

Over-expression of Neuron-derived Orphan Receptor-1 (NOR-1) exacerbates neointimal hyperplasia after vascular injury

Ricardo Rodríguez-Calvo¹, Anna Guadall¹, Olivier Calvayrac¹, María A. Navarro², Judith
Alonso¹, Beatriz Ferrán¹, Alicia de Diego³, Pedro Muniesa², Jesús Osada², Cristina
Rodríguez¹, José Martínez-González^{1*}

1. Centro de Investigación Cardiovascular, CSIC-ICCC. IIB-Sant Pau. 08025 Barcelona, Spain.
2. Facultad de Veterinaria, Universidad de Zaragoza. CIBEROBN. 50013 Zaragoza, Spain.
3. Unidad de Transgénesis. Instituto Aragonés de Ciencias de la Salud. 50009 Zaragoza, Spain.

* Corresponding author: Centro de Investigación Cardiovascular (CSIC-ICCC), Hospital de la Santa Creu i Sant Pau (pabellón N° 11), Avda. Sant Antoni Maria Claret 167. 08025 Barcelona. Spain. TEL: +34 93 5565896; FAX: +34 93 5565559; E-mail: jmartinez@csic-iccc.org

ABSTRACT

Background and Objective: We have previously shown that NOR-1 (NR4A3) modulates the proliferation and survival of vascular cells in culture. However, in genetically modified animal models somewhat conflicting results have been reported concerning the involvement of NOR-1 in neointimal formation after vascular injury. The aim of this study was to generate a transgenic mouse model over-expressing NOR-1 in smooth muscle cells (SMC) and assess the consequence of a gain-of-function of this receptor on intimal hyperplasia after vascular injury.

Methods and Results: The transgene construct (SM22-NOR1) was prepared by ligating the full-length human NOR-1 cDNA (hNOR-1) and a mouse SM22 α minimal promoter able to drive NOR-1 expression to SMC. Two founders were generated and two stable transgenic mouse lines (TgNOR-1) were established by backcrossing the transgene-carrying founders with C57BL/6J mice. Real-time PCR and immunohistochemistry confirmed that hNOR-1 was mainly targeted to vascular beds such as aorta and carotid arteries, and was similar in both transgenic lines. Vascular SMC from transgenic animals exhibit increased NOR-1 transcriptional activity (assessed by EMSA and luciferase assays), increased mitogenic activity (determined by [³H]-thymidine incorporation; 1.58-fold induction, $p < 0.001$) and increased expression of embryonic smooth muscle myosin heavy chain (SMemb) than wild-type cells from control littermates. Using the carotid artery ligation model we show that neointima formation was increased in transgenic versus wild-type mice (2.36-fold induction, $p < 0.01$).

Conclusions: Our *in vivo* data support a role for NOR-1 in VSMC proliferation and vascular remodelling. This NOR-1 transgenic mouse could be a useful model to study fibroproliferative vascular diseases.

INTRODUCTION

Abnormal vascular smooth muscle cell (VSMC) proliferation is an important component of the chronic inflammatory response that promotes atherosclerosis [1,2], and is the main responsible of vessel re-narrowing (restenosis) in patients undergoing percutaneous coronary intervention and stent implantation [3,4]. Both atherosclerosis and restenosis can therefore be viewed as hyperproliferative diseases. Consistent with this idea, strategies aimed to inhibit genes positively involved in cell proliferation or to up-regulate growth suppressors have been shown to reduce neointimal thickening in animal models of atherosclerosis and restenosis [1,2,5]; and importantly, the incidence of clinical restenosis is significantly diminished by the use of stents that locally deliver antiproliferative drugs [3,4].

Neointimal thickening requires the activation of gene networks which control major changes in the migratory, proliferative and secretory properties of VSMC [5,6]. We and others have shown that the nuclear receptor NOR-1 (Neuron-derived Orphan Receptor-1) is up-regulated in VSMC induced by mitogens and serum growth factors such as platelet-derived growth factor [7,8]. NOR-1 (NR4A3 according to the unified nomenclature system for nuclear receptors) is a member of the NR4A subfamily of nuclear receptors that also includes Nur77 (NR4A1) and Nurr1 (NR4A2) [9-11]. These nuclear receptors seem to be constitutively active, ligand-independent transcription factors [12], expressed at low levels in resting vascular cells but quickly induced by extracellular cues, acting as early-response genes [9-11].

NR4A receptors are over-expressed in atherosclerotic plaques from human coronary arteries (7,8,13-16), and are up-regulated in arteries from porcine and mouse models subjected to vascular injury [7,17]. In both VSMC [7,8,13,18,19] and endothelial cells (16,20-22), NOR-1 expression is induced by mitogenic stimuli, and inhibition of NOR-1 expression efficiently reduced mitogen-induced vascular cell proliferation [7,18,19,21,22].

However, studies from genetically modified animal models have yielded somewhat conflicting results. Indeed, while carotid artery ligation increased neointima formation in transgenic mice expressing a dominant-negative variant that suppressed the activity of all three NR4A receptors [13], recent studies in NOR-1 knockout mice showed a decrease in neointima formation after femoral artery denudation injury [17]. Furthermore, it should be noted that early studies reported that NOR-1 deficiency led to embryonic lethality [23]. In this study, we used SMC specific-transgenesis to generate a mouse model that over-expresses NOR-1 in VSMC to assess the effect of a gain-of-function of this receptor on intimal hyperplasia after vascular injury.

RESULTS

Generation and characterization of a mouse model over-expressing human NOR-1 in smooth muscle cells

To target hNOR-1 expression into SMC, we inserted the hNOR-1 cDNA downstream of a mouse SMC-specific promoter (SM22 α proximal promoter). The structure of the transgene is shown in figure 1A. This construct was able to efficiently drive NOR-1 expression (both mRNA and protein) when it was transiently transfected in rat VSMC (Figure 1 B and C). Transgenic animals were generated by microinjection of the resulting expression cassette into fertilized mouse eggs. Twenty percent offspring carried the transgene. F1 transgenic mice (TgNOR-1) grew to adulthood normally and were phenotypically indistinguishable from their wild-type littermates. To assess germline transmission, transgenic founders were crossed with non-transgenic mice. Resulting pups had genotypes with the expected Mendelian ratios, indicating that the hNOR-1 transgene had no effect on embryonic development or viability. TgNOR-1 mice were mated with C57BL/6J mice up to tenth generation to guarantee the 99.9 % of purity on this genetic background. TgNOR-1 mice survived without developing any

malformation and had no problems in procreation. Two stable transgenic mouse lines (family a and b) were established (Figure 2A). Family a and b exhibited similar expression levels of hNOR-1 in aorta (Figure 2B). NOR-1 transgenesis did not modify the endogenous expression of mouse NR4A receptors (Figure 2C). NOR-1 protein levels were also similarly increased in the aorta and carotid arteries of both transgenic families as determined by Western blot (Figure 3A) and immunohistochemistry (Figure 3B and data not shown). Thus, for further experiments we indifferently used animals from both transgenic lines. As expected, hNOR-1 was also highly expressed in other SMC-rich tissues such as uterus and stomach (Figure 3C).

Aortic VSMC from TgNOR-1 mice over-express functional hNOR1-transgenic protein

VSMC were isolated from pooled aortas of TgNOR-1 and their wild-type littermate mice. VSMC from transgenic animals exhibited significant expression of hNOR-1 mRNA levels (Figure 4A), and increased NOR-1 protein levels (Figure 4 B and C). The over-expression of NOR-1 did not alter the endogenous expression of mouse NR4A receptors (Figure 4D). These cell cultures were used to check the ability of the hNOR-1 transgenic protein to drive the expression of a NOR-1-sensitive promoter (minimal pro-opiomelanocortin [POMC] promoter plus three NBRE) [31]. As shown in figure 5A, in transient transfection assays, the activity of this reporter construct was significantly higher in the VSMC obtained from transgenic animals than in control cells (53.4-fold, $p < 0.001$). Consistent with this, the EMSA study showed that nuclear extracts from NOR1-transgenic VSMC exhibited higher binding capacity to a consensus NBRE site than those from control cells (Figure 5B).

Aortic VSMC from TgNOR-1 mice exhibit higher mitogenic activity than cells from wild-type mice

Next we determined the incorporation of [6-³H]-thymidine into *de novo* DNA synthesis as an index of the mitogenic activity of VSMC [7,18]. As shown in figure 6, VSMC from TgNOR-1 mice exhibited a higher rate of DNA synthesis than VSMC from wild-type mice, in both growing unsynchronized cultures (Figure 6A) and in growth-arrested cells stimulated with foetal calf serum (FCS) (Figure 6B). Cultures of VSMC from transgenic mice also exhibited higher levels of the embryonic smooth muscle myosin heavy chain (SMemb/ non-muscle myosin heavy chain (MHC) isoform-B) than wild-type cells from control littermates (Figure 6C).

Neointimal hyperplasia after vascular injury is increased in TgNOR-1: results from the mouse carotid artery ligation model

To determine the effect of hNOR-1 over-expression on neointimal formation after vascular injury, the mouse carotid artery ligation model was used. Serial section data show a vascular remodeling reliably persistent up to 2400 μ m from ligature (Figure 7A). TgNOR-1 exhibited an exaggerated vascular remodelling (Figure 7A). Indeed, quantitative measurements revealed a significant higher thickening of neointimal hyperplasia (2.36-fold induction, $p<0.01$), a higher ratio of intimal to medial area and a higher percentage of stenosis in TgNOR-1 than in wild-type mice (Figure 7 B-E).

DISCUSSION

Our previous studies using transient silencing of NOR-1 in knockdown experiments in human VSMC [7,18], and those from Bruemmer's group in NOR-1-deficient mouse VSMC [8,17], point to a role for this nuclear receptor in the gene network regulating VSMC proliferation. However, studies from genetically modified animal models have yielded somewhat

conflicting results. Early studies reporting that NOR-1 deficiency led to embryonic lethality [23] were not subsequently corroborated by others. Indeed, later studies showed that NOR-1-deficient mice were perfectly viable and exhibited a decrease in neointima formation after femoral artery denudation injury [17]. However, in apparent contradiction with these results, carotid artery ligation increased neointima formation in transgenic mice expressing a dominant-negative variant that suppressed the activity of all three NR4A receptors [13]. In the present study, we have generated a mouse model that over-express NOR-1 in SMC to assess the effect of a gain-of-function of this receptor on intimal hyperplasia after vascular injury.

To drive the expression of human NOR-1 to vascular smooth muscle cells, we generated transgenic mice that express the cDNA encoding human NOR-1 under the control of the mouse SM22alpha proximal promoter. SM22 α (also known as transgelin, WS3-10, or p27) is a 22-kDa protein that is considered one of the earliest markers of SMC differentiation, exclusively and abundantly expressed in the SMCs of adult vertebrates [33]. The 5'-flanking sequence of the SM22alpha promoter used (-591/+62) has been found to be sufficient to direct transgene expression to SMC. Indeed, in this region the mouse (and human) proximal SM22 α promoter contains two CArG boxes (CC[A/T]₆GG, at 150- and 273-bp upstream of the transcription initiation site) recognized by the serum response factor (SRF), which are critical for the specific transcription of SM22 α in SMC [24,34]. This ability of SM22 α promoter has been successfully used to generate transgenic animals that over-express the protein of interest in the vascular wall [13,24,35]. Using this strategy, we generated a construct that was validated *in vitro* in transient transfections of rat VSMC, and was subsequently used to generate a transgenic mouse by standard procedures. Two stable transgenic mouse lines were established. NOR-1 over-expression had no effect on embryonic development or viability, and TgNOR-1 mice did not show obvious phenotypic differences

compared with their wild-type littermates. We confirmed the anticipated pattern of SM22 α -directed transgene expression in TgNOR-1 mice: mainly in aorta and SMC-rich tissues such as uterus and stomach that typically express high levels of SM22 α [24,34,36]. Therefore, SM22 α promoter-directed transgenesis of NOR-1 led to viable animals which were born at the expected frequency and exhibited a tissue-expression pattern of hNOR-1 compatible with that described for SM22 α .

As a first approach to assess the functionality of hNOR-1-transgenic protein in TgNOR-1, we comparatively analyzed the ability of extracts from aortic VSMC from wild-type and TgNOR-1 to bind to a NBRE consensus and to activate the transcription of a luciferase reporter plasmid containing several elements responsive to NOR-1 [31]. Indeed, VSMC from TgNOR-1 exhibited marked levels of hNOR-1 mRNA and NOR-1 protein levels, and increased NOR-1 transcriptional activity. More interestingly, DNA synthesis was increased in VSMC from TgNOR-1. **Furthermore, VSMC from transgenic mice exhibited higher expression levels of a synthetic SMC marker (SMemb) that is up-regulated in proliferating VSMC [37].** Thus, hNOR-1 over-expression positively modulates VSMC mitogenic activity, confirming and expanding our previous studies showing that the negative modulation of NOR-1 expression impairs VSMC mitogenic activity [9,18].

To evaluate the effect of a gain-of-function of NOR-1 on intimal hyperplasia after vascular injury, we used the mouse model of carotid artery ligation. In this model, the vascular wall experiences a profound remodelling, leading to neointima formation and carotid occlusion in a few weeks [38]. TgNOR-1 exhibited an exaggerated neointimal hyperplasia compared with their wild-type littermates. These results are in agreement with those reported in NOR-1 knockout mice [17], and further support a differential role of individual NR4A receptors in vascular remodelling. Indeed, although Nurr1 and Nur77 are induced in vascular cells by stimuli similar to those which up-regulate NOR-1, they seem to prevent vascular cell

proliferation and to attenuate neointima formation after vascular injury [13,14,39,40]. The significant effects of NOR-1 on vascular remodelling observed after transgenesis and gene targeting also suggest that this nuclear receptor participate in the regulation of gene networks governing cell cycle progression [41]. In this regard, several growth-related genes targeted by NOR-1 in VSMC have been recently identified, among them cyclins D1 and D2 [17] and S phase kinase-associated protein 2 (SKP2) [42]. These genes could be responsible, at least in a part, of the increased vascular remodelling response observed in transgenic mice undergoing vascular injury. Interestingly, our preliminary data suggest that basal expression of these cyclins could be increased in the aorta from NOR-1 transgenic mice (cyclin D2 1.34-fold induction, $p < 0.05$). However, the regulation of cyclins through the cell cycle is complex and involves different mechanisms. Further studies are required to identify key NOR-1 target genes regulating VSMC proliferation and vascular remodelling, and to determine whether NOR-1 could also control the expression of VSMC phenotype markers such as SMemb that is quickly and markedly up-regulated in proliferating VSMC.

In conclusion, we have generated a novel mouse model that over-expresses human NOR-1 in SMC-rich organs, particularly in vascular beds such as aorta and carotid arteries. Human NOR-1-transgenic protein was functional in aortic VSMC, and NOR-1 transgenesis increased VSMC mitogenic activity and exacerbated neointima formation after mechanical injury in a mouse model of carotid artery ligation. Therefore, the TgNOR-1 mouse could be a powerful tool for further studies aimed to clarify the molecular mechanism involved in NOR-1 in vascular function and disease.

MATERIALS AND METHODS

Animal Handling

Animals were bred in the Animal Experimentation Unit (CSIC-ICCC, Barcelona, Spain). Mice were housed in a controlled, specific pathogen-free environment in rooms maintained on a standard light-dark cycle (12-h light/dark cycle) and temperature ($21\pm 1^\circ\text{C}$) conditions and were fed *ad libitum* with a standard commercial diet (Harlan Ibérica SL, Barcelona, Spain). Animal handling and disposal were performed in accordance with the principles and guidelines established by the American Physiological Society for animal research, and all procedures were approved by the Ethical Committee as stated in Law 5/1995, 21st June, passed by the Generalitat de Catalunya. Animals were taken care of and used according to the Spanish Policy for Animal Protection RD1201/05, which meets the European Union Directive 86/609 on the protection of animals used for experimental and other scientific purposes.

Generation of an expression plasmid for human NOR-1

An expression plasmid was generated by cloning a full-length human NOR-1 (hNOR1) cDNA downstream the mouse SM22 α promoter the mammalian vector pCAGGS. In brief, the proximal promoter of SM22 α (653 bp; GenBank Accession No. U36589; positions 2145 to 2797) was amplified by PCR using as a template a construct containing this sequence (pSM2735-luc; kindly provided by Dr. E Olson, Department of Molecular Biology, University of Texas Southwestern Medical Center, Dallas, USA) [24], using the following primers: 5'-CACTAGTCGACATGTTCTGCCACGCACTTGG-3' (forward primer; *SalI* restriction site is underlined) and 5'-TAGATCTCGAGGCCTTGGTCGTTTGTGGACT-3' (reverse primer; *XhoI* restriction site is underlined). The pGAGGS promoter was excised by digestion with *SalI* and *XhoI* and replaced by the SM22 α promoter generated by PCR (digested with *SalI* and *XhoI*), obtaining the pCAGGS-SM construct. Subsequently, a full-length hNOR1 cDNA (GenBank Accession No. D78579; position 513 to 2872), excised by

digestion with *EcoRI* from a pBlueScript-NOR1 construct (kindly provided by Dr. N Ohkura, Growth Factor Division, National Cancer Center Research Institute, Tokyo, Japan) [25], was cloned into the *EcoRI* site of pCAGGS-SM. In the resulting construct (pCAGGS-SM/hNOR1), hNOR-1 (downstream fused to a rabbit poly (A) tail sequence) was under the control of a SMC-specific promoter (Figure 1). To validate the construct, rat VSMC were transfected with pCAGGS-SM/hNOR1 and NOR-1 expression was analyzed (mRNA and protein levels).

Generation of transgenic mice

The expression cassette (3.6-kb) (Figure 1A) was recovered by double digestion with *SalI* and *HindIII*, purified by QIAquick™ Gel Extraction Kit (Qiagen), and used for pronuclear injection of zygotes as previously described [26]. All procedures were carried out in the Transgenesis Unit of the Aragon Health Science Institute under the Project Licenses PI29/08 and PI 30/08 approved by the in-house Ethics Committee for Animal Experiments from the University of Zaragoza.

Embryos were obtained from B6D2F1/J mice mated to B6D2F1/J males (Charles River). Four- to 6-week-old females were superovulated by an intraperitoneal (i.p.) injection of pregnant mare's serum gonadotropin (PMSG) (5 IU), followed 48 h later by an i.p. injection of human chorionic gonadotropin (hCG; 7.5 IU). After mating, fertilized eggs were harvested at 20 h post-hCG. After removing cumulus cells with hyaluronidase, zygotes were thoroughly washed, selected for good morphology and collected. Fertilized eggs (1-cell embryos) were cultured in EmbryoMax™ KSOM medium (Millipore MR-020P) at 37°C in an atmosphere of 5% CO₂, 90% relative humidity. One-cell embryos were microinjected into the pronucleus in an M-2 medium (Sigma M7167) and transferred into the reproductive tract of pseudopregnant recipients (CrI:CD-1) to complete their development. We screened the

resulting offspring (3 weeks after birth) for transgene transmission by PCR analysis of genomic DNA (extracted from mouse tails) using specific primers. Genomic DNA was prepared from tail biopsies following the DNeasy[®] Blood & Tissue kit (Qiagen) instructions and used for PCR. Specific primers selected to amplify a fragment of hNOR-1 cDNA (579 bp) were: 5'-GACAGACTGCTCCAACTTGG-3' and 5'-GGTTCATGATCTCCGTGGTG-3'. PCR was performed in a volume of 25 µl containing genomic DNA (100 ng), 1X MyTaq[™] Reaction Buffer (Biolone), 0.4 µM of each primer and 1.25 U of MyTaq[™] polymerase. PCR was run in a thermocycler (GeneAmp[®] PCR System 9700, Applied Biosystems) programmed for 30 cycles: denaturation (94°C for 1 min), annealing (54°C for 45 s) and polymerization (72°C for 1 min). PCR products were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide, and were visualized under UV light. Images were captured using the BIO-RAD Gel Doc 1000 Multi-Analyst 1.1 (Bio-Rad). Stable transgenic mouse lines were established by backcrossing the transgene-carrying founders with C57BL/6J mice up to the tenth generation to guarantee the 99.9% of purity in this genetic background.

Cell culture

VSMC from rat or mouse (TgNOR-1 and wild-type) aorta were obtained by the explant technique [27]. Briefly, the aorta was excised immediately following euthanasia, was perfused with saline and surrounding connective and fatty tissue was dissected away from around the artery. Then, the vessel was cut open longitudinally and the endothelium was removed by scraping the cell layer off with a sterile scalpel blade. Endothelium-denuded medial tissue was stripped from the adventitia and cut into 1 to 2 mm cubes that were transferred to a 25 cm² culture flask containing 2 mL of pre-warmed Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 20% FCS (Biological Industries) and

antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). Explants were left undisturbed for 4 days to avoid detachment and half the medium was replaced every 4 days. VSMC migrate out from the explants within 1 to 2 weeks. Then, after removing the explants from the flask surface, the cells were trypsinized, used as P1 stage cells, and routinely subcultured. VSMC between passages 3 to 6 were seeded in multiwell plates to be used in transfection experiments and to isolate RNA and protein extracts as described below.

Analysis of mRNA levels

Total RNA was isolated from cell cultures using the Ultraspec™ reagent (Biotecx Laboratories), and from mouse tissues using the RNeasy Micro kit (Qiagen). RNA integrity was determined by electrophoresis in agarose gel. RNA (1 µg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) in the presence of random hexamers. Quantification of mRNA levels was performed by real-time PCR using the ABI PRISM 7900HT sequence detection system (Applied Biosystems) and specific primers and probes provided by the Assay-on-Demand system for human NOR-1 (Hs00175077_m1), mouse NOR-1 (Mm00450074_m1), mouse Nurr1 (Mm00443056_m1) and mouse Nur77 (Mm00439358_m1). TATA-binding protein (TBP) expression was used as reference gene (Mm00446973_m1 and Rn01455646_m1) [28]. Each sample was amplified in duplicate.

Immunohistochemical and immunocytochemical analysis

Mouse aortas were perfused with phosphate-buffered saline (PBS), removed, fixed in 4% paraformaldehyde/0.1 M PBS (pH 7.4) for 24 hours, embedded in paraffin and prepared in 5-µm cross sections with a microtome (Jung RM2055, Leica) [29]. Sections from aorta of TgNOR-1 and wild-type mice were deparaffinised in xylene and rehydrated in graded ethanol

solutions. Slides were then rinsed in distilled water and treated with 10% hydrogen peroxide in methanol for 30 min to remove endogenous peroxidase activity. Sections were blocked with a 10% of normal serum in PBS-Tween 0.1% for 30 min and incubated with an anti-human NOR-1 antibody (H00008013-M06, Abnova) overnight at 4°C. After washing 3 times in PBS-Tween 0.1%, samples were incubated for 1 h with a biotinylated secondary antibody (Vector Laboratories). After rinsing 3 times in PBS, standard Vectastain (ABC) avidin-biotin peroxidase complex (Vector Laboratories) was applied, and the slides were incubated for 30 min. Colour was developed using 3,3'-diaminobenzidine (DAB) and sections were counterstained with haematoxylin before dehydration, clearing, and mounting. Negative controls in which the primary antibody was omitted were included to test for non-specific binding. For immunocytochemical analysis, VSMC from TgNOR-1 and wild-type mice were seeded in Permanox® Chamber Slides (Lab-Tek®) and serum starved for 48 h. Cells were fixed in 4% paraformaldehyde/0.1 M PBS (pH 7.4) for 10 min and endogenous peroxidase was blocked with 3% hydrogen peroxide for 30 min. Then, slides were treated with 1% BSA in PBS for 20 min to block unspecific binding of the antibody and permeabilized with 0.4% Triton X-100 for 5 min. After extensive washing, the primary antibody (H00008013-M06, Abnova) was added and the Vectastain ABC (HRP-DAB) protocol progressed as described above. **Levels of SMemb were analyzed in VSMC from TgNOR-1 and wild-type mice using a specific antibody (ab684, Abcam).**

Isolation of nuclear extracts

Nuclear extracts were obtained from VSMC of TgNOR-1 and wild-type mice. Cells were scraped into 1.5 ml of cold PBS, pelleted for 15 s and resuspended in 300 µl of cold Buffer A (10 mM HEPES pH 7.8 at 4°C, 1.5 mM MgCl₂, 10 mM KCl, 1 mM DTT, and 1X CIP, Calbiochem). Cells were allowed to swell on ice for 15 min, and then added 0.6% NP-40 and

vortexed for 15 s. Samples were then centrifuged for 30 s and the supernatant fraction was removed. Pellets were then resuspended in 50 μ l of cold Buffer C (25 mM HEPES pH 7.8 at 4°C, 10% glycerol, 400 mM NaCl, 1.5 mM MgCl₂, 50 mM KCl, 1 mM DTT and 1X CIP, Calbiochem) and incubated on ice for 30 min in a rocker. The chromatin-rich nuclear debris was removed by centrifugation for 5 min at 4°C and the supernatant fraction (containing nuclear extracts proteins) was stored at -80°C. Protein concentration was determined by the BCA method (Pierce).

Immunoblotting

Whole-cell extracts were obtained from vascular cell cultures. Briefly, cells were washed twice with PBS and lysed with a lysis buffer containing 50 mM Hepes pH 7.4, 150 mM NaCl, 100 mM NaF, 10 mM NaPPi, 10 mM EDTA, 2 mM Na₃VO₄ 1 mM PMSF, 5 μ M leupeptin and 0.5% SDS [30]. Proteins from nuclear and whole-cell extracts (10-20 μ g/lane) were separated under reducing conditions on SDS-polyacrylamide gels (10%) and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore). Blots were incubated with antibodies directed against human NOR-1 (H00008013-M06, Abnova), β -actin (A5441, Sigma), nucleolin (ab22758, Abcam), **SMemb (ab684, Abcam) and smooth muscle myosin heavy chain (MHC) isoforms SM1 and SM2 (ab97539, Abcam)**. Bound antibodies were detected after incubation with appropriate HRP-conjugated secondary antibodies (Dako) and using the SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). The size of detected proteins was estimated using protein molecular-mass standards (Fermentas). Equal loading of protein in each lane was verified by Ponceau staining and by β -actin (for whole-cell extracts) or nucleolin (for nuclear extracts) signal [28].

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were obtained from VSMC of TgNOR-1 and wild-type mice as described above. A double-stranded probe containing the nerve growth factor-induced gene-B (NGFI-B) response element (NBRE) [31] was generated from the annealing of single-stranded complementary oligonucleotides: 5'-GATCCTCGTGCGAAAAGGTCAAGCGCTA-3' and its complementary. Purified probe was end-labelled with [γ - 32 P]-ATP (Perkin Elmer) and T4 polynucleotide kinase (Roche) for 2 h at 37°C [28]. EMSA assays were performed using the Novagen's EMSA Accessory kit. Briefly, nuclear proteins (5 μ g) were incubated for 15 min on ice with 0.01 U Poly(dI-dC)•Poly(dI-dC) in 20 mM HEPES (pH 8), 0.2 mM EDTA, 100 mM KCl, 20% glycerol, and 0.5 mM DTT. Approximately 50,000 cpm of the 32 P end-labelled probe were then added to the reaction mixture and incubated for 30 min on ice in a final volume of 20 μ l. Protein-DNA complexes were resolved by electrophoresis at 4°C on 5% polyacrylamide, 5% glycerol gels in 0.5X TBE. The gels were dried and subjected to autoradiography using a Storage Phosphor Screen (GE Healthcare). Shifted bands were detected using a Typhoon 9400 scanner (Amersham).

Transient transfection and luciferase assay

Transfection experiments were carried out using LipofectamineTM LTX and PlusTM Reagent (Invitrogen) according to the manufacturer's instructions. Rat VSMC were transfected with the construct (pCAGGS-SM/hNOR1) used to generate transgenic mice (containing human NOR-1 cDNA under the control of the SM22 proximal promoter). Alternatively, VSMC from TgNOR-1 and wild-type mice were transfected with a luciferase reporter plasmid (JA982) containing the minimal (-34 to +63) POMC promoter and three NBRE (5'-GATCCTCGTGCGAAAAGGTCAAGCGCTA-3') (kindly provided by Dr. J Drouin, Laboratory of Molecular genetics, Institut de Recherches Cliniques de Montréal, Canada) [31]. In luciferase assays, the plasmid containing the Renilla luciferase (E2271, Promega)

was included to normalize for the transfection efficiency. Transient transfection assays of subconfluent cells (60-80% confluence) were performed in 6-well plates. DNA was diluted in 200 μ l of serum-free Opti-MEM, mixed with PLUSTM Reagent (1 μ l PLUSTM Reagent: 1 μ g DNA), and incubated at room temperature for 10 min. Afterwards, LipofectamineTM LTX was added, mixed gently and incubated for 30 min at room temperature to facilitate the formation of DNA-liposome complexes. The ratio DNA/liposome used was as follows: 1 μ g of the indicated constructs (+ 0.05 μ g of pRL-null vector in luciferase assays) and 10 μ l of LipofectamineTM LTX/well. The culture plates were washed with PBS, and 800 μ l of antibiotic-free medium (DMEM/20%FCS) was added. The plasmid/liposome mixture (200 μ l) was then gently added to each well and incubated overnight. Following transfection, rat VSMC monolayers were growth arrested by serum starvation for 48 h, and finally they were processed for either total RNA or protein isolation. For luciferase assays, protein extracts from transfected mouse VSMC were prepared by rinsing each plate twice with PBS and lysing in 100 μ L of Reporter Assay Lysis Buffer (Promega). Luciferase activity was measured in cell lysates using the Dual-LuciferaseTM Reporter Assay System (Promega) and a luminometer (Orion I, Berthold Detection Systems).

Determination of DNA synthesis

The incorporation of [6-³H]-thymidine into de novo DNA synthesis was used as an index of mitogenic activity of VSMC [7,18,19]. VSMC were plated in 48 well-plates and grown to subconfluency. The incorporation of [6-³H]-thymidine into DNA was determined in two sets of cell cultures: cultures maintained in complete medium, and cultures incubated with a growth factor-poor medium (0.2% FCS for 24 h) to arrest cell proliferation and then stimulated with complete medium (20% FCS). [6-³H]-thymidine (23 Ci/mmol; Amersham) was added to the cells at a concentration of 0.5 μ Ci/mL as described elsewhere. Briefly, 24

hours later, cultures were washed with PBS, fixed with 95% methanol, treated with 10% TCA at 4°C and dissolved in 0.3 N NaOH. Aliquots were counted on a β -counter (BECKMAN CULTER™, LS 6500 Multipurpose Scintillation Counter).

Carotid artery ligation experiments and morphometric analysis

Permanent ligation of the left common carotid artery was performed in 4-month-old TgNOR-1 and wild-type mice. Animals were anesthetized with intraperitoneal injection of ketamine (45 mg/kg body weight) and medetomidine (0.6 mg/kg body weight). A midline neck incision was made and the left common carotid artery was carefully dissected under a dissecting microscope and ligated with a 7-0 silk suture just proximal to its bifurcation. After closing the wound, mice were allowed to recover and maintained on standard chow diet for 3 weeks. Each experimental group included 7 animals. Following euthanasia, the carotid arteries were excised, fixed in 4% paraformaldehyde for 24 h and embedded in paraffin for morphometry and immunohistochemistry analysis [32]. Serial sections (5 μ m thick) of the paraffin-embedded arteries were cut using a microtome (Leica JUNG RM 2055). **Cross-sections at different distances from ligature were cut and stained with hematoxylin and eosin.** **Images corresponding** to cross-sections at 1.5, 1.7 and 1.9 mm from the ligation site were captured with a microscope (Olympus Vanox AHB3) at 100X magnification and analyzed using the image analysis software ImageJ (NIH, Bethesda, MD). Three sections for each carotid artery were measured, and the data were averaged. The circumferences of the lumen, internal elastic lamina (IEL) and external elastic lamina (EEL) were obtained by tracing their contours on digitalized images. Medial, intimal and lumen areas were calculated from these parameters. The media area is the area between EEL and IEL, and the intima area was calculated by subtracting the lumen area from the IEL area. The % stenosis was calculated as: $[\text{intima area} / (\text{intima area} + \text{lumen area})] \times 100$.

Statistical Analyses

Results are shown as mean \pm standard deviation (unless otherwise stated), or median and interquartile range (IQR), as appropriate. Significant differences were established by Student's *t*-test, or Mann–Whitney *U*-test for unpaired data. Analysis of data was done using the GraphPad InStat programme (GraphPad Software V2.03). Differences were considered significant at $p < 0.05$.

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CONFLICT OF INTEREST STATEMENT

None

REFERENCES

1. Dzau, V.J., Braun-Dullaeus, R.C., Sedding, D.G. (2002) Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat. Med.*, **8**, 1249-1256.
2. Fuster, J.J., Fernandez, P., Gonzalez-Navarro, H., Silvestre, C., Abu Nabah, Y.N., Andres, V. (2010) Control of cell proliferation in atherosclerosis: insights from animal models and human studies. *Cardiovasc Res.*, **86**, 254-264.
3. Daemen, J., Serruys, P.W. (2007) Drug-eluting stent update 2007: part I. A survey of current and future generation drug-eluting stents: meaningful advances or more of the same? *Circulation.*, **116**, 316-328.
4. Wessely, R. (2010) New drug-eluting stent concepts. *Nat Rev Cardiol.*, **7**, 194-203.
5. Andres, V. (2004) Control of vascular cell proliferation and migration by cyclin-dependent kinase signalling: new perspectives and therapeutic potential. *Cardiovasc Res.*, **63**, 11-21.
6. Kumar, M.S., Owens, G.K. (2003) Combinatorial control of smooth muscle-specific gene expression. *Arterioscler Thromb Vasc Biol.*, **23**, 737-747.
7. Martinez-Gonzalez, J., Rius, J., Castello, A., Cases-Langhoff, C., Badimon, L. (2003) Neuron-derived orphan receptor-1 (NOR-1) modulates vascular smooth muscle cell proliferation. *Circ Res.*, **92**, 96-103.
8. Nomiya, T., Nakamachi, T., Gizard, F., Heywood, E.B., Jones, K.L., Ohkura, N., Kawamori, R., Conneely, O.M., Bruemmer, D. (2006) The NR4A orphan nuclear receptor NOR1 is induced by platelet-derived growth factor and mediates vascular smooth muscle cell proliferation. *J Biol Chem.*, **281**, 33467-33476.

9. Martinez-Gonzalez, J., Badimon, L. (2005) The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. *Cardiovasc Res.*, **65**, 609-618.
10. Bonta, P.I., Pols, T.W., de Vries, C.J. (2007) NR4A nuclear receptors in atherosclerosis and vein-graft disease. *Trends Cardiovasc Med.*, **17**, 105-111.
11. Zhao, Y., Bruemmer, D. (2009) NR4A Orphan Nuclear Receptors in Cardiovascular Biology. *Drug Discov Today Dis Mech.*, **6**, e43-e48.
12. Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N.P., Perlmann, T. (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature.*, **423**, 555-560.
13. Arkenbout, K., de Waard, V., van Bragt, M., van Achterberg, T., Grimberg, J., Pichon, B., Pannekoek, H., de Vries, C.J. (2002) Protective function of transcription factor TR3 orphan receptor in atherogenesis: decreased lesion formation in carotid artery ligation model in TR3 transgenic mice. *Circulation.*, **106**, 1530-1535.
14. Arkenbout, E.K., van Bragt, M., Eldering, E., van Bree, C., Grimbergen, J.M., Quax, P.H., Pannekoek, H., de Vries, C.J. (2003) TR3 orphan receptor is expressed in vascular endothelial cells and mediates cell cycle arrest. *Arterioscler Thromb Vasc Biol.*, **23**, 1535-1540.
15. Bonta, P.I., van Tiel, C.M., Vos, M., Pols, T.W., van Thienen, J.V., Ferreira, V., Arkenbout, E.K., Seppen, J., Spek, C.A., van der Poll, T., *et al.* (2006) Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses. *Arterioscler Thromb Vasc Biol.*, **26**, 2288-2294.
16. Zhao, Y., Howatt, D.A., Gizard, F., Nomiya, T., Findeisen, H.M., Heywood, E.B., Jones, K.L., Conneely, O.M., Daugherty, A., Bruemmer, D. (2010) Deficiency of the

- NR4A orphan nuclear receptor NOR1 decreases monocyte adhesion and atherosclerosis. *Circ Res.*, **107**, 501-511.
17. Nomiya, T., Zhao, Y., Gizard, F., Findeisen, H.M., Heywood, E.B., Jones, K.L., Conneely, O.M., Bruemmer, D. (2009) Deficiency of the NR4A neuron-derived orphan receptor-1 attenuates neointima formation after vascular injury. *Circulation.*, **119**, 577-586.
 18. Rius, J., Martinez-Gonzalez, J., Crespo, J., Badimon, L. (2004) Involvement of neuron-derived orphan receptor-1 (NOR-1) in LDL-induced mitogenic stimulus in vascular smooth muscle cells: role of CREB. *Arterioscler Thromb Vasc Biol.*, **24**, 697-702.
 19. Crespo, J., Martinez-Gonzalez, J., Rius, J., Badimon, L. (2005) Simvastatin inhibits NOR-1 expression induced by hyperlipemia by interfering with CREB activation. *Cardiovasc Res.*, **67**, 333-341.
 20. Liu, D., Jia, H., Holmes, D.I., Stannard, A., Zachary, I. (2003) Vascular endothelial growth factor-regulated gene expression in endothelial cells: KDR-mediated induction of Egr3 and the related nuclear receptors Nur77, Nurr1, and Nor1. *Arterioscler Thromb Vasc Biol.*, **23**, 2002-2007.
 21. Rius, J., Martinez-Gonzalez, J., Crespo, J., Badimon, L. (2006) NOR-1 is involved in VEGF-induced endothelial cell growth. *Atherosclerosis.*, **184**, 276-282.
 22. Martorell, L., Martínez-González, J., Crespo, J., Calvayrac, O., Badimon, L. (2007) Neuron-derived orphan receptor-1 (NOR-1) is induced by thrombin and mediates vascular endothelial cell growth. *J Thromb Haemost.*, **5**, 1766-1773.
 23. DeYoung, R.A., Baker, J.C., Cado, D., Winoto, A. (2003) The orphan steroid receptor Nur77 family member Nor-1 is essential for early mouse embryogenesis. *J Biol Chem.*, **278**, 47104-47109.

24. Li, L., Miano, J.M., Mercer, B., Olson, E.N. (1996) Expression of the SM22alpha promoter in transgenic mice provides evidence for distinct transcriptional regulatory programs in vascular and visceral smooth muscle cells. *J Cell Biol.*, **132**, 849-859.
25. Ohkura, N., Ito, M., Tsukada, T., Sasaki, K., Yamaguchi, K., Miki, K. (1996) Structure, mapping and expression of a human NOR-1 gene, the third member of the Nur77/NGFI-B family. *Biochim Biophys Acta.*, **1308**, 205-214.
26. Nagy, A., Gertsenstein, M., Vintersten, K., Behringer, R. (2003) Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. Churchman, A.T., Siow, R.C. (2009) Isolation, culture and characterisation of vascular smooth muscle cells. *Methods Mol Biol.*, **467**, 127-138.
28. Guadall, A., Orriols, M., Rodriguez-Calvo, R., Calvayrac, O., Crespo, J., Aledo, R., Martinez-Gonzalez, J., Rodriguez, C. (2011) Fibulin-5 is up-regulated by hypoxia in endothelial cells through a HIF-1{alpha} dependent mechanism. *J Biol Chem.*, **286**, 7093-7103.
29. Fuster, J.J., González-Navarro, H., Vinué, A., Molina-Sánchez, P., Andrés-Manzano, M.J., Nakayama, K.I., Nakayama, K., Díez-Juan, A., Bernad, A., Rodríguez, C., *et al.* (2011) Deficient p27 phosphorylation at serine 10 increases macrophage foam cell formation and aggravates atherosclerosis through a proliferation-independent mechanism. *Arterioscler Thromb Vasc Biol.*, **31**, 2455-2463.
30. Martorell, L., Gentile, M., Rius, J., Rodriguez, C., Crespo, J., Badimon, L., Martinez-Gonzalez, J. (2009) The hypoxia-inducible factor 1/NOR-1 axis regulates the survival response of endothelial cells to hypoxia. *Mol Cell Biol.*, **29**, 5828-5842.

31. Maira, M., Martens, C., Philips, A., Drouin, J. (1999) Heterodimerization between members of the Nur subfamily of orphan nuclear receptors as a novel mechanism for gene activation. *Mol Cell Biol.*, **19**, 7549-7557.
32. Zhang, L.N., Wilson, D.W., da Cunha, V., Sullivan, M.E., Vergona, R., Rutledge, J.C., Wang, Y.X. (2006) Endothelial NO synthase deficiency promotes smooth muscle progenitor cells in association with upregulation of stromal cell-derived factor-1alpha in a mouse model of carotid artery ligation. *Arterioscler Thromb Vasc Biol.*, **26**, 765-772.
33. Assinder, S.J., Stanton, J.A., Prasad, P.D. (2009) Transgelin: an actin-binding protein and tumour suppressor. *Int J Biochem Cell Biol.*, **41**, 482-486.
34. Solway, J., Seltzer, J., Samaha, F.F., Kim, S., Alger, L.E., Niu, Q., Morrissey, E.E., Ip, H.S., Parmacek, M.S. (1995) Structure and expression of a smooth muscle cell-specific gene, SM22 alpha. *J Biol Chem.*, **270**, 13460-13469.
35. Kakkar, R., Ye, B., Stoller, D.A., Smelley, M., Shi, N.Q., Galles, K., Hadhazy, M., Makielski, J.C., McNally, E.M. (2006) Spontaneous coronary vasospasm in KATP mutant mice arises from a smooth muscle-extrinsic process. *Circ Res.*, **98**, 682-689.
36. Li, L., Miano, J.M., Cserjesi, P., Olson, E.N. (1996) SM22 alpha, a marker of adult smooth muscle, is expressed in multiple myogenic lineages during embryogenesis. *Circ Res.*, **78**, 188-195.
37. Rensen, S.S., Doevendans, P.A., van Eys, G.J. (2007) Regulation and characteristics of vascular smooth muscle cell phenotypic diversity. *Neth Heart J.*, **15**, 100-108.
38. Kumar, A., Lindner, V. (1997) Remodeling with neointima formation in the mouse carotid artery after cessation of blood flow. *Arterioscler Thromb Vasc Biol.*, **17**, 2238-2244.
39. Pires, N.M., Pols, T.W., de Vries, M.R., van Tiel, C.M., Bonta, P.I., Vos, M., Arkenbout, E.K., Pannekoek, H., Jukema, J.W., Quax, P.H., *et al.* (2007) Activation of nuclear

receptor Nur77 by 6-mercaptopurine protects against neointima formation. *Circulation.*, **115**, 493-500.

40. Bonta, P.I., Pols, T.W., van Tiel, C.M., Vos, M., Arkenbout, E.K., Rohlena, J., Koch, K.T., de Maat, M.P., Tanck, M.W., de Winter, R.J., *et al.* (2010) Nuclear receptor Nurrl is expressed in and is associated with human restenosis and inhibits vascular lesion formation in mice involving inhibition of smooth muscle cell proliferation and inflammation. *Circulation.*, **121**, 2023-2032.
41. Owens, G.K., Kumar, M.S., Wamhoff, B.R. (2004) Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol Rev.*, **84**, 767-801.
42. Gizard, F., Zhao, Y., Findeisen, H.M., Qing, H., Cohn, D., Heywood, E.B., Jones, K.L., Nomiya, T., Brummer, D. (2011) Transcriptional regulation of S phase kinase-associated protein 2 by NR4A orphan nuclear receptor NOR1 in vascular smooth muscle cells. *J Biol Chem.*, **286**, 35485-35493.

FIGURE LEGENDS

Figure 1. Structure of the human NOR-1 transgene. (A) The transgene consisted of a full-length human NOR-1 cDNA (shown in grey; (GenBank Accession No. D78579; from position 513 to 2872) fused to the rabbit β -globin poly (A) tail (shaded fragment) and driven by the proximal mouse SM22 α promoter (shown in black; GenBank Accession No. U36589; position 2145 to 2797). Light grey correspond to hNOR-1 cDNA untranslated regions. Purified *Sall*-*Hind*III fragment was used for microinjection into pro-nuclei of mouse zygotes. (B) Human NOR-1 (hNOR-1) mRNA levels (analyzed by real-time PCR) in rat VSMC transfected with the resulting construct pCAGGS-SM/hNOR1 (pNOR-1 in the figure). In rat VSMC transfected with the empty vector (control) hNOR-1 mRNA levels were undetectable

but an arbitrary value of 1 was attributed for comparative purposes only. Data (relative to control) are expressed as mean \pm SD (n= 6). *p<0.001 vs. control. (C) NOR-1 protein levels in rat VSMC transfected with pCAGGS-SM/hNOR1 (pNOR-1 in the figure). Levels of β -actin are shown as a loading control.

Figure 2. Expression of NR4A receptors in the aorta of NOR-1 transgenic mice. Two stable transgenic mouse lines (TgNOR-1) were established: family (a) and (b). (A) An ethidium bromide-agarose gel showing the fragment of hNOR-1 (579 bp) amplified by PCR using genomic DNA from transgenic mice of each family. The amplicon was absent in genomic DNA from wild-type (WT) animals. (B) Bar graphs showing human NOR-1 (hNOR-1) mRNA levels (analyzed by real-time PCR) in the aorta of mice from both transgenic families. In wild-type mice hNOR-1 mRNA levels were undetectable but an arbitrary value of 1 was attributed for comparative purposes only. Data (relative to levels in WT) are expressed as mean \pm SEM from at least 9 animals. *p<0.001 vs. WT. (C) Endogenous expression of mouse NR4A receptors in aorta from wild-type (white bars) and transgenic mice (black bars) was analyzed by real-time PCR. Data (relative to levels in WT) are expressed as mean \pm SEM from at least 9 animals.

Figure 3. Expression of NOR-1 in mouse tissues. (A) Representative immunoblot showing NOR-1 protein levels in aorta from wild-type (WT) and transgenic mice (families a and b). Levels of β -actin are shown as a loading control. (B) Immunohistochemical analysis of NOR-1 in aorta and carotid arteries from both NOR-1 transgenic (TgNOR-1) and WT animals. Squares indicate the areas showed a high magnification. [Bar = 50 μ m]. (C) Human NOR-1 (hNOR-1) mRNA levels (analyzed by real-time PCR) in several mouse organs. For

comparative purposes, an arbitrary value of 100 was attributed to hNOR-1 mRNA levels in aorta. Data are expressed as mean \pm SEM from at least 9 animals. [S. Intes: small intestine].

Figure 4. NOR-1 is over-expressed in aortic VSMC from transgenic mice. (A) Human NOR-1 (hNOR-1) mRNA levels (analyzed by real-time PCR) in aortic VSMC from wild-type (WT) and transgenic mice (TgNOR-1). In aortic VSMC from WT mice hNOR-1 mRNA levels were undetectable but an arbitrary value of 1 was attributed for comparative purposes only. Data (relative to levels in WT) are expressed as mean \pm SD (n= 6). *p<0.001 vs. WT. (B) Representative immunoblot showing NOR-1 protein levels in nuclear extracts from aortic VSMC. Levels of nucleolin are shown as a loading control. (C) Representative microphotography showing NOR-1 levels in aortic VSMC from WT and transgenic mice. (D) Endogenous expression levels of mouse NR4A receptors in aortic VSMC from WT (white bars) and transgenic mice (black bars) were analyzed by real-time PCR. Data (relative to levels in WT) are expressed as mean \pm SD (n= 6).

Figure 5. NOR-1 transgenic mice (TgNOR-1) express a functional human NOR-1 protein. (A) Luciferase activity in aortic VSMC from WT and transgenic mice transfected with a reporter construct (clone JA982) containing several functional elements responsive to NOR-1 (minimal pro-opiomelanocortin [POMC] promoter and three NBRE). Data (relative to levels in WT) are expressed as mean \pm SD (n= 6). *p<0.001 vs. WT. (B) Representative autoradiogram of an EMSA performed using a ³²P-labelled probe containing a NBRE consensus and nuclear protein extracts from WT (or transgenic) mouse aortic VSMC. The position of one complex (I), which is enhanced in lanes corresponding to protein extracts from TgNOR-1 VSMC and that is competed by a molar excess of unlabelled probe (100-fold [100X]) is indicated. [NE: nuclear extracts; UP: unlabelled probe]

Figure 6. Mitogenic activity in aortic VSMC from NOR-1 transgenic mice. (A) [³H]-thymidine incorporation into DNA synthesis in aortic VSMC from wild-type (WT) and NOR-1 transgenic (TgNOR-1) mice cultured in complete medium. Data (relative to WT cells) are expressed as a mean ± SD (n= 6). *p<0.001 vs. WT. (B) [³H]-thymidine incorporation into DNA synthesis in aortic VSMC from WT and TgNOR-1 mice that were growth-arrested by incubation with a growth factor-poor medium (0.2% FCS for 24 h) and subsequently induced with 20% FCS. White bars correspond to cells maintained in arrest medium and black bars to FCS-induced cells. Data (relative to WT arrested cells) are expressed as mean ± SD of (n= 6). *p<0.001 vs. arrested WT cells; #p<0.001 vs. arrested TgNOR-1 cells. (C) **Representative immunoblot and microphotography showing levels of the embryonic smooth muscle myosin heavy chain (SMemb) in aortic VSMC from WT and transgenic mice. Unchanged levels of the smooth muscle myosin heavy chain (MHC) isoforms SM1 and SM2 (SM1/2) are shown as a loading control.**

Figure 7. Transgenesis of NOR-1 exacerbates neointimal hyperplasia in the mouse carotid ligation model. Permanent ligation of the left common carotid artery was performed in wild-type (WT) and NOR-1 transgenic (TgNOR-1) mice. (A) **Representative hematoxylin-and-eosin-stained sections of ligated carotid arteries from WT and TgNOR-1 mice at different distances from ligature.** (B-E) Graphs showing the quantification of the intima area (B), the media area (C), the intima/media ratio (D) and the percentage of stenosis (E) in carotid segments from WT and TgNOR-1 undergoing vascular remodelling after carotid ligation (**as indicated in Materials and Methods**). Data are expressed as median with interquartile range and whiskers (B and D) or mean ± SD (C and E). *p<0.001 and †p<0.01 vs. WT.

ABBREVIATIONS

ABC: avidin-biotin peroxidase complex; DAB: 3,3'-diaminobenzidine; DMEM: Dulbecco's modified Eagle's medium; EEL: external elastic lamina; EMSA: electrophoretic mobility shift assay; FCS: foetal calf serum; IEL: internal elastic lamina; **myosin heavy chain (MHC)**; NGFI-B: nerve growth factor-induced gene-B; NBRE: NGFI-B response element; NOR-1: neuron derived orphan receptor-1; NR4A3: nuclear receptor subfamily 4, group A, member 3; PMSG: pregnant mare's serum gonadotropin; POMC: pro-opiomelanocortin; **SMemb: embryonic smooth muscle myosin heavy chain**; VSMC: vascular smooth muscle cells; WT: wild-type.

Figure 1

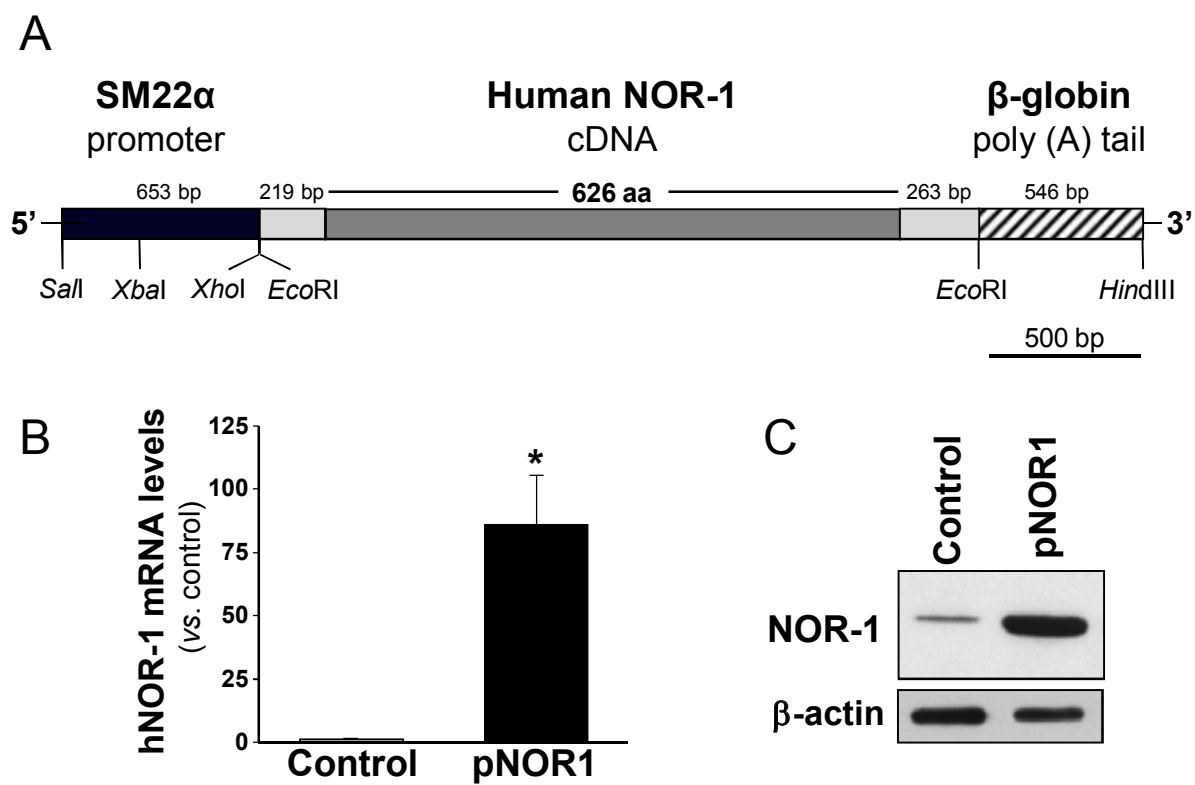


Figure 2

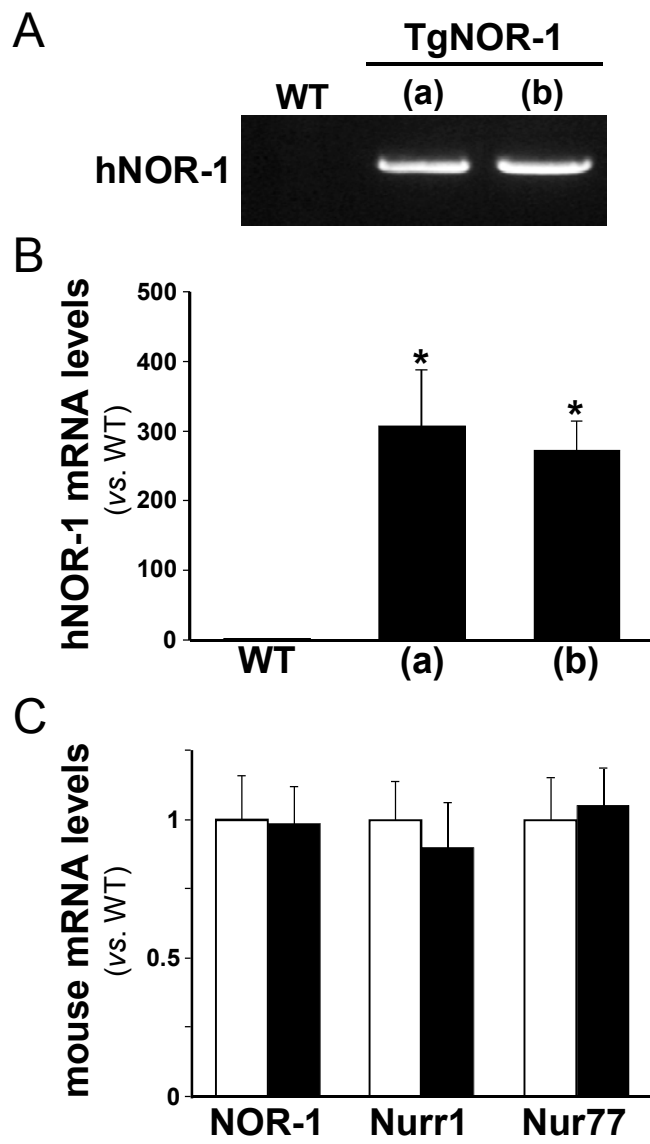


Figure 3

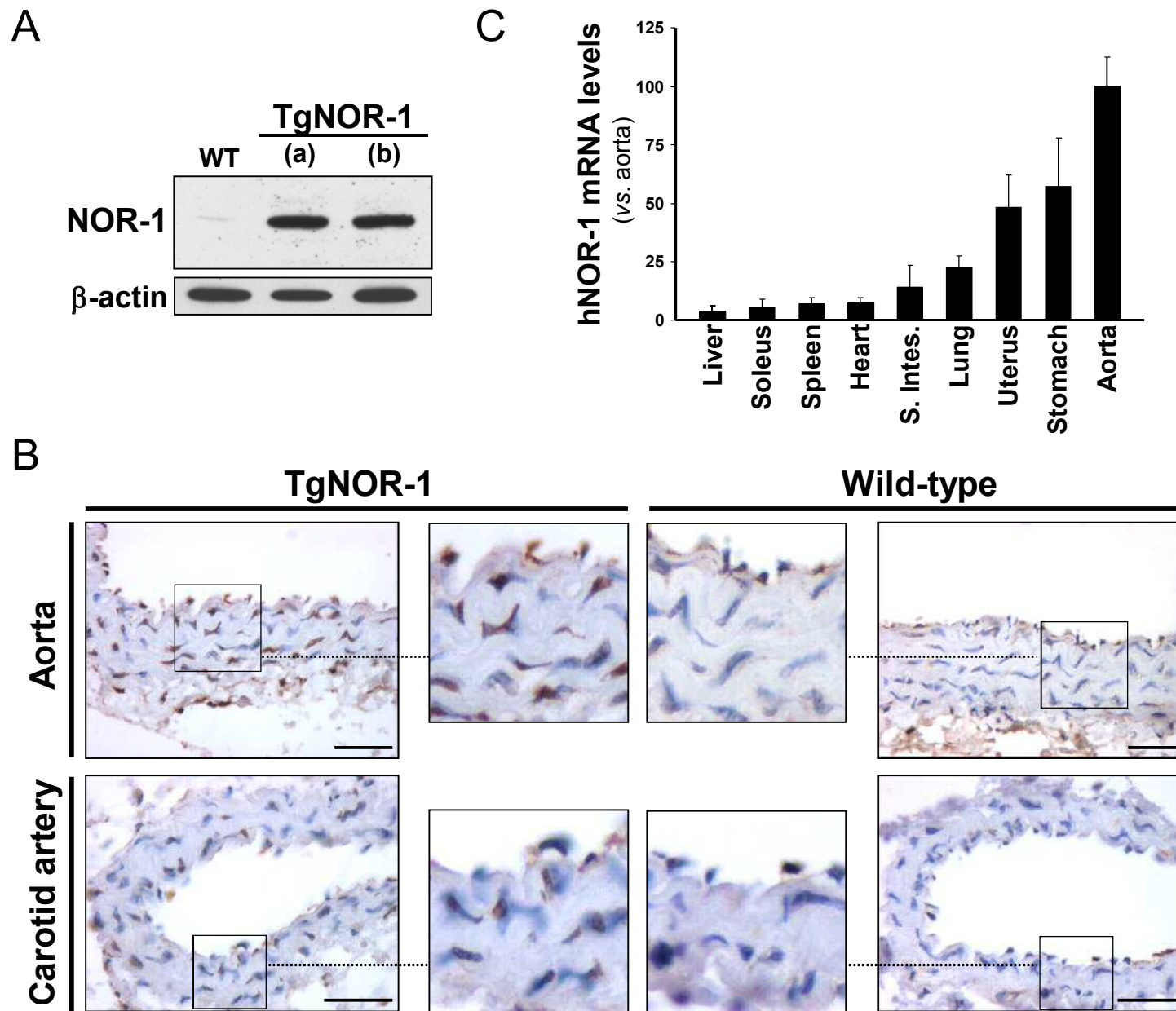


Figure 4

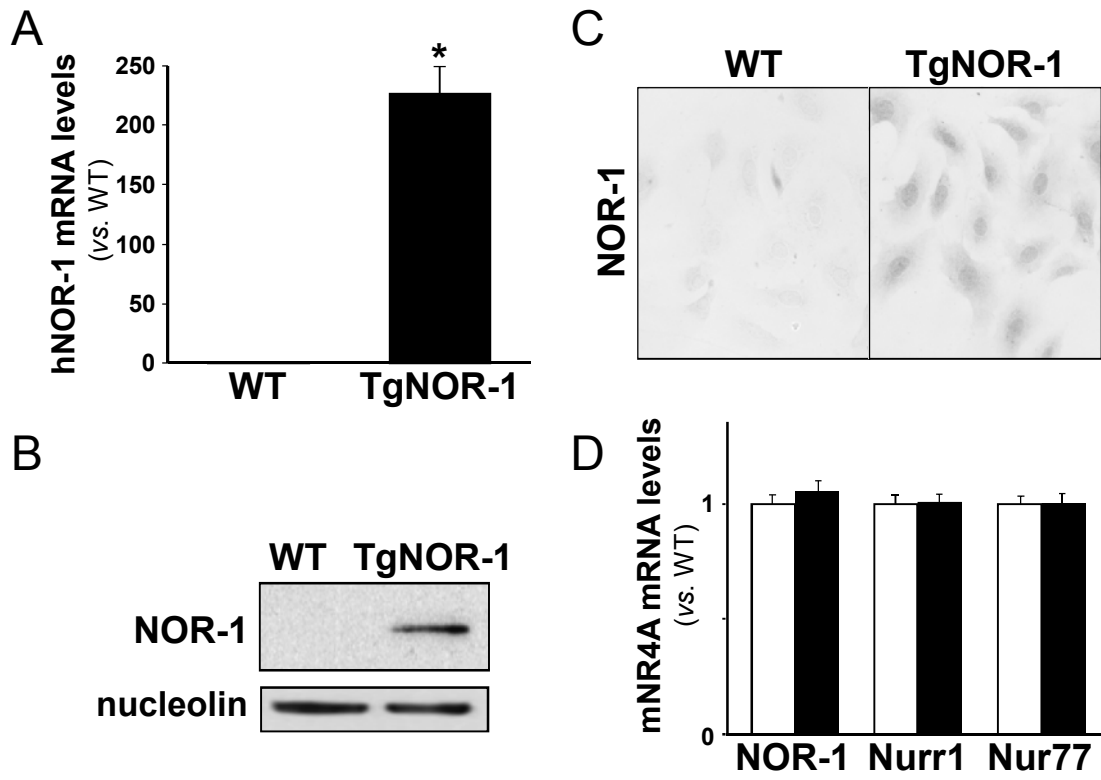


Figure 5

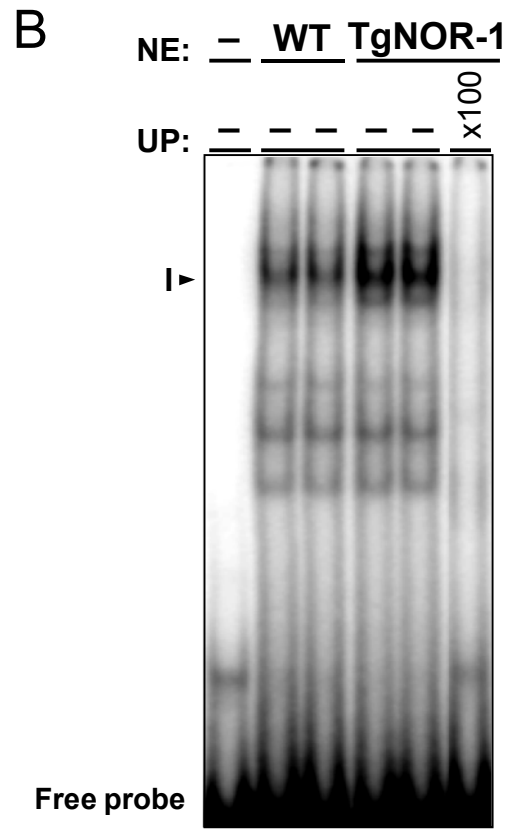
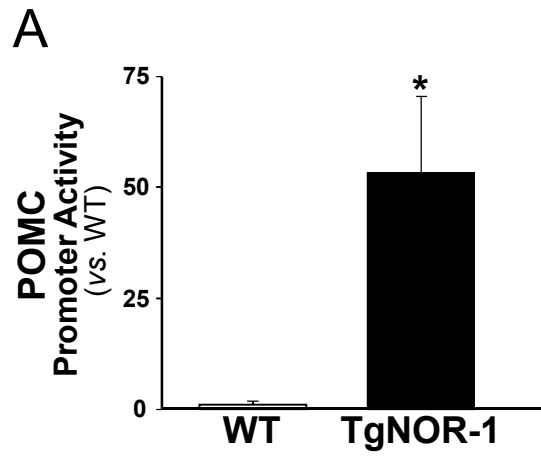


Figure 6

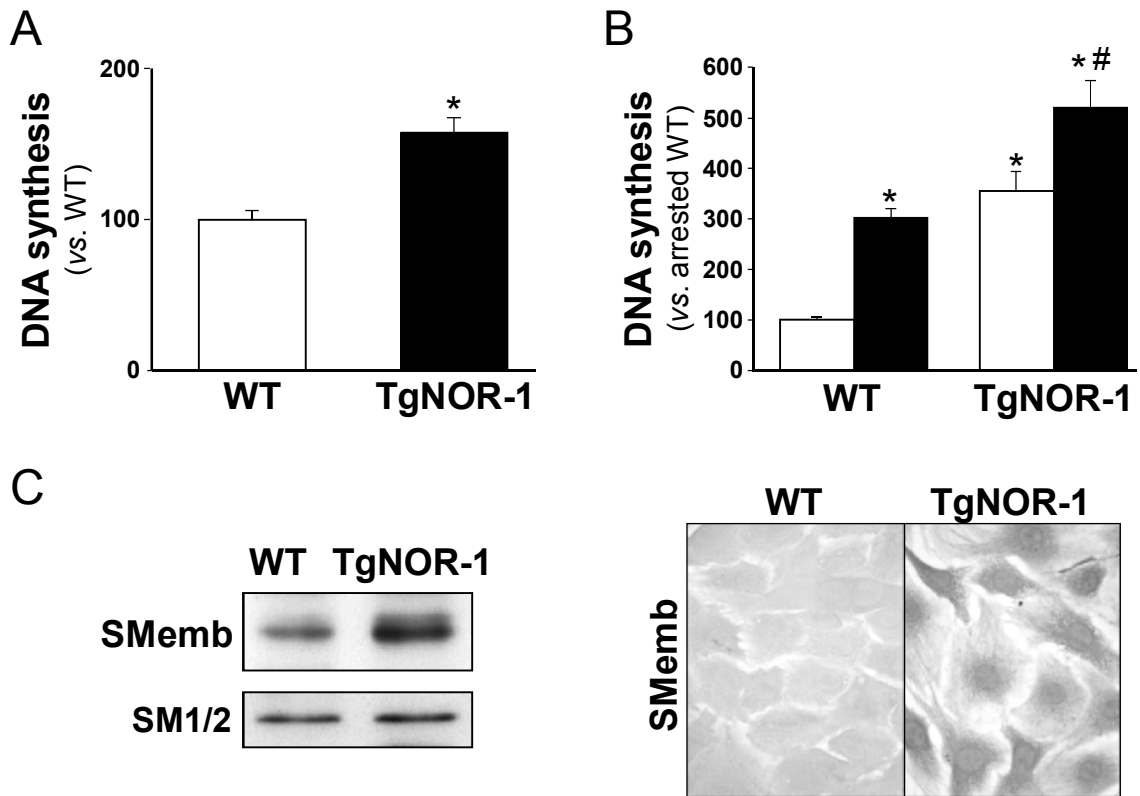


Figure 7

