Supplementary Material to Rodríguez-Calvo, Ferrán et al. “NR4A receptors up-regulate the antiproteinase alpha-2 macroglobulin (A2M) and modulate MMP-2 and MMP-9 in vascular smooth muscle cells” (Thromb Haemost 2015; 113.6)

Suppl. Material and Methods

Animals

A transgenic animal model that over-expresses NOR-1 in VSMC was used (1). Both transgenic animals (TgNOR-1) and control littermates (wild-type) on a C57BL/6J genetic background were bred in the Animal Experimentation Unit (CSIC-ICCC). Fourteen TgNOR-1 and 14 wild-type male mice (2 months old) were randomly distributed into four experimental groups: TgNOR-1 mice which received lipopolysaccharide (LPS; 0.5 mg/kg; n=7) or vehicle (saline; n=7), and control mice which received LPS (0.5 mg/kg; n=7) or vehicle (saline; n=7). After 24 h of treatment, mice were euthanized under ketamine/medetomidine anaesthesia (75 mg/kg and 1 mg/kg, respectively). Then, aorta was excised, frozen in liquid nitrogen and stored at -80°C. 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, June 21st, passed by the Generalitat de Cataluny. Animals were taken care of and used according to the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63/UE on the protection of animals used for experimental and other scientific purposes.

Generation of lentiviral expression vectors for human NR4A receptors and viral particles

The full-length human NOR-1 (hNOR1) cDNA (GenBank Accession No. D78579) was excised by digestion with EcoRI from a pBlueScript-NOR1 construct (kindly provided by
Dr. N Ohkura) (2). The human cDNAs for Nurr1 and Nur77 were amplified by PCR from VSMC stimulated with 10% FCS. The primers used were: human Nurr1: 5’-AATGACTCGAGACAGACGCCGGAAGTCCTA-3’ (forward; XhoI site is underlined) and 5’-ATTCCCCACTAGAGCTGAGACTGCTCACACG-3’ (reverse; XbaI site is underlined); human Nur77: 5’-TCAGCGAATTCAGCGAACAGGTGCAAG-3’ (forward; EcoRI site is underlined) and 5’-TGCATTCTAGAGCGGAGGGTGGAG-3’ (reverse; XbaI site is underlined). Human NR4A cDNAs were cloned into pLVX-puro lentiviral vector. Alternatively, lentiviral constructs containing NR4A mouse cDNAs linked to a FLAG sequence were generated from the plasmids pCMV5/NOR-1-FLAG, pCMV5/Nurr1-FLAG and pCMV5/Nurr77 kindly provided by Dr. J Hastie (Protein Phosphorylation Unit, Medical Research Council, University of Dundee, Dundee, Scotland) (3). HEK 293T cells were transfected with these constructs or the empty pLVX-puro vector using Lenti-X™ Lentiviral Expression System kit (Clontech) according to manufacturer’s instructions. After 48 h, culture supernatants containing viral particles were collected and filtered. Lentiviral transduction of VSMC was performed in the presence of polybrene (8 μg/ml). After puromycin selection, RNA, whole protein or nuclear extracts were obtained. Lentiviral constructs containing or not FLAG sequences yielded similar results, but the later were preferably used in Western blot, immunocytochemistry, chromatin immunoprecipitation and electrophoretic mobility shift assay (EMSA) analysis to facilitate the detection of the overexpressed proteins by using anti-FLAG antibodies. Empty pLVX and pLVX-EGFP lentivirus were used as controls obtaining similar results.

**Constructs, transient transfection and luciferase assays**

A 2.0 kb fragment of human A2M promoter (nucleotides -1999 to +93) was generated by PCR and cloned into pGL3 vector (Promega) (pGL3/A2M-1999). The primers used were: 5’-ATGACTCACAGGGCATGTGTT-3’ (forward; placed upstream an internal
Nhel at -1999/-1993) and 5’-AGGAGCTCGAGGAGAACAGAC-3’ (reverse; Xhol site is underlined). The PCR product was digested with Nhel and Xhol and cloned into pGL3 vector. A promoter deletion construct (-163 to +93; pGL3/A2M-163) was generated using the reverse primer indicated above and the following forward primer: 5’-AGCTGCTGTACGGTAAAAAGTGAG-3’ (The three first bases of a SacI site that is extended in A2M promoter is underlined). The putative NBRE site (-71/-64) present in A2M promoter was mutated using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. The primers used were: forward 5’-TGACTATAAATAGGCCATCAATGttCTTTCCAGAGAATGTTTAGAGAC-3’, and reverse 5’-GTCTCTGAACATTCTCTCTGAAAAGaaCTTGATGGCCTATTTATAGTCA-3’ (the putative NBRE site is underlined and changes are indicated in lower case letters). In silico analysis of the mutated sequence confirm that no new response elements were introduced using this strategy. Wild-type and mutated sequences were confirmed by DNA sequencing. A 2.0 kb fragment of mouse A2M promoter (nucleotides -2089 to -86, from the translation start site) was generated by PCR, similarly to the human A2M promoter construction. The primers used were: 5’-TTGGTACCTGGAATGATCCTTTTCTTGGGACT-3’ (forward) and 5’-ATGCTAGCAGTTTTATGCAACAGCGGGA-3’ (reverse). The PCR product was digested with KpnI and Nhel (restriction sites are underlined), and cloned into pGL3 vector (Promega). Sequences were confirmed by DNA sequencing.

NIH/3T3 cells were transfected using Lipofectamine™ (Invitrogen) according to the manufacturer’s protocol (4). EGFP (pCMV5-EGFP) or NR4A expression plasmids (pCMV5-NOR-1, pCMV5-Nurr1 or pCMV5-Nur77) were co-transfected together with luciferase reporter plasmids containing A2M promoter fragments. In competition assays, a dominant negative Nurr1 derivative (pCMX/Nurr1EngR) or its mutated version without dominant negative activity (pCMV5/Nurr1EngR/R334A) were used (5). Briefly,
transient transfections were performed in subconfluent cells seeded in 12 well/plates using 0.05 µg/well of the EGFP or NR4A expression plasmids, 0.5 µg/well of the luciferase reporter plasmid, 0.05 µg/well of pRL-SV40 (Promega) as an internal control, and 1.6 µl/well of Lipofectamine™ Reagent (Invitrogen). The DNA/liposome complexes were added to the cells for 6 h. Cells were washed once and maintained in growth medium for 24 h. The activities of firefly and renilla luciferases were determined in cell lysates using the Dual-Luciferase® Reporter Assay System (Promega) and a luminometer (Orion I, Berthold Detection Systems) according to the manufacturer. Results were expressed as the ratio of firefly to renilla activity.

**Chromatin immunoprecipitation (ChIP) assay**

VSMC transduced with lentiviral vector expressing NR4A receptors or EGFP linked to a FLAG sequence were cross-linked with 1% formaldehyde for 10 min. The cross-link reaction was stopped by adding glycine (100 mM). Then, cells were extensively washed and lysed in LB1 buffer (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% NP-40, 0.3% Triton X-100, 10% glycerol) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). After washing, nuclei were collected and resuspended in LB2 buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine). Chromatin was sheared by sonication using Bioruptor™ UCD-200 and an aliquot was saved and stored as input DNA. Supernatants were then immunoprecipitated with 5 µg of anti-FLAG antibody (F1804, Sigma) or an IgG as a control. Immune complexes were recovered by addition of A/G-Agarose beads (Santa Cruz Biotechnologies). After washing, immune complexes were extracted in elution buffer (1% SDS, 100 mM NaHCO₃, 200 mM NaCl), cross-link was reversed and the DNA was purified and concentrated using the QIAquick PCR Purification kit (QIAGEN). Purified DNA was analyzed by conventional and real-time PCR with primers designed to amplify the
human A2M gene from -183 to -12; upper primer: 5’-AGGTGCTGTACGGTAAAAGTGAG-3’; lower primer: 5’-CCACCCACACAAGATCTCTAAAC-3’. Real-time PCR from two independent experiments was performed by triplicate with the Quantifast™ SYBR® Green PCR kit (QIAGEN). Quantifications were analyzed by the ΔΔCt method and corrected to account for 4% inputs (4).

Suppl. References
Supplemental Figure 1

**Suppl. Figure 1.** Expression levels of NR4A receptors in control VSMC and in cells transduced to over-express NR4A receptors. VSMC were transduced with lentiviral pLVX vectors expressing each NR4A receptor (pLVX-NOR-1, NOR; pLVX-Nurr1, Nurr1; pLVX-Nur77, Nur77) or with pLVX-EGFP (GFP). The expression of each receptor was determined by real-time PCR (A) and Western blot (B) in non-transduced cells (NT) and in lentiviral transduced VSMC. Data are expressed as a mean ± SD (n = 6). *p<0.0001 vs. NT and pLVX-EGFP cells.
Supplemental Figure 2

Suppl. Figure 2. NR4A knockdown attenuates basal alpha-2-macroglobulin (A2M) expression in human VSMC. VSMC were transfected with siRNAs against NOR-1 (A) Nurr1 (B), Nur77 (C) (black bars) or a scrambled sequence as a control (siRandom; white bars). Real-time PCR confirms the specific blockade of each NR4A receptor by the indicated siRNAs. As shown, A2M mRNA levels were significantly downregulated by the knockdown of any of these receptors. Data are expressed as a mean ± SD (n=6). *p<0.05; †, vs. siRandom transfected cells.
Supplemental Figure 3

Suppl. Figure 3. Alpha-2-macroglobulin (A2M) knockdown attenuates the inhibition of MMP activity induced by NOR-1 over-expression. VSMC were transduced with pLVX-EGFP or pLVX-NOR-1 and transfected with an A2M specific siRNA (siA2M; black bars) or a scrambled sequence as a control (siRandom; white bars). Real-time PCR (A) and Western blot analysis (B) confirm the blockade of A2M mRNA and protein levels. (C) Gelatinolytic activity was assessed in cell supernatants using the DQ-gelatin assay. Data are expressed as a mean ± SD (n=6). p<0.05: * vs. pLVX-EGFP cells transduced with the siRandom; p<0.05: #, vs. pLVX-NOR-1 transduced cells transfected with the siRandom.
Supplemental Figure 4

**Consensus NBRE: TGACCTT**

- Human A2M promoter: .86TAAGGGCCTCATTGACCTTTCCCAAGGAAACCTTC .39
- Mouse A2M promoter: .85TAAGGGCCTCATTGACCCCTTGCAAGGAAACCTTC .17

**A**

![Bar chart showing A2M promoter activity](image)

**B**

![Bar chart showing EGFP and NOR-1](image)

**C**

![Bar chart showing A2M mRNA levels](image)

**D**

![Bar chart showing A2M mRNA levels](image)

**Supp. Figure 4.** Alpha-2-macroglobulin (A2M) is not regulated by NR4A receptors in mouse. (A) Alignment of the proximal promoter regions corresponding to human and mouse A2M. As observed, the NBRE consensus binding site (upper panel) is not conserved in mouse A2M promoter. (B) Luciferase activity evaluated in NIH/3T3 cells co-transfected with a luciferase reporter vector (pGL3) containing the mouse A2M promoter (from -2069 to -96 relative to ATG) and pCMV5-expression vectors for EGFP (Control; white bar) and NOR-1 (black bar). Bars graph shows the inability of NOR-1 over-expression to modulate mouse A2M promoter (n=4). (C, D) A2M expression was not regulated by NOR-1 transgenesis in mice. A2M mRNA levels were analysed by real-time PCR in mouse aorta from wild-type (n=7) and transgenic mice that specifically over-express human NOR-1 in VSMC (TgNOR-1; n=9) (C), and in cultures of VSMC from these animals (n=5) (D). Data are expressed as mean ± SD.
Suppl. Figure 5. NOR-1 attenuates the up-regulation of MMPs induced by pro-inflammatory stimuli in the vascular wall. MMP-2 (A) and MMP-9 (B) mRNA levels were analysed by real-time PCR in mouse aorta from wild-type mice (WT; n=14) and from transgenic animals that specifically over-express human NOR-1 in VSMC (TgNOR-1; n=14). Half of the animals of each group were treated with LPS (n=7) or saline (Control; n=7). As observed, over-expression of NOR-1 in VSMC ameliorated the LPS-induced increase in the expression of these MMPs. Data are expressed as mean±SD. *p<0.01; † vs. non-stimulated WT mice; ‡ vs. LPS-stimulated WT mice.