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NOS3 prevents MMP-9, and MMP-13 induced extracellular matrix proteolytic degradation through specific microRNA-targeted expression of extracellular matrix metalloproteinase inducer in hypertension-related atherosclerosis

Rafael Ramírez-Carracedo[a,*], Ignacio Hernández[a,b], Rafael Moreno-Gómez-Toledano[a,c], Javier Díez-Mata[a], Laura Tesoro[a], Claudia González-Cucharero[a], Beatriz Jiménez-Guirado[a], Nunzio Alcharani[a], Laura Botana[a], Marta Saura[b,c], Jose L. Zamorano[b,d], and Carlos Zaragoza[a,b,*]

Background: Endothelial nitric oxide synthase (NOS3) elicits atheroprotection by preventing extracellular matrix (ECM) proteolytic degradation through inhibition of extracellular matrix metalloproteinase inducer (EMMPRIN) and collagenase MMP-13 by still unknown mechanisms.

Methods: C57BL/6 mice lacking *ApoE*, *NOS3,* and/or *MMP13* were fed with a high-fat diet for 6 weeks. Entire aortas were extracted and frozen to analyze protein and nucleic acid expression. Atherosclerotic plaques were detected by ultrasound imaging, Oil Red O (ORO) staining, and Western Blot. RNA-seq and RT-qPCR were performed to evaluate EMMPRIN, MMP-9, and EMMPRIN-targeting miRNAs. Mouse aortic endothelial cells (MAEC) were incubated to assess the role of active MMP-13 over MMP-9. One-way ANOVA or Kruskal–Wallis tests were performed to determine statistical differences.

Results: Lack of NOS3 in *ApoE* null mice fed with a high-fat diet increased severe plaque accumulation, vessel wall widening, and high mortality, along with EMMPRIN-induced expression by upregulation of miRNAs 46a-5p and 486-5p. However, knocking out *MMP-13* in *ApoE/NOS3*-deficient mice was sufficient to prevent mortality (66.6 vs. 26.6%), plaque progression (23.1 vs. 8.8%), and MMP-9 expression, as confirmed in murine aortic endothelial cell (MAEC) cultures, in which MMP-9 was upregulated by incubation with active recombinant MMP-13, suggesting MMP-9 as a new target of MMP-13 in atherosclerosis.

Conclusion: We describe a novel mechanism by which the absence of NOS3 may worsen atherosclerosis through EMMPRIN-induced ECM proteolytic degradation by targeting the expression of miRNAs 146a-5p and 485-5p. Focusing on NOS3 regulation of ECM degradation could be a promising approach in the management of atherosclerosis.

Graphical abstract: http://links.lww.com/HJH/C406

Keywords: atherosclerosis, , Extracellular Matrix MetalloPRotease INducer, microRNA, MMP-13

Abbreviations: ApoE, apolipoprotein E; ECM, extracellular matrix; EMMPRIN, Extracellular Matrix MetalloPRotease INducer; KO, knock out; MAEC, mouse aortic endothelial cells; miRNA, microRNA; MMP, matrix metalloprotease; NO, nitric oxide; NOS3, nitric oxide synthase 3; ORO, Oil Red O

INTRODUCTION

Targeting extracellular matrix (ECM) degradation is a promising strategy for managing atherosclerosis, as matrix metalloproteases, including MMP-9 and MMP-13, play a major role on plaque formation by still unknown mechanisms. MMP-9 is a strong predictor of plaque instability, whereas MMP-13 may act in both ways: in early stages of atherosclerosis, MMP-13 cleaves ICAM-1, thereby preventing circulating monocyte adhesion to the lesion site [1], but at the end, MMP-13 contributes to ECM degradation. Others found that reducing MMP-9 and MMP-13 activity separately may attenuate plaque burden [2], but no studies have been conducted so far on the possible relationship between the two enzymes in atherosclerosis.

Journal of Hypertension 2024, 42:685–693

[a]Unidad Mixta de Investigación Cardiovascular, Departamento de Cardiología, Universidad Francisco de Vitoria, Hospital Ramón y Cajal (IRYCIS), [b]Centro de Investigación Biomédica en Red de Enfermedades Cardiovasculares (CIBERCV), Av. Monforte de Lemos, [c]Universidad de Alcalá, Unidad de Fisiología, Departamento de Biología de Sistemas, Alcalá de Henares and [d]Departamento de Cardiología, Hospital Universitario Ramón y Cajal (IRYCIS), Madrid, Spain

Correspondence to Rafael Ramírez-Carracedo, Universidad Francisco de Vitoria, Spain. Tel: +34 648294920; e-mail: rrcarracedo@hotmail.com; c.zaragoza.prof@ufv.es

*R.R.-C. and C.Z. equally contributed to this work.

Received 31 July 2023 **Revised** 8 January 2024 **Accepted** 22 January 2024

DOI:10.1097/HJH.0000000000003679

Hypertension increases the risk for atherosclerosis, and in this context, we and others have shown the protective effect of the endothelial Nitric Oxide Synthase (NOS3, eNOS) in preventing the formation of atherosclerotic plaques [2–4]. Endothelial nitric oxide is known to alter the ECM composition and metabolism in atherosclerosis by regulating the synthesis of collagen I and III in vascular smooth muscle cells (VSMCs) [5,6] and the expression of the tissue inhibitor of MMPs, TIMP-2 [7]. Also, by modulating the Extracellular Matrix MetalloProtease Inducer (EMMPRIN), NOS3 expression is related to low levels MMP-9, and MMP-13 in the atherosclerotic plaque by means yet to be identified [1,8].

In this context, recent studies have shown the role of microRNAs (miRNAs), small noncoding RNA molecules that regulate gene expression. Some miRNAs are known to modulate EMMPRIN expression in cancer, such as miRNA-22 [9,10]; miR-125a-5p [11]; miR-146a-5p [12–14]; and miRNA-485-5p [15], which also are related to a decrease in MMP expression [13,16–18]. These miRNAs also have a role in atherosclerosis, as miRNA-22 and miRNA-146a regulates cellular migration [19], miRNA-125a-5p mediates in the transition of macrophages into foam cells [20] and miRNA-485-5p is associated to hypertriglyceridemia by targeting *APOA5* [21]. Hence, we cannot exclude the role of specific miRNAs, which may help to explain the protective effect of NOS3 against atherosclerosis by preventing ECM degradation through inhibition of EMMPRIN.

In this study, we describe novel mechanisms by which the absence of NOS3 may worsen atherosclerosis through EMMPRIN-induced ECM proteolytic degradation by targeting the expression of miRNAs 146a-5p and 485-5p in mice. To validate the translational relevance of our findings, we collected and analyzed human carotid endarterectomy samples with atherosclerotic lesions to ascertain the involvement of ECM degradative proteins in the pathogenesis of atherosclerosis. Elevated levels of EMMPRIN, MMP-9, and MMP-13 were found, serving as proof of concept for the present study.

MATERIAL AND METHODS

Animal models of atherosclerosis and sample extraction

Animal research was performed according to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, NIH Publication No. 85-23, and revised 1996). *NOS3*, *ApoE* and *MMP13* knockout (KO) mice (C57BL/6 strain) were acquired from The Jackson Laboratory (Bar Harbor, Maine, USA). Selective breeding was performed to acquire the double KO *ApoE/MMP13, ApoE/NOS3* and the triple KO *ApoE/NOS3/MMP13* genotypes. Animal genotypes were determined using the KAPA Mouse Genotyping Kit from Merck (Darmstadt, Germany) by traditional PCR. The primer sequences for mouse MMP-13 gene were: forward 5′-GGA AAG TCT GAG ACA AGT AAC GTG C-3′ and reverse 5′-ATG GAC CTT CTG GTC TTC TGG C-3′. For Apoe-/- verification, the primers sequences were: common 5′-GCC TAG CCG AGG GAG AGC CG-3′, wild type reverse 5′-TGT GAC TTG GGA GCT CTG CAG C-3′ and mutant reverse 5′-GCC GCC CCG ACT GCA TCT-3′. Mutant shows a band at 245 bp, wild type at 155 bp and heterozygote at 155 and 245 bp. For NOS-/- confirmation, the primers sequences were: common 5′- CTT GTC CCC TAG GCA CCT CT-3′, wild type forward 5′-AGG GGA ACA AGC CCA GTA GT-3′ and mutant 5′-AAT TCG CCA ATG ACA AGA CG-3′. PCR bands were detected using a ChemiDoc Imaging System from Bio-rad (Hercules, California, USA). Mutant shows a band at 300 bp, wild type at 337 bp and heterozygote at 300 and 337 bp.

Wild type animals were used as control. A total of 15 animals of each condition were bred and fed for 6 weeks with high-fat diet and then euthanized to extract the entire aortas. After complete perfusion with sterile PBS, the aortas were extracted and immediately frozen in liquid nitrogen and then stored at −80 °C or immersed in formalin solution.

Cell culture

Mouse aortic endothelial cells (MAEC) were cultured in Dulbecco's modified Eagle's medium F-12 (DMEM F-12) from Lonza (Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) from Merck and 50 mg/ml of penicillin and 50 mg/ml streptomycin (Invitrogen, Carlsbad, California, USA), and incubated at 37 °C in humidified atmosphere of 5% CO_2 and 95% oxygen. After reaching 70% confluence, cells were treated with 1 μg MMP-13 (Merck) previously activated in APMA solution (0.04 mol/l in DMSO) or 10 μmol/l CL-82198, an MMP-13 inhibitor (Abcam, Cambridge, UK).

Artery wall thickness and lipid deposition analysis

Animals were anesthetized with isoflurane (Abbot, Chicago, Illinois, USA) prior to acquire the echography images and then longitudinal sections of the carotid artery were visualized using a Vivid Q ultrasound system from GE Healthcare (General Electric, Chicago, Illinois, USA) 6 weeks after starting high-fat diet. Artery wall thickness was measured in centimeters using the system's built-in software.

On the other hand, Oil Red O (ORO) staining was performed to determine the lipid content within the aortic wall. Aortas immersed in formalin solution were rinsed three times with PBS and incubated for 1 h in 60% Isopropanol (Merck Millipore). Afterwards, aortas were stained in ORO solution (6 mg/ml in 60% Isopropanol) for 1 h, and then were rinsed for 30 min in 60% isopropanol (Merck Millipore). Pictures were taken using a binocular loupe and lipid quantification was performed using ImageJ software by relating the red-stained lipids to the total artery area.

Histological procedures

Endarterectomy samples were obtained according to the rules of the Declaration of Helsinki of 1975, revised in 2008, and the study was approved by the ethics committee from the University Francisco de Vitoria, Spain. 24 patients (16 men: mean age, 70 ± 5 years; 8 women: mean age, 75 ± 8 years) provided written consent, and carotid endarterectomies were selected by angiography and Doppler ultrasound.

Immunoblotting

Total protein from aortic samples or MAEC cells was extracted using RIPA Buffer (Merck Millipore) and 20 μg of total protein were loaded into 10% polyacrylamide gels to measure EMMPRIN, MMP-9, and MMP-13 expression. Proteins were transferred to PVDF membranes and blocked with 5% BSA in T-TBS. Incubations were performed for 1 h with anti-EMMPRIN, anti-MMP-9 or anti-MMP-13 antibodies (Abcam) and then for 1 h with anti-Rabbit HRP-conjugated secondary antibody (Abcam). Protein bands were visualized by chemiluminescence, and band density was analyzed using ImageJ. The activity of EMMPRIN was determined based on the ratio between the expression of high glycosylated (HG; 45-66 kDa) or low glycosylated (LG; 25–34 kDa) bands. MMP-9 and MMP-13 expression was determined by measuring band density, and GAPDH was used as housekeeping protein. Also, protein expression for each condition was related to WT or CT and presented as a percentage.

Small RNASeq and micro-RNA expression analysis by RT-qPCR

Aortic samples were processed to extract and isolate total miRNA content using the miRNA isolation kit mirVana (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's instructions.

Small RNASeq was performed using CUTADAPT to preprocess the sequencing reads and to eliminate Illumina adaptor remains. Afterwards, sequencing reads were aligned against mature miRNA sequences extracted from miRbase 22 to determine miRNA expression. Only those miRNAs expressed at least one counts-per-million (CPM) in at least two samples per condition were considered for differential analysis.

An adjusted P value (Benjamini–Hochberg method) less than 0.05 was used to detect significant differential expression. Subsequently, a Venn diagram was developed to identify specifically regulated miRNAs after *NOS3* deletion.

Bioinformatics analysis using miRWalk webtool predicted target genes of the selected miRNAs employing TargetScan and miRDB databases (score 1.0).

FunRich open software (v.3.1.4) [22–24] was used to develop enrichment analysis using Gene Ontology mouse database. A threshold of P value less than 0.001 was selected to filter significant Gene Ontology terms, and a posterior analysis using ShinyGO 0.77, was performed for KEGG mapping (Kyoto Encyclopedia of Genes and Genomes) and Gene Ontology enrichment analysis.

RT-qPCR was performed in triplicate on a Quant Studio 7 Flex PCR System (Applied Biosystems, Foster City, California, USA) using commercial TaqMan probes (Thermo Fisher). The obtained amplification results were analyzed with QuantStudio Real-Time PCR software 1.3 (Thermo Fisher) using miR92b-3p a housekeeping miRNA. The n-fold differences were calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, California, USA) and SPSS 26 (IBM, Armonk, California, USA). The D'Agostino–Pearson omnibus normality test and Shapiro–Wilk normality test were used to analyze data distribution. All results are represented as median ± interquartile range because of small number of samples. Student t-test or Mann–Whitney test was used in the comparative analysis of two groups. For multiple comparative analysis, one-way ANOVA or Kruskal–Wallis followed by a Bonferroni or Dunn's test, were carried out. GraphPad Prism software and SPSS from IBM (Armonk) were used to perform statistical analysis. Differences were considered significant at P less than 0.05.

RESULTS

EMMPRIN, MMP-9 and MMP-13 are overexpressed in human endarterectomy samples

EMMPRIN is the main inducer of matrix metalloproteases (MMPs) and is known to be overexpressed in atherosclerosis, as well as MMPs. Therefore, as a proof of concept, we confirmed the increased expression of EMMPRIN, MMP-9 and MMP-13 in human endarterectomies by immunohistochemistry (Fig. 1).

NOS3 induces the expression of EMMPRIN-targeting micro-RNAs

Endothelial NO inhibits the expression of EMMPRIN by still unexplored mechanisms. Recent findings on miRNA-mediated regulation of specific targets in atherosclerosis, prompted us to explore whether endothelial NO may play a role on miRNA-regulated expression of EMMPRIN. RNA-seq studies from aortas of wild-type, *ApoE and NOS3* null mice fed with high-fat diet, led us to find a significant number of miRNAs differentially expressed between WT and *NOS3-/-* (229 miRNAs), and between *ApoE-/-* and *NOS3-/-* (255 miRNAs), in which 123 miRNAs were differentially regulated in both studies, and hence, represent the NOS3 miRNA footprint (Fig. 2a). The validated targets of selected miRNAs were studied to assess the implication of NOS3 in a KEGG and GO enrichment analysis (see methods for details), showing a significant regulation of pathways including TGF-beta signaling, involved in transcriptional regulation of NOS3 [6], autophagy [25] and apoptosis signaling [26], as well as PI3K-AKT signaling pathway, involved in NOS3 activation [27] or lipid and atherosclerosis-related genes (Fig. 2b). GO enrichment analysis of biological processes revealed the regulation MMP-related ECM degradation by the absence of NO, altering pathways related to ECM organization or atherosclerosis-related processes as angiogenesis, cell migration and cell-adhesion (Fig. 2c).

Among these 123 differentially expressed miRNAs, two specific reported EMMPRIN-inhibitor miRNAs mIR-146a-5p and mIR-485-5p were expressed in wild type, and *ApoE* null mice (Fig. 2d), but in the absence of *NOS3*, *ApoE/NOS3* double and *ApoE/NOS3/MMP13* triple knockout mice (Fig. 3a) exhibited low levels of both miRNAs (Fig. 3b). The role of MMP-13 in the NO-mediated prevention of atherosclerosis was previously reported [1], and here we found that in *ApoE/MMP-13* null mice expressing NOS3, miRNA-146a-5p and miRNA-485-5p, were not downregulated (Fig. 3b), suggesting a new finding by which NOS3 might prevent EMMPRIN-mediated ECM degradation in atherosclerosis. Interestingly, high mortality rates were found in mice *ApoE/NOS3* expressing MMP-13 but were reduced in triple *ApoE/NOS3/MMP-13* (Fig. 2c).

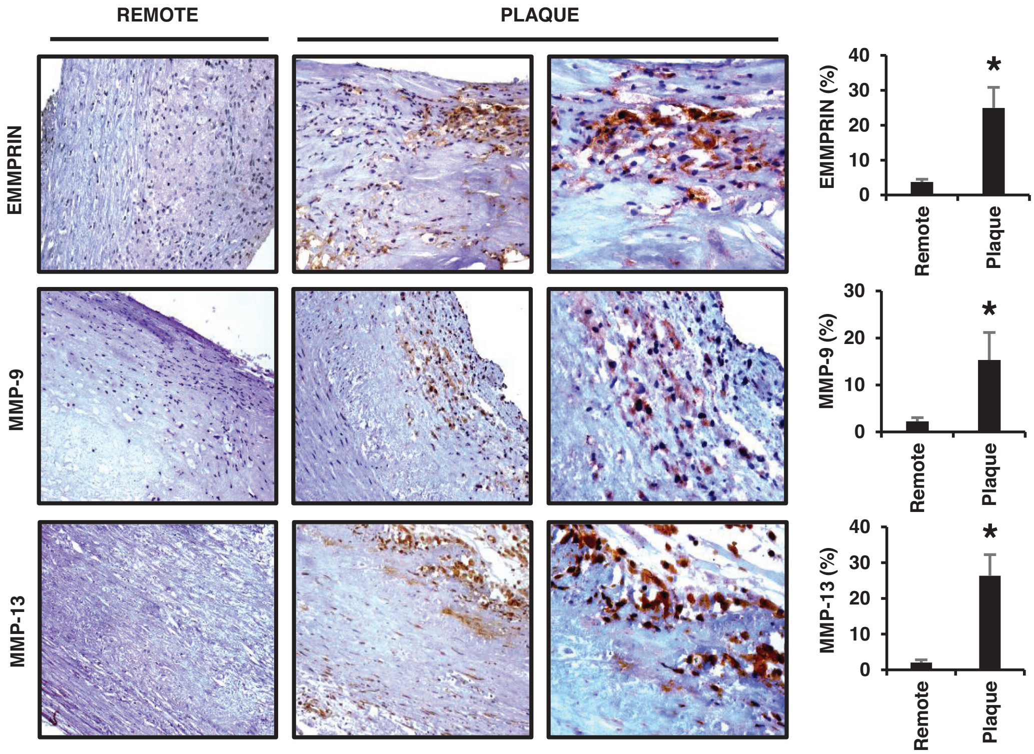

FIGURE 1 Extracellular matrix metalloproteinase inducer, MMP-9 and MMP-13 expression in human endarterectomies. IHQ showing EMMPRIN, MMP-9 and MMP-13 expression in human endarterectomies ($N=5$ EMMPRIN and MMP-9; $N=3$ MMP-13; $^{*}P<0.001$ vs. remote). EMMPRIN, extracellular matrix metalloproteinase inducer.

Lack of MMP-13 prevents aortic lipid accumulation and arterial wall thickening in NOS3 null mice

Our data point to EMMPRIN as a target of endothelial NO to prevent atherosclerosis. Immunoblot of protein lysates from the aortas of mice lacking *NOS3* show increased EMMPRIN levels in *ApoE/NOS3*, and *ApoE/NOS3/MMP-13* null mice, compared with *ApoE*, and *ApoE/MMP-13*-deficient mice (Fig. 4a), suggesting that in both strains, the absence of endothelial NO might worsen atherosclerosis. Indeed, the aortas of mice fed with high cholesterol diet for 6 weeks and stained with ORO indicated severe plaque accumulation in *ApoE/NOS3*, compared with *ApoE* and *ApoE/MMP-13* null mice, whereas in the *ApoE/NOS3/MMP-13* triple null genotype, the absence of MMP-13 implied lower lipid deposition (Fig. 4b). This was also evidenced when measuring aortic wall thickness from the same mice (Fig. 4c) pointing towards for the first time MMP-13 as a new therapeutic target against atherosclerosis.

The expression of MMP-9 is regulated by MMP-13 in atherosclerotic mice

NOS3 regulates the levels of EMMPRIN *in vivo*. MMP induction by EMMPRIN relies in its glycosylation, being the high glycosylated (45–66 kDa) forms of EMMPRIN more active than the low glycosylated (25–34 kDa) forms. Mice lacking NOS3 alone or in combination with the absence of MMP13 show elevated HG-EMMPRIN (Fig. 4a). However, in the absence of NOS3 and MMP-13, atherosclerotic burden is much lower, when compared with *NOS3-/-* mice expressing MMP-13 (Fig. 4b and c), suggesting that MMP-13 might at least represent a target of EMMPRIN in atherosclerotic mice. In addition to MMP-13, gelatinase MMP-9 is also regulated by EMMPRIN and plays a key role in the proteolytic breakdown of ECM in murine and human atherosclerosis. As expected, lack of NOS3 induced MMP-9 expression in atherosclerotic mice, but surprisingly, in *ApoE/NOS3/MMP-13* triple KO, MMP-9 levels were much lower compared with MMP-13 expressing mice (*ApoE/NOS3* double KO) (Fig. 4d), suggesting for the first time, the implication of collagenase MMP-13 in the expression of MMP-9 in atherosclerosis. To test this hypothesis, incubation of murine aortic endothelial cells (MAEC) with 10 μmol/l MMP-13 inhibitor had a very modest effect on MMP-9 expression, whereas in the presence of 1 μg recombinant MMP-13 active form (see methods for details), MAEC expressed MMP-9 over control cells (Fig. 4e).

In conclusion, our results unveil for the first time a molecular link between NOS3 and EMMPRIN that explains how endothelial NO prevents ECM degradation in atherosclerosis, as depicted in Fig. 5.

DISCUSSION

ECM-degrading MMPs play a key role in the development and progression of atherosclerosis [28]. Therefore, several pharmaceutical companies have focused on the potential therapeutic properties of targeting selected MMPs against plaque development and progression. However, these efforts have seen moderate to limited success, possibly because of a lack of understanding of the molecular complexity of ECM degradation in atherosclerosis or compensatory activities among different MMPs, where the

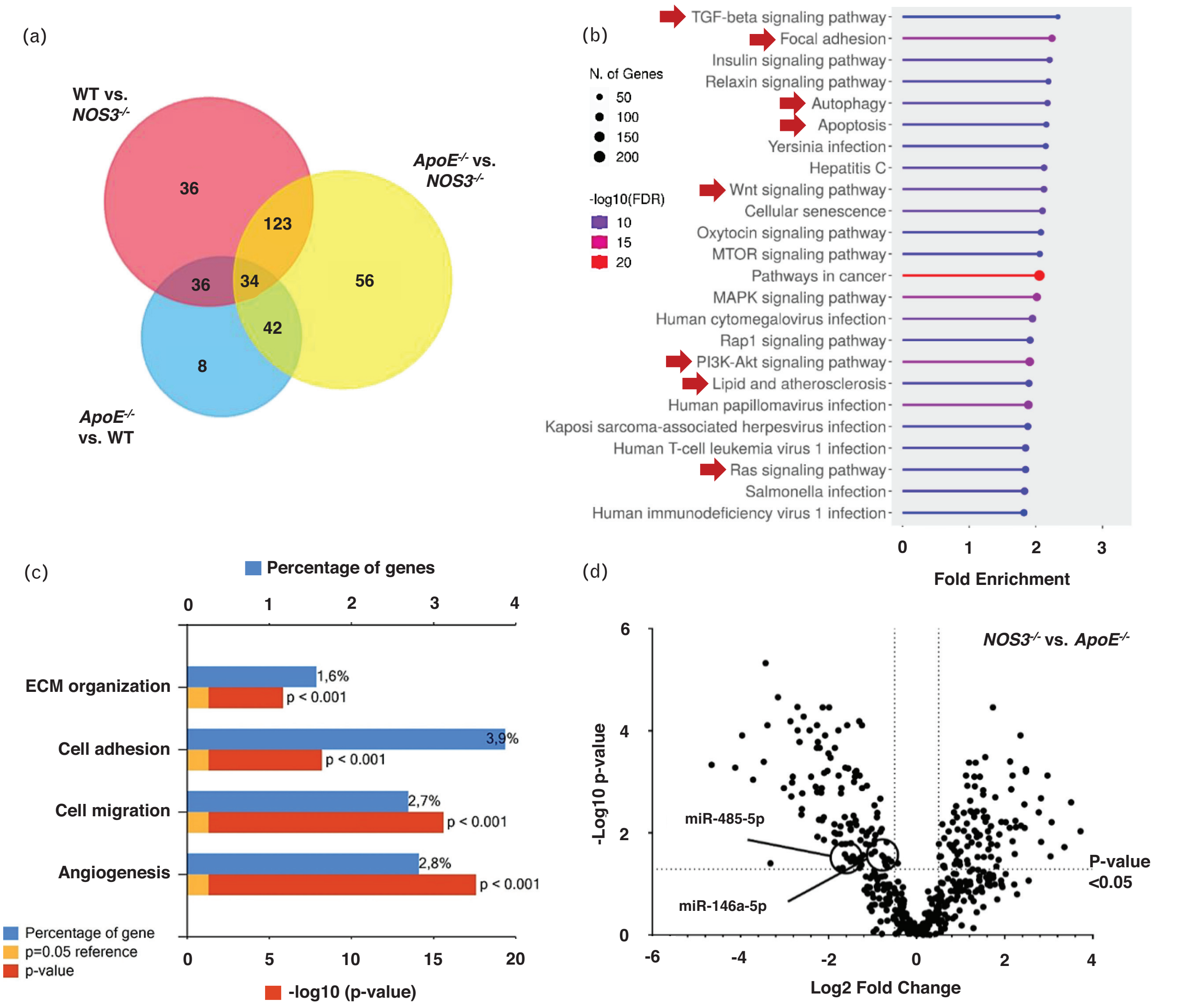

FIGURE 2 Small RNAseq determination of extracellular matrix metalloproteinase inducer-targeting miRNAs. (a) WT vs. *NOS3*$^{-/-}$ and *NOS3*$^{-/-}$ vs. ApoE$^{-/-}$ miRNA expression in mouse arteries expressed in a Venn diagram (*N* = 3/condition). (b) KEGG pathways enrichment analysis showing the validated molecular pathways targeted by *NOS3*$^{-/-}$ differentially expressed miRNAs. Red arrows point to atherosclerosis-related pathways. (c) GO enrichment analysis showing the validated biological processes targeted by *NOS3*$^{-/-}$ differentially expressed miRNAs. Blue bar refers to the number of genes differently expressed in *NOS3*$^{-/-}$ animals associated to each biological process and red bar indicates the $-\log_{10}$ (*P* value) of the result. (d) Volcano plot representing decreased (left) or increased (right) expression of miRNAs from *NOS3*$^{-/-}$ samples compared with *ApoE*$^{-/-}$ (*N* = 3/condition). GO, gene ontology.

inactivation of one is offset by others [29]. Hence, one of the proposed alternatives consists of inhibiting upstream MMP-inducing mechanisms.

In this context, human endarterectomy samples with atherosclerotic lesions shown in this work act as proof of concept showing that not only EMMPRIN but also MMPs are overexpressed in atherosclerotic plaques, suggesting that ECM degradative proteins have indeed a role in the progression of the disease. However, the origin of this increased expression remains unclear. Therefore, our current work provides evidence that NOS3 may confer atheroprotection through a novel distinct pathway, with EMMPRIN playing a major role.

It is established that endothelial NO, synthesized by NOS3, induces atheroprotection by decreasing arterial blood pressure, preventing oxidative stress and inflammation [4], and preventing early plaque development through MMP-13-mediated monocyte protein adhesion ICAM-1 proteolytic degradation [1]. In later stages, endothelial NO also inhibits the activation and expression of the Extracellular Matrix Metalloproteinase INducer EMMPRIN, by still unknown mechanisms, whose overexpression leads to plaque weakening [2,8,30]. Here, we describe how NOS3-derived NO regulates the expression of 123 miRNAs, some of which are implicated in key biological processes and mechanisms in atherosclerosis, such as TFG-β, Wnt and PI3K-akt pathways, cell adhesion or ECM organization. These results are in accordance with previous studies showing the NOS3 implication in some of those routes, by interfering with the TGF-β/Smad2 signaling [6], inhibiting endothelial cell proliferation by activating Wnt/β-catenin signaling [31] or regulating cell adhesion in atherosclerosis [1]. Concerning ECM degradation, several EMMPRIN-targeting miRNAs have been described and proven to participate in different

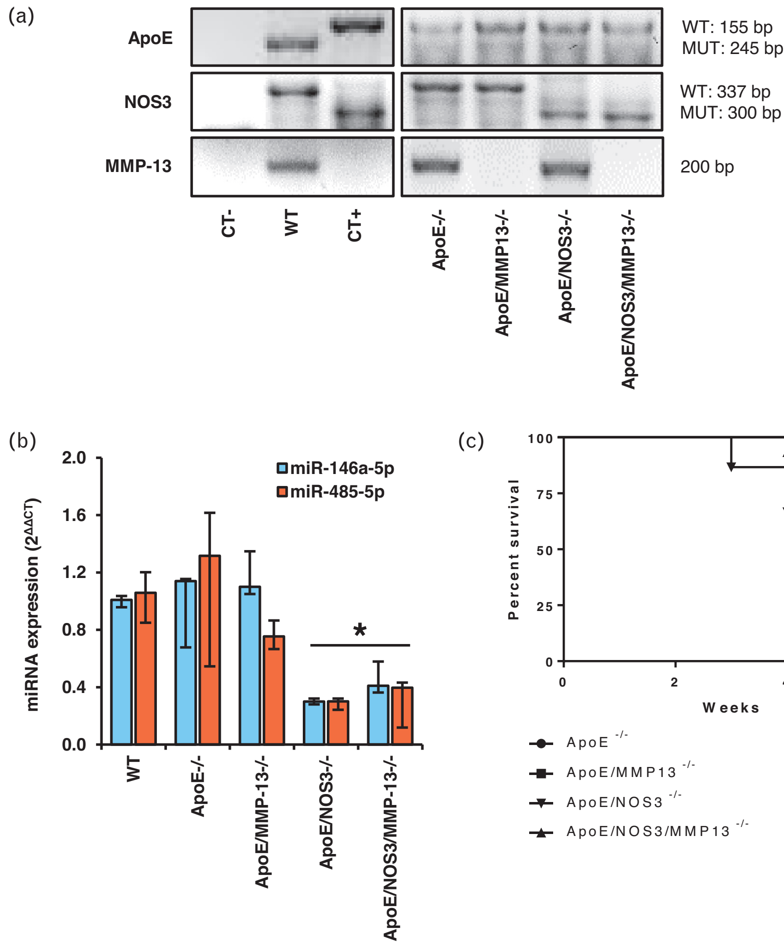

FIGURE 3 Extracellular matrix metalloproteinase inducer-targeting miRNA expression in mutant C57BL/6 mice. (a) PCR genotyping of mutant mice, lacking *ApoE*, *MMP13* and/or *NOS3* genes. (b) miRNA 146a-5p and 485-5p expression in aortas of mutant mice determined by RT-qPCR ($N = 5$/condition; $^*P < 0.0001$ vs. WT). (c) Kaplan–Meier survival plot. Survival rate is shown in percentage for each condition (initial $N = 15$/condition; $P < 0.0001$ determined by log-rank test). WT, wild-type.

disorders, including atherosclerosis [13,15,32]. Accordingly, we found that NOS3 reduced the expression of EMMPRIN through specific EMMPRIN-targeted miRNAs miRNA-146a-5p and miRNA-485-5p, thereby reducing collagenolytic MMP activity, suggesting that endothelial NO prevents MMP expression at least through miRNA-mediated EMMPRIN inhibition, which also helps to understand the atheroprotective effect of NO by preventing platelet aggregation and monocyte adhesion [33].

In accordance with others [34–36], *ApoE/NOS3* deficiency increased systemic blood pressure, together with higher mortality rates even in the absence of MMP-13 (Figure S1, http://links.lww.com/HJH/C405). This could imply cross-talk in atherosclerosis, as knocking out MMP-13 expression in the *ApoE/NOS3* double-deficient mouse causes partial recovery of arterial wall thickness and plaque extension parameters, as well as reduced mortality, pointing towards MMP-13 as a target of NOS3 in atheroprotection. However, and even though MMP-13 is well known to increase plaque instability through collagen degradation [2,37], little is known about the relationship between NOS3 and MMP-13 in this context. We previously found that macrophage-derived NO induces the expression of MMP-13 in vascular endothelial cells and in later atherosclerosis [8,38,39], while in the onset of inflammation, lack of *NOS3* reduces MMP-13 in *ApoE/NOS3*-double vs. *ApoE*-single null mice [1].

Matrix metalloproteinases are zinc-dependent ECM-degrading endopeptidases whose expression as inactive zymogens is transcriptionally regulated by a plethora of pro-inflammatory signals. Additionally, zymogens became active proteases by means of several stimuli, which may release the zinc from its catalytic domain [38], or by factors that may disrupt the complex with other proteins. Such is the case of MMP-13, which is bound to caveolae component Caveolin-1 [40], and protein tyrosine nitration induces MMP-13-dependent endothelial cell migration and wound healing [39,41]. In addition, other MMPs, may act as zymogen activators [42,43], but for the first time, we report the contribution of MMP-13 regulating MMP-9 expression. Further studies will aim to fully characterize the MMP-induced expression of other metalloproteases, and its functional consequences.

In conclusion, endothelial NO exerts atheroprotective effects by regulating adverse extracellular matrix (ECM) degradation in the vessel wall through mechanisms that are still not fully understood. Although additional studies are needed to delve into the NO-mediated regulation of miRNAs and MMPs, this research provides an exciting

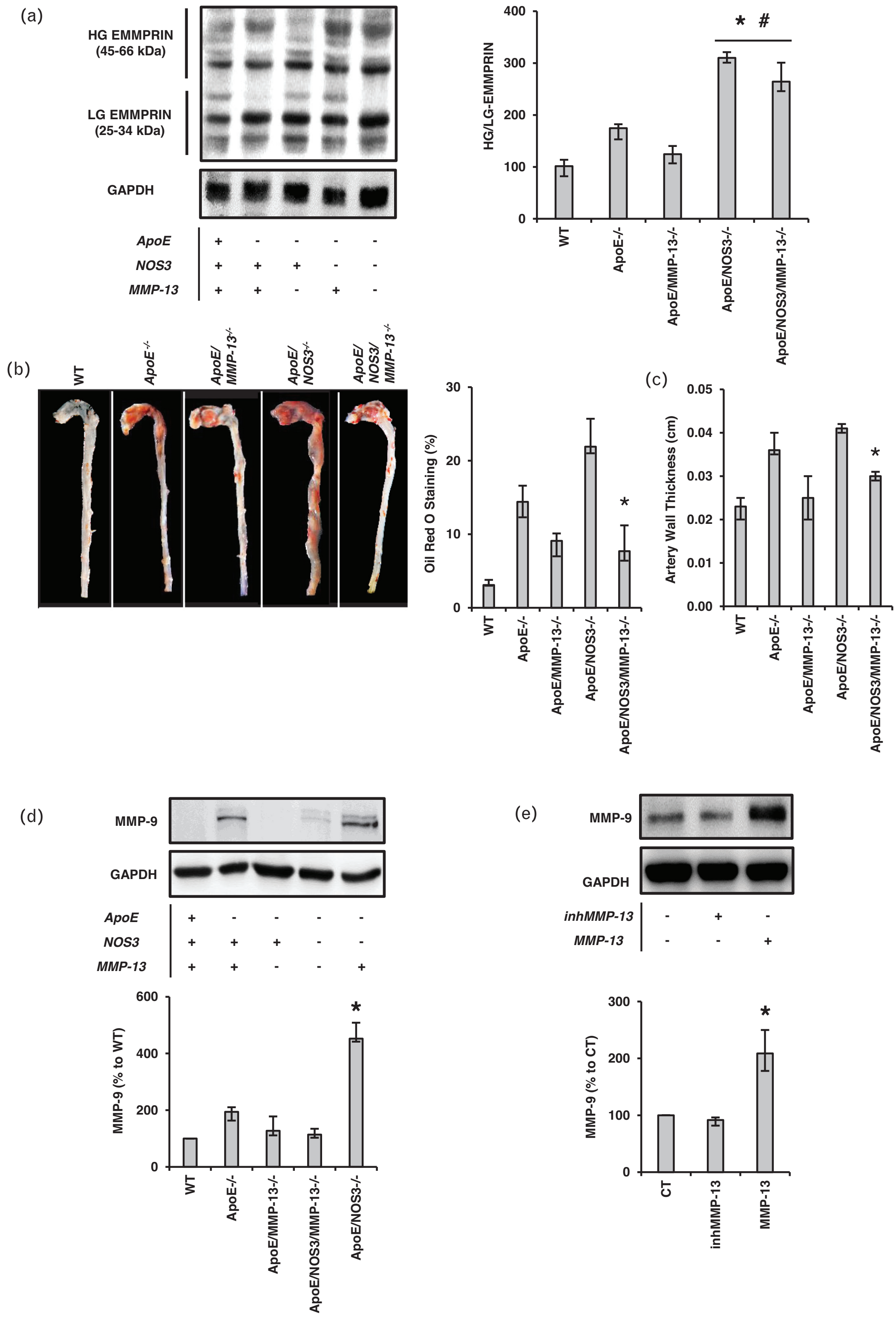

FIGURE 4 Extracellular matrix metalloproteinase inducer and MMP-9 expression in atherosclerotic aortas and endothelial cells. (a) EMMPRIN expression in atherosclerotic aortas of mutant mice ($N=5$/condition; $^*P<0.0001$ vs. WT; $^{\#}P<0.0001$ vs. $ApoE^{-/-}$). (b) Quantification of ORO staining of lipid accumulation indicative of atheroma plaque formation in whole aortas ($N=5$/condition; $^*P<0.01$ vs. $ApoE/NOS3^{-/-}$). (c) Ultrasound measurement of left common carotid artery wall thickness ($N=5$/condition; $^*P<0.001$ vs. $ApoE/NOS3^{-/-}$). (d) MMP-9 expression in atherosclerotic aortas of mutant mice ($N=5$/condition; $^*P<0.0001$ vs. WT). (e) MMP-9 expression in MAEC treated with 10 μmol/l MMP-13 inhibitor or 1 μg activated MMP-13 ($N=3$/treatment; $*P<0.01$ vs. CT). EMMPRIN, extracellular matrix metalloproteinase inducer. EMMPRIN, extracellular matrix metalloproteinase inducer; ORO, Oil Red O; WT, wild-type.

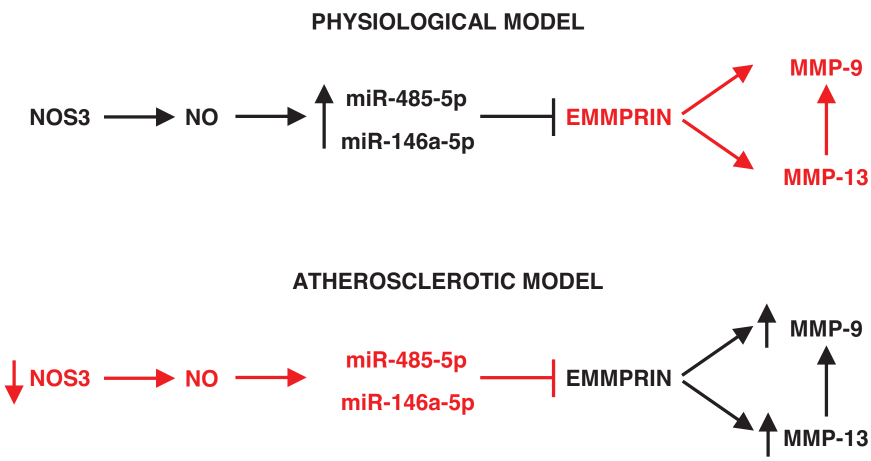

FIGURE 5 Graphical depiction of the molecular inhibition of extracellular matrix metalloproteinase inducer by nitric oxide.

opportunity for developing novel therapeutic approaches to overcome the persistent challenges in managing atherosclerosis.

ACKNOWLEDGEMENTS

Financial support: this research was funded by Carlos Zaragoza 'Instituto de Salud Carlos III, PI14/02022 co-financed by the European Development Regional Fund A way to achieve Europe (ERDF)' and 'Universidad Francisco de Vitoria, Grant number 2019'.

Dataset availability: the datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

Conflicts of interest

There are no conflicts of interest.

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