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Original Research Article

Upregulation of NOR-1 in calcified human vascular tissues: impact on osteogenic differentiation and calcification

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ABSTRACT

Cardiovascular calcification is a significant public health issue whose pathophysiology is not fully understood. NOR-1 regulates critical processes in cardiovascular remodeling, but its contribution to ectopic calcification is unknown. NOR-1 was overexpressed in human calcific aortic valves and calcified atherosclerotic lesions colocalizing with RUNX2, a factor essential for osteochondrogenic differentiation and calcification. NOR-1 and osteogenic markers were upregulated in calcifying human valvular interstitial cells (VICs) and human vascular smooth muscle cells (VSMCs). Gain- and loss-of-function approaches demonstrated that NOR-1 negatively modulates the expression of osteogenic genes relevant for the osteogenic transdifferentiation (RUNX2, IL-6, BMP2, and ALPL) and calcification of VICs. VSMCs from transgenic mice overexpressing NOR-1 in these cells (TgNOR-1^{VSMC}) expressed lower basal levels of osteogenic genes (IL-6, BMP2, ALPL, OPN) than cells from WT littermates, and their upregulation by a high-phosphate osteogenic medium (OM) was completely prevented by NOR-1 transgenesis. Consistently, this was associated with a dramatic reduction in the calcification of both transgenic VSMCs and aortic rings from TgNOR-1^{VSMC} mice exposed to OM. Atherosclerosis and calcification were induce in mice by the administration of AAV-PCSK9^{D374Y} and a high-fat/high-cholesterol diet. Challenged-TgNOR-1^{VSMC} mice exhibited decreased vascular expression of osteogenic markers, and both less atherosclerotic burden (assessed in whole aorta and lesion size in aortic arch and brachiocephalic artery) and less vascular calcification (assessed either by near-infrared fluorescence imaging or histological analysis) than WT mice. Our data indicate that NOR-1 negatively modulates the expression of genes critically involved in the osteogenic differentiation of VICs and VSMCs, thereby restraining ectopic cardiovascular calcification.

INTRODUCTION

Cardiovascular calcification is a process in which mineral deposits form in heart valve leaflets and vessel walls increasing tissue stiffness, disrupting their normal biomechanical functions and, ultimately, leading to major clinical complications. Indeed, calcific aortic valve disease (CAVD) drives aortic valve stenosis which can result in left ventricular dysfunction and heart failure. CAVD is becoming a growing public health concern due to the ageing of the population.[1,2] Because there are no effective pharmacological therapies, the management of CAVD patients relies primarily on costly surgical or transcatheter aortic valve replacement procedures. Regarding the vasculature, calcification

Abbreviations: AAV, adeno-associated virus vector; ALPL, alkaline phosphatase, biomineralization associated; BMP2, bone morphogenetic protein 2; BGLAP, bone gamma-carboxyglutamic acid-containing protein; CAVD, calcific aortic valve disease; DKK-1, Dickkopf-related protein 1; HC, high cholesterol; HF, high fat; LDLR, low-density lipoprotein receptor; NBRE, nerve growth factor-responsive element; NIRF, near-infrared fluorescence; NOR-1, neuron-derived orphan receptor-1; NR4A, nuclear receptor subfamily 4A; OM, osteogenic media; OPG, osteoprogeterin; OPN, osteopontin; ORO, Oil Red O; PCSK9, proprotein convertase subtilisin/kexin type 9; RUNX2, Runt-related transcription factor 2; siRNA, small interfering RNAs; SMA, α-alpha-smooth muscle actin; TgNOR-1VSMC, transgenic mice overexpressing NOR-1 in VSMC; TNAP, tissue non-specific alkaline phosphatase; VICs, valvular interstitial cells; VSCMs, vascular smooth muscle cells; WT, wild-type

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At A Glance Commentary

Ballester-Servera C, et al.

Background

Cardiovascular calcification is a significant public health issue whose pathophysiology is not fully understood. The nuclear receptor NOR-1 regulates critical processes in cardiovascular remodeling, but its contribution to ectopic calcification is unknown.

Translational Significance

Studies in human calcific aortic valves and human atheromas, gain-and loss-of-function strategies in cell cultures, and *in vivo* approaches in atherosclerosis-challenged transgenic mice have allowed us to decipher the functional role of NOR-1 as a negative modulator of the gene expression program underlying osteogenic transdifferentiation and calcification of VICs and VSMCs in cardiovascular calcific diseases. These results open up new expectations for fighting against cardiovascular calcification.

deposition associated to atherosclerosis might affect plaque stability, favoring plaque rupture and leading to complications such as myocardial infarction and stroke. In fact, coronary artery calcium score is an independent predictor of major cardiovascular outcomes.[3,4]

CAVD and atherosclerosis are different disease entities; however, in both calcification is an active process with some similarities to bone formation.[5-8] Multiple mechanisms contribute to CAVD progression including cell differentiation, inflammation, oxidative/mechanical stress, fibrosis, and finally calcification.[5,9-12] Valvular interstitial cells (VICs), the major cell type within the aortic valve leaflets, are responsible for maintaining valve homeostasis and structural integrity along with valvular endothelial cells and are also crucial in the development of CAVD. In fact, VICs can undergo osteogenic transdifferentiation into an active phenotype which promotes tissue remodeling and calcification.[5,9-12] This requires the activation of intricate signaling pathways and the upregulation of osteogenic genes controlled by a small number of transcription factors not fully elucidated.[5,9-12] Similarly, in atherosclerosis, intimal calcification is a tightly regulated process driven by the transdifferentiation of vascular smooth muscle cells (VSMCs) into osteoblast-like cells.[9,11,13] A number of key genes/proteins have been involved in the osteogenic reprogramming and calcification process of both VICs (in CAVD) and VSMCs (in calcification associated to atherosclerosis). Among them, RUNX2, a transcription factor essential for osteochondrogenic differentiation and cell calcification,[13-15] stands out along with IL-6, a cytokine critically involved in the switch of the osteogenic program as well as in mineralization.[5,10,12,16]

Neuron-derived orphan receptor-1 (NOR-1) is a member of the NR4A subfamily of nuclear receptors that regulates diverse cellular processes and has been implicated in the pathophysiological mechanisms of cardiovascular diseases.[17-19] Indeed, NOR-1 has been involved, for instance, in intimal hyperplasia and restenosis following vascular injury,[20-22]

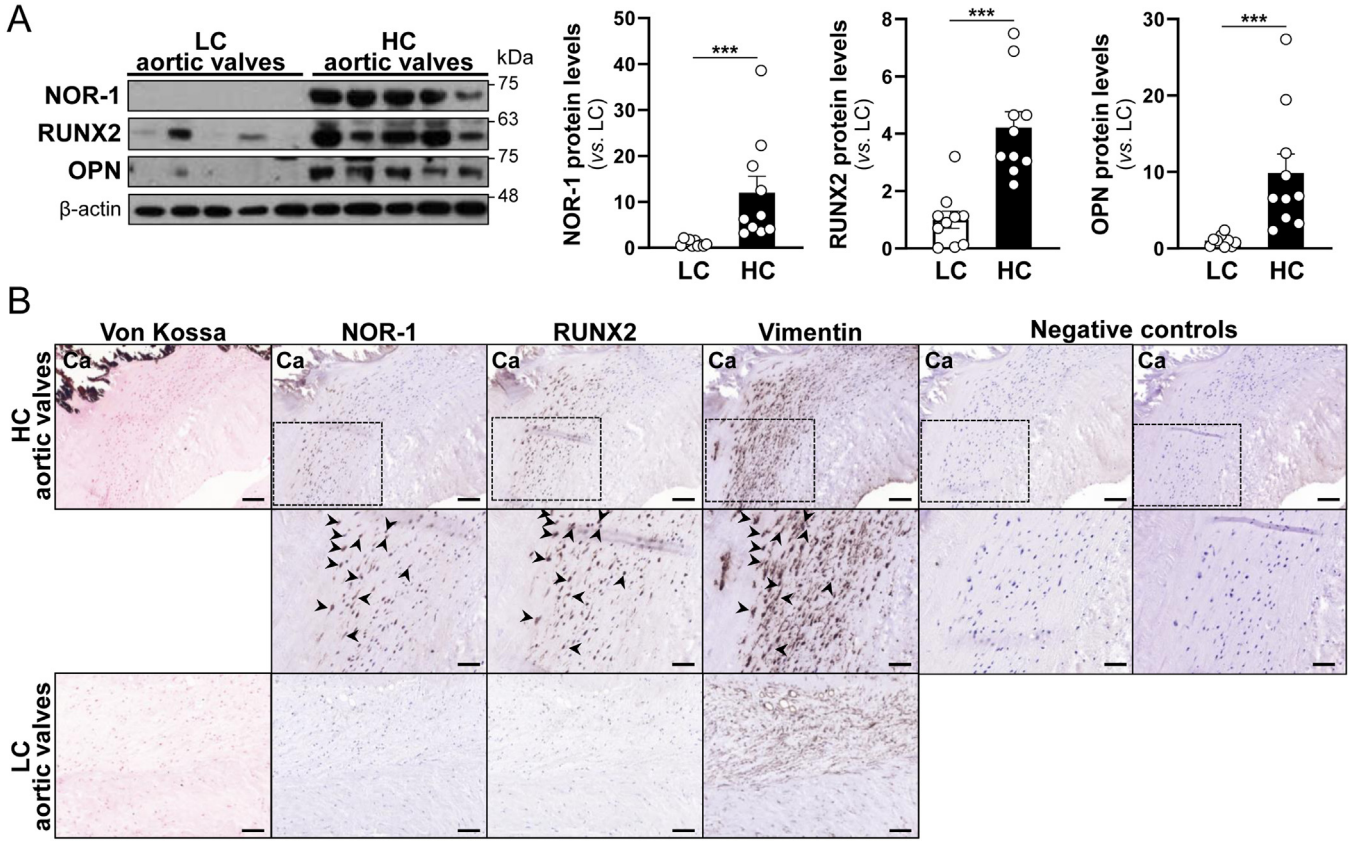


Fig 1. NOR-1 expression is upregulated in calcified human aortic valves. (**A**) Representative immunoblot images showing NOR-1, RUNX2 and osteopontin (OPN) protein levels in low (LC) and high calcified (HC) human aortic valves, and bar graphs showing the result of the densitometric quantification of western blots. NOR-1 band (68 kDa) was significantly increased in HC aortic valves. The levels of β-actin are shown as a loading control. The migration of protein molecular markers is indicated on the right. Data are mean ± SEM (n = 10). Significant differences were determined by Mann−Whitney test. ***$P < 0.001$. (**B**) Representative images of Von Kossa staining and immunostainings for NOR-1, RUNX, and vimentin in HC and LC aortic valves. The indicated areas are magnified in middle panels. Black arrow heads indicate positive cells for NOR-1, RUNX2, and vimentin detected in consecutive sections. The location of the calcium deposit in consecutive sections is indicated as Ca (calcium) in the upper left corner. Negative immunostaining controls for highly calcified aortic valves are shown. The primary antibody was omitted and either anti-mouse (left panel) or anti-rabbit (right panel) biotinylated secondary antibodies were used. Bars: 100 μm (upper and lower panels) and 50 μm (central panels). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

abdominal aortic aneurysm triggered by angiotensin II,[23,24] and cardiac hypertrophy induced by pressure overload or β-adrenergic stimulation.[25,26] NOR-1 has also been associated with atherosclerosis, although there has been some controversy about its pro- or anti-atherogenic role.[20,27,28] We and others have reported how this nuclear receptor modulates the dedifferentiation of VSMC into a migratory and proliferative phenotype in response to different stimuli,[20,29-32] and its role in the activation and in the balance survival/apoptosis balance of vascular endothelial cells.[33-35] As a transcription factor NOR-1 regulates the expression of genes involved in cellular processes which impact on cardiovascular remodeling including cell phenotypic differentiation,[20,29-33] inflammation,[36-38] oxidative stress,[23,39] and extracellular matrix remodeling and fibrosis.[25,40,41] However, whether NOR-1 regulates gene expression associated with cardiovascular calcification is currently unknown. We hypothesize that NOR-1 might play a role in the complex gene network involved in cellular osteogenic transdifferentiation and calcification. To investigate this hypothesis, analysis of human calcified aortic valve leaflets and human atherosclerotic plaques, gain-and loss-of-function studies in VICs, studies in human (hVSMCs) and mouse VSMCs (mVSMCs), and *ex vivo* and *in vivo* approaches in a genetically modified mouse model that specifically overexpresses human NOR-1 in VSMCs were carried out. Or results provide evidence about the role of NOR-1 as a negative modulator of osteogenic genes involved in ectopic cardiovascular calcification.

MATERIALS AND METHODS

A more detailed description of Materials and methods can be found in the Supplemental Materials and Methods.

Human samples

Human aortic valve leaflets were obtained from patients undergoing aortic valve replacement at the Hospital de la Santa Creu i Sant Pau (HSCSP; Barcelona, Spain) according to the Research Ethics Committee (approval number: 19/267). Supplementary Table S1 shows the characteristics of these patients. They were patients with severe aortic valve regurgitation or severe aortic stenosis who were diagnosed and underwent surgical intervention following the recommendations of the European Society of cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS) guidelines.[42] We obtained aortic valves clearly differentiated by their macroscopic degree of calcification, which were classified as low and high-calcified (Supplementary Fig S1). Low calcified aortic valves came from patients with severe aortic valve regurgitation, while those highly calcified came from patients with severe aortic stenosis. Once collected, samples were dissected for VICs isolation, rapidly stored at -80°C for subsequent RNA/protein extraction or processed for immunohistochemical analysis. Human coronary arteries were collected from patients undergoing heart transplant at the HSCSP (approval number: 19/267). Human femoral artery samples were obtained from patients undergoing cardiovascular surgery at the Hospital Clínico Universitario Virgen de la Arrixaca (Murcia, Spain) according to the Research Ethics Committee (approval number: 02/10). These patients underwent femoropopliteal bypass surgery to treat femoral artery disease. They were men, smokers with a mean age of 62 ± 5 years. The use of human discarded tissue was performed in accordance with the Declaration of Helsinki of 1975, revised in 2013. A written informed consent was obtained from all patients or legal representatives.

VSMC and VICs isolation and culture under osteogenic conditions

VICs were isolated as described.[43] Calcification was induced by culturing VICs in an osteogenic media (OM; high glucose DMEM supplemented with 5% FBS, 2 mmol/L Na_2HPO_4 and 50 μg/mL L-ascorbic acid). hVSMCs from coronary artery and aorta were obtained from nonatherosclerotic arteries of hearts removed in transplant surgeries at the HSCSP,[41,39,44] while aortic mVSMC were isolated from wild-type (WT) mice and transgenic mice overexpressing human NOR-1 specifically in smooth muscle cells (TgNOR-1^{VSMC}).[21] The research was approved by the HSCSP Research Ethics Committee (approval number: 19/267) and was performed in accordance with the Declaration of Helsinki. Osteogenesis was induced in both hVSMCs and mVSMC by culturing cells in

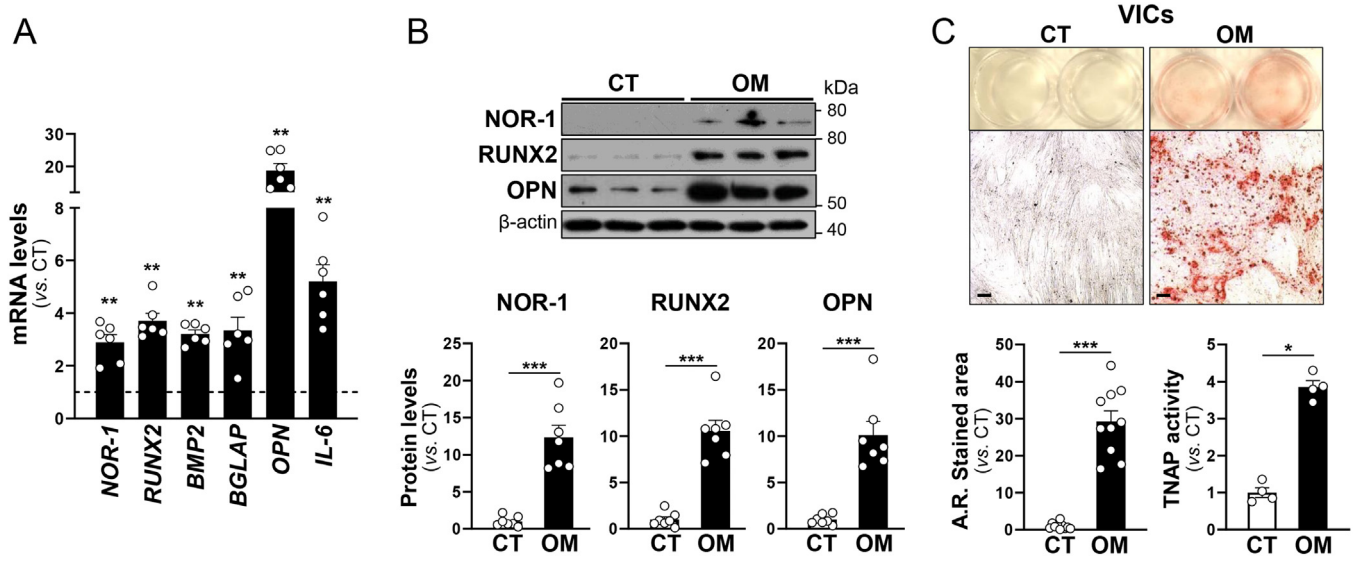


Fig 2. NOR-1 expression is enhanced in calcifying valvular interstitial cells (VICs). Human VICs were cultured in control media (CT, white bars) or in osteogenic media (OM, black bars). (**A**) mRNA levels of NOR-1 and osteogenic genes were quantified by real-time PCR in cells maintained under osteogenic conditions for 6 days. The dotted line indicates the expression of cells cultured under control conditions (CT, normalized to 1). Data are mean ± SEM (n = 6). $^{**}P < 0.01$ vs controls (dotted line). (**B**) Representative immunoblot images showing NOR-1, RUNX2, and OPN protein levels (after 6 days in OM), and bar graphs showing the results of the densitometric quantification of western blots. NOR-1 band (68 kDa) was significantly increased in OM-treated cells. The levels of β-actin are shown as a loading control. The migration of protein molecular markers is indicated on the right. Data are mean ± SEM (n = 7). (**C**) Representative images of Alizarin Red (A.R) stainings from VICs 14 days after osteogenic induction (Bars: 100 μm), and bar graphs showing the quantification of both the A.R. stained area (n = 10) and TNAP activity (n = 4). Data are mean ± SEM. Significant differences were determined by Mann−Whitney test (A−C) or unpaired t-test (A.R stained area in C). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

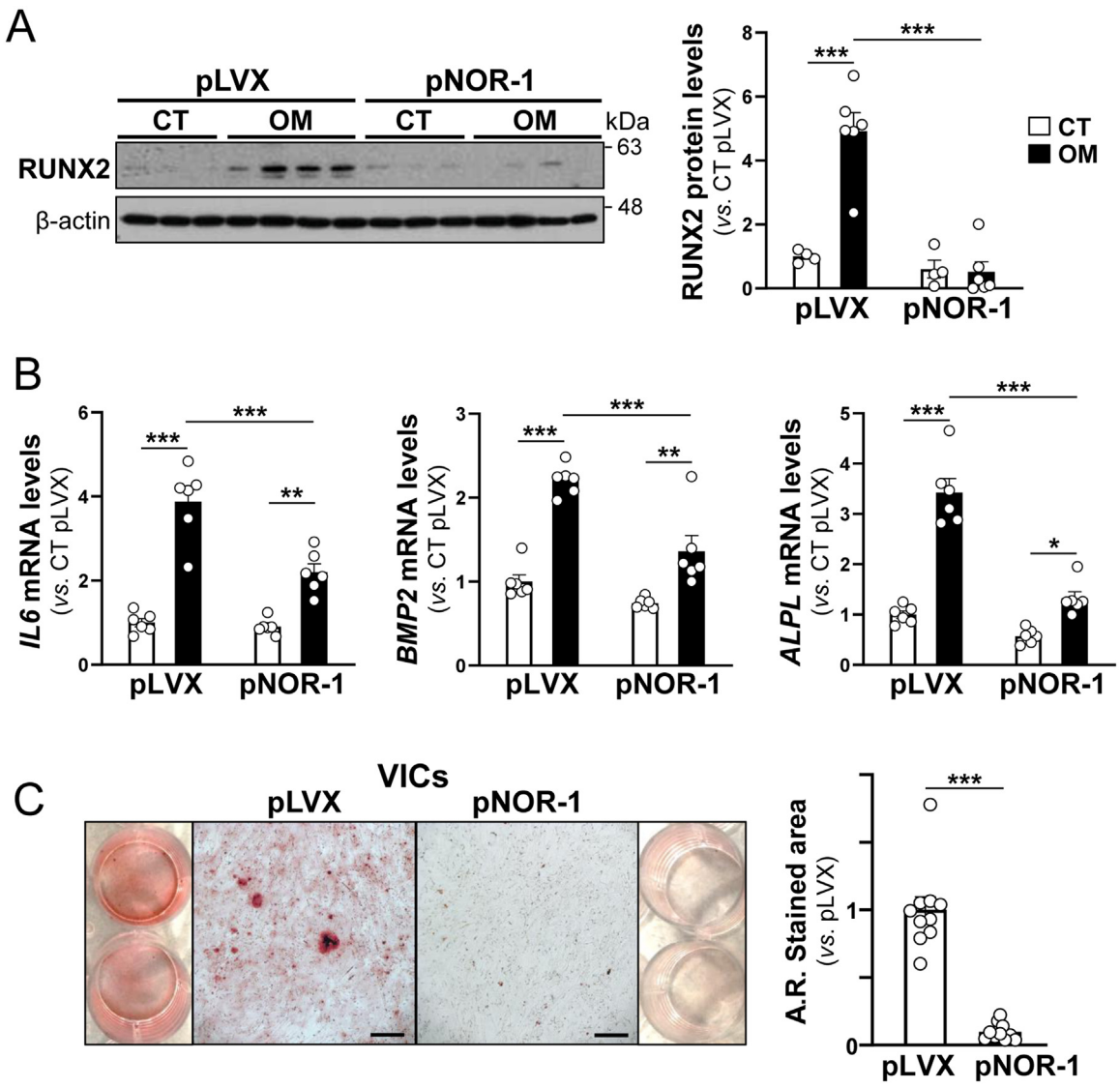


Fig 3. Effect of NOR-1 overexpression on gene expression and valvular interstitial cells (VICs) calcification. Human VICs were transduced with pLVX/NOR-1 (pNOR-1) or with the control lentiviral vector pLVX and incubated under control (CT, white bars) or osteogenic conditions (OM; black bars). (**A**) Representative immunoblot images showing RUNX2 protein levels in these cells (after 6 days in OM), and bar graph showing the result of the densitometric quantifications. Levels of β-actin are shown as a loading control. The migration of protein molecular markers is indicated on the right. Data are mean ± SEM (CT n = 4; OM n = 6). (**B**) *ALPL* and *BMP2* mRNA levels assessed by real-time PCR 6 days after osteogenic induction. Results are expressed as mean ± SEM (n = 6). (**C**) Representative images of Alizarin Red (A.R) stainings from these cells 14 days after osteogenic induction (Bars: 200 μm), and bar graph showing the quantification of the A.R. stained area (n = 10). Data are mean ± SEM. Significant differences were determined using 2-way ANOVA followed by Tukey's post-hoc test (A and B) or Mann−Whitney test (C). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

DMEM high glucose supplemented with 10% FBS, 10 mM β-glycerophosphate, 50 μg/mL L-ascorbic acid and 10 nM dexamethasone.

Calcium assessment in cell culture

Calcium deposition in cell cultures was visualized by Alizarin Red S staining as described.[44] Tissue non-specific alkaline phosphatase (TNAP) activity was measured in cell lysates using the Alkaline Phosphatase Activity Colorimetric Assay Kit (Ref K412; BioVision, Abcam, Waltham, USA). Alizarin Red staining and TNAP activity were determined after 14 days in OM (unless otherwise stated).

Western blotting

Protein lysates were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (IPVH00010, Immobilon, Merck-Millipore; Burlington, USA,). Membranes were incubated with antibodies against NOR-1 (H00008013-M06, Abnova, Taipei, Taiwan), osteopontin (OPN; NB-600-1043, Novus Biologicals), Runt-related transcription factor 2 (RUNX2; ab23981, Abcam) and LDL receptor (LDLR; 3839-30, BioVision). ß-actin (for whole-cell and tissue extracts; A5441, Merck) or nucleolin (for nuclear extracts; sc-9893, Santa Cruz Biotechnology Inc., Dallas, USA) were used to verify equal loading of protein on each lane.

Gene silencing and overexpression in VICs

Transient NOR-1 knockdown was carried out in VICs using siRNAs supplied by the On-Target Plus SmartPool. Lentiviral transduction of VICs was performed using the pLVX/NOR-1 construct, as reported.[36]

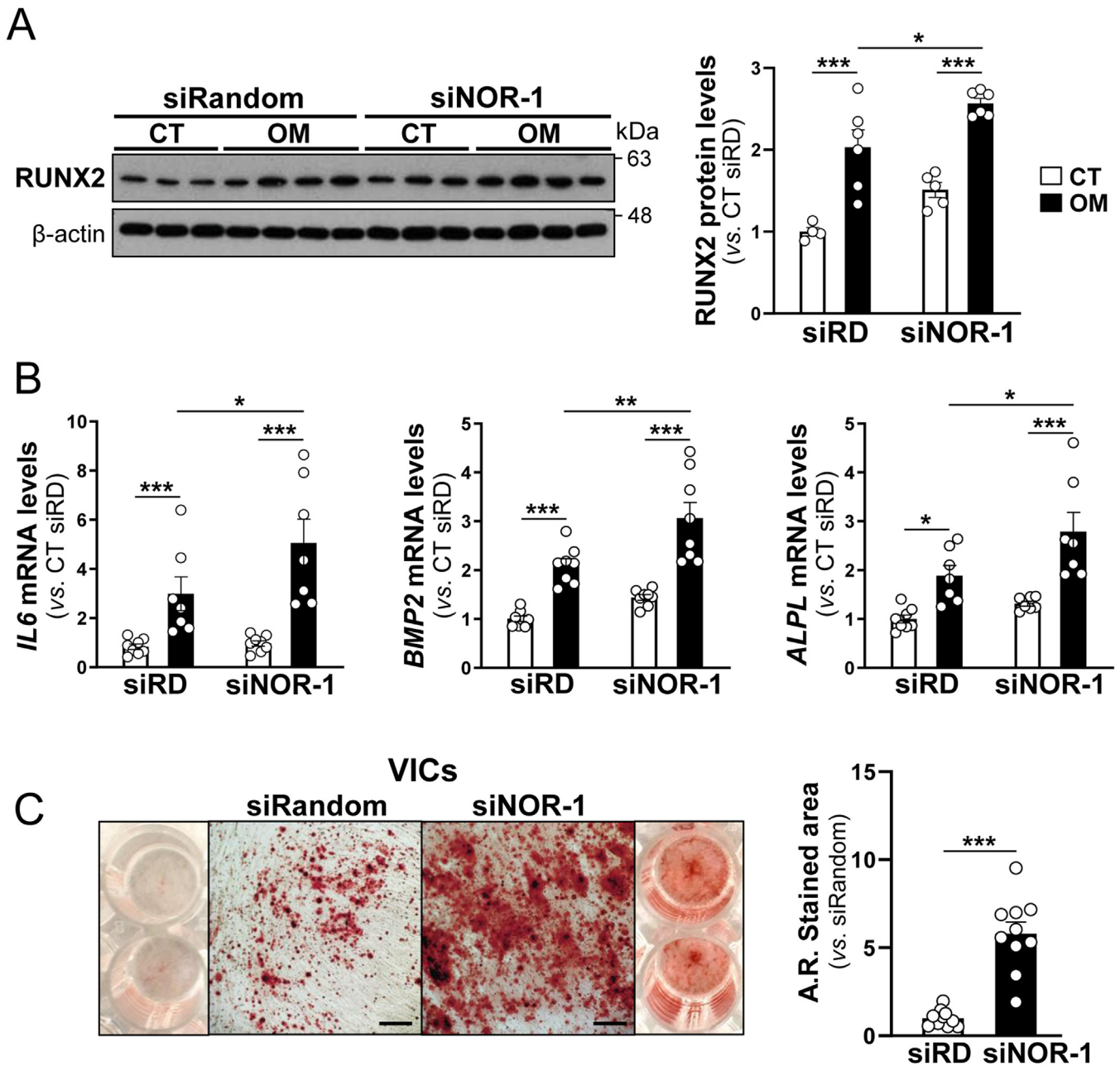


Fig 4. Effect of NOR-1 silencing on gene expression and calcification in valvular interstitial cells (VICs). Human VICs were transfected with a siRNA against NOR-1 (siNOR-1) or a Random siRNA (siRandom, siRD) and subjected to control (CT, white bars) or osteogenic conditions (OM, black bars). (**A**) Representative immunoblots showing RUNX2 protein levels in these cells (after 6 days in OM), and bar graph showing the result of the densitometric quantifications. The levels of β-actin are shown as a loading control. The migration of protein molecular markers is indicated on the right. Data are mean ± SEM (CT n = 4-5; OM n = 6). (**B**) mRNA levels for *ALP* and *BMP2* assessed by real-time PCR 6 days after osteogenic induction. Data are mean ± SEM (n = 7−8). (**C**) Representative images of Alizarin Red (A.R) staining from siRNA-transfected cells 10 days after osteogenic induction (Bars: 100 μm), and bar graph showing the quantification of the A.R. stained area (n = 10). Data are mean ± SEM. Significant differences were determined using 2-way ANOVA followed by Tukey's post-hoc test (A and B) or unpaired t-test (C). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Generation of human IL-6 promoter constructs, and transient transfections and luciferase assays

A construct containing a fragment of the IL-6 proximal promoter[45] was generated by PCR and cloned in a luciferase reporter plasmid (pIL6-1322). The NF-κB response element (−51/−42) located in IL-6 promoter was mutated using the QuickChange Site-Directed Mutagenesis Kit (Agilent, Santa Clara, USA) to generate the construct pIL6-1322-mut. VICs were transfected using Lipofectamine LTX and Plus Reagent (Invitrogen) with the IL-6 luciferase reporter plasmids, and a p65 expression vector (pMT7-p65), and co-transfected or not with a NOR-1 expresion vector (pCMV5/NOR-1).[39] The pRL-SV40 was added as internal control (Promega) Firefly and Renilla luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega).

Animal handling

The studies were carried out in compliance with the Spanish Policy for Animal Protection RD53/2013 and the European Union Directive 2010/63/UE and were approved by the IRHSCSP ethical committee (Law 5/June 21, 1995; Generalitat de Catalunya). Male transgenic mice over-expressing the human nuclear receptor NOR-1 in the vascular wall

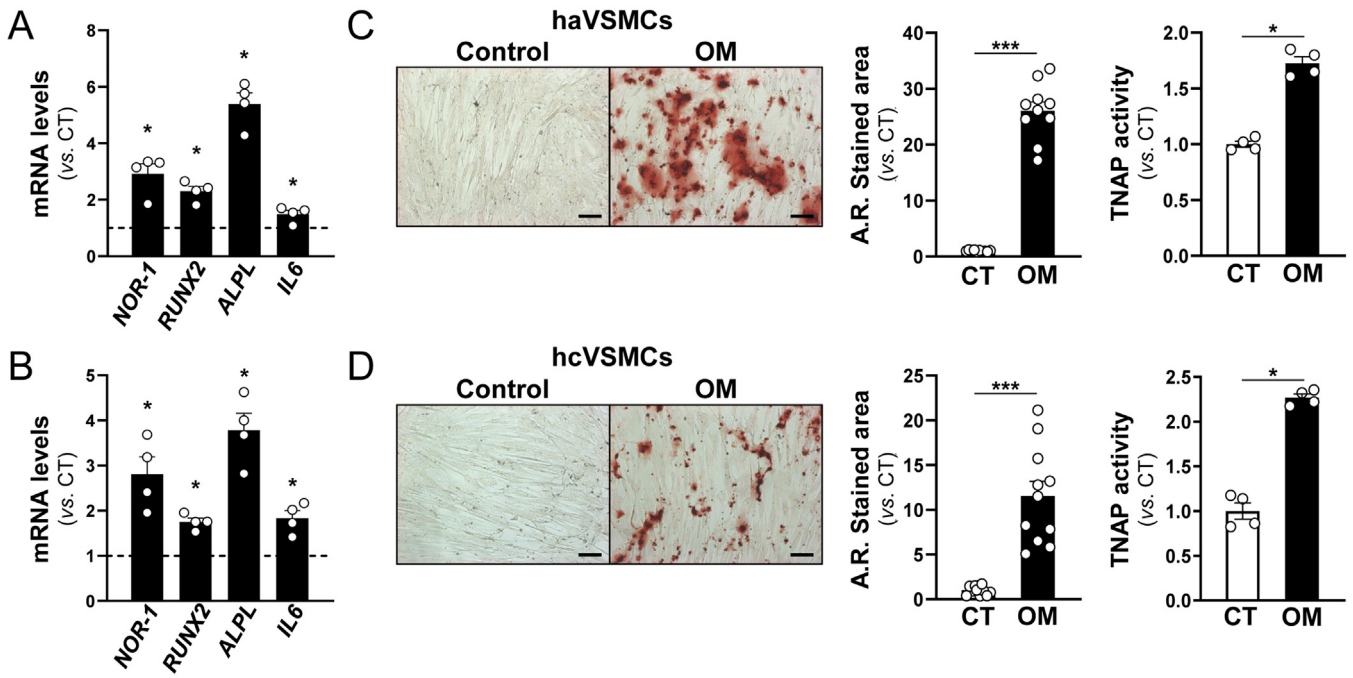


Fig 5. NOR-1 is upregulated in human vascular smooth muscle cells (hVSMCs) exposed to osteogenic conditions. Human aortic VSMCs (haVSMCs, A and C) and human coronary artery VSMCs (hcVSMCs, B and D) were maintained in control conditions (CT, white bars) or exposed to an osteogenic media (OM, black bars). (**A** and **B**) Expression levels of *NOR-1, RUNX2, ALPL* and *IL-6* determined by real-time PCR in haVSMCs (**A**) and hcVSMCs (**B**) 6 days after osteogenic induction. The dotted line indicates the expression levels in cells cultured under control conditions (normalized to 1). Data are mean ± SEM (n = 4). $^{*}P < 0.05$ vs controls (dotted line). (**C** and **D**) Representative images of Alizarin Red (A.R) staining, and bar graphs showing the quantification of the A.R. stained area (n = 10) and tissue-nonspecific alkaline phosphatase (TNAP) activity (n = 4) in cells cultured under control conditions or exposed to OM for 14 days. Data are mean ± SEM. Significant differences were determined by Mann−Whitney test (A to D) and unpaired t-test (A.R. Stained area in C and D). $^{*}P < 0.05$ and $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

(TgNOR-1^{VSMC}) and their control littermates, both on C57BL/6J genetic background,[21,23,24,36] were subjected to a single tail vein injection of an adeno-associated virus vector (AAV) encoding for the pathological human gain-of-function mutant of PCSK9 (AAV-PCSK9^{D374Y}; CNIC, Madrid, Spain).[46,47] Control mice received saline. The following day animals were switched to a high fat (HF; 21%) and high cholesterol diet (HC; 1.25%) (D12108C, Research Diets, New Brunswick, USA) for 20 weeks. At the end of the experimental period, vascular samples were harvested and properly processed for subsequent analysis.

Ex vivo *vascular calcification*

Calcification was induced in cultured aortic rings from WT or TgNOR-1^{VSMC} mice by the addition of 3 mM high-phosphate media, as described.[44]

Plasma analysis

Levels of total cholesterol (Wako Cholesterol E, Wako Pure Chemicals), triglyceride (L-type Triglyceride M, Wako Pure Chemicals), human PCSK9 (Human PCSK9 Quantikine ELISA Kit), osteoprogeterin (OPG; Mouse Osteoprogeterin Quantikine ELISA Kit), Dickkopf-related protein 1 (DKK-1; Mouse Dkk-1 Quantikine ELISA Kit, R&D Systems, Biotechne, Minneapolis, USA), calcium (Quanti chrom Calcium Assay, Bioassay Systems, Hayward, USA) and phosphate (Phosphate Colorimetric Assay Kit, BioVision) were assessed.

Oil Red O staining of en face aortas

Atherosclerosis burden was determined on whole aorta by ORO staining.[48] Images of whole aortas were captured and planimetric analysis was performed using the Image J software. Results were expressed as integrity density/aortic area.

Analysis of macroscopic calcification by near-infrared fluorescence imaging

Macroscopic calcifications were quantified by near-infrared fluorescence (NIRF) after an intravenous injection of Osteosense 680EX (2 nmol/100 μL; PerkinElmer, Waltham, USA). Total radiance efficiency was captured by an IVIS Spectrum *In Vivo* Imaging System (PerkinElmer).

Histology and immunohistochemistry

Vascular sections were incubated with antibodies against α-SM actin (SMA; ab5694, Abcam), CD68 (ab125212, Abcam), RUNX2 (ab23981, Abcam), OPN (NB-600-1043, Novus Biologicals, Littleton, CO, USA), IL-6 (GTX110527, Genetex, Irvine, USA), NOR-1 (H00008013-M06, Abnova) and vimentin (ab92547, Abcam). Slides were incubated with a biotinylated secondary antibody (Vector Laboratories, Burlingame, USA) and stained using the DAB chromogenic method (Roche). The histological characterization was performed by hematoxylin-eosin and the modified Russell-Movat pentachrome (Movat's) stainings (Electron Microscopy Sciences, Hatfield, PA). Lesion area was established by a blinded operator on hematoxylin-eosin stained sections using Image J software. Calcification was assessed by Von Kossa staining (Silver plating kit acc. to von Kossa, Merck, Darmstadt, Germany)[44] or using the Osteosense 680EX fluorescent probe (200 nM). Collagen content and crosslinking was evaluated by Picrosirus Red staining (Merck) visualized under polarized light.[48]

Statistical analysis

Results are shown as mean ± standard error of the mean (SEM). Significant differences were determined using the unpaired *t*-test, 2-way ANOVA with repeated measures or 2-way ANOVA followed by the Tukey's post-hoc test or the 2-stage step-up method of Benjamini,

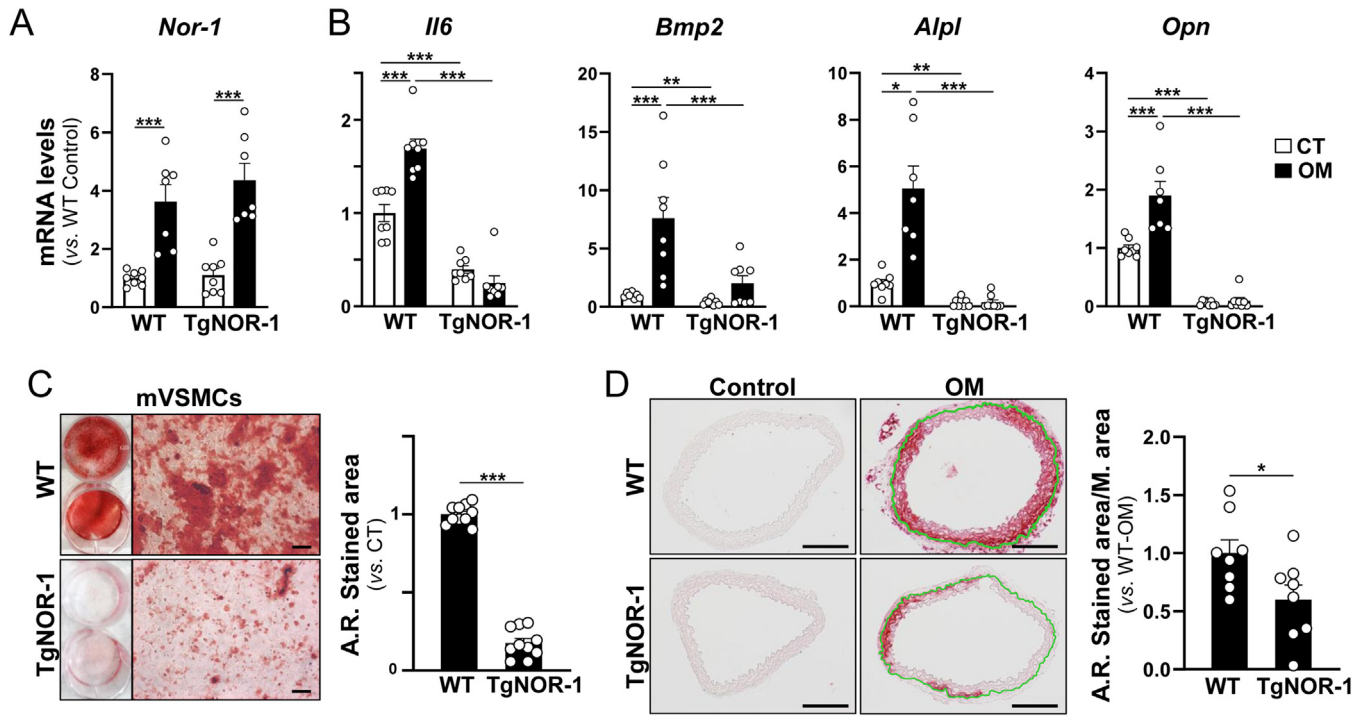


Fig. 6. NOR-1 transgenesis attenuates the calcification of mouse VSMCs (mVSMcs) and mouse aortic rings exposed to osteogenic media. (**A to C**) mVSMCs from wild-type (WT) and NOR-1 transgenic mice (TgNOR-1) were cultured under control (CT, white bars) or osteogenic conditions (OM; black bars). (**A**) Up-regulation of endogenous (mouse) NOR-1 mRNA levels by OM, and (**B**) impact of transgenic NOR-1 overexpression on mRNA levels of *IL-6 and* osteogenic markers (*Bmp2, Opn and Alpl*) assessed by real time PCR 6 days after osteogenic induction. Data are mean ± SEM (n = 8). (**C**) Representative images of Alizarin Red (A.R) stainings from these cells 10 days after osteogenic induction (Bars: 100 μm), and bar graph showing the quantification of the A.R stained area (n = 10). Data are mean ± SEM. (**D**) Representative images of A.R staining of aortic rings from WT and TgNOR-1 mice exposed to OM for 6 days, and bar graphs showing the quantification of the A.R stained area relative to the total area of the media (M. area) and referred to the control group (WT exposed to OM, WT-OM). Green lines delimited the area considered for the quantitative analysis (Bars: 200 μm). Data are mean ± SEM (n = 8). Significant differences were determined by 2-way ANOVA followed by 2-stage step-up method of Benjamini, Krieger, and Yekutieli False Discovery Rate post-hoc test (A and B) or the unpaired t-test (C and D). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Krieger, and Yekutieli False Discovery Rate post-hoc test. When the distribution of data failed the Shapiro–Wilk test, the Mann–Whitney U test was performed. Incidence of calcification was assessed by Fisher's exact test. Data were analyzed with the GraphPad Prism version 8.0.2. Differences were considered significant at $P < 0.05$.

RESULTS

NOR-1 expression is enhanced in calcified human aortic valves

Western-blot analysis of aortic valve leaflets from patients referred for aortic valve replacement revealed high NOR-1 levels in extensively calcified aortic valves, while they were virtually undetectable in low-calcified valves (Fig 1, A). Highly calcified valves also exhibited great protein levels of both RUNX2 and the osteogenic marker OPN. Consistently, in these samples, in the vicinity of calcified regions (positive to von Kossa staining) an intense NOR-1 immunostaining was detected (Fig 1, B). Further, analysis in consecutive sections evidenced that NOR-1 colocalizes with RUNX2 in areas enriched in VICs (Vimentin +). Conversely, NOR-1 and RUNX2 immunostaining was scarce or absent in calcium free areas of poorly calcified valves.

NOR-1 is upregulated in calcifying VICs incubated under osteogenic conditions

The exposition of VICs to an osteogenic medium (OM) upregulated the expression of NOR-1, RUNX2 and other osteogenic markers (Bone morphogenetic protein 2 [BMP2], Bone gamma-carboxyglutamic acid-containing protein [BGLAP] and OPN) as well as IL-6 (Fig 2, A). Consequently, VICs exposed to OM exhibited higher protein levels of NOR-1, RUNX2 and OPN than those exposed to control medium (Fig 2, B). The activation of this osteogenic program, which was detected within the first days of cell exposure to OM, preceded the calcification of VICs. Fig 2, C shows calcium deposition revealed by alizarin red S staining and TNAP activity in VICs exposed to OM for 14 days.

NOR-1 negatively regulates the expression of osteogenic genes in VICs protecting against calcification induced by high-phosphate medium

The transcription factor NOR-1 commonly upregulates gene expression, but it is also able to prevent gene expression through different mechanisms.[17-19,36,39] To gain more insight into the potential role of NOR-1 as a modulator of key genes involved in the osteogenic transdifferentiation and calcification of VICs, we carried out gain- and loss-of-function approaches. The effective over-expression of NOR-1 in VICs by lentiviral transduction (Supplementary Fig S2, A), abolished the upregulation of RUNX2 promoted by OM (Fig 3, A), significantly attenuated the induction of IL-6, BMP2 and ALPL (alkaline phosphatase, biomineralization associated) (Fig 3, B), and virtually prevented calcium deposition (Fig 3, C). Conversely, NOR-1 silencing in VICs (Supplementary Fig S2, B), significantly exacerbated the OM-induced upregulation of RUNX2, IL-6, BMP2 and ALPL (Fig 4, A and B), and coherently aggravated VICs calcification (Fig 4, C).

As a first approach to understand the molecular mechanism through which NOR-1 negatively regulates the expression of osteogenic genes, we focus on IL-6. The addition of IL-6 to OM favors and accelerates the calcification of VICs (Supplementary Fig S3, A) suggesting that the

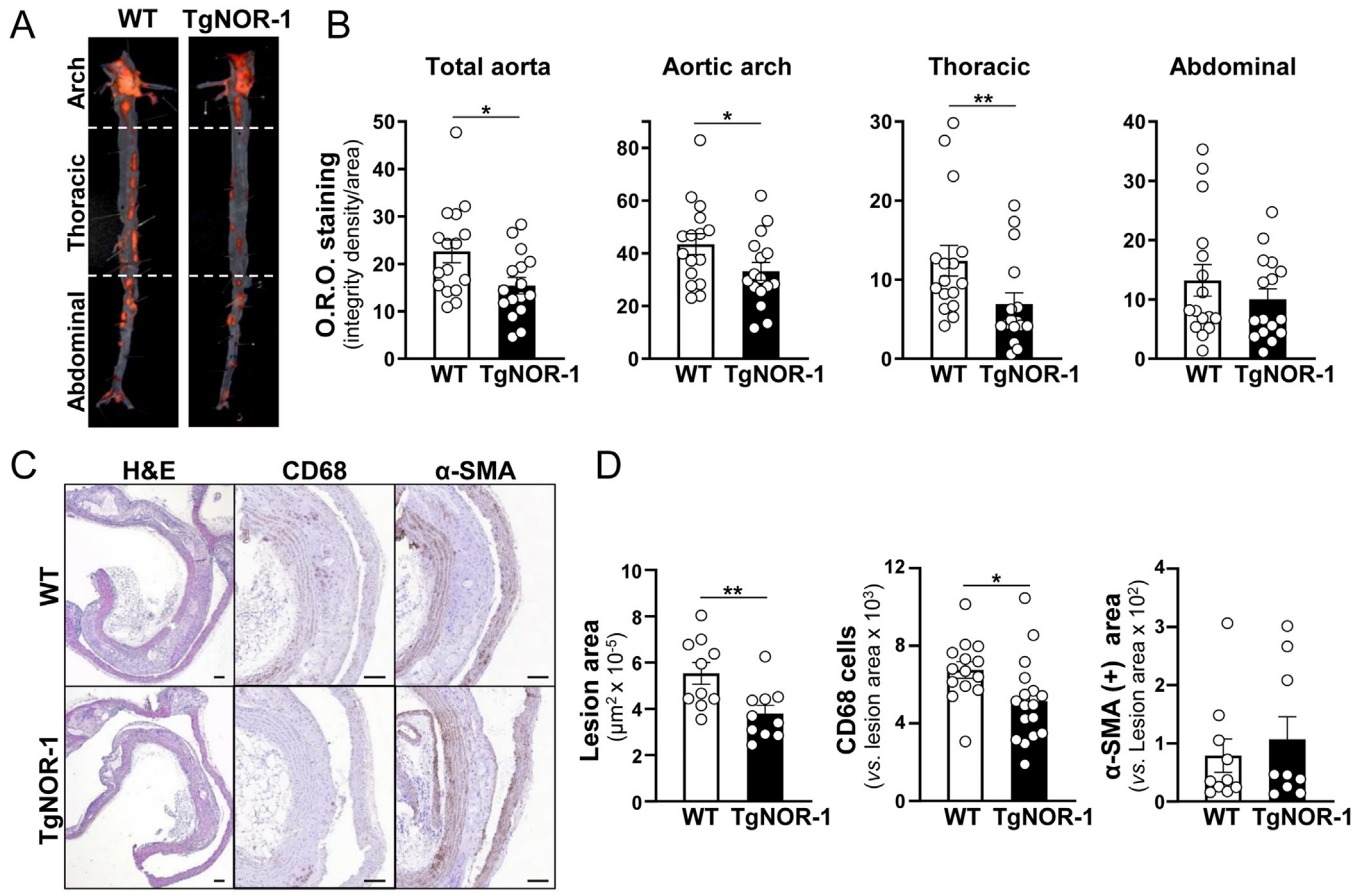


Fig 7. NOR-1 transgenesis attenuates atherosclerosis. Wild-type (WT) and NOR-1 transgenic mice (TgNOR-1) were subjected to a single tail vein injection of adeno-associated viruses (AAV) encoding for PCSK9^{D374Y} (AAV-PCSK9^{D374Y}) combined with a high fat/high cholesterol diet for 20 weeks. (**A**) Representative images of en face Oil Red O (O.R.O.) aorta stainings and dotted lines indicating the areas corresponding to aortic arch, thoracic aorta and abdominal aorta, and (**B**) bar graphs showing the results of the quantitative planimetric analysis for the whole aorta and these areas. Results are mean ± SEM (WT, n = 16; TgNOR-1, n = 16). (**C**) Representative images of hematoxylin & eosin (H&E) and immunohistochemical staining of aortic arch sections from these animals used to determine lesion area (n = 10) and to estimate the content of macrophages (CD68; WT [n = 14] and TgNOR-1 [n = 17]) and VSMCs (α-alpha-smooth muscle actin [α-SMA] positive cells; WT [n = 10] and TgNOR-1 [n = 9]). Bars: 100 μm. (**D**) Bar graphs showing the result of the quantitative analyses indicated in C. Results are mean ± SEM. Significant differences were determined using the unpaired t-test. *$P < 0.05$ and **$P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

modulation of IL-6 levels *per se* significantly impact on calcium deposition. Interestingly, transient co-transfections assays in VICs, evidenced that NOR-1 antagonized the increase in IL-6 transcriptional activity induced by the binding of p65 to an NFκB site present in IL-6 promoter (Supplementary Fig S3, B and C).

NOR-1 is detected in calcified human atherosclerotic arteries and is upregulated under osteogenic conditions that promotes hVSMC calcification

NOR-1 immunostaining was detected in human calcified femoral arteries colocalizing with RUNX2 in areas enriched in VSMC (SMA + cells) adjacent to calcium deposits (positive to von Kossa staining) (Supplementary Fig S4).

Consistent with our findings in VICs, in hVSMCs from both coronary artery (hcVSMCs) and aorta (haVSMCs) OM also upregulated NOR-1, as well as RUNX2, ALPL and IL-6 (Fig 5, A and B), and subsequently led to increased TNAP activity and exacerbated calcium deposition (Fig 5, C and D).

Transgenic overexpression of NOR-1 reduced mVSMC calcification both in vitro *and* ex vivo

To gain more insights into the impact of NOR-1 on the gene expression program leading to VSMC osteogenic transdifferentiation and calcification, we isolated mVSMCs from a transgenic mouse model that overexpresses human NOR-1 in these cells (TgNOR-1^{VSMC})[21,24,36] and from WT littermates (Supplementary Fig S5). In WT cells, OM upregulated NOR-1 and induced the expression of genes involved in osteogenic transdifferentiation and cell calcification such as IL-6 and several osteogenic markers (Fig 6, A and B). Endogenous NOR-1 mouse transcript levels were similarly induced by OM in WT and transgenic mVSMCs (Fig 6, A). However, in transgenic mVSMCs, which strongly overexpress the human NOR-1 transcript (Supplementary Fig S5), basal expression levels both of IL-6 and osteogenic markers were significantly attenuated, and their upregulation by OM completely prevented. The great impact of NOR-1 transgenesis on the expression pattern of these pro-osteogenic genes was consistent with a dramatic reduction of calcification both in transgenic mVSMCs in culture (Fig 6, C) and *ex vivo*, in aortic rings from transgenic mice exposed to OM (Fig 6, D).

Transgenic overexpression of NOR-1 in mVSMC reduced both atherosclerosis and the calcification of the intima of atherosclerotic lesions

These data prompted us to explore the impact of the specific overexpression of NOR-1 in mVSMC on atherosclerosis and on intimal calcification of atherosclerotic lesions *in vivo*. Hypercholesterolemia and atherosclerosis were induced in WT and TgNOR-1^{VSMC} mice by a single injection of AAV-PCSK9^{D374Y} combined with a HF/HC diet for 20 weeks,

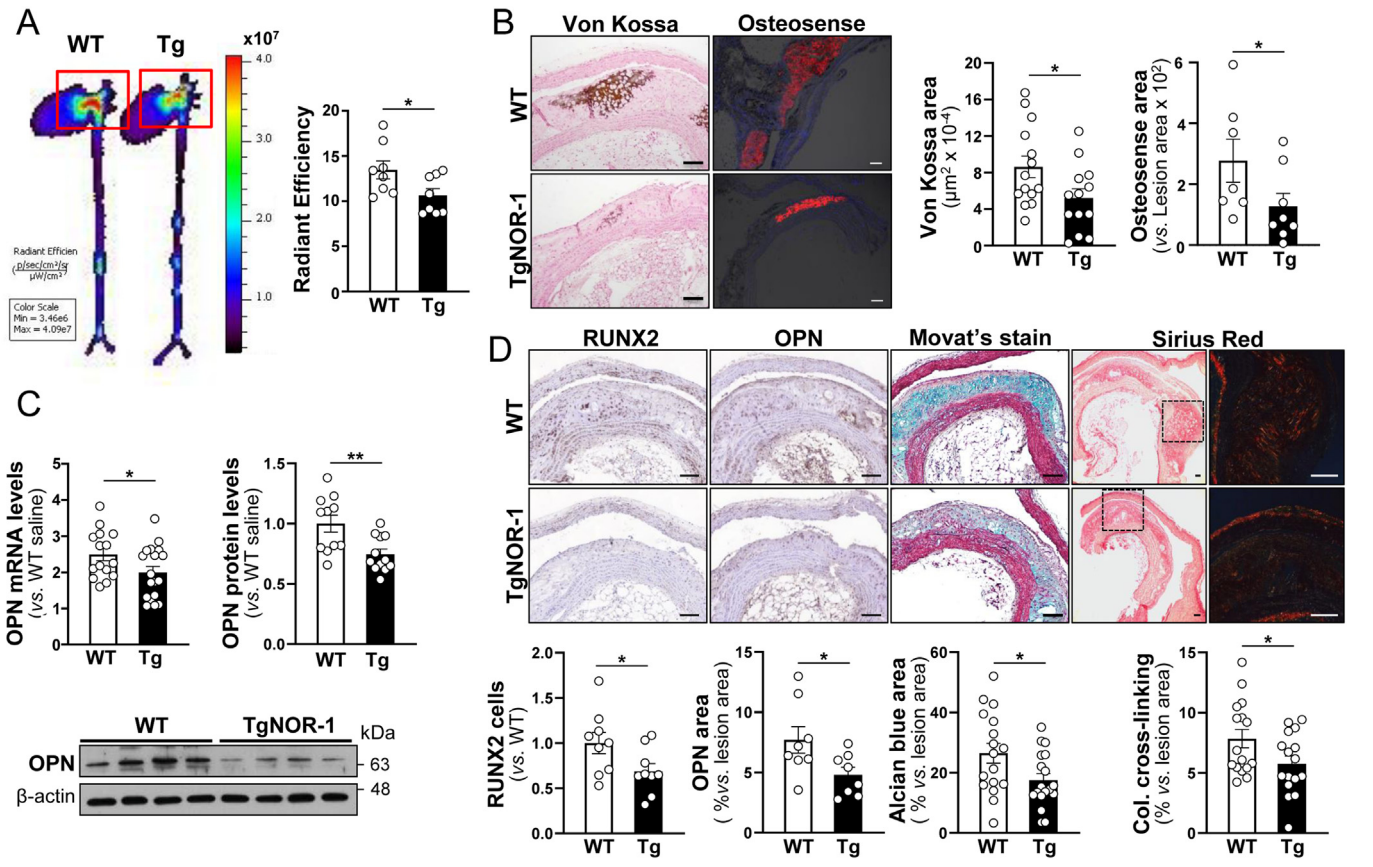


Fig 8. NOR-1 transgenesis ameliorates atherosclerosis-associated vascular calcification. Wild-type (WT) and NOR-1 transgenic mice (TgNOR-1, Tg) were subjected to a single tail vein injection of adeno-associated viruses (AAV) encoding for PCSK9^{D374Y} (AAV-PCSK9^{D374Y}) combined with a high fat/high cholesterol diet for 20 weeks. (**A**) Representative images of the *ex vivo* near-infrared fluorescence reflectance imaging (NIRF) analysis of aortas from WT and TgNOR-1 mice transduced with AAV-PCSK9^{D374Y} and injected with the fluorescence calcium tracer OsteoSense 680EX 24 h before the end of the experimental protocol. Bar graphs on the right show the result of the quantification of NIRF images. Results are mean ± SEM (n = 8). (**B**) Representative images of von Kossa (WT, n = 14; TgNOR-1, n = 13) and OsteoSense 680EX stainings (WT, n = 7; TgNOR-1, n = 8) used to determine vascular calcification in aortic arch sections from WT and TgNOR-1 mice transduced with AAV-PCSK9^{D374Y}. Bar graphs on the right show the result of the quantitative analysis. Results are mean ± SEM. (**C**) Aortic levels of osteopontin (OPN) assessed by real time PCR (mRNA; WT n = 5; Tg n = 18) and Western blot (Protein; WT n = 11; Tg n = 12), and a representative Western blot image showing OPN levels. Results are mean ± SEM. The levels of β-actin are shown as a loading control. (**D**) Representative images of immunostainings for RUNX2 (n = 9) and OPN (n = 8), and Movat's (WT n = 17; TgNOR-1 n = 18) and Sirius Red stainings (n = 16) of aortic arch sections from these animals. Sirius Red staining was captured under both bright field (left panel) and polarized light to estimate collagen cross-linking (Col. Cross-linking) (right panel). Bar graphs on the bottom show the result of the quantitative analyses. Data are mean ± SEM. Significant differences were determined by the Mann−Whitney test (A) or the unpaired t-test (B to D). *$P < 0.05$ and **$P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

while control groups were injected with saline (Supplementary Fig S6, A). Body weight increased without differences among experimental groups, all of them maintained on a HF/HC diet (Supplementary Fig S6, B). We confirmed that the AAV-PCSK9^{D374Y} injection enhanced circulating PCSK9 levels and that this parameter was not influenced by the vascular overexpression of the human NOR-1 transcript (Supplementary Fig S6, C). Consistently, PCSK9^{D374Y} transduction triggered an extreme reduction of hepatic LDLR protein levels in both WT and TgNOR-1^{VSMC} mice (Supplementary Fig S6, D) and a comparable increase in fasting plasma levels of total cholesterol and triglycerides (Supplementary Fig S6, E and F).

Before sacrifice, we used ultrasound imaging at the level of the innominate artery to confirm that the administration of AAV-PCSK9 ^{D374Y} and the atherogenic diet triggered a significant luminal stenosis indicative of the formation of atherosclerotic lesions (Supplementary Fig S7, A). Indeed, atherosclerotic plaques in the aortic arch were macroscopically apparent at sacrifice (Supplementary Fig S7, B). Interestingly, the analysis of whole en face mounted aortas stained with ORO revealed that atherosclerosis burden was significantly lower in TgNOR-1^{VSMC} mice than in WT control littermates (15.41 ± 1.72 vs 22.61 ± 2.41) (Fig 7, A), and the sectorized analysis showed that the reduction was significant and more pronounced in the aortic arch-thoracic aorta than in the abdominal region (Fig 7, B). This was associated with a lower expression of inflammatory markers in the aorta of transgenic animals (Supplementary Fig S8). Consistently, the atherosclerotic lesion area and macrophage infiltration in the aortic arch were lower in PCSK9^{D374Y}-transduced TgNOR-1^{VSMC} mice than in WT animals (Fig 7, C and D), while a non-significant trend to higher content of VSMC (SMA + cells) was observed in the former. Similarly, both the extent of atherosclerosis and the content of macrophages were lower in brachiocephalic artery lesions from transgenic mice than in those from WT animals (Supplementary Fig S9).

We investigated whether the specific NOR-1 overexpression in mVSMC modulates intimal calcification associated to atherosclerosis. In both WT and TgNOR-1^{VSMC} mice transduced with AAV-PCSK9^{D374Y} and fed an atherogenic diet for 20 weeks, plasma levels of DKK-1 decreased and those of phosphate and OPG increased, without significant differences between both groups, while circulating levels of calcium remain unchanged (Supplementary Fig S10). To analyze aortic calcification, the fluorescent probe Osteosense 680EX was administered 24 hours before mice sacrifice and its uptake by the aortas was imaged *ex vivo* by NIRF reflectance imaging. Interestingly, this probe, which specifically binds

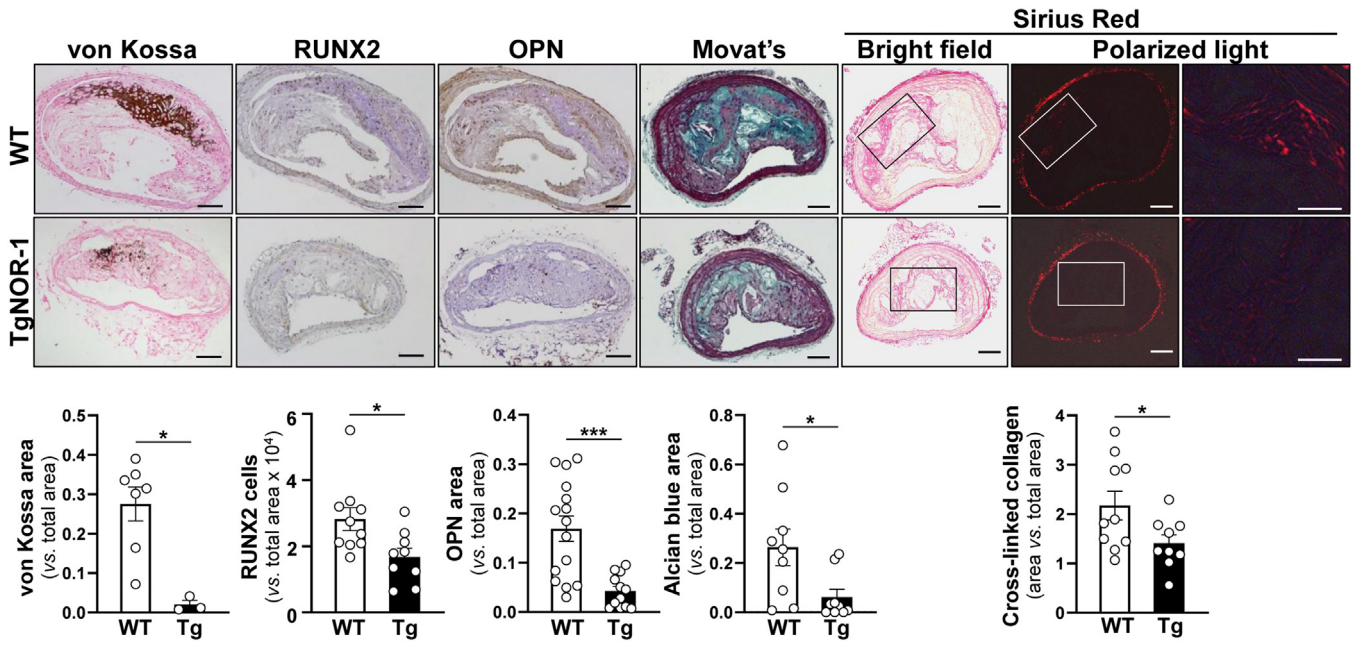


Fig 9. Impact of NOR-1 transgenesis on calcification in the brachiocephalic artery. WT and TgNOR-1 (Tg) mice were subjected to a single tail vein injection of an AAV vector encoding for PCSK9^{D374Y} (AAV-PCSK9^{D374Y}) combined with a HF/HC diet for 20 weeks and the brachiocephalic artery was dissected. Calcification was assessed by von Kossa stainings (WT n = 7; TgNOR-1 n = 3). RUNX2 positive cells (WT n = 10; TgNOR-1 n = 9), OPN stained area (WT n = 15; TgNOR-1 n = 12), and both Movat's (n = 9) and Sirius Red stainings (WT n = 10; TgNOR-1 n = 9) are shown. Bars: 100 μm. Sirius Red staining was visualized under both bright field and polarized light and the indicated area is shown magnified on the right panels (Bars: 50 μm). Histograms show the quantitative analysis. Results are mean ± SEM. Significant differences were determined by the Mann–Whitney test (von Kossa, RUNX2 and Alcian Blue stainings) or by the unpaired t-test (OPN and collagen crosslinking). *$P <$ 0.05 and ***$P < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

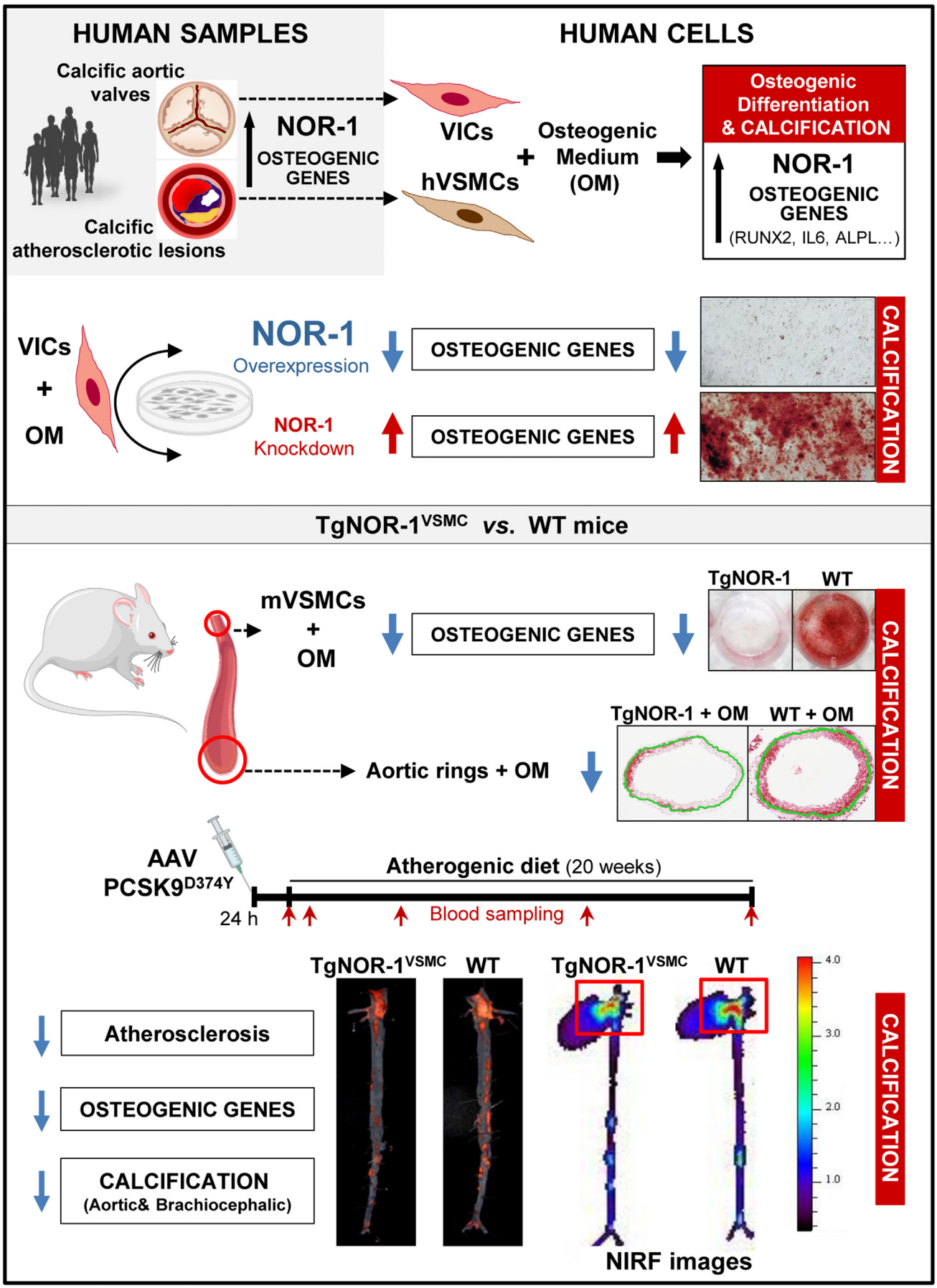
to solid-phase calcium phosphate and preferentially to crystalline phases (eg, hydroxyapatite), showed that calcium phosphate deposition was lower in aortic tissues from transgenic animals (Fig 8, A). Moreover, analysis of aortic arch sections using von Kossa or Osteosense 680EX stainings showed less calcium deposition in the aortic arches of TgNOR-1^{VSMC} mice (Fig 8, B). Consistently, this was associated with lower levels of OPN and RUNX2 (Fig 8, C and D), reduced content of mucoid material (alcian blue area in Movat's staining) and a significant decrease in the amount of mature collagen (Sirius red staining visualized under polarized light) (Fig 8, D). Although calcification in the brachiocephalic arteries was only observed in some samples, in the transgenic group we noted a lower incidence of calcification (10% in transgenic vs 20% in WT) and less mineralization in those samples that did calcify (Fig 9). The immunohistochemical analysis of calcified specimens showed that NOR-1 transgenesis led to attenuated expression of RUNX2 and OPN, lower content of mucoid material and less plaque collagen cross-linking (Fig 9).

DISCUSSION

Cardiovascular calcification has become a growing healthcare issue. The search and development of pharmacological tools for preventing disease progression responds an unmet public health need that requires a better knowledge of the underlying mechanisms.[9,11] NOR-1 plays a role in the pathophysiology of several cardiovascular diseases[17-28]; however, its involvement in cardiovascular calcification has not been previously approached. In this study, we show that NOR-1 is strikingly expressed in human calcified aortic valves and femoral arteries and, complementary *in vitro* and in *vivo* approaches (Fig 10), uncover that under osteogenic conditions this transcription factor is co-upregulated with osteogenic genes. However, NOR-1 actually acts as a negative modulator that counteracts the induction of the osteogenic transdifferentiation program, thus attenuating calcification in both VICs and VSMCs.

Several transcription factors have been involved in the intricate response triggering the osteoblastic transition of VICs that drives CAVD, among them RUNX2, which is considered the archetypical osteogenic transcription factor in both valvular and vascular calcification.[13-15,49] Nowadays, however, this picture is incomplete. NOR-1 modulates the dedifferentiation of VSMC into a migratory and proliferative phenotype in response to different stimuli,[20,29-32] and NOR-1 and RUNX2 have been shown to be co-regulated in mesenchymal stem cell differentiation,[50] but a potential role for NOR-1 in VICs mineralization or CAVD has not been previously reported. We observed that while NOR-1 expression was virtually undetectable in low calcified human valves, it was strongly up-regulated in extensively calcified valves, mostly in areas enriched in RUNX2-positive VICs, in which co-expression of both transcription factors was detected. Moreover, in calcifying human VICs exposed to high-phosphate osteogenic medium NOR-1 was co-upregulated along with RUNX2, IL-6 and other osteogenic genes. NOR-1 is a constitutively-active nuclear receptor that does not require any ligand to be active, and the main mechanism by which cells regulate its activity as a transcription factor is by modulating its expression levels.[17-19] For this reason, to investigate how NOR-1 expression levels could impact on VIC calcification we undertook both gain- and loss-of-function approaches. Interestingly, in human VICs exposed to an osteogenic stimulus, while NOR-1 silencing slightly potentiated the up-regulation of osteogenic genes, NOR-1 lentiviral over-expression limited its induction, including that of RUNX2 and IL-6. These results point to NOR-1 as a negative modulator that counteracts the upregulation of genes relevant for the osteogenic transdifferentiation and calcification of VICs.

As a NR4A receptor, NOR-1 usually regulates gene expression by binding to a NBRE (nerve growth factor-responsive element) on the promoter of its target genes inducing their transcriptional activity.[17-19] However, these receptors can also repress gene expression by binding to specific NBREs that function as negative regulatory elements.[51] Unfortunately, the search for functional regulatory elements that directly respond to NOR-1 is hampered by the fact that nuclear receptors often bind to remote regions of the DNA located well upstream of the transcriptional initiation site of their target promoters,[52] and this also appears to be the case for nuclear receptors of the NR4A subfamily.[53] Interestingly, NR4A receptors can further inhibit gene expression,


HUMAN SAMPLES
Calcific aortic valves
Calcific atherosclerotic lesions
NOR-1
OSTEOGENIC GENES
HUMAN CELLS
VICs
hVSMCs
Osteogenic Medium (OM)
Osteogenic Differentiation & CALCIFICATION
NOR-1
OSTEOGENIC GENES
(RUNX2, IL6, ALPL…)
VICs + OM
NOR-1 Overexpression
OSTEOGENIC GENES
NOR-1 Knockdown
OSTEOGENIC GENES
CALCIFICATION
TgNOR-1VSMC vs. WT mice
mVSMCs + OM
OSTEOGENIC GENES
TgNOR-1
WT
CALCIFICATION
Aortic rings + OM
TgNOR-1 + OM
WT + OM
AAV PCSK9D374Y
24 h
Atherogenic diet (20 weeks)
Blood sampling
TgNOR-1VSMC
WT
TgNOR-1VSMC
WT
4.0
3.0
2.0
1.0
CALCIFICATION
Atherosclerosis
OSTEOGENIC GENES
CALCIFICATION (Aortic& Brachiocephalic)
NIRF images

through functional antagonism with other transcription factors. This negative cross-talk has been primarily described for NFκB. NR4A receptors can bind with low-affinity to NFκB response elements or interfere with the NFκB signaling pathway at various levels including physical association with p65, as we and others have shown.[36,39,54-56] In order to shed some light on the mechanism by which NOR-1 negatively regulates gene expression, as a first approach we focused on IL-6, a cytokine critically engaged in VIC osteogenic differentiation and mineralization,[5,10,12,16] that has been associated to the genetic susceptibility underlying CAVD.[57] IL-6 was significantly modulated in human VICs when NOR-1 was either overexpressed or silenced, and the addition of IL-6 to osteogenic medium favors and accelerates VICs calcification, suggesting that the modulation of IL-6 levels *per se* has a significant repercussion on calcium deposition in these cells. In fact, accumulating evidence indicates that fibrocalcific remodeling is associated with the increase of IL-6, mediated by the NF-κB pathway, which promotes the activation of an osteogenic program as well as the mineralization of VICs[5,10,12,16] and VSMCs.[58-61] In this regard, transient co-transfections assays in VICs, showed that NOR-1 antagonized the increase in IL-6 transcriptional activity induced by the binding of p65 to a NFκB site present in IL-6 promoter. Taken together, these data suggest that the antagonism between NOR-1 and the NFkB signaling pathway may contribute, at least in part, to the regulation of the complex gene network involved in cellular osteogenic transdifferentiation and calcification. Therefore, our results allow us to postulate NOR-1 as a new negative modulator of the balance between these pro- and anti-calcific factors that operates in CAVD.

As the calcification of VSMCs also entails osteogenic reprogramming,[9,11,13] we analyzed whether NOR-1 plays a role in vascular calcification associated to atherosclerosis. Similar to that found in calcific aortic valves, in human calcified atherosclerotic plaques we observed a significant upregulation of NOR-1 in areas enriched in VSMC positive for RUNX2. Likewise, when we induced calcification of hVSMC, either from coronary artery or from aorta, NOR-1 was up-regulated along with RUNX2, IL-6 and other osteogenic genes. To gain more insights into the impact of NOR-1 on the gene expression program leading to VSMC osteogenic transdifferentiation and calcification, we used mVSMCs and aortic rings from a transgenic mouse model that overexpresses human NOR-1 in these cells (TgNOR-1^{VSMC}). The response of both mVSMCs and aortic rings from NOR-1 transgenic mice cultured under osteogenic conditions was similar to that observed in NOR-1 transduced VICs, namely NOR-1 prevented the up-regulation of genes associated to the osteoblastic differentiation of mVSMC *in vitro* (IL-6, BMP2, ALPL, OPN) and limited calcium deposition both *in vitro* and *ex vivo*. This response triggered by NOR-1 on VSMC calcification is essentially opposite to that reported for Nur77 (NR4A3), a NR4A receptor which frequently exerts antagonistic effects to those mediated by NOR-1,[62-64] that contributes to lactate-induced accelerated VSMC calcification.[65] The present study expands our knowledge about the influence of NR4A receptors on VSMC phenotypic plasticity.

Early studies from our group demonstrated the increase of NOR-1 mRNA levels in coronary arteries from patients with ischemic heart disease,[20] and recently have shown that NOR-1 is up-regulated in inflamed atherosclerotic lesions.[38] However, the relationship between NOR-1 and vascular calcification in atherosclerosis has not been previously addressed. To establish the pathophysiological relevance of our insights *in vivo* we induced atherosclerosis in WT and TgNOR-1^{VSMC} mice through the administration of AAV-PCSK9^{D374Y} combined with a HF/HC diet, a strategy that has been previously validated for the study of vascular calcification.[66] The specific overexpression of NOR-1 in mVSMC decreased atherosclerosis and significantly reduced the formation of calcium deposits in the intima of atherosclerotic lesions. NOR-1 transgenesis decreased atherosclerosis burden in the whole aorta and markedly reduced atherosclerotic lesion area in both the aortic arch and the brachiocephalic artery. Previous studies by Zhao el al.[27] concluded that the ubiquitous deficiency of NOR-1 reduces hypercholesterolemia-induced atherosclerosis in ApoE-KO. Our results, however, are more consistent with those subsequently reported by the same group showing that the specific deletion of NOR-1 in hematopoietic stem cells aggravated atherosclerosis.[28] Recently, we have also reported that NOR-1 modulates Treg/Th17 balance and CD69-dependent immunomodulatory functions thereby mediating anti-inflammatory/anti-atherosclerotic effects.[37,38] Thus, in the last years it has become increasingly apparent that the impact of NOR-1 on atherosclerosis and vascular remodeling operates in a cell type−specific manner. This is the first study showing that the mVSMC-specific overexpression of NOR-1 reduced experimental atherosclerosis. More noteworthy, challenged-TgNOR-1^{VSMC} mice exhibited decreased vascular expression of osteogenic genes and less vascular calcification (assessed either by NIRF imaging or histological analysis) than WT mice. The effect was mainly observed in the aortic arch, where calcification was more pronounced, but also in the brachiocephalic artery, despite calcification in this vascular bed was less consistent. Coherently, atherosclerotic lesions from transgenic mice, less prone to calcify than WT animals, also exhibit lesser collagen crosslinking and a lesser content of mucoid material that those from WT.

In summary, our findings highlight the contribution of NOR-1 as a negative modulator of the complex osteogenic reprogramming process underlying the phenotypic transdifferentiation experienced by VICs and VSMCs, which boosts both aortic valves and atherosclerotic arteries calcification. Further research is warranted to better characterize the molecular mechanisms through which NOR-1 modulates the expression of these osteogenic genes critically involved in such processes, as they could provide grounds for potential new therapeutic strategies to fight against cardiovascular calcification.

Acknowledgments

Conflict of interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare. Moreover, all authors have read the journal's authorship agreement and reviewed and gave their final approval of the submitted version. There is no financial or personal relationship with organizations that could potentially be perceived as influencing the described research.

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Author contributions are as follows: C.R. and J.M.-G. conceived the study, designed the experiments, acquired the financial support and wrote the manuscript. C.B.-S, L.C., J.A., and M.T. performed the *in vivo* studies and acquired data. C.B.-S, L.P-U., and LC carried out cell culture and *ex vivo* studies P.V.-S carried out transient transfection and

Fig 10. NOR-1 in human and experimental ectopic calcification. The scheme summarizes the experimental strategies approached to analyze the role of NOR-1 in the calcification of both aortic valves and VSMCs. Studies in (i) human aortic valves and human atherosclerotic lesions, (ii) human cells (VICs and hVSMCs), mouse cells (mVSMCs) and (iii) atherosclerosis-challenged mice that overexpress NOR-1 in VSMCs (TgNOR-1^{VSMC}) evidence that the nuclear receptor NOR-1 negatively modulates the expression of genes critically involved in the osteogenic transdifferentiation of VICs and VSMCs thereby attenuating calcium deposition. The figure was partly generated using Servier Medical Art provided by Servier, licenced under a Creative Commons Attribution 3.0 unported license. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

luciferase analysis. M.T., E.R., and F.M. participated in patient recruitment and supervised the clinical aspects of the research. All the authors gave the approval to the final version of the manuscript.

Data availability statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2023.09.004.

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