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Ricardo Rodríguez-Calvo, Anna Guadall, Olivier Calvayrac, María A Navarro, Judith Alonso, et al.. Over-expression of Neuron-derived Orphan Receptor-1 (NOR-1) exacerbates neointimal hyperplasia after vascular injury. *Human Molecular Genetics*, 2013, 22 (10), pp.1949-1959. 10.1093/hmg/ddt042 . hal-03026064

HAL Id: hal-03026064

<https://hal.science/hal-03026064v1>

Submitted on 26 Nov 2020

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

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Received November 9, 2012; Revised and Accepted January 30, 2013

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 (SMCs) and assess the consequence of a gain of function of this receptor on intimal hyperplasia after vascular injury. 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 electrophoretic mobility shift assay 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, $P < 0.01$). 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

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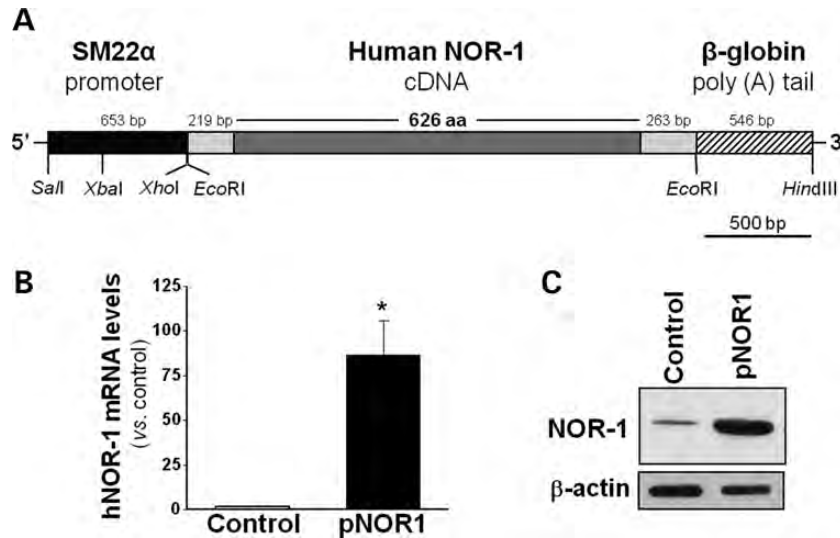


Figure 1. Structure of the human NOR-1 transgene. (A) The transgene consisted of a full-length human NOR-1 cDNA [shown in grey; (GenBank Accession No. D78579; from position 513 to 2872)] fused to the rabbit β -globin poly (A) tail (shaded fragment) and driven by the proximal mouse SM22 α promoter (shown in black; GenBank Accession No. U36589; position 2145 to 2797). Light grey correspond to hNOR-1 cDNA untranslated regions. Purified *Sall-HindIII* fragment was used for microinjection into pro-nuclei of mouse zygotes. (B) Human NOR-1 (hNOR-1) mRNA levels (analysed 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 ($n = 6$). * $P < 0.001$ versus 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.

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 (Fig. 1B 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 (families a and b) were established (Fig. 2A). Families a and b exhibited similar expression levels of hNOR-1 in the aorta (Fig. 2B). NOR-1 transgenesis did not modify the endogenous expression of mouse NR4A receptors (Fig. 2C). NOR-1 protein levels were also similarly increased in the aorta and carotid arteries of both transgenic families as determined by western blot (Fig. 3A) and immunohistochemistry (Fig. 3B and data not shown). Thus, for further experiments we

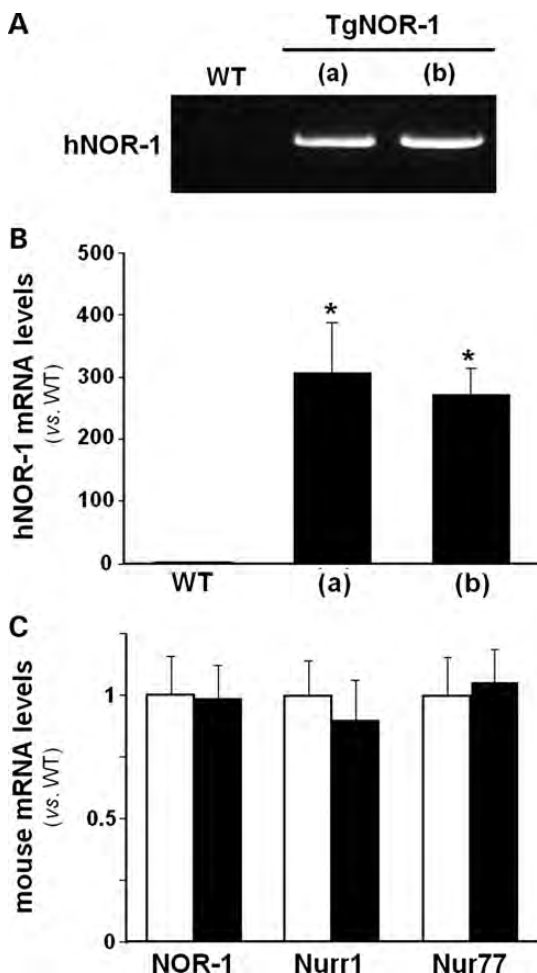


Figure 2. Expression of NR4A receptors in the aorta of NOR-1 transgenic mice. Two stable transgenic mouse lines (TgNOR-1) were established: families (a) and (b). (A) An ethidium bromide-agarose gel showing the fragment of hNOR-1 (579 bp) amplified by PCR using genomic DNA from transgenic mice of each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

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 (Fig. 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 (Fig. 4A), and increased NOR-1 protein levels (Fig. 4B and C). The over-expression of NOR-1 did not alter the endogenous expression of mouse NR4A receptors

(Fig. 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] (24). 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 Electrophoretic mobility shift assay (EMSA) study showed that nuclear extracts from NOR1-transgenic VSMC exhibited higher binding capacity to a consensus NBRE site than those from control cells (Fig. 5B).

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

Next we determined the incorporation of [3 H]-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 (Fig. 6A) and in growth-arrested cells stimulated with fetal calf serum (FCS) (Fig. 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 (Fig. 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 remodelling reliably persistent up to 2400 μ m from ligation (Fig. 7A). TgNOR-1 exhibited an exaggerated vascular remodelling (Fig. 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 (Fig. 7B–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

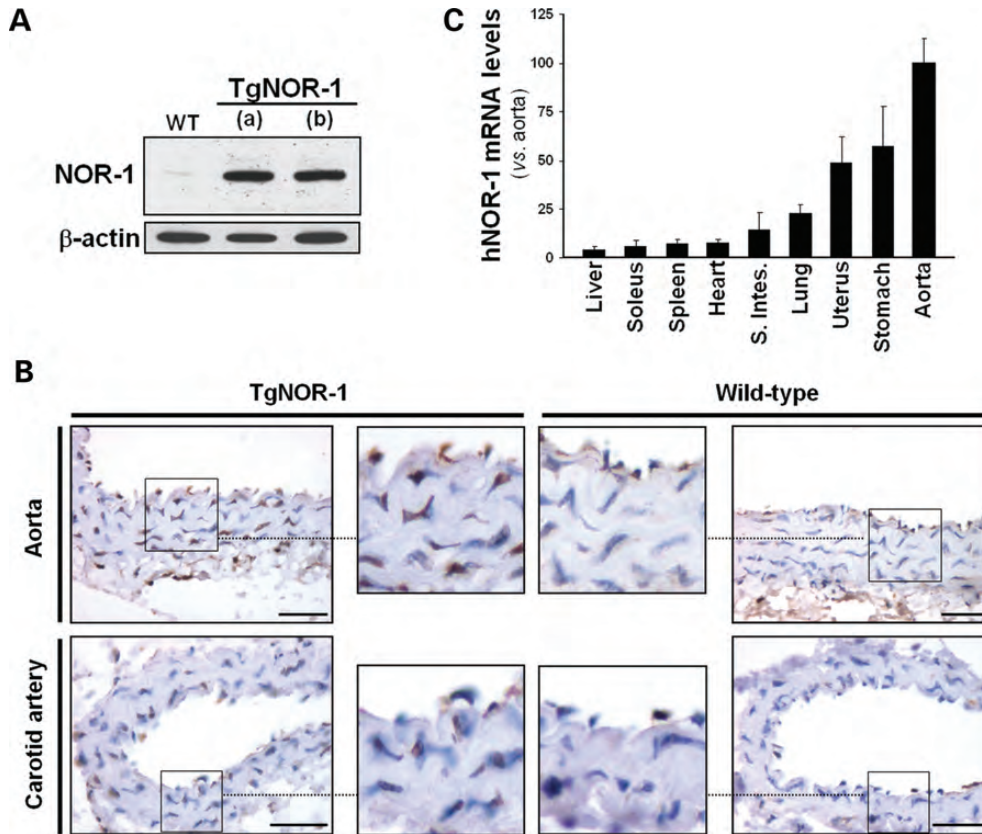


Figure 3. Expression of NOR-1 in mouse tissues. **(A)** Representative immunoblot showing NOR-1 protein levels in the aorta from wild-type (WT) and transgenic mice (families a and b). Levels of β -actin are shown as a loading control. **(B)** Immunohistochemical analysis of NOR-1 in the aorta and carotid arteries from both NOR-1 transgenic (TgNOR-1) and WT animals. Squares indicate the areas shown at high magnification (bar = 50 μ m). **(C)** Human NOR-1 (hNOR-1) mRNA levels (analysed by real-time PCR) in several mouse organs. For comparative purposes, an arbitrary value of 100 was attributed to hNOR-1 mRNA levels in the aorta. Data are expressed as mean \pm SEM from at least nine animals (S. Intes, small intestine).

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 VSMCs, 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 (25). 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, which are critical for the specific transcription of SM22 α in SMC (26,27). 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 (13,26,28). 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 SM22alpha-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 (26,27,29). 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 the hNOR-1-transgenic protein in TgNOR-1, we comparatively analysed 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 (24). 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

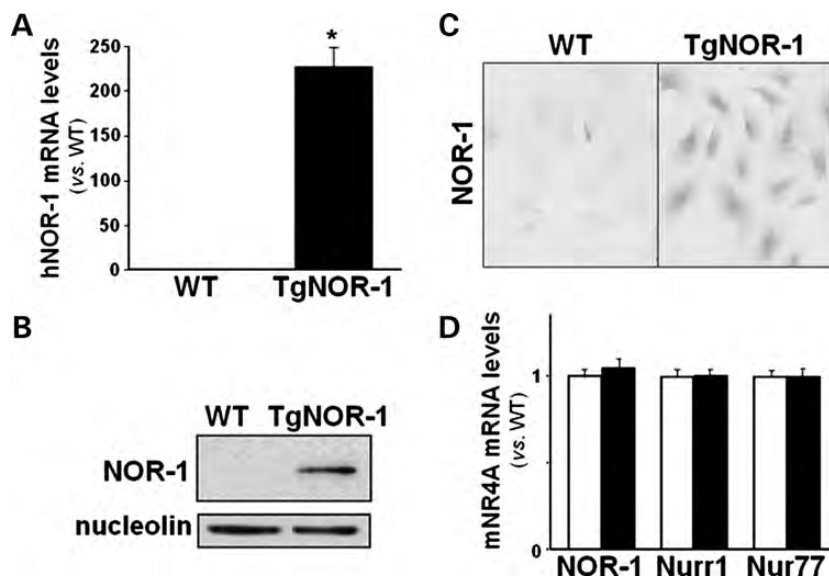


Figure 4. NOR-1 is over-expressed in aortic VSMC from transgenic mice. **(A)** Human NOR-1 (hNOR-1) mRNA levels (analysed by real-time PCR) in aortic VSMC from wild-type (WT) and transgenic mice (TgNOR-1). In aortic VSMC from WT mice, hNOR-1 mRNA levels were undetectable but an arbitrary value of 1 was attributed for comparative purposes only. Data (relative to levels in WT) are expressed as mean \pm SD ($n = 6$). * $P < 0.001$ versus 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 analysed by real-time PCR. Data (relative to levels in WT) are expressed as mean \pm SD ($n = 6$).

proliferating VSMC (30). 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 (31). 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 (13,14,32,33). The significant effects of NOR-1 on vascular remodelling observed after transgenesis and gene targeting also suggest that this nuclear receptor participates in the regulation of gene networks governing cell cycle progression (34). 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) (35). These genes could be responsible, at least in a part, for 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, 21 June, passed by the Generalitat de

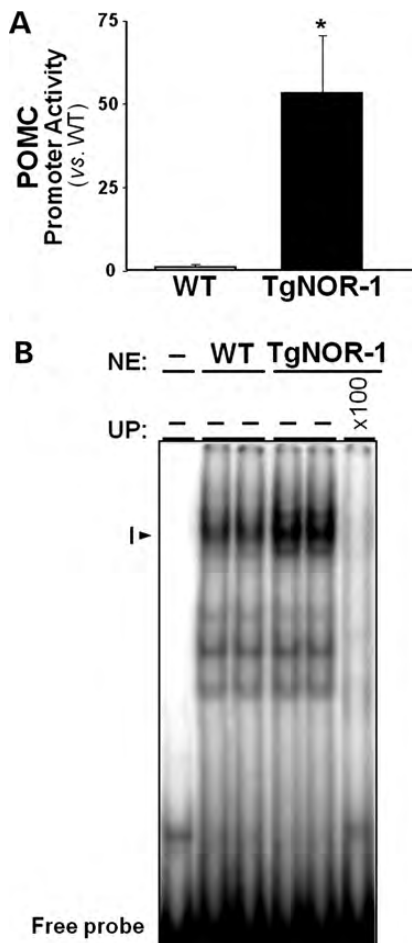


Figure 5. NOR-1 transgenic mice (TgNOR-1) express a functional human NOR-1 protein. (A) Luciferase activity in aortic VSMC from WT and transgenic mice transfected with a reporter construct (clone JA982) containing several functional elements responsive to NOR-1 [minimal pro-opiomelanocortin (POMC) promoter and three NBRE]. Data (relative to levels in WT) are expressed as mean \pm SD ($n = 6$). * $P < 0.001$ versus WT. (B) Representative autoradiogram of an EMSA performed using a 32 P-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.

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 into the mammalian vector pCAGGS. In brief, the proximal promoter of SM22 α (653 bp; GenBank Accession No. U36589; positions 2145–2797) 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, TX, USA) (26), using the following primers: 5'-CACTAGTCGACATGTTCTGCCACGCACTT GG-3' (forward primer; *SalI* restriction site is underlined) and 5'-TAGATCTCGAGGCTTGGTCGTTTGTGGACT-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 513–2872), 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) (36), 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 an SMC-specific promoter (Fig. 1). To validate the construct, rat VSMC were transfected with pCAGGS-SM/hNOR1 and NOR-1 expression was analysed (mRNA and protein levels).

Generation of transgenic mice

The expression cassette (3.6 kb) (Fig. 1A) was recovered by double digestion with *SalI* and *HindIII*, purified by QIAquickTM Gel Extraction Kit (Qiagen) and used for pronuclear injection of zygotes as previously described (37). 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 (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 EmbryoMaxTM 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'-GACAGACTGCTCCAACCTTGG-3' and 5'-GGTTCATGATCTCCGTGGTG-3'. PCR was performed in a volume of 25 μ l containing genomic DNA (100 ng), 1X MyTaqTM Reaction Buffer (Biolone), 0.4 μ M of each primer and 1.25 U of MyTaqTM 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

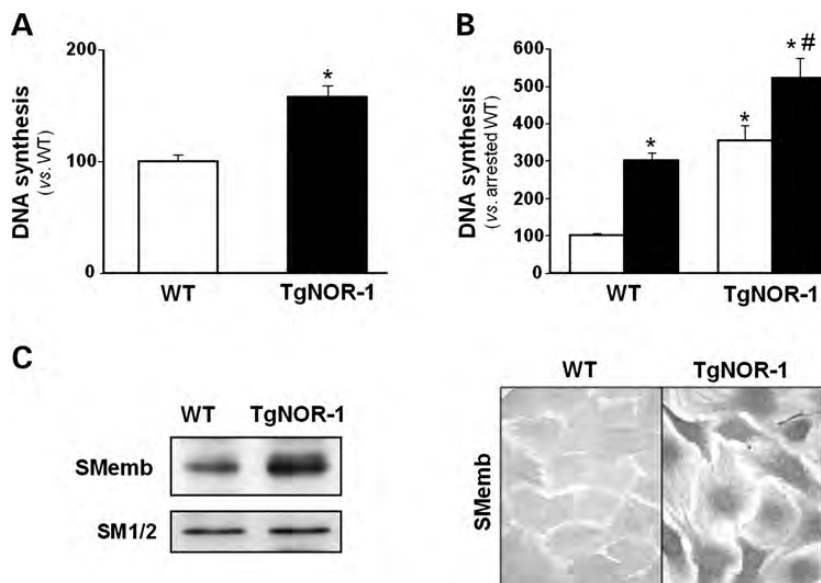


Figure 6. Mitogenic activity in aortic VSMC from NOR-1 transgenic mice. (A) [3 H]-thymidine incorporation into DNA synthesis in aortic VSMC from wild-type (WT) and NOR-1 transgenic (TgNOR-1) mice cultured in the complete medium. Data (relative to WT cells) are expressed as a mean \pm SD ($n = 6$). $*P < 0.001$ versus WT. (B) [3 H]-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 \pm SD of ($n = 6$). $*P < 0.001$ versus arrested WT cells; $\#P < 0.001$ versus 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.

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 the rat or mouse (TgNOR-1 and wild-type) aorta were obtained by the explant technique (38). 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 1–2 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 1–2 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 and 6 were seeded in multi-well 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 Ultra-spectTM reagent (Biotecx Laboratories), and from mouse tissues using the RNeasy Micro kit (Qiagen). RNA integrity was determined by electrophoresis in agarose gel. RNA (1 μ g) 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 expression was used as reference gene (Mm00446973_m1 and Rn01455646_m1) (39). Each sample was amplified in duplicate.

Immunohistochemical and immunocytochemical analyses

Mouse aortas were perfused with phosphate-buffered saline (PBS), removed, fixed in 4% paraformaldehyde/0.1 M PBS (pH 7.4) for 24 h, embedded in paraffin and prepared in 5 μ m cross-sections with a microtome (Jung RM2055, Leica) (40). Sections from the 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,

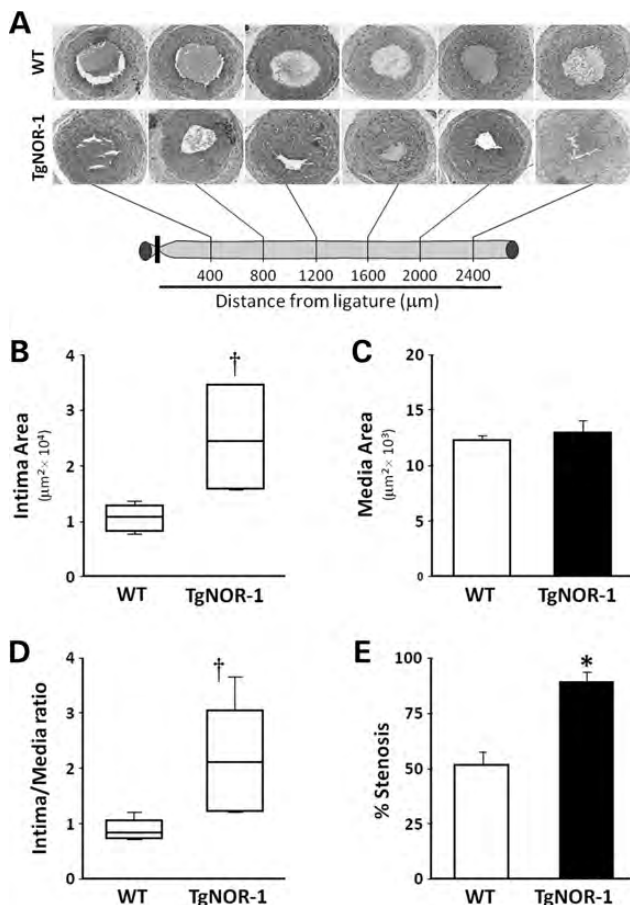


Figure 7. Transgenesis of NOR-1 exacerbates neointimal hyperplasia in the mouse carotid ligation model. Permanent ligation of the left common carotid artery was performed in wild-type (WT) and NOR-1 transgenic (TgNOR-1) mice. (A) Representative haematoxylin-and-eosin-stained sections of ligated carotid 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 \pm SD (C and E). * $P < 0.001$ and † $P < 0.01$ versus WT.

Abnova) overnight at 4°C. After washing three times in PBS–Tween 0.1%, samples were incubated for 1 h with a biotinylated secondary antibody (Vector Laboratories). After rinsing three 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 un-specific 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 analysed 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 μl 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 1 × 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 μM leupeptin and 0.5% SDS (41). Proteins from nuclear and whole-cell extracts (10–20 μg/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 (39).

Electrophoretic mobility shift assay

Nuclear extracts were obtained from VSMC of TgNOR-1 and wild-type mice as described above. A doubled stranded probe containing the nerve growth factor-induced gene-B (NGFI-B) response element (NBRE) (24) 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 (39). EMSAs 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 0.5 \times 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) (24). 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 six-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- 3 H]-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- 3 H]-thymidine into DNA was determined in two sets of cell cultures: cultures maintained in the 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 the complete medium (20% FCS). [6- 3 H]-thymidine (23 Ci/mmol; Amersham) was added to the cells at a concentration of 0.5 μ Ci/ml as described elsewhere. Briefly, 24 h 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 CULTETM, 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 seven animals. Following euthanasia, the carotid arteries were excised, fixed in 4% paraformaldehyde for 24 h and embedded in paraffin for morphometry and immunohistochemistry analysis (42). 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 ligation were cut and stained with haematoxylin 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 \times 100 magnification and analysed using the image analysis software ImageJ (NIH, Bethesda, MD, USA). 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: [intima area / (intima area + 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 $P < 0.05$.

ACKNOWLEDGEMENTS

We are indebted to Prof. Naganari Ohkura, Prof. Eric Olson and Prof. Jacques Drouin for kindly providing us with the human NOR-1 cDNA, the SM22 α promoter and the JA982 construct, respectively. Authors are grateful for the technical assistance to Silvia Aguiló and Marta Sanz.

Conflict of Interest statement. None declared.

FUNDING

This study was partly supported by grants SAF2009-11949, SAF2012-40127, PI12/01952 and Red de Investigación Cardiovascular (RD12/0042/0053) from the Spanish Ministerio de Economía y Competitividad (MINECO)—Instituto de Salud Carlos III (ISCIII). R.R.-C. was supported by a Juan de la Cierva grant from the Spanish MINECO. J.A. is supported by a PhD fellowship (FPU) from the Spanish Ministerio de Educación, and B.F. by a JAE-Pre from the Consejo Superior de Investigaciones Científicas (CSIC).

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