NOR1 is required for NOR1 stability. COS1 cells were co-transfected with FLAG-tagged wild-type NOR1 and NOR1 deletion mutants (Δ-39, Δ-79, Δ-138). The lysates from cells transfected with NOR1 constructs were immunoblotted (IB) with anti-Flag antibody. The experiments were independently repeated three times. (*C*) N-terminal part of NOR1 is required for full transcriptional activation of NOR1. COS1 cells were co-transfected with NBRE reporter plasmid and either wild-type NOR1 or NOR1 mutants. After stimulation with FCS (10%), NBRE-dependent gene expression was quantified by measuring luciferase activity. Data represent the mean of three independent experiments performed in triplicate. *$P < 0.05$. (*D*) After treatment with the DNA-PK inhibitor NU7026 (1 μmol/L) and FCS (10%) for different time periods, expression of NOR1 was analysed by quantitative RT−PCR. Error bars represent S.D. of three experiments (each two measurements). *$P < 0.05$. (*E*) To detect ubiquitination of endogenous NOR1, vascular SMC were treated with NU7026 (1 μmol/L) and MG132 (5 μmol/L) for 3 h prior to stimulation with FCS. The lysates were immunoprecipitated with anti-NOR1 antibody. Ubiquitination of immunoprecipitated NOR1 was examined using anti-ubiquitin antibody. The experiments were independently repeated three times.

in COS1 cells resulted in a considerable FCS-induced luciferase activity (*Figure 5C*). Deletion of the N-terminal part of NOR1 progressively reduced the ability of NOR1 to become activated indicating that the N-terminal part of NOR1 is required for full transcriptional activation of NOR1 (*Figure 5C*).

Using real-time PCR, we were surprised to find that inhibition of DNA-PK resulted in elevated NOR1 mRNA levels in a time-dependent manner (*Figure 5D*). Conversely, DNA-PKcs transcript levels were not altered (data not shown). Post-transcriptional ubiquitination and proteasomal degradation has been shown to control many transcription factor protein levels. To further test for post-transcriptional regulation, we performed an ubiquitination assay. For this purpose, NOR1 protein was immunoprecipitated from human vascular SMC cells pretreated with the DNA-PK inhibitor NU7026 and then immunoblotted with anti-ubiquitin antibody (*Figure 5E*). Treatment with the DNA-PK inhibitor increased ubiquitination of NOR1. When proteasomal degradation was inhibited by MG132, accumulation of ubiquitinated forms of NOR1

further increased. Addition of MG132 prevented down-regulation of NOR1 in control cells, and more importantly, in cells in which DNA-PK was blocked (*Figure 5E*).

3.6 DNA-PK inhibition attenuates neointimal formation in a mouse model of arterial injury

Human carotid endatherectomy specimens were analysed for DNA-DSB and phosphorylation of DNA-PKcs that results from DSBs.[17,18] The immunoblot detected phosphorylation of either DNA-PKcs or gammaH2AX indicating an active DNA-PK within atherosclerotic plaques (*Figure 6A*). As well, expression of NOR1 was observed. The atherosclerotic tissue specimens were also analysed by immunohistochemistry. Both, the Ku70 subunit (representative for the DNA-PK complex) and NOR1, were found co-localized
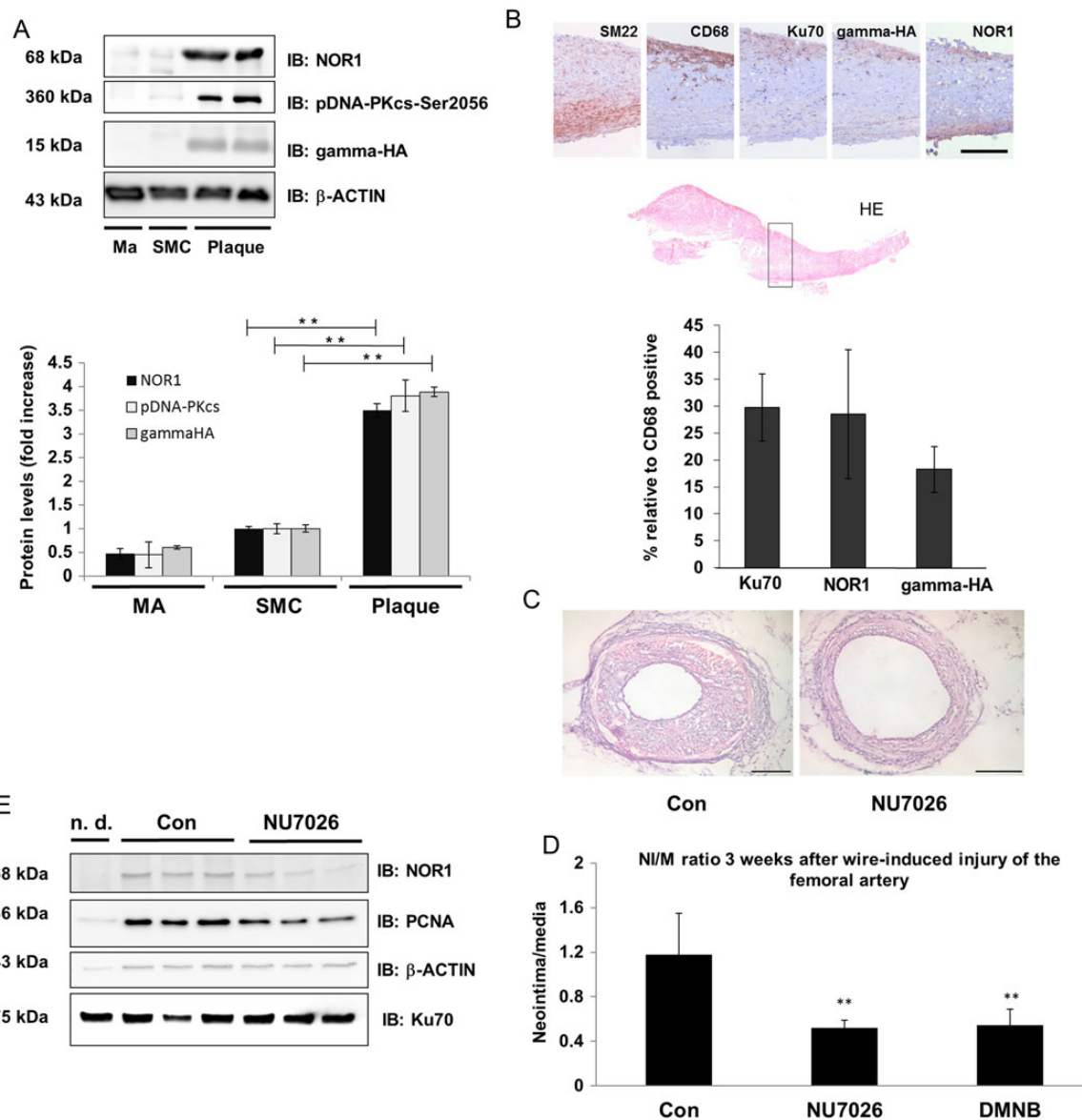
Figure 6 DNA-PK inhibition attenuates neointimal formation in a mouse model of arterial injury. (*A*) Lysates of homogenates of atherosclerotic tissue specimens were immunoblotted (IB) with indicated antibodies (upper panel). Lysates of vascular smooth muscle cells (SMC) or macrophages (MA) served as controls. Quantitative analysis (bottom panel) of NOR1 (black), pDNA-PKcs (grey), and gamma-HA (white). (Normalized to β-actin, the ratio at control vascular SMC was set as 1.) $n = 6$; **$P < 0.01$. (*B*) Representative cross-sections of an atherosclerotic plaque after staining for CD-68 (macrophages), SM22 (SMC), γHASer39 (DSB), Ku70 (representatitve for DNA-PK), NOR1, and haematoxylin eosin. Images are representatives of staining performed on $n = 6$ tissues of patients (size bar 100 μm). Quantitative analysis (bottom panel) of Ku70, NOR1, and gamma-HA. (Relative to CD68 positive area, $n = 6$ tissues of patients). (*C*) DNA-PK inhibition attenuates neointimal formation. Representative cross-sections of femoral arteries from control mice (con) and mice treated with the DNA-PK inhibitor NU7026 are shown 3 weeks after wire-injury (size bar 100 μm); (*D*) Neointima/media (NI/M) ratio of control mice (con) and mice treated with DNA-PK inhibitors (NU7026 or DMNB) was quantified 3 weeks after injury (each $n = 6$, **$P < 0.01$). (*E*) DNA-PK inhibition leads to reduced expression of NOR1. Twenty-one days after injury, NOR1 protein expression in mice treated with DNA-PK inhibitor was significantly reduced compared with controls ($n = 3, P < 0.05$). Lysates of homogenates of femoral arteries from control mice (con) and mice treated with the DNA-PK inhibitor NU7026 were immunoblotted (IB) with indicated antibodies. $P < 0.05$.

predominantly within the neointima as was phosphorylated gamma-H2AX indicating DSB and active DNA-PK (*Figure 6B*).

To determine whether DNA-PK inhibition influences neointima formation, we wire-injured the femoral artery of mice and applied 50 μL of a thermosensitive pluronic F-127 gel containing NU7026 or DMNB (each 50 μg/mL) vs. vehicle as control around the dilated region of the artery.[13] Twenty-one days post injury, a significant concentric neointimal lesion had developed (*Figure 6C* and *D*), which was significantly attenuated in NU7026 or DMNB-treated mice. The immunoblot detected NOR1 and PCNA protein expressions were reduced in femoral artery samples from DNA-PK inhibitor treated mice in comparison to control treated mice (*Figure 6E*).

4. Discussion

In this study, we identified the serine/threonine kinase DNA-PK as regulator of human vascular SMC cell proliferation. Furthermore, we identified the transcription factor NOR1 as a new DNA-PK substrate that is phosphorylated at multiple serine/threonine residues located within the N-terminal domain of the NOR1. It was, however, impossible to determine the exact phosphorylation sites within the NOR1 protein. We had generated several NOR1 mutants where serines and threonines were mutated to alanine. In addition, we performed mass spectrometric analyses. Yet, it turned out that mutation of one site led to compensatory phosphorylation of NOR1 at another site, since the NOR1 N-terminal domain contains unusual multiple serine and threonine residues. A variety of transcription factors are phosphorylated by DNA-PK.[19,20] Even nuclear receptors were shown to be target of DNA-PK. For example, we were able to identify oestrogen receptor as a substrate of DNA-PK.[8] Furthermore, the nuclear orphan receptor NR4A2 is regulated and phosphorylated by DNA-PK increasing its ability to assemble with DSB.[21]

In this study, we report that DNA-PK phosphorylates NOR1, as well. Our data imply that this phosphorylation leads to NOR1 stabilization as blockade of DNA-PK using specific siRNA or DNA-PK inhibitors led to increased ubiquitination and degradation of NOR1 protein. Our data fit the accepted view of NR4A receptors functioning as constitutively active and ligand-independent receptors, whose transcriptional activity is primarily dependent on the expression of the receptor and its post-translational modification.[9] All NR4A receptors are potently induced by a variety of pro-atherogenic stimuli such as growth factors and cytokines,[22] indicating their importance in control of vascular gene expression and different aspects of vascular remodelling, including proliferation. Still NR4A receptors seem to act differently. While Nur77 augments SMC proliferation,[23] reduced proliferation has been observed in NOR1-deficient SMC, demonstrating that NOR1 expression is required for vascular SMC proliferation in response to mitogenic stimulation.[11] These findings are consistent with data demonstrating impaired proliferation of cells treated with NOR1 antisense oligonucleotides.[24] Furthermore, knocking down NOR-1 suppressed DNA synthesis in vascular SMCs, suggesting that NOR1 mediates cycle activity.[25] Accordingly, serum-stimulated proliferation of human SMC was significantly impaired in our study when NOR1 protein levels were reduced following DNA-PK blockade.

In addition to DNA-DSB repair, DNA-PK plays an important role in cell-cycle progression through mitosis.[26] DNA-PK has been implicated to control cell cycle through nuclear receptors before. Recently, we were able to demonstrate that the oestrogen-induced activation of the oestrogen receptor and subsequent cell-cycle progression of breast cancer cells is, at least, partially dependent on DNA-PK.[8] A pro-proliferative role of the kinase is generally implicated by the fact that knock-out animals of DNA-PK subunits, e.g. Ku70 or Ku80, are retarded in growth, as well.[27,28]

Local inflammation, but also hypoxia and ROS formation imply that the atherosclerotic plaque milieu exerts genotoxic stress. Indeed, there is evidence that DNA defects within the cells considerably contribute to atherogenesis.[29,30] The defects found within the plaque range from chromosomal deletions to DNA breaks and single-base mutations. According to these findings, premature atherosclerosis and vascular death dominate so called Progeroid-Syndromes, which are characterized by disturbed DNA repair mechanisms.[31] Most DNA defects within the atherosclerotic plaque arise, however, through generation of ROS via NAD(P) oxidase, xanthinoxidase, and lipoxigenase.[32] ROS are able to induce DNA cross-links, base modifications, and DNA single-strand breaks and DSB. Increased DSB and increased expression of DNA-PK within atherosclerotic plaque have, indeed, been reported.[33] Increased expression of NOR1 in human atherosclerotic tissue specimens has been reported, as well.[34] It is tempting to speculate that DSB within atherosclerotic plaques trigger activation of DNA-PK, resulting in phosphorylation and stabilization of NOR1 subsequent vascular SMC proliferation. Our results do not, however, completely exclude the possibility that blockade of DNA-PK may inhibit other factors involved in vascular SMC proliferation, too.

To study the *in vivo* relevance of our findings, two different DNA-PK inhibitors[14,15] were locally applied around the femoral arteries in mice, which had been wire-dilated. Either was able to significantly reduce local NOR1 expression and subsequent neointima formation.

Collectively, our data suggest that the function of NOR1 is positively regulated by its phosphorylation by DNA-PK. These data add to our understanding of vascular remodelling processes and opens new avenues for treatment of vascular proliferative diseases

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