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THE ROLE OF THE NR4A ORPHAN NUCLEAR RECEPTOR NOR1 IN VASCULAR CELLS AND ATHEROSCLEROSIS

Yue Zhao
University of Kentucky, zh1983y@hotmail.com

ABSTRACT OF DISSERTATION

Yue Zhao

The Graduate School

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the

requirements for the degree of Doctor of Philosophy in the

College of Medicine, Graduate Center for Nutritional Sciences

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By

Yue Zhao

Lexington, Kentucky

Director: Dr. Dennis Bruemmer, Professor of Endocrinology and Molecular Medicine

Lexington, Kentucky

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ABSTRACT OF DISSERTATION

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The neuron-derived orphan receptor 1 (NOR1) belongs to the NR4A nuclear receptor subfamily. As an immediate early response gene, NOR1 is rapidly induced by a broad spectrum of physiological and pathological signals. Functional studies demonstrate NOR1 as a constitutively active ligand-independent nuclear receptor whose transcriptional activity is dependent on both expression level and posttranslational modifications. To date, an increasing number of studies have demonstrated a pivotal role of NOR1 in the transcriptional control of metabolism and the development of cardiovascular diseases.

In this dissertation, we demonstrate NOR1 expression in endothelial cells and sub-endothelial cells of human atherosclerotic lesions. In response to inflammatory stimuli, NOR1 expression is rapidly induced in endothelial cells through an NF-κB-dependent signaling pathway. Functional studies reveal that NOR1 increases monocyte adhesion by inducing the expression of adhesion molecules VCAM-1 and ICAM-1 in endothelial cells. Transient transfection and chromatin immunoprecipitation assays identify VCAM-1 as a *bona fide* NOR1 target gene in endothelial cells. Finally, we demonstrate that NOR1-deficiency reduces hypercholesterolemia-induced atherosclerosis formation in apoE-/- mice by decreasing the macrophage content of the lesion.

In smooth muscle cells (SMC), NOR1 was previously established as a cAMP response element binding protein (CREB) target gene in response to platelet-derived growth factor (PDGF) stimulation. CREB phosphorylation and subsequent binding of phosphorylated CREB to the NOR1 promoter play a critical role in inducing NOR1 expression. In this dissertation, we further demonstrate that histone deacetylase (HDAC) inhibition potentiates and sustains PDGF-induced NOR1 mRNA and protein expression

in SMC. This augmented NOR1 expression is associated with increased phosphorylation of CREB, recruitment of phosphorylated CREB to the NOR1 promoter, and *trans*-activation of the NOR1 promoter. Additionally, HDAC inhibition also increases NOR1 protein half-life in SMC.

Collectively, these findings identify a novel pathway in endothelial cells underlying monocyte adhesion and expand our knowledge of the epigenetic mechanisms orchestrating NOR1 expression in SMC. Finally, we establish a previously unrecognized atherogenic role of NOR1 in positively regulating monocyte recruitment to the vascular wall.

KEYWORDS: nuclear receptor; endothelial cells; atherosclerosis; smooth muscle cells; epigenetic mechanism

Yue Zhao

Student's Signature

March 23, 2011

Date

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By

Yue Zhao

Dennis Bruemmer
Director of Dissertation

Geza Bruckner
Director of Graduate Studies

March 25, 2011
Date

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF FILES

YueZhaoDissertation.pdf……Size: 3.28 MB

Chapter one: Introduction

1.1. Background

1.1.1. Introduction on atherosclerosis

Atherosclerosis is a chronic inflammatory and proliferative disease, in which immune mechanisms interact with arterial risk factors to initiate early fatty streaks[1, 2]. Continued lesion progression results in a mature lipid-enriched necrotic core surrounded by a cap of activated smooth muscle cells[1, 3]. In this process, endothelial cells, smooth muscle cells and monocyte-derived macrophages constitute the primary cell types responsible for atherosclerosis development[1, 2].

The endothelium, a barrier between circulating blood and tissues, does not support leukocyte binding under normal conditions[2, 4]. However, at the early stage of atherosclerosis, endothelial cells undergo inflammatory activation/dysfunction in response to atherogenic factors, such as inflammatory cytokines and hypercholesterolemia[5, 6]. Increased expression of chemokines and adhesion molecules leads to monocyte recruitment into the arterial wall, which is the key initial step for atherosclerotic lesion formation[1]. Numerous studies revealed that monocyte homing is a tightly regulated process, including initial rolling along the endothelium, triggering adhesion, strengthening adhesion and finally transmigrating across the endothelium. Many chemokines and adhesion molecules are involved in this process, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), selectins and integrins[7]. *In vivo* mouse model studies demonstrate that reduced expression of adhesion molecules or chemokines protects against the development of atherosclerosis. For example, the deficiency of monocyte chemoattractant protein-1 (MCP-1) reduces macrophage recruitment and atherosclerotic lesion formation in low density lipoprotein receptor (LDLR)-deficient mice and human

apolipoprotein (apo) B transgenic mice[8, 9]. Consistently, deletion of MCP-1 receptor CCR2 markedly decreases aortic macrophage accumulation and lesion formation in apoE knockout mice[10]. While whole body VCAM-1 deficient mice are not viable, studies using different knockdown strategies demonstrate a VCAM-1 gene dosage-dependent effect on atherosclerosis development[11, 12]. In addition, ICAM-1 deficiency has been shown to protect against atherosclerosis formation in apoE knockout mice[13].

The continuous retention of atherogenic lipoproteins, recruitment of monocytes, and lipid uptake by monocyte-derived macrophages promote fatty streak formation[1, 2]. The role of macrophage accumulation in promoting lesion initiation and progression was initially demonstrated by the observation that hypercholesterolemic mice become resistant to atherosclerosis when they are bred to macrophage-deficient mice[14]. Recent advances in this area demonstrate that monocytes are highly heterogeneous and each subtype plays distinct roles in atherosclerosis development[15]. $CCR2^{+}CX3CR1^{+}Ly\text{-}6C^{hi}$ and $CCR2^{-}CX3CR1^{++}Ly\text{-}6C^{lo}$ monocytes constitute two major subtypes, and data suggest that these monocytes utilize different receptors to infiltrate into the vascular wall[16]. While $CCR2^{+}$ monocytes are more efficiently recruited into the sub-endothelial space, $CCR2^{-}$ monocytes exhibit a higher potential in developing into plaque cells[16]. In addition to monocytes, other leukocyte subsets have also been observed in atherosclerotic lesions at various stages[17], such as neutrophils[18], mast cells[19], dendritic cells[20, 21] and T lymphocytes[17]. Although mostly found in the arterial adventitia, B lymphocytes are thought to confer a protective immunity during atherogenesis[17].

Further lesion progression advances by continued macrophage accumulation and a broad range of inflammatory responses[1, 2]. Recently, emerging evidence recapitulates the concept of inflammation resolution, which was considered only as a passive process. However, recent findings suggest that this process in fact represents a large number of actively regulated anti-inflammatory responses[22, 23]. Inflammation resolution in

atherosclerotic lesions can be achieved through several steps, including prevention of macrophage recruitment, promotion of anti-inflammatory cytokine expression, and efficient efferocytosis (clearance of apoptotic inflammatory cells)[22, 23].

The observation that increased macrophage apoptosis reduces early lesion formation is indicative of effective clearance of apoptotic cells in early atherogenesis[24]. On the contrary, another study demonstrates that phagocytosis of apoptotic cells is impaired in advanced atherosclerotic lesions[25]. These findings point to a protective role of efferocytosis in early atherogenesis. The maintenance of efficient efferocytosis in later stages may provide a novel strategy to attenuate atherosclerosis progression. Given the important role of macrophages in efferocytosis[23], these findings elicit an interesting concept that macrophages promote atherogenesis by producing inflammatory cytokines, while play a protective role through the clearance of apoptotic cells. Some evidence has implied that macrophage phenotype can be triggered to switch between classically (M1) and alternatively (M2) activated macrophages[26]. An increasing number of studies suggest that M2 macrophages are less inflammatory and have a greater phagocytic capacity[27]. Therefore, future studies on monocyte/macrophage subtypes may explain the discrepancies observed in macrophages.

Progression of early fatty streaks into complex atherosclerotic lesions is accompanied by SMC activation, migration and proliferation[1, 2]. Vascular SMCs constitute the major component of arteries and maintain a quiescent contractile phenotype in normal physiological conditions. In atherosclerotic lesions, inflammatory cytokines and mitogens trigger SMC migration into the arterial intima. Intimal SMCs undergo a phenotypic transition which is associated with a higher proliferation rate and greater synthesis capacity[28]. In addition, intimal SMCs take up lipids and secrete extracellular matrix proteins[1, 2]. A key role of these intimal SMCs is to form a fibrous cap under the endothelial cell layer, stabilizing lesions and preventing the necrotic lipid core from interacting with

blood[1, 2]. However, the continuous proliferation of SMCs and formation of extracellular matrix further promote lipoprotein retention and contribute to the narrowing of the vessel lumen[1, 2]. Another element of advanced atheroma is increased secretion and activation of matrix metalloproteinases (MMPs). MMPs function to degrade extracellular matrix, ultimately resulting in a thinner and more fragile fibrous cap prone to rupture[1-3]. While plaque rupture and thrombosis usually lead to acute cardiovascular diseases such as myocardial infarction and stroke, massive SMC proliferation plays a major role in the failure of occlusive vascular disease interventions, such as in-stent restenosis.[29]

1.1.2. Introduction on epigenetics

It was proposed by Conrad Waddington in the 1950s that "An epigenetic trait is a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence"[30]. This original definition of epigenetics emphasizes the heritability of a phenotype through cell divisions. Intensive studies have revealed that cellular responses to physiological/pathological signals involve a large number of transient chromatin modifications. In fact, these chromatin changes in response to environmental cues exactly reflect the ability of a cell to remember its past state[31]. Therefore, a broader definition of epigenetics has evolved to include these transient events happening to the chromosome.

Factors involved in epigenetic regulation include DNA methylation, histone modifications, transcription factors, non-coding RNAs, nucleosome position, and 3D organization of genes in the nucleus[31, 32]. There are a variety of histone post-translational modifications (PTM) which can be categorized into histone lysine acetylation, methylation, ubiquitinization and sumoylation, serine/threonine phosphorylation, arginine methylation and proline isomerization[33]. DNA methylation and histone PTM are most well characterized epigenetic markers, which can be read and translated into signals dictating gene activation and repression[33, 34]. For instance, acetylation of histone H4 on lysine 16 (AcH4-K16) is associated with increased gene expression[35] and has been shown to disrupt

higher chromatin structure in nucleosomal arrays[36]. This example represents a direct mechanism in which chromatin modifications activate gene transcription by increasing DNA accessibility to transcription factors[33]. Another mechanism by which chromatin modifications indirectly influence gene transcription involves the recruitment of chromatin binding proteins[33]. For example, acetylated histone H3 and H4 peptides only function to recruit other bromodomain-containing transcription co-activators whose acetyl-transferase activity is required for gene activation[37].

Both DNA methylation and histone PTM are reversible[33]. The addition and removal of these chromatin modifications are carried out by specific enzymes[33]. For example, histone acetyl-transferases (HAT) add acetyl-groups to lysine residues while histone deacetylases (HDAC) remove the acetylation[33, 38]. Gcn5 *N*-acetyltransferases (GNAT) and MYST HATs are the two predominant families of HAT. A number of transcription co-activators such as p300/CBP possess intrinsic acetyl-transferase activity and are categorized as HAT[39]. In addition, eighteen human HDACs have been identified with different substrate selectivity[38]. Similarly, all other chromatin modification enzymes consist of a large number of family members[40, 41]. Therefore, the timely and sequential actions of various chromatin modification enzymes are essential for orchestrating gene expression.

Cardiovascular disease, which involves dysregulated gene expression in the cardiovascular system [116], is one of the leading causes of death in the world[1-3]. Since every single cell in the body is subjected to epigenetic regulation, understanding the underlying mechanisms will provide further insight on the development, physiology and disease of cardiovascular system[42].

1.1.3. Introduction on NR4A orphan nuclear receptors

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1.1.3.1. Molecular biology of NR4A orphan nuclear receptors

Nuclear receptors (NR) share a common structure consisting of a ligand-independent AF-1 transactivation domain in the N-terminal region, a highly conserved DNA-binding domain (DBD) composed of two zinc fingers recognizing specific DNA sequences, and a ligand-binding domain (LBD) that contains a ligand-dependent AF-2 transactivation domain in its C-terminal portion (Figure 1.1A)[43]. NR4A receptors share this common NR structure, and the three members reveal a high degree of homology in their genomic structure and conservation of their DNA binding domain (degree of conservation > 90%)[44]. However, several lines of evidence indicate that NR4A receptors may represent a distinct group of transcription factors that do not function in a classical manner. Mutational analysis indicated that NR4A receptors function as constitutively-active receptors whose transcriptional activity is independent on the LBD[45, 46]. Instead, their transcriptional activity and co-activator recruitment appear to be dependent on the N-terminal AF-1 domain[46-48], which constitutes a common distinction of ligand-independent transcriptional activation by NR[49, 50]. This initial observation was supported by the finding that the LBD of NR4A contains hydrophilic surfaces instead of the classical hydrophobic cleft that mediates co-activator recruitment of other NR[48]. Finally, this unusual structure of the NR4A LBD has been recently confirmed by X-ray crystallography demonstrating that the Nurr1 LBD contains no cavity as a result of hydrophobic residues in the region normally occupied by ligands[51]. Considering these observations, NR4A receptors are currently thought to function as constitutively-active and ligand-independent receptors, whose transcriptional activity is primarily dependent on the expression of the receptor and its posttranslational modification.

NR4A receptors are early immediate response genes, which are induced by a pleiotropy of stimuli including growth factors, inflammatory stimuli, cytokines, peptide hormones, and cellular stress (Table 1.1)[52]. Once their expression is induced, NR4A receptors activate transcription by binding as monomers or homodimers to canonical DNA target sites, the NGFI-B-responsive element (NBRE) consisting of an octanucleotide AAAGGTCA motif (Figure 1.1B)[53, 54]. NR4A homodimers preferentially bind to the Nur-responsive element (NurRE), which constitutes an everted repeat of the NBRE-related sequence (AAAT(G/A)(C/T)CA) found in the pro-opiomelanocortin (POMC) gene promoter[55]. In addition, Nur77 and Nurr1 (but not NOR1) heterodimerize with RXR and activate transcription through a DR-5 element in a 9-cis retinoic acid-dependent manner[56, 57]. This heterodimerization of Nurr1 with RXR is isotype-specific since Nurr1 interacts only with RXRα and RXRγ but not with RXRβ [58]. Furthermore, different NR4A receptors can form heterodimers to synergistically activate transcription[59]. While it was initially thought that NR4A receptors only activate genes, a recent study has provided first evidence that Nurr1 can also repress inflammatory gene promoters by recruiting co-repressor complexes[60].

In addition to the rapid expression as early response genes, the transcriptional activity of NR4A receptors is regulated by posttranslational modification. All three NR4A receptors are phosphorylated at serine residues in response to growth factor-dependent activation of various kinases, including MAPK, PI3K, Akt, JNK, and RSK[58, 61-64]. For example, Nur77 is phosphorylated at Ser-350 and Ser-354 within the DNA binding domain, which inhibits the *trans*-activation activity[65, 66]. Furthermore, phosphorylation of Nur77 at Ser-105 induces nuclear export of the Nur77/RXR heterodimer complex[67], providing an additional mechanism by which phosphorylation may inhibit the transcriptional activity of Nur77. In addition to phosphorylation, all NR4A receptors contain sumoylation consensus sites, and sumoylation of Nurr1 induces or inhibits the transcriptional activity in a

sumoylation site-specific manner[68]. Although still in its infancy, these posttranslational modifications regulate the transcriptional activity and may represent a major mode of the control of gene expression by NR4A receptors.

1.1.3.2. NR4A nuclear receptors in metabolism and energy balance

Carbohydrate metabolism

All three NR4A receptors are potently induced in the liver in response to physiological stimuli, including fasting and glucagon stimulation (Figure 1.2)[69, 70]. Furthermore, hepatic NR4A receptor expression is increased in diabetic mice as a model of pathologic gluconeogenesis[69]. Functional studies further demonstrated that adenoviral over-expression of Nur77 increases the expression of genes involved in gluconeogenesis and stimulates hepatic glucose production in mice[69]. Interestingly, Nur77 over-expression induces several gluconeogenic genes including G6pc, Fbp1 and Fbp2, and enolase 3, which all contain NBRE consensus sites in their promoters[69]. Therefore, this study has provided the first experimental evidence that NR4A receptors regulate gluconeogenesis and may serve to link hormonal stimulation to downstream metabolic gene expression.

In skeletal muscle, NR4A receptors are induced by growth factors, β-adrenergic signaling, and endurance exercise[71-74]. Maxwell et al. first demonstrated that knock-down of Nur77 in muscle cells results in decreased lipolysis and expression of genes regulating energy expenditure and lipid homeostasis, including AMP-activated protein kinase, UCP3, Glut4, CD36, adiponectin receptor 2, and caveolin-3[72]. Conversely, Cao et al. reported that overexpression of Nur77 in C2C12 muscle cells increases the expression of genes involved in glucose and glycogen metabolism while Nur77 deficiency in mice reduces the expression of genes involved in skeletal muscle glucose utilization *in vivo*[75]. Consistent with this role of Nur77 to promote glucose utilization was the observation that Nur77-deficient mice develop skeletal muscle insulin resistance when fed a high fat diet

due to altered insulin signaling and reduced GLUT4 expression[76]. Although glucose metabolism has not been studied in NOR1-deficient mice, knock-down of NOR1 in skeletal muscle cells attenuates the expression of genes that control fatty acid oxidation and pyruvate use (i.e. PGC-1α, PGC-1β, lipin-1α, PDP1r and PDP1c) indicating that NOR1 may be necessary for oxidative metabolism[77]. Finally, NOR1 has recently been demonstrated to also promote insulin-stimulated glucose uptake in adipocytes by augmenting insulin signaling and GLUT4 translocation[78]. In concert, these intriguing observations point to a key role of NR4A receptors in the transcriptional control of glucose homeostasis and oxidative metabolism.

Lipid metabolism

Accumulating evidence indicates that NR4A receptors regulate various aspects of lipid metabolism. As noted earlier, initial experiments by Maxwell et al. demonstrated that Nur77 promotes lipolysis in muscle[72]. Subsequently, Pols et al. revealed that Nur77 modulates plasma lipoprotein profiles and hepatic lipid metabolism in mice[79]. In this study, adenoviral-mediated over-expression of Nur77 increased plasma LDL cholesterol and decreased HDL cholesterol while reducing hepatic triglyceride levels, which was thought to be due to a repression of the lipogenic transcription factor SREBP1c[79]. Consistent with these data, Chao et al. noted hepatic steatosis and increased SREBP1c expression in Nur77-deficient mice fed a high fat diet[76]. However, since Nur77 did not directly affect SREBP1c activity in reporter assays, the authors concluded that the hepatic steatosis in Nur77-deficient mice was likely secondary to the lipogenic effect of hyperinsulinemia[76].

In 3T3-L1 preadipocytes, NR4A receptors expression is induced during adipogenesis and initiating of the differentiation program[80, 81]. Initial studies using siRNA approaches and over-expression of a Nur77 mutant lacking the N-terminal AF-1 *trans*-activation domain indicated that Nur77 is not required for adipocyte differentiation[82]. However, a functional role for NR4A receptors in adipogenesis was suggested by two recent *in vitro*

studies, which have demonstrated that constitutive NR4A receptor expression in 3T3-L1 preadipocytes inhibits adipocyte differentiation[83, 84]. One of the mechanisms proposed for this negative regulation of adipogenesis by NR4A receptors has been the inhibition of the mitotic clonal expansion of preadipocytes[83]. However, considering that the initial mitotic expansion step is primarily a prerequisite for 3T3-L1 preadipocyte differentiation, further studies seem required and there are likely additional mechanisms involved by which NR4A receptors inhibit adipogenesis. These may include a direct regulation of target genes affecting adipogenesis, including extracellular matrix genes[83]. In addition, NR4A receptors may cross-talk with adipogenic signalling and transcriptional programs, particularly since Nurr1 and Nur77 have been reported to interact with Wnt signaling pathways or the glucocorticoid receptor, which both play important roles in adipogenesis[85-87].

Energy homeostasis

Brown adipose tissue plays a key role in energy balance and is the primary organ involved in thermogenesis through uncoupling of mitochondrial respiration by the action of uncoupling proteins (UCP). Early studies have demonstrated that Nur77 expression is highly induced in response to β-adrenergic stimulation of brown adipocytes while transcript levels of all three NR4A receptors are induced during cold-exposure[88, 89]. Kanzleiter et al. demonstrated a repressive effect of Nur77 on the UCP-1 promoter in brown adipocytes, which was likely indirect since Nur77 did not directly interact with the UCP-1 promoter[88]. Despite this repression of UCP-1, nonshivering thermogenesis was not affected by Nurr77 deficiency in mice[88]. In contrast, Kumar et al. observed that NOR1 transcriptionally up-regulates UCP-1 expression by binding to an NBRE site on the UCP-1 promoter[89]. Furthermore, over-expression of a Nur77 mutant lacking the N-terminal AF-1 *trans*-activation domain prevented UCP-1 transcription induced by β-adrenergic signaling[89]. The reasons underlying these seemingly conflicting two studies remain unclear but are likely due to a differential regulation of UCP-1 by Nur77 and NOR1. Moreover,

NR4A receptors may affect the central regulation of energy homeostasis since injection of NOR1 siRNA into the third cerebral ventricle significantly suppresses food intake and body weight in mice[90]. In concert, these intriguing studies characterize NR4A receptors as important regulators of energy balance and food intake, although the underlying mechanisms remain elusive and warrant further studies in gene-targeted mice.

1.1.3.3. NR4A nuclear receptors in vascular biology

An increasing number of studies have demonstrated that all three members of the NR4A subfamily are expressed in the developing neointima and in advanced atherosclerotic lesions[91-97]. Moreover, accumulating evidence indicates that NR4A receptors constitute important transcription factors in the control of vascular gene expression and play critical roles in essentially all aspects of vascular remodeling, including cell viability, proliferation, and inflammation (Figure 1.3). In the following section, we will briefly summarize these studies pointing to a previously unrecognized function of NR4A receptors in vascular biology.

Cell viability and proliferation

Endothelial cell injury followed by the expression of adhesion molecules and the subsequent recruitment of circulating monocytes constitute critical events for the initiation of atherosclerosis[98]. All three NR4A receptors are potently induced by a variety of pro-atherogenic stimuli in endothelial cells, including atherogenic lipoproteins, inflammatory cytokines, growth factors, and hypoxia (Figure 1.3)[92, 99-105]. The transcriptional mechanisms governing this inducible expression have been primarily studied in the context of growth factor and hypoxia-induced NOR1 expression. While the former mechanisms involve a cAMP response element binding protein (CREB)-dependent activation of the NOR1 promoter[101, 105], NOR1 expression in response to hypoxia is dependent on hypoxia-inducible factor 1 (HIF-1) binding to a hypoxia response element in

the promoter[103]. Arkenbout et al. performed the first functional experiments in endothelial cells and demonstrated that adenoviral over-expression of Nur77 inhibits proliferation of this cell type by up-regulating $p27^{Kip1}$ and down-regulating cyclin A[92]. However, the role of Nur77 for endothelial cell proliferation remains controversial since Zeng et al. reported that Nur77 induces proliferation and cell cycle gene expression[102]. Moreover, this report noted that angiogenesis is induced by over-expression of Nur77 and decreased in Nur77-/- mice[102]. With respect to the sibling NOR1, Rius et al. identified a mitogenic role for this receptor by demonstrating that antisense oligonucleotides against NOR1 inhibit endothelial cell growth and wound repair after injury[101]. Consistent with these observations, NOR1 has recently been characterized as a pro-survival gene in endothelial cells exposed to hypoxia by inducing the expression of cellular inhibitor of apoptosis protein 2[103]. Collectively, these studies establish a role for Nur77 and NOR1 in regulating endothelial cell survival and proliferation; however, little is known about the transcriptional target genes and molecular mechanisms. At present, only two direct NR4A target genes have been identified in endothelial cells. Gruber et al. characterized PAI-1 as a Nur77 target gene, which is activated by the receptor through direct binding to an NBRE site in the promoter[99]. In addition, You et al. demonstrated that Nur77 overexpression prevents NF-κB nuclear translocation in endothelial cells by enhancing the expression of IκBα, which is mediated through a direct *trans*-activation of a NBRE site in the IκBα promoter[104]. Interestingly, the functional relevance of Nur77-dependent IκBα expression was confirmed by the finding that Nur77 inhibited the expression of VCAM-1 and ICAM-1 in endothelial cells[104].

Similarly as in endothelial cells, NR4A receptor expression is rapidly induced in response to atherogenic stimulation of smooth muscle cells (SMC), including lipoproteins, cyclic stretch, and mitogenic stimuli[91, 93-95, 106]. The transcriptional induction of NOR1 in SMC is mediated through mitogen-induced CREB binding to CRE sites in the NOR1

promoter and can be pharmacologically inhibited by simvastatin[93, 94, 106, 107]. Consistent with the earlier described growth-inhibitory function of Nur77 in endothelial cells, Nur77 over-expression inhibits SMC proliferation *in vitro* by stabilizing of p27[Kip1][91, 108] and reduces neointima formation *in vivo*[91]. Interestingly, data from the same group has further recently suggested that SMC-specific over-expression of Nur77 inhibits pathological outward remodeling in response to carotid artery ligation, which was associated with decreased macrophage accumulation and MMP expression[109]. While these studies clearly indicate that Nur77 prevents SMC proliferation, NOR1 has been reported to act mitogenic suggesting a function that is distinct from that of Nur77. A proliferative role of NOR1 was first reported by Martínez-González et al. using antisense NOR1 oligonucleotides[93]. Consistent with these initial observations, data from our group has demonstrated a proliferative defect and an increased propensity for apoptosis in SMC isolated from NOR1-deficient mice[94, 95]. *In vivo*, the proliferative response and neointima formation following endovascular femoral artery guide wire injury was decreased in NOR1-deficient mice[95]. At a molecular level, this mitogenic activity of NOR1 was at least in part mediated by a *trans*-activation of a canonical NBRE site in the cyclin D1 promoter, characterizing cyclin D1 as a *bona fide* NOR1 target gene in SMC[95]. Furthermore, DNA microarray profiling revealed a lower expression of NOR1 in elongated SMC while NOR1 knock-down suppressed DNA synthesis, further supporting the mitogenic function of NOR1 and pointing to a potential role of NOR1 in regulating cell shape[110]. In concert, these studies establish not only an important but also distinct role for Nur77 and NOR1 in the control of vascular cell proliferation and remodeling. Continued investigation will be required to define the transcriptional target genes and the molecular basis underlying the differential function of NOR1 and Nur77 in SMC biology.

Inflammation

The first evidence linking NR4A expression with inflammatory signaling was

reported by Woronicz et al. and Liu et al., who noted that Nur77 is induced in apoptotic T-cells and that inhibition of Nur77 function prevented apoptosis[111, 112]. However, mice deficient in Nur77 exhibit unimpaired T-cell apoptosis, and functional redundancy of Nur77 and NOR1 in T-cell apoptosis has been suggested[113, 114]. Similarly to T-cells, Nur77 expression is increased in apoptotic macrophages and, in contrast to the experiments performed in T-cells, peritoneal macrophages isolated from Nur77-deficient mice reveal a phenotype of reduced cell death[115]. In response to inflammatory activation, all three NR4A receptors are potently induced in macrophages[96, 97]. This inducible expression of NR4A receptors in macrophages depends on the activation of NF-κB signaling, as exemplified by the recruitment of NF-κB to response elements in the Nur77 promoter[96]. Functional studies have indicated that NR4A receptors both activate and repress inflammatory genes in macrophages[60, 97, 116]. An initial microarray analysis by Pei et al. discovered that NR4A overexpression in macrophages induces proinflammatory gene expression[116]. Interestingly, among the identified direct Nur77 target genes was the inducible kinase IKKi/IKKepsilon, which functions as a NF-κB activating kinase, providing a potential mechanism for the activation of inflammatory gene expression by Nur77 in macrophages[116]. In contrast to these studies, Bonta et al. revealed that lentiviral over-expression of each NR4A member reduces certain inflammatory genes (i.e. IL-1β, IL-6, IL-8, MIP1α and 1β and MCP-1) and the uptake of oxidized LDL[97]. Finally, a recent study by Saijo et al. identified that Nurr1 transcriptionally represses the inflammatory genes TNFα, iNOS, and IL-1β in microglia and the murine RAW264.7 cell line[60]. This *trans*-repression was mediated through a Nurr1-dependent recruitment of the co-repressor for element-1-silencing transcription factor (CoREST) complex to the target promoter and the subsequent clearance of NF-κB[60]. While these studies indicate that NR4A receptors function as important transcriptional regulators of inflammatory gene expression, further *in vivo* evidence using animal models deficient for either of the NR4A receptors seems required, particularly with respect to the development of atherosclerosis.

1.2. General hypothesis and specific aims

In conclusion, emerging evidence indicates that NR4A nuclear receptors have multifaceted, even contradictory, effects in vascular cells and macrophages. The induction of NOR1 in endothelial cells and smooth muscle cells elicit a proliferative effect on cell growth. Whereas accelerated endothelial cell growth benefits revascularization after vascular injury, increased smooth muscle cell proliferation leads to intimal thickening and ultimately vascular occlusion. Additionally, the role of NOR1 in endothelial cell activation and inflammatory signaling still needs to be investigated. In macrophages, recent studies indicate that NOR1 can be either pro- or anti-inflammatory dependent upon the choice of target genes and specific assays. Therefore, it is intriguing and necessary to further explore the functions of NOR1 in vascular cells and to identify the net effect of NOR1 on atherosclerosis development.

At the beginning of this study, we found that NOR1 expression is induced by inflammatory stimuli in endothelial cells and increases adhesion molecule expression. In addition, it was reported by our group that mitogen induced NOR1 expression promotes vascular smooth muscle cell proliferation. Based on these findings, it was hypothesized that **NOR1 plays an atherogenic role in atherosclerosis development**. In order to test this hypothesis, two **specific aims** were proposed:

Specific aim I. To investigate how transcriptional induction of NOR1 during endothelial cell activation contributes to monocyte recruitment and atherosclerosis development.

Specific aim II. To characterize the epigenetic regulation of NOR1 expression in vascular smooth muscle cells.

Table 1.1. NR4A orphan nuclear receptors are induced by pleiotropic physiological and pathological signals*

	Stimuli	Expression locations	Effects	Potential partners
NR4A1 (Nur77)	parathyroid hormone[117]	bone	bone metabolism	
	Palmitate, oleate[118]	pancreatic beta-cells	beta-cell adaptation to hyperlipidemia	
	nerve growth factor, membrane depolarization[119]	PC12 cells	neuron survival during development	
	antipsychotic drugs: raclopride, olanzapine[120]	CA1 region of the hippocampus	antipsychotic drug effects in the central nervous system	
	9-cis retinoic acid[56]	human chorion carcinoma JEG-3 cells	crosstalk between retinoid and growth factor signaling pathways	Retinoid X Receptor (RXR)
	T-cell receptor activation[114]	thymocytes	thymocyte apoptosis	
	deficiency of Nur77 and NOR1[121, 122]	hematopoietic stem cells, myeloid progenitors	mixed myelodysplastic/myeloproliferative neoplasms, acute myeloid leukemia	
NR4A2 (Nurr1)	Nurr1 deficiency [123]	mouse embryo midbrain	midbrain dopaminergic cell differentiation	
	antipsychotic drugs: clozapine; fluphenazine; haloperidol; risperidone;[120]	CA1 region of the hippocampus	antipsychotic drug effects in the central nervous system	
	parathyroid hormone[124]	bone	bone metabolism	
	9-cis retinoic acid[56]	human chorion carcinoma JEG-3 cells	crosstalk between retinoid and growth factor signaling pathways	Retinoid X Receptor (RXR)

Table 1.1. NR4A orphan nuclear receptors are induced by pleiotropic physiological and pathological signals* (continued)

	Stimuli	Expression locations	Effects	Potential partners
NR4A2 (Nurr1)	normal Nurr1 expression[125]	hematopoietic stem cells	hematopoietic stem cell quiescence	
NR4A3 (NOR1)	antipsychotic drugs: risperidone, clozapine[120]	CA3 region of the hippocampus	antipsychotic drug effects in the central nervous system	
	NOR1 deficiency[126]	semicircular canal during early otic development	inner ear defect, partial bidirectional circling behavior	
	parathyroid hormone[127]	osteoblasts	crosstalk between parathyroid hormone and steroid hormone receptor signaling pathways	
	T-cell receptor activation[114]	thymocytes	thymocyte apoptosis	
	deficiency of Nur77 and NOR1[121, 122]	hematopoietic stem cells, myeloid progenitors	mixed myelodysplastic/myeloproliferative neoplasms, acute myeloid leukemia	

* The induction of NR4A orphan nuclear receptors in the liver, muscle, adipose tissues, and vascular cells is summarized in Figure 1.2 and Figure 1.3.

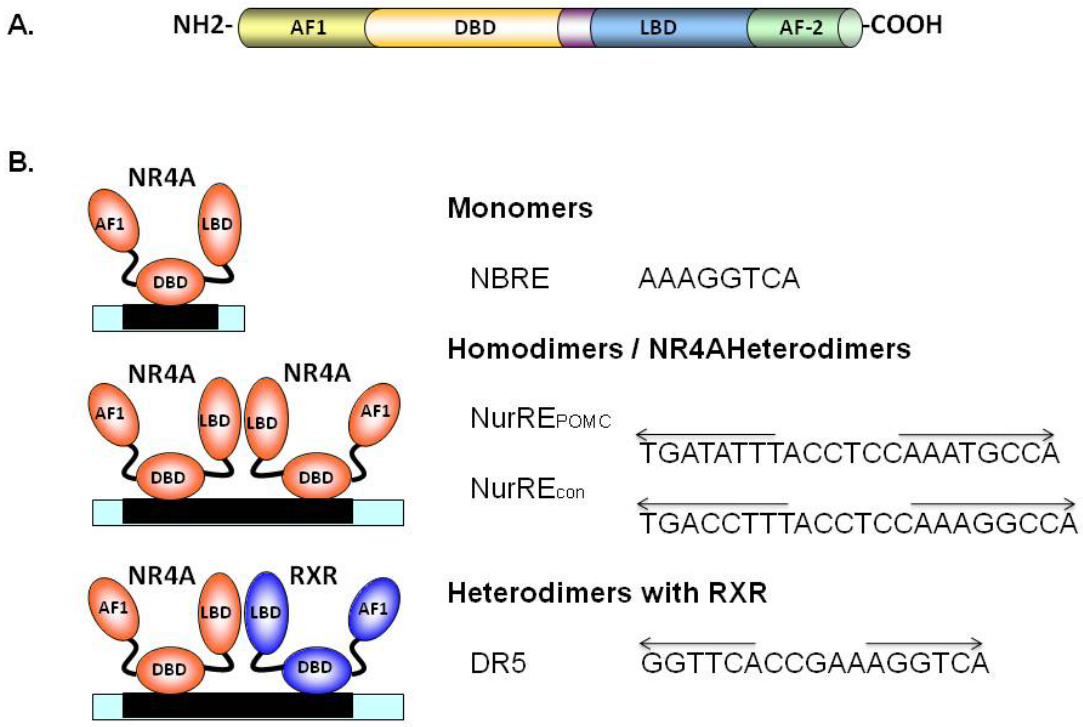

Figure 1.1. Molecular biology of NR4A orphan nuclear receptors

(A) NR4A nuclear receptors share a conserved molecular structure consisting of an N-terminal AF-1 domain, a central DNA-binding domain (DBD), and a C-terminal ligand binding domain (LBD) and AF-2 domain. (B) NR4A receptors induce gene expression by binding as monomers to the NBRE site and as homodimers or heterodimers to the NurRE site in the promoter of their regulated target genes. $NurRE_{POMC}$ represents the binding sequence demonstrated in the pro-opiomelanocortin (POMC) promoter, while $NurRE_{CON}$ represents the NurRE site comprising consensus NBRE sites. Nur77 and Nurr1, but not NOR1, heterodimerize with RXR and bind to the direct repeats of nuclear receptor binding motif separated by five nucleotides (DR5).

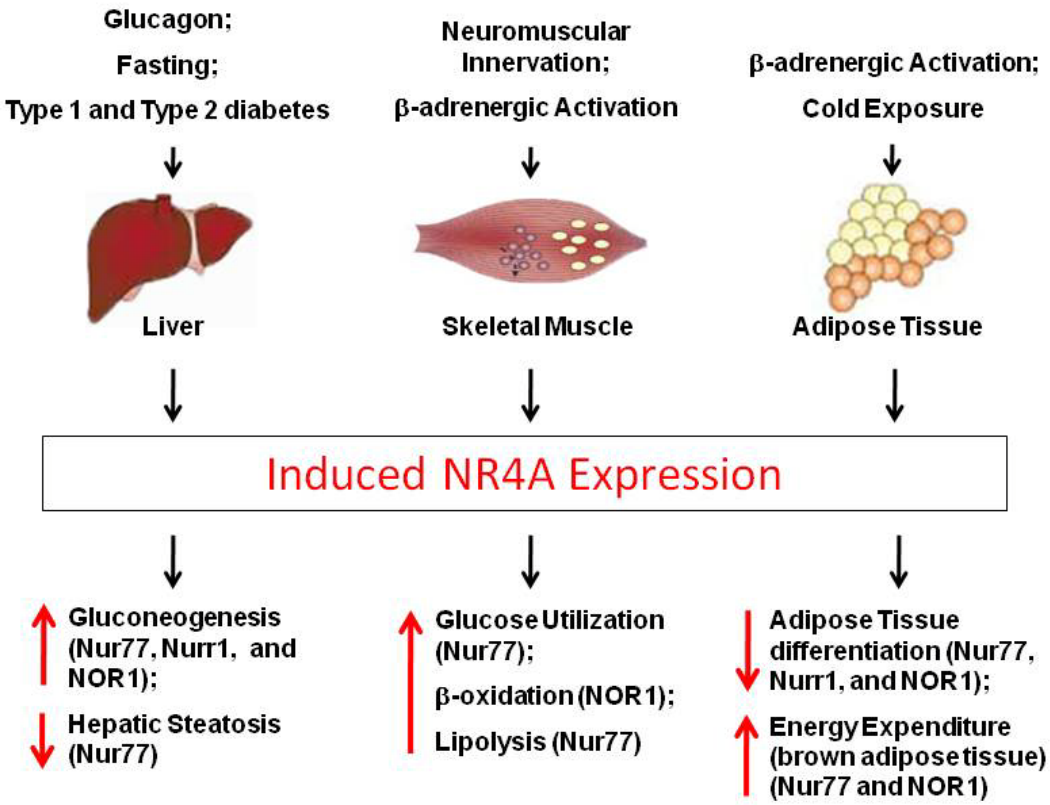

Figure 1.2. NR4A receptor function in metabolism and energy balance

NR4A orphan nuclear receptors are potently induced by physiological and pathological stimuli in the liver, muscle and adipose tissues. In these tissues, NR4A orphan receptors function as transcriptional regulators of gene expression programs involved in the control of glucose homeostasis, lipid metabolism, and energy expenditure (see text for details).

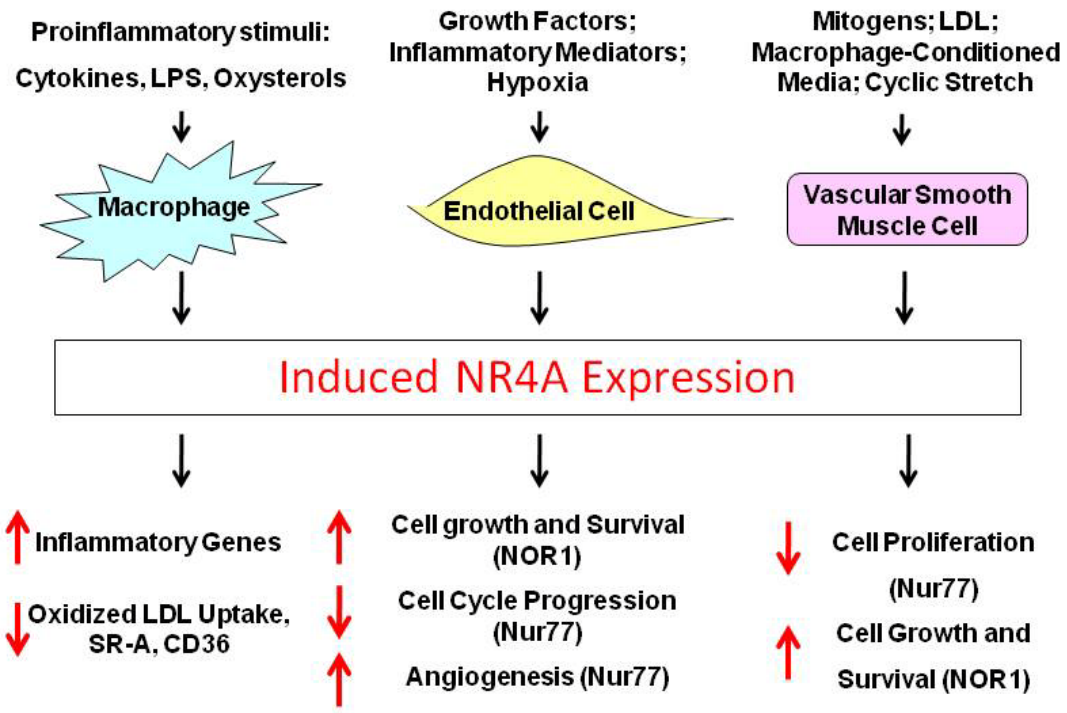

Figure 1.3. Expression and function of NR4A receptors in vascular cells

NR4A orphan nuclear receptors are induced in vascular cells by a variety of stimuli, including inflammatory mediators, cytokines, hypoxia, and growth factors. In response to these pathophysiological environmental cues, NR4A receptors modulate gene expression leading to cell-specific processes (see text for details).

Chapter two: Deficiency of the NR4A orphan nuclear receptor NOR1 decreases monocyte adhesion and atherosclerosis

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2.1. Synopsis

The orphan nuclear receptor NOR1 is a member of the evolutionary highly conserved and ligand-independent NR4A subfamily of the nuclear hormone receptor superfamily. Members of this subfamily have been characterized as early response genes regulating essential biological processes including inflammation and proliferation; however the role of NOR1 in atherosclerosis remains unknown. In the present study, we demonstrate expression of NOR1 in endothelial cells of human atherosclerotic lesions. In response to inflammatory stimuli, NOR1 expression is rapidly induced in endothelial cells through a NF-κB-dependent *trans*-activation of the NOR1 promoter. Over-expression of NOR1 in human endothelial cells increased the expression of VCAM-1 and ICAM-1 while NOR1 deficiency altered adhesion molecule expression in response to inflammatory stimuli. Transient transfection experiments and chromatin immunoprecipitation assays revealed that NOR1 induces VCAM-1 promoter activity by binding to a canonical response element for NR4A receptors in the VCAM-1 promoter. Further functional studies confirmed that NOR1 mediates monocyte adhesion by inducing VCAM-1 and ICAM-1 expression in endothelial cells. Finally, we demonstrate that NOR1-deficiency reduces hypercholesterolemia-induced atherosclerosis formation in apoE-/- mice by decreasing the macrophage content of the lesion. In concert, these studies identify a novel pathway underlying monocyte adhesion and establish that NOR1 serves a previously unrecognized atherogenic role in mice by positively regulating monocyte recruitment to the vascular wall.

2.2. Introduction

The transcription factor NOR1 (NR4A3) belongs to the highly conserved NR4A subfamily of orphan nuclear hormone receptors[52, 128]. Members of this subgroup are classified as early response genes, which are induced by a pleiotropy of stimuli, including mitogens and inflammatory signals[93, 129]. In contrast to other members of the nuclear receptor superfamily, NR4A receptors function as constitutively-active and ligand-independent transcription factors[45, 130]. Therefore, the transcriptional activity of NR4A receptors is determined by the expression level and by posttranslational modifications of the receptor[131]. NR4A receptors positively regulate target gene expression by binding as monomer or homodimer to different variations of the canonical 5'-A/TAAAGGTCA NGFI-B response element (NBRE)[53]. In addition, Nur77 (NR4A1) and Nurr1 (NR4A2) exhibit transcriptionally distinct mechanisms, as both are able to *trans*-activate target genes as a heterodimer with RXR[132]. Consistent with the pleiotropic stimuli that induce the expression of NR4A receptors, these transcription factors have been implicated in regulating key cellular functions, including inflammation, proliferation, and cell survival[52, 128].

NOR1 was first cloned from neuronal cells, and its deletion in mice results in hippocampal dysgenesis and inner ear defects[133, 134]. In addition to neurons, NOR1 is highly expressed in atherosclerotic lesions[93, 94, 135, 136]. NOR1 is induced by mitogens in smooth muscle cells (SMC) and required for proliferative remodeling following vascular injury[93-95]. In macrophages, NOR1 is induced during inflammation and represses cytokine secretion[129, 136]. Finally, NOR1 expression is increased by vascular endothelial growth factor (VEGF) in endothelial cells[101]. However, the functional role of NOR1 in endothelial cells and its contribution to atherosclerosis remain to be investigated.

In the present study, we provide first evidence that NOR1 plays an essential role in the regulation of monocyte adhesion to the endothelium by positively regulating VCAM-1 and

ICAM-1 expression. *In vivo*, loss of NOR1 function in apoE-deficient mice reduces atherosclerosis formation and macrophage recruitment to the arterial wall. These studies identify NOR1 as a previously unrecognized key component of a transcriptional cascade regulating monocyte adhesion during atherogenesis.

2.3. Material and Methods

Immunostaining

Immunostaining of human atherosclerotic lesions was performed as described previously using primary antibodies against NOR1 (1:100 dilution; IMG-71915; Imgenex, Inc.) or von Willebrand Factor (vWF) (1:100 dilution; 115-01, SIGNET)[94, 95]. For immunofluorescent co-localization studies, sections were incubated with primary antibodies against NOR1 (1:50 dilution; ab56340; Abcam) and vWF (1:100 dilution; 115-01, SIGNET). Sections were subsequently incubated with Alexa 488-conjugated goat anti-mouse antibody (1:1000 dilution, A11001, Invitrogen) and Alexa 594-conjugated goat anti-rabbit antibody (1:1000, A11012, Invitrogen), respectively. All studies on human tissues were performed with the approval of the University of Kentucky Institutional Review Board.

For immunohistochemical analysis of mouse aortic atherosclerotic lesions, the ascending aortae were embedded in OCT (Tissue-Tek; Miles Inc., Elkhart, Illinois, USA) and snap-frozen. Transverse cryosections (10 μm) were collected from the aortic arch, fixed in cold acetone, and immunostained using macrophage anti-sera (AI-AD 31240, Accurate Chemicals) or a VCAM-1 antibody (BD 550547). All experiments on mice were approved by the University of Kentucky Institutional Animal Care and Use Committee.

Cell culture

Human umbilical vein endothelial cells (HUVEC, Lonza) and human aortic endothelial cells (HAEC, Cascade Biologics) were cultured as directed by the

manufacturer. Human THP-1 monocytes (ATCC) were maintained in RPMI-1640 medium supplemented with 10% FBS. Murine WEHI-274.1 premyelocytic cells (ATCC) were cultured in DMEM supplemented with 10% FBS. Confluent endothelial cells were stimulated with different cytokines or proinflammatory factors as indicated in the Figure Legends. The reagents used in this study included human TNFα (R & D), human IL-1β (R & D), human IL-6 (R & D), human IFNγ (R & D), human oxidized low-density-lipoprotein (ox-LDL) (INTRACEL), mouse TNFα (R & D), mouse IL-1β (R & D) and LPS (Sigma). All experiments were performed with cells between passages 2 to 8, and each experiment was repeated at least three times with different preparations of cells.

Isolation of murine endothelial cells

Mouse aortic endothelial cells (MAEC) were isolated from aortae of littermate NOR1+/+ and NOR-/- mice using an explant technique. Aortic segments were placed on Cultrex Basement Membrane Extract gel (R & D Systems) and incubated in low-glucose DMEM supplemented with 15 % FBS, 180 μg/ml heparin and 20 μg/ml endothelial cell growth supplement. Migrated endothelial cells were passaged using dispase (BD Sciences) and cultured for 2 days in media containing D-valine to limit fibroblast contamination. Once confluent, MAEC were incubated with TNFα (R & D Systems) as indicated.

Western blotting

Western blotting was performed as described using antibodies against NOR1 (PP-H7833, R & D Systems), human VCAM-1 (BD Pharmingen), human ICAM-1 (Abcam), mouse VCAM-1 (R & D Systems), mouse ICAM-1 (Abcam), □-actin (Sigma), and GAPDH (FL 335) (Santa Cruz)[94, 95].

Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol® (Invitrogen) and reverse transcribed into cDNA using SuperScript II (Invitrogen). RNA expression levels of target genes were

quantified using an iQ^{TM} SYBR Green Supermix (BioRad) and 5 pmol of the indicated primer pairs (Table 2.1). PCR reactions were performed on an iCycler (BioRad) using the following PCR cycles: 1 cycle of 95 °C 10 min; 40 cycles of 95 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec; 1 cycle of a final extension at 72°C for 10 min. Each sample was analyzed in triplicate and normalized to expression values of the house-keeping gene TBP or TFIIB. Data were calculated using the $2^{-\Delta\Delta CT}$ method[137].

Adenovirus-mediated over-expression in HUVEC

The adenovirus over-expressing the dominant-negative IκBα mutant (IκBα-S32A/S36A) (Ad-CMV-IκB(DN)) was purchased from Vector Biolabs. The adenovirus over-expressing human NOR1 (Ad-CMV-NOR1) was generously provided by Dr. Peter Tontonoz (University of California, Los Angeles, CA). Sub-confluent HUVEC were infected with 25 PFU Ad-CMV-IκB(DN) for 3 hours and 50 PFU Ad-CMV-NOR1 for 6 hours, respectively. Adenoviruses over-expressing GFP (Ad-CMV-GFP) or an empty vector (Ad-CMV-null) were used as controls.

Plasmids, transient transfections and luciferase assay

The human NOR1 promoter constructs have previously been described[94]. The NF-κB response elements located at -198bp to -190bp and -595bp to -496bp from the transcription initiation site were mutated from GGAGTTTCC to AGAGTTTAA and from GGGATTAGCC to ATGATTAGAA using the QuickChange II XL site–directed mutagenesis kit (Stratagene). HAEC were transiently transfected with NOR1 promoter constructs using promofectin (PromoKine). Following transfection, cells were recovered overnight and stimulated with TNFα. The human VCAM-1 promoter construct was commercially obtained from Epoch Biolabs Company. The NBRE site located at -2618 bp to -2611 bp from the transcription initiation site was mutated from TGACCTTT to TCGGAGTT. For overexpression of NOR1, HUVEC were infected with 50 PFU

Ad-CMV-Null or Ad-CMV-NOR1 for 6 hours and recovered for 24 hours. Infected cells were subsequently transfected with luciferase reporter constructs driven by the VCAM-1 promoter. Following transfection, cells were recovered in growth media for 2 days. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay (Promega). Transfection efficiency was normalized to renilla luciferase activities generated by cotransfection of 5 ng pRLCMV.

siRNA experiments

siRNA experiments were performed using the SMARTpool technology (L003428, Dharmacon RNA Technologies), which provides a mix of four different proprietary siRNAs specific against human NOR1. HUVEC were seeded at a density of 1.2×10^5 cells/well in 6-well plates and transiently transfected for 3 h with 30 nM NOR1 siRNA or scrambled siRNA using promofectin (PromoKine). Following transfection, cells were recovered in complete growth media overnight and subsequently stimulated with TNFα for 6 h. Fluorescently labeled THP-1 monocytes were added onto the HUVEC monolayers and adhesion was quantified after 30 min as indicated in the section "Adhesion assay".

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the EZ-ChIP kit (Millipore) as described[94, 95]. Briefly, HUVEC were stimulated with TNFα and sheared chromatin was immunoprecipitated using 5 μg antibodies directed against NF-κB p65 (sc-372x, Santa Cruz) or NOR1 (PP-H7833, R & D Systems). Target DNA product was amplified by PCR using primer pairs covering the NF-κB binding sites in the NOR1 promoter or the NBRE site in the VCAM-1 promoter (Table 2.1).

Adhesion assay

HUVEC were infected with 50 PFU Ad-CMV-Null or Ad-CMV-NOR1 for 6 hours and recovered for 48 hours. HUVEC treated with TNFα were employed as a positive

control. THP-1 monocytes were pre-stained with 5 μM calcein-AM (Sigma-Aldrich) at 37 °C for 30 minutes. After washing in PBS, fluorescently labeled THP-1 monocytes were added onto the HUVEC monolayers at the density of 10^6 cells/ml. To block VCAM-1 and ICAM-1 function, HUVEC monolayers were incubated with blocking antibodies against VCAM-1 (25 μg/ml, BBA5, R & D Systems) and ICAM-1 (10 μg/ml, BBA3, R & D Systems) for 1 hour prior to the addition of THP-1 monocytes. Non-adherent monocytes were washed off after 30 minutes. For mouse monocyte adhesion assays the thoracic aortae of littermate NOR1+/+ or NOR1-/- mice were cut into segments, pinned on dental wax, and maintained in MAEC growth media supplemented with TNFα (1 ng/ml) or vehicle (PBS). After 6 hours, aortae were washed with PBS, and 700 μl of fluorescently labeled WEHI-274.1 cells were added at the density of 10^6 cells/ml. Non-adherent monocytes were removed after 30 minutes by washing with PBS. For both experiments, six pictures were taken for each condition, and adhesion was quantified by counting fluorescent monocytes attached to the endothelium.

Mice

Littermate NOR1-/- and NOR1+/+ mice on a mixed C57BL/6J/129Sv background were used as previously described[94, 95]. ApoE-/- mice on a C57BL/6J background (N10) were obtained from The Jackson Laboratory (stock #002052) and interbred with NOR1-/- mice to obtain NOR1-/-apoE-/- mice. At 8 to 10 weeks of age, littermate female NOR1+/+apoE-/-, NOR1+/-apoE-/- and NOR1-/-apoE-/- mice were fed a saturated fat enriched diet (TD88137; Harlan Teklad) for 12 weeks for atherosclerosis analysis or for 2 weeks to analyze NR4A gene expression.

Atherosclerosis quantification

Atherosclerosis was quantified as described and reviewed recently in detail[138, 139]. Briefly, after exsanguination aortic tissue was removed from the ascending aorta to the

ileal bifurcation and fixed by perfusion with freshly prepared 4 % paraformaldehyde in PBS overnight at room temperature. After tissue fixation, the aorta was dissected from the adventitia. The intimal surface was exposed by a longitudinal cut through the inner curvature of the aortic arch that extended down the whole length of the aortic tree. To permit the arch region to be laid out flat, the greater curvature was cut down to the level of the subclavian artery. The tissue was laid out, and an image of the aorta was recorded. To quantify the extent of intimal surface covered by grossly discernible lesions, image analysis was performed with Image-Pro (Media Cybernetics). The extent of atherosclerotic lesions was quantified in the arch as defined from the ascending arch to 4 mm distal to the left subclavian artery. The data were presented as the percentage of lesion area on the aortic arch.

Quantification of macrophage accumulation

Macrophage content was quantified in a 2-mm segment as defined from the beginning of the ascending aortic arch using a modified technique described by Mach et al[140]. From this segment 180 serial transverse cryosections (10 μm) were collected and placed onto twenty slides per mouse. Slides were immunostained for macrophages as described above, and the macrophage content was quantified in nine sections 200 μm apart. Macrophage accumulation was determined by quantifying the total area positive for macrophage staining using computer-assisted image analysis (Image-Pro, Media Cybernetics). Each section was quantified by two observers blinded to the experimental design. The data were presented as mean area ± SEM positive for macrophage staining for each of the nine measurements and their cumulative sum.

Lipoprotein resolution and quantification

Lipoproteins were resolved using size exclusions chromatography as described[138, 139]. Briefly, serum samples were centrifuged and placed onto a single Sepharose 6 HR 10/30 column (300 x 10 mm, Pharmacia) with a mobile phase of saline/EDTA run at 0.5

ml/min. Fractions (0.5 ml) were collected and cholesterol concentrations were determined by placing 100 μl of each fraction into an equivalent volume of cholesterol reagent (Wako Chemical Company) that was diluted to half the manufacturer's instructions. Assays were performed in a 96 well format and absorbance was determined at 600 nm.

Statistical analysis

Results were represented as means or medians depending on the distribution of data. Unpaired Student's t-test was utilized to compare the means between two independent groups on a single variable. One-way or Two-way ANOVA was used to compare groups. The effect of NOR1 on atherosclerosis was compared using the Kruskal-Wallis test followed by Dunn Test post-hoc analysis. P values < 0.05 were considered to be statistically significant.

2.4. Results

NOR1 is expressed in endothelial cells of human coronary atherosclerotic lesions

To characterize the distribution of NOR1 expression in atherosclerosis, human coronary arteries were immunostained for NOR1. NOR1 protein was readily detectable in atherosclerotic lesions (Figure 2.1A) but negligible in normal arteries (data not shown). In these atherosclerotic sections, high levels of NOR1 immunostaining were observed in the endothelial cell layer and in sub-endothelial cells of advanced atherosclerotic lesions. Colocalization experiments using confocal microscopy confirmed a typical nuclear expression pattern of NOR1 in endothelial cells staining positive for the endothelial cell marker vWF (Figure 2.1B).

NOR1 is induced by inflammatory stimuli in endothelial cells

In vitro, stimulation of HUVEC with the inflammatory mediators IL-1β, oxidized LDL, LPS, or TNFα highly induced NOR1 mRNA expression (Figure 2.2A). In contrast,

IL-6 or IFN-γ did not significantly induce NOR1 transcript levels in HUVEC. Considering that TNFα elicited a maximal increase in NOR1 expression, subsequent experiments focused on the regulation of NOR1 by TNFα. Consistent with previous reports characterizing NOR1 as an early response gene[93, 129], time-course experiments confirmed a maximal induction of NOR1 mRNA after 3 h of TNFα stimulation (Figure 2.2B). This increase in NOR1 transcript levels was followed by a maximal induction of NOR1 protein expression after 6 h (Figure 2.2C-D). The observed regulation of NOR1 mRNA and protein expression by TNFα was dose-dependent revealing a maximal increase of NOR1 expression with 1 ng/ml TNFα (Figure 2.2E-G, respectively). Finally, TNFα induced a similar induction of NOR1 mRNA expression in primary MAEC isolated from NOR1 wildtype mice (Figure 2.2H). In contrast, the other two members of the NR4A subfamily Nur77 and Nurr1 were only modestly induced in NOR1 wildtype cells, and this induction was lost in NOR1-/- MAEC.

NOR1 expression in endothelial cells is mediated through an NF-κB-dependent *trans*-activation of the NOR1 promoter

NOR1 is primarily regulated through transcriptional mechanisms, and sequence analysis of the NOR1 promoter identified several putative NF-κB binding sites. To address the functional relevance of these NF-κB sites, endothelial cells were infected with an adenoviral construct over-expressing a dominant-negative IκBα mutant. In cells over-expressing GFP as control, TNFα treatment resulted in a significant increase of NOR1 mRNA expression (Figure 2.3A). In contrast, over-expression of the dominant-negative IκBα mutant resulted in an almost complete inhibition of TNFα-induced NOR1 mRNA expression. These experiments indicate that TNFα-induced NOR1 expression is primarily mediated via NF-κB signaling in endothelial cells.

To further confirm an NF-κB-dependent transcriptional regulation of NOR1 expression in endothelial cells, we performed transient transfection assays using a NOR1

promoter construct. This 4.0 kb full-length human NOR1 promoter construct includes two putative NF-κB binding sites at -595 to -586 and -198 to -190 from the transcription initiation site (Figure 2.3B). Stimulation of endothelial cells with TNFα resulted in a significant activation of the full-length NOR1 promoter (Figure 2.3C). This induction of the NOR1 promoter was maintained upon 5'-deletion to 1.7 kb but completely abolished following mutation of the two NF-κB binding sites, confirming that TNFα induces NOR1 promoter activity via an NF-κB-dependent *trans*-activation of the promoter. As depicted in Figure 2.3D and E, ChIP assays revealed the recruitment of p65 to the NF-κB site in the endogenous NOR1 promoter in response to TNFα. Maximal promoter occupancy was observed after 3 h, which is consistent with the kinetics of NOR1 mRNA expression in response to inflammatory stimuli. In concert, these data demonstrate that TNFα induces NOR1 expression through a NF-κB-dependent signaling pathway resulting in the subsequent *trans*-activation of the proximal NOR1 promoter.

NOR1 positively regulates VCAM-1 and ICAM-1 in endothelial cells

To explore whether NOR1 is involved in the transcriptional control of endothelial cell responses, we next infected HUVEC with an adenovirus overe-xpressing human NOR1 (Figure 2.4A). As depicted in Figure 2.4B-E, NOR1 over-expression resulted in a prominent induction of VCAM-1 and ICAM-1 mRNA and protein expression levels, respectively.

We next employed a murine model to address whether NOR1 is also required for VCAM-1 and ICAM-1 expression in endothelial cells. In these experiments, MAEC were isolated from littermate NOR1 wildtype and NOR1-deficient mice[94, 95]. As depicted in Figure 2.5A-E, TNFα stimulation profoundly increased VCAM-1 and ICAM-1 mRNA and protein expression in NOR1 wildtype MAEC. In contrast, the induction of both adhesion molecules was markedly reduced in MAEC isolated from NOR1-deficient mice. Similar data were obtained when cells were stimulated with IL-1β or LPS (Figure 2.5F-H).

NOR1 *trans*-activates the VCAM-1 promoter by binding to a canonical NBRE consensus site

We next performed transient transfection assays to investigate the underlying mechanism by which NOR1 regulates VCAM-1 expression in endothelial cells. NOR1 induces transcription by binding to NBRE consensus sites in target gene promoters[53]. Interestingly, sequence analysis of the human VCAM-1 promoter identified a canonical NBRE site at -2618 bp from the transcription initiation site (Figure 2.6A). Transient transfection of HUVEC with a luciferase reporter construct driven by the human 3.0 kb VCAM-1 promoter revealed that over-expression of NOR1 increases VCAM-1 promoter activity (Figure 2.6B). However, this transcriptional induction was significantly altered upon site-directed mutagenesis of the canonical NBRE motif. ChIP assays subsequently confirmed that TNFα induced the recruitment of NOR1 to this NBRE site in the VCAM-1 promoter (Figure 2.6C). These experiments demonstrate that NOR1 *trans*-activates the VCAM-1 promoter by binding to a NBRE consensus site in the promoter and characterize VCAM-1 as NOR1-regulated target gene.

NOR1 mediates monocyte adhesion by regulating VCAM-1 and ICAM-1 expression

VCAM-1 and ICAM-1 have both been well characterized to mediate monocyte adhesion to the endothelium leading to the infiltration of monocytes into the sub-endothelial area and atherosclerosis development[141]. To investigate whether the transcriptional induction of VCAM-1 and ICAM-1 by NOR1 is sufficient to promote monocyte adhesion, we analyzed THP-1 monocyte adhesion to HUVEC over-expressing NOR1. Stimulation of a HUVEC monolayer with TNFα profoundly increased monocyte adhesion, confirming the validity of the assay (Figure 2.7A, upper panel). Compared to HUVEC infected with an adenovirus over-expressing an empty vector as control, overexpression of NOR1 significantly increased monocyte adhesion in the absence of TNFα stimulation (Figure 2.7A and B). Furthermore, monocyte adhesion induced by

NOR1 over-expression was almost completely abolished by pre-incubation of HUVEC with VCAM-1 and ICAM-1 blocking antibodies (Figure 2.7A and 2.7B). Conversely, acute knock-down of NOR1 in HUVEC using siRNA significantly decreased monocyte adhesion (Figure 2.7C-D). Collectively, these studies indicate that NOR1 is necessary and sufficient for monocyte adhesion and that this activity is mediated primarily by inducing the expression of VCAM-1 and ICAM-1.

To further confirm a causal contribution of NOR1-dependent VCAM-1 and ICAM-1 expression for monocyte adhesion e*x vivo*, we performed adhesion assays with aortae isolated from NOR1+/+ and NOR1-/- mice. Incubation with TNFα significantly increased the adhesion of monocytes to the aortic endothelial cell layer of NOR1 wildtype mice (Figures 2.7E-F). However, this inducible adhesion was completely abolished on aortae of NOR1-deficient mice. From these findings we infer that NOR1 expression in resident endothelial cells is necessary for mediating monocyte adhesion.

NOR1 deficiency decreases atherosclerosis formation and reduces macrophage recruitment in apoE-/- mice

Considering that monocyte adhesion constitutes a critical initial step for atherogenesis[141]. We next investigated whether NOR1-deficiency decreases atherosclerotic lesion development. Consistent with the important function of NOR1 to regulate monocyte adhesion *ex vivo*, homozygous deletion of NOR1 in apoE-/- mice resulted in a 52 % reduction of atherosclerosis compared to their wildtype littermates (NOR1+/+apoE-/- 14.3 % (n=14), NOR1+/-apoE-/- 14.0 % (n=15), and NOR1-/-apoE-/- 6.8 % (n=15) median atherosclerotic lesion area of aortic arches; $P < 0.05$; Figure 2.8A-B). NOR1-deficiency revealed no overt effect on cholesterol distribution confirming a direct effect of NOR1 on lesion formation (Figure 2.8C). Immunostaining of atherosclerotic tissues and quantification of the macrophage content confirmed macrophage-enriched lesions in NOR1+/+apoE-/- mice that were considerably less with decreased macrophage

content in NOR1-/-apoE-/- mice (Figure 2.8D-F). Finally, while VCAM-1 was readily detectable in atherosclerotic lesions from NOR1+/+apoE-/- mice, there was a paucity of immunoreactivity for this adhesion molecule noted in the vascular wall of NOR1-/-apoE-/- mice (Fig. 2.8G).

2.5. Discussion

Monocyte adhesion constitutes a critical event for the initiation of atherosclerosis[141]; however, the molecular mechanisms that orchestrate monocyte-endothelial cell interactions are incompletely understood. In the present study, we report a previously unrecognized role for the nuclear receptor NOR1 to serve as a transcriptional regulator of adhesion molecule expression and monocyte recruitment during atherosclerosis. In endothelial cells, NOR1 is rapidly induced by inflammatory stimuli via an NF-κB-dependent *trans*-activation of the NOR1 promoter. Loss and gain-of-function studies establish that NOR1 positively regulates VCAM-1 and ICAM-1 expression in endothelial cells leading to increased monocyte adhesion. Consistent with the key role of NOR1 to promote monocyte adhesion to the endothelium, our studies further demonstrate that NOR1 deficiency results in decreased atherosclerosis development and macrophage recruitment in apoE-deficient mice.

Consistent with recent studies, we identified abundant NOR1 expression in endothelial cells[135] as well as in cells of the sub-endothelial space likely representing macrophages and SMC[91, 94, 96, 97, 135]. However, the transcriptional mechanisms governing inducible NOR1 expression in endothelial cells remain elusive. Our data provides evidence for an NF-κB-dependent induction of NOR1 transcription during endothelial cell inflammation. Inhibition of NF-κB signaling in endothelial cells repressed inducible NOR1 expression in response to inflammatory stimulation. Using site-directed mutagenesis and ChIP assays, we identified two functional NF-κB sites in the NOR1 promoter, to which p65 is recruited during inflammatory activation of endothelial cells.

Earlier studies have demonstrated that NOR1 expression in endothelial cells is highly induced by mitogens through a cAMP response element binding protein (CREB)-dependent activation of the proximal region of NOR1 promoter[101]. Furthermore, hypoxia has recently been reported to induce NOR1 in endothelial cells through a mechanism involving the hypoxia-inducible factor (HIF) family of transcription factors[142]. These studies, in concert with our data characterizing NOR1 as an NF-κB target gene in endothelial cells, point to distinct transcriptional mechanisms regulating the rapid NOR1 induction in response to various environmental cues.

Compared with the well-studied early-response genes encoding proteins of the AP-1 complex, little is known about the physiological function of NOR1 and its regulated target genes, yet the high degree of conservation points to an important role in the control of gene expression. A previous study has provided initial evidence to support a functional role of NOR1 in endothelial cells by demonstrating that NOR1 regulates growth of this cell type[101]. The data presented here extends these findings and points to an unsuspected function of NOR1 to serve as a positive regulator of monocyte adhesion. In experiments using adenoviral-mediated over-expression, NOR1 induced VCAM-1 and ICAM-1 expression resulting in increased monocyte adhesion. The observation that this inducible adhesion was abolished when VCAM-1 and ICAM-1 function were blocked, suggests that the induction of both adhesion molecules by NOR1 constitutes a primary mechanism by which NOR1 induces monocyte adhesion. Conversely, the inducible expression of both adhesion molecules in response to inflammatory activation was attenuated in NOR1-deficient endothelial cells. Furthermore, consistent with these findings, TNFα-induced monocyte adhesion to HUVEC transfected with NOR1 siRNA or to the endothelium of NOR-deficient mice was altered *ex vivo*, suggesting that vascular NOR1 expression is not only sufficient but also required for monocyte adhesion.

An intriguing question that arises from the observation that NOR1 induces the

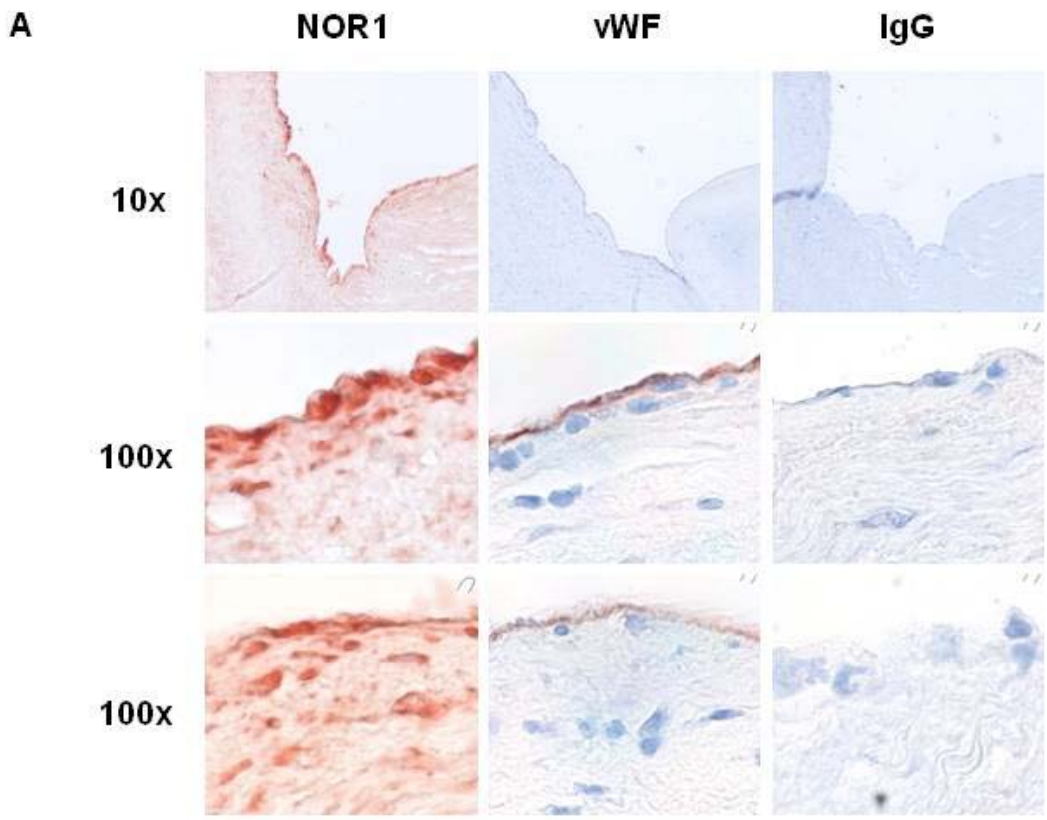
expression of VCAM-1 and ICAM-1 relates to the mechanisms by which NOR1 positively regulates these genes. Initial sequence analysis identified putative NBRE consensus sites in both the VCAM-1 and ICAM-1 promoters. Exemplified by the VCAM-1 promoter, our studies demonstrate that the molecular mechanisms underlying this novel function of NOR1 involve at least in part a direct *trans*-activation of the VCAM-1 promoter by NOR1. In response to inflammatory activation NOR1 is recruited to a canonical NBRE site in the VCAM-1 promoter. Moreover, the observation that NOR1-dependent VCAM-1 promoter *trans*-activation was attenuated upon mutation of this NBRE site confirms the functionality of this NBRE motif. However, the residual induction of the VCAM-1 promoter bearing a mutation of this NBRE site suggests that additional transcriptional mechanisms may regulate NOR1 expression. NOR1 has recently been shown to *trans*-activate the inducible IκB kinase (IKKi/IKKepsilon) promoter, which phosphorylates IκBα and induces NF-κB activation[143]. Therefore, in addition to a direct *trans*-activation, NOR1 may function as a positive upstream regulator of NF-κB signaling and indirectly activate the VCAM-1 and ICAM-1 promoters. Alternatively, NOR1 deficiency may affect other transcriptional networks acting on these promoters, including for example the AP-1 complex. We have recently demonstrated that the combined deficiency of NOR1 and its sibling Nur77 decreases the expression of AP-1 transcription factors[121], which may regulate adhesion molecule expression[144]. Clearly, the findings presented here provide justification for further investigating the transcriptional mechanisms by which NOR1 regulates endothelial cell gene expression and promotes monocyte adhesion.

The protein products of the VCAM-1 and ICAM-1 genes are well established to participate in atherogenesis by promoting macrophage accumulation in the arterial intima[141]. Consistent with this evidence and with the observed regulation of endothelial cell adhesion molecule expression by NOR1, we provide the first evidence that NOR1 deficiency decreases atherosclerosis in apoE-deficient mice. Considering that all three

members of the NR4A receptor subfamily bind to an NBRE site, functional redundancy in certain cell types between Nur77 and NOR1 has been suggested[114]. However, NOR1-deficiency did not result in a compensatory upregulation of the siblings Nur77 and Nurr1 in endothelial cells (Figure 2.2H) or in the aortae of NOR1-/-apoE-/- mice (Figure 2.9). Therefore, the previously reported phenotypes in NOR1-deficient mice[95, 133, 134]. In concert with the decreased atherosclerosis in NOR1-/-apoE-/- mice presented in this study, point to a function of NOR1 that is distinct and not compensated by Nur77 or Nurr1. As evidenced by the decreased accumulation of macrophages in the vascular wall of NOR1-/-apoE-/- mice, monocyte recruitment represents at least one plausible mechanism by which NOR1 acts atherogenic. However, it is possible if not likely that there are additional mechanisms by which NOR1 promotes atherosclerosis development. NOR1 induces neointimal proliferation of SMC [95] and inflammatory gene expression in macrophages[143], which both could affect lesion development. Therefore, characterization of the cell-specific role of NOR1 in atherosclerosis will be necessary and will have to rely on tissue-specific gene targeting strategies. In conclusion, data presented here characterize the orphan nuclear receptor NOR1 as a novel positive regulator of monocyte adhesion by inducing VCAM-1 and ICAM-1 transcription. Continued investigation of the transcriptional networks regulated by NOR1 will provide new insights into how this orphan nuclear receptor participates in the development of vascular diseases.

Table 2.1. Oligonucleotides used in this study

Name	Use	Sequence
human NOR1	Real-time RT-PCR	F : 5’-GGGCTTTTTCAAGAGAACAGTG-3’ R: 5’-ATCTCTGGGTGTTGAGTCTGTT-3’
human VCAM-1	Real-time RT-PCR	F: 5’-TGAGGGGACCAATTCCAC-3’ R: 5’-ATTCACGAGGCCACCACT-3’
human ICAM-1	Real-time RT-PCR	F: 5’-ACCGTGAATGTGCTCTCC-3’ R: 5’-GGCTTGTGTGTTCGGTTT-3’
murine VCAM-1	Real-time RT-PCR	F: 5’-TCAAAGAAAGGGAGACTG-3’ R: 5’-GCTGGAGAACTTCATTATC-3’
murine ICAM-1	Real-time RT-PCR	F: 5’-AGATCACATTCACGGTGCTG-3’ R: 5’-CTTCAGAGGCAGGAAACAGG-3’
human TBP	Real-time RT-PCR	F: 5’-GGAGAGTTCTGGGATTGTACCGC-3’ R: 5’-ATATTCGGCGTTTCGGGCAC-3’
murine TFIIB	Real-time RT-PCR	F: 5’-CTCTCCCAAGAGTCACATGTCC-3’ R: 5’-CAATAACTCGGTCCCCTACAAC -3’
murine NOR1	Real-time RT-PCR	F: 5’-GGCCGCAGCTGCACTCAGTC -3’ R: 5’-GCGGAGGGAAGGTCAGCGTG -3’
murine Nur77	Real-time RT-PCR	F: 5’-TTGATGTTCCCGCCTTTG-3’ R: 5’-GGTAGCCATGTGCTCCTTC-3’
murine Nurr1	Real-time RT-PCR	F: 5’-TCACCTCCGGTGAGTCTGATC-3’ R: 5’-TGCTGGATATGTTGGGTATCATCT-3’
hNOR1-NFKB	ChIP PCR	F: 5’-CCATCTGCATCCCTGTGT-3’ R: 5’-GCTGCACTTTCCTCTTGC-3’
hVCAM-1-NOR1	ChIP PCR	F: 5’-CTGTACTCAAACATTGGAAACATT -3’ R: 5’-CCTTAGAGATGAGAGAAGCAAGA-3’


A
NOR1
vWF
IgG
10x
100x
100x

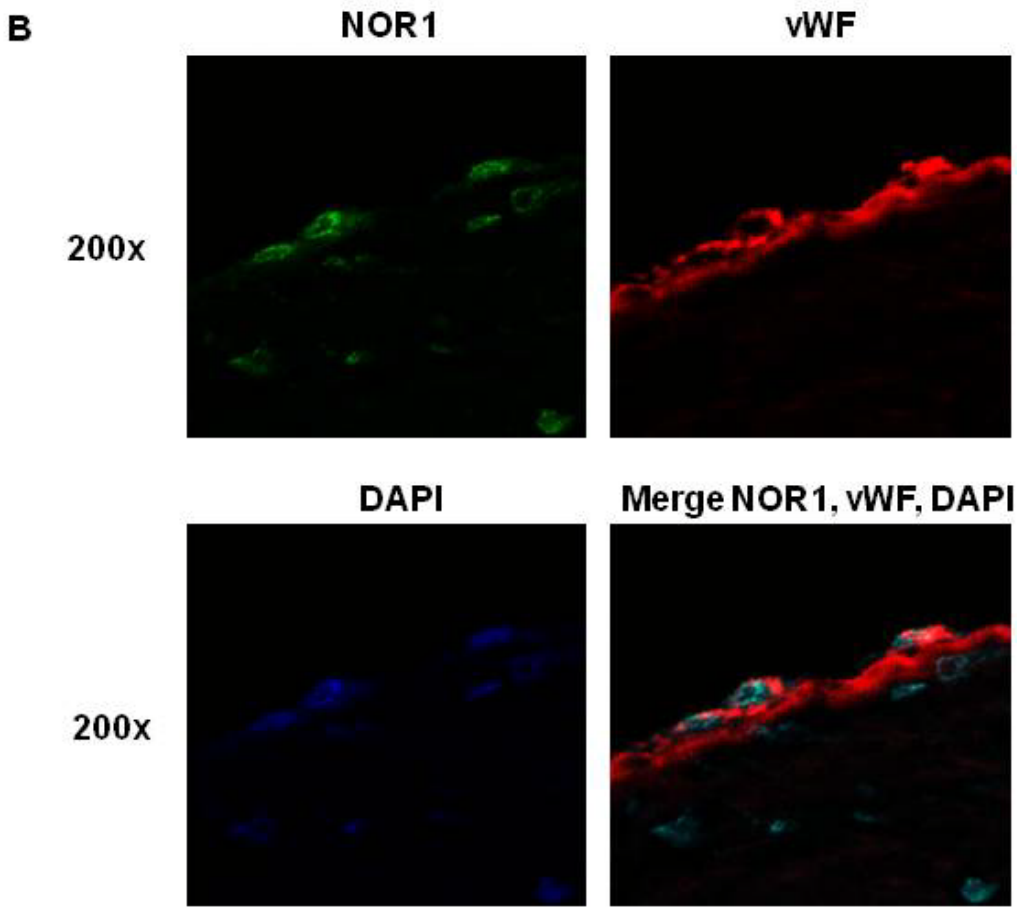
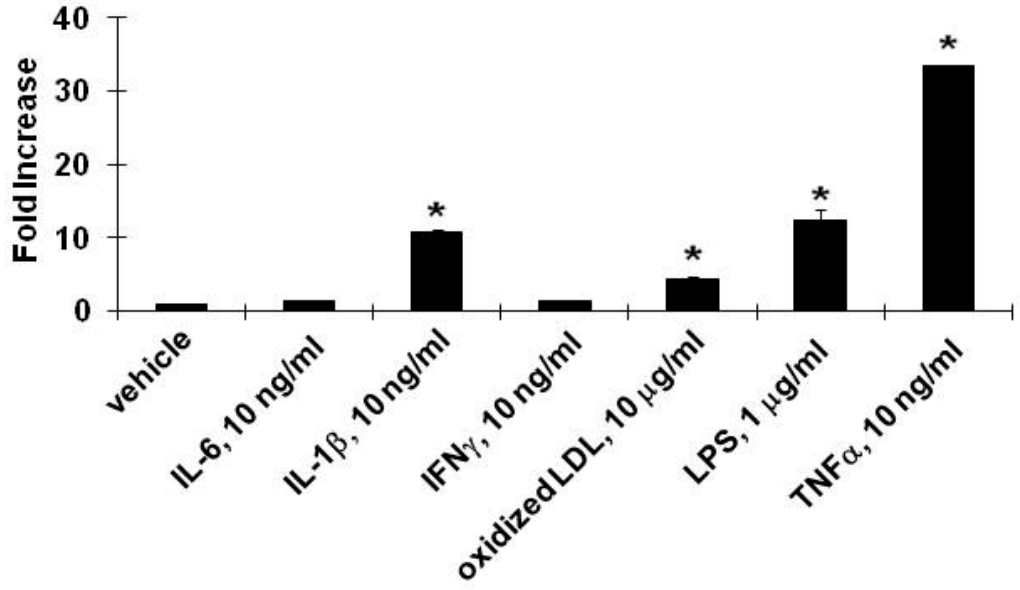
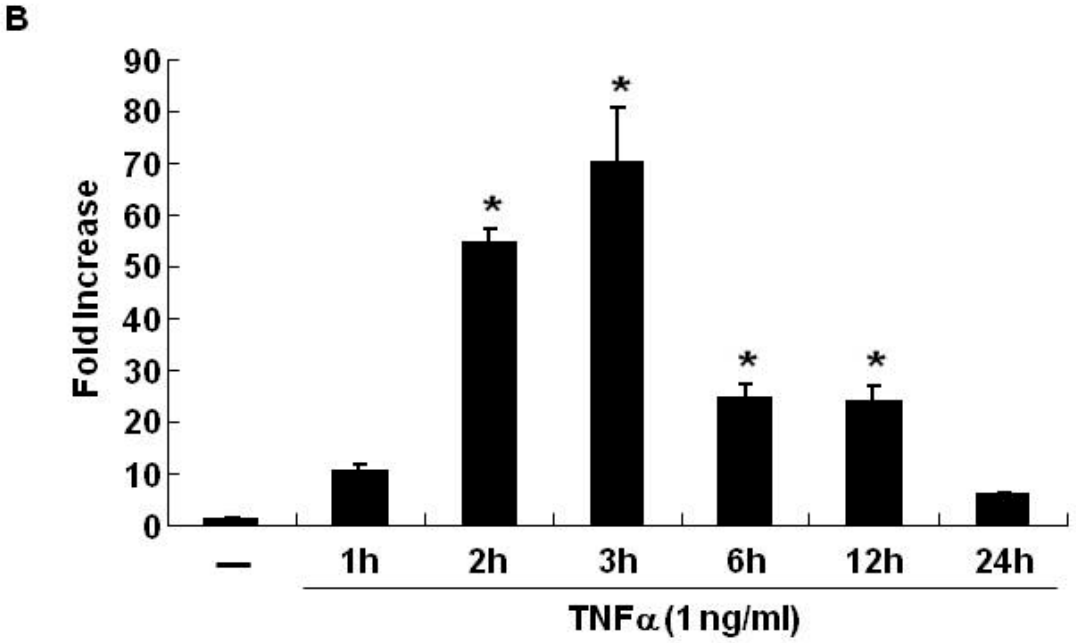


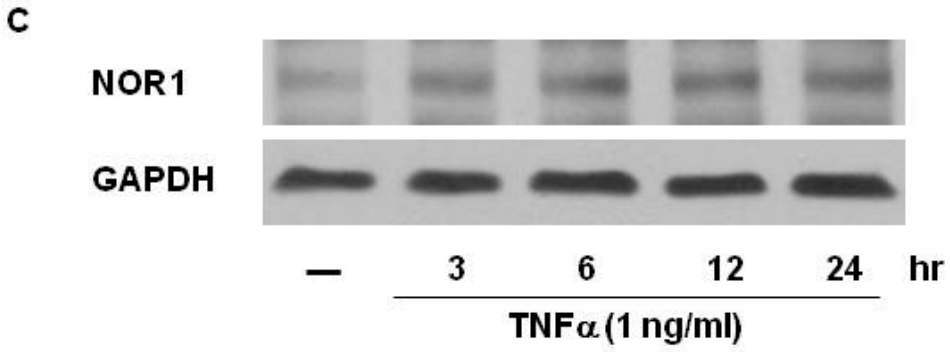
Figure 2.1. NOR1 is present in endothelial cells of human coronary atherosclerotic lesions

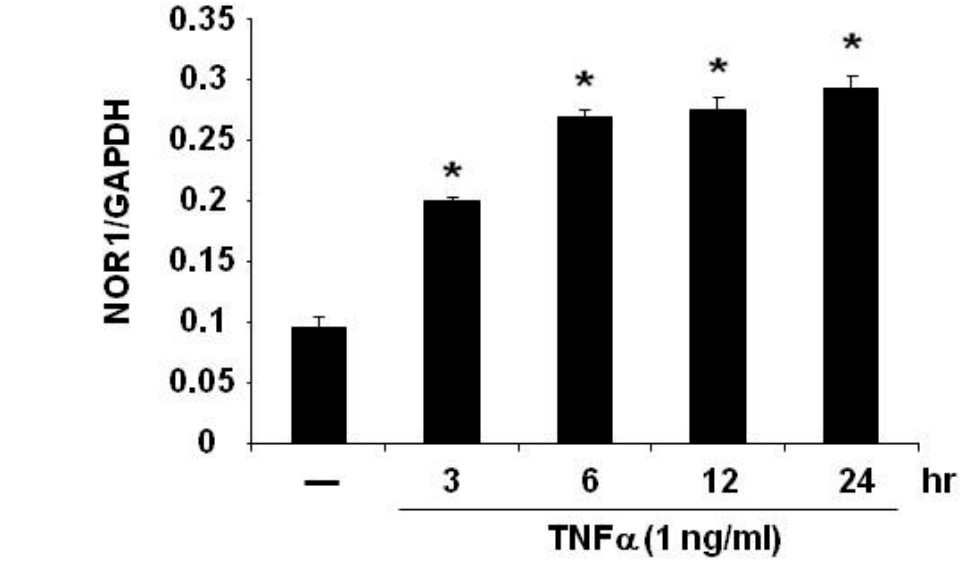
Sections of atherosclerotic human coronary arteries were immunostained for NOR1 and von Willebrand Factor (vWF) to identify endothelial cells. Species-matched normal IgG served as negative control. Images were acquired using (A) light microscopy and (B) confocal microscopy. In (B) stainings for NOR1, vWF and DAPI were merged into one image as indicated. Objective magnifications are indicated.

A

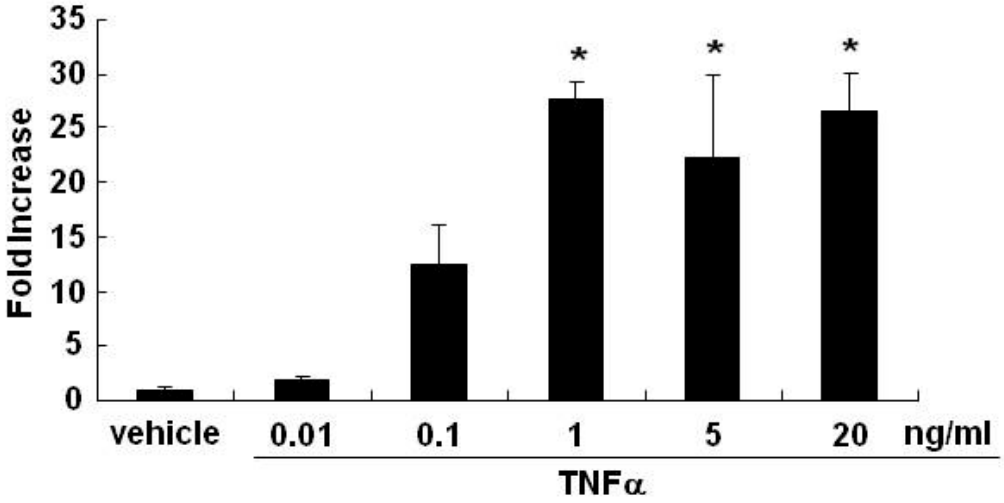

Fold Increase
40
30
20
10
0
*
*
*
*
vehicle
IL-6, 10 ng/ml
IL-1β, 10 ng/ml
IFNγ, 10 ng/ml
oxidized LDL, 10 μg/ml
LPS, 1 μg/ml
TNFα, 10 ng/ml


B
Fold Increase
90
80
70
60
50
40
30
20
10
0
*
*
*
*
—
1h
2h
3h
6h
12h
24h
TNFα (1 ng/ml)

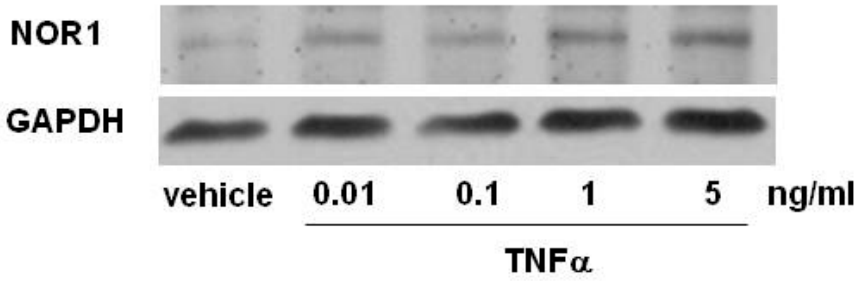

C
NOR1
GAPDH
—
3
6
12
24
hr
TNFα (1 ng/ml)


D
NOR1/GAPDH
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
*
—
3
6
12
24
hr
TNFα (1 ng/ml)

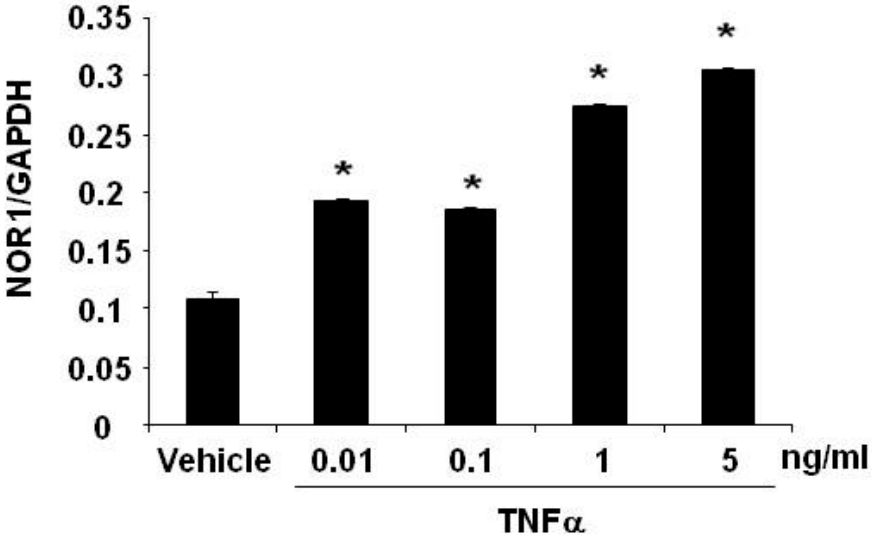
E


35
30
25
20
15
10
5
0
Fold Increase
*
*
*
vehicle
0.01
0.1
1
5
20
ng/ml
TNFα

F


NOR1
GAPDH
vehicle
0.01
0.1
1
5
ng/ml
TNFα

G


0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
NOR1/GAPDH
*
*
*
*
Vehicle
0.01
0.1
1
5
ng/ml
TNFα

H

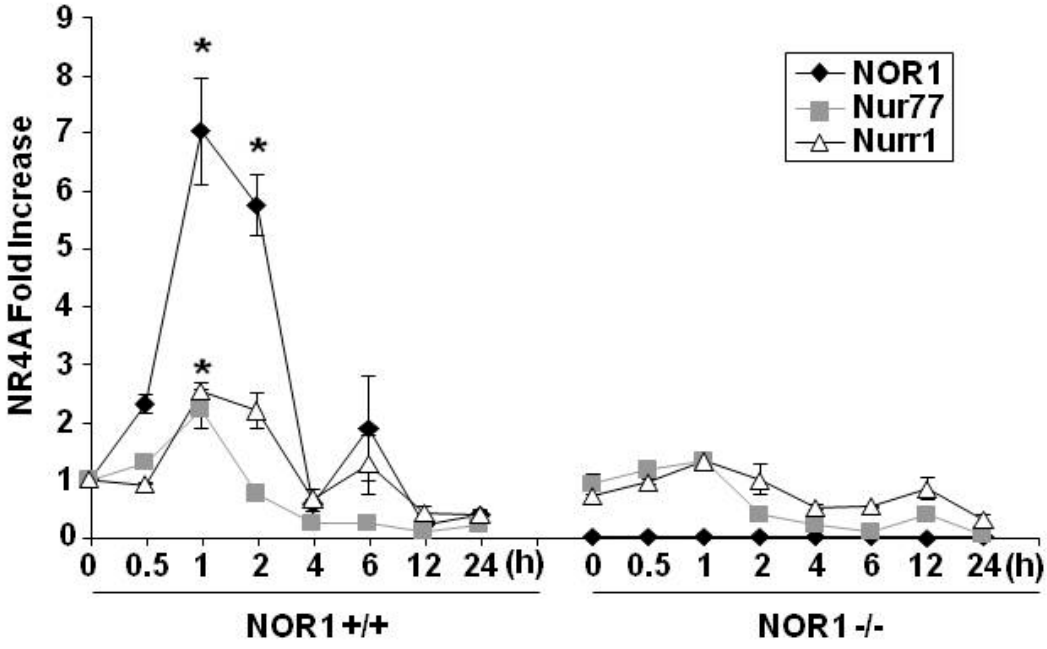
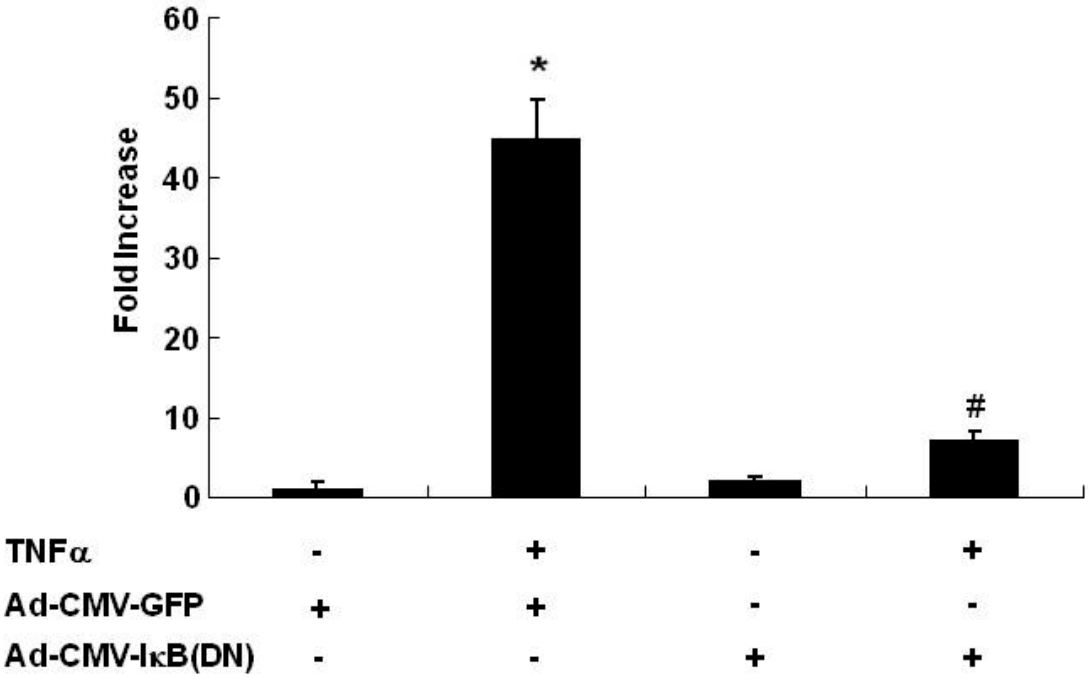
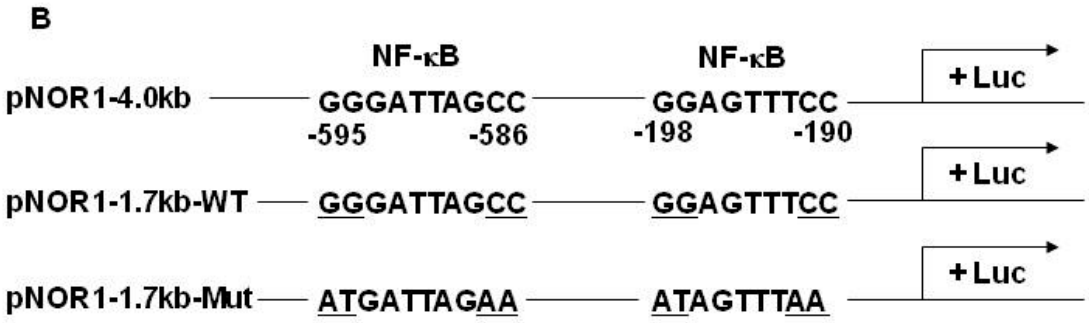

NR4A Fold Increase
9
8
7
6
5
4
3
2
1
0
*
*
*
NOR1
Nur77
Nurr1
0 0.5 1 2 4 6 12 24 (h)
0 0.5 1 2 4 6 12 24 (h)
NOR1+/+
NOR1-/-

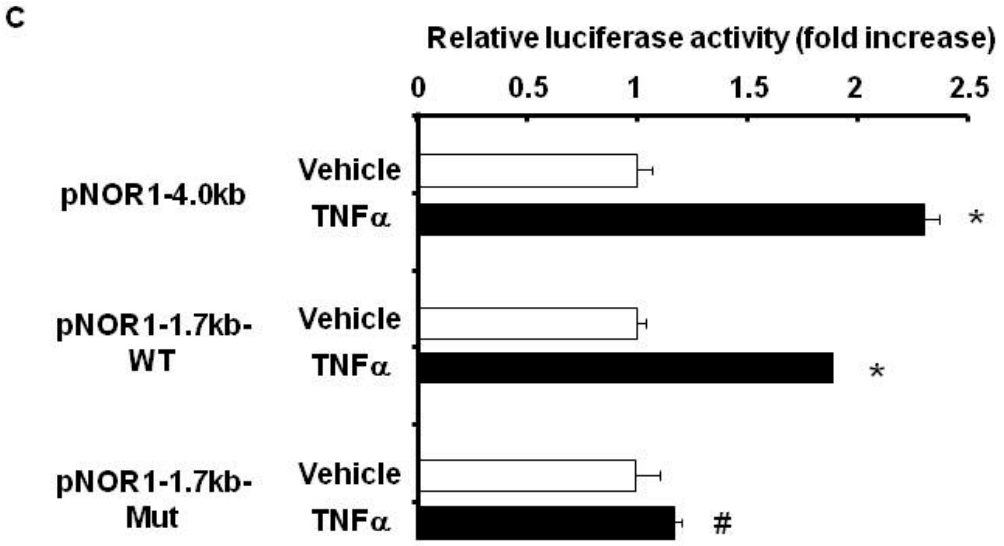
Figure 2.2. NOR1 is induced by inflammatory stimuli in endothelial cells

(A) HUVEC were stimulated as indicated for 2 hours and NOR1 mRNA expression was analyzed. (B-D) HUVEC were stimulated with TNFα (1 ng/ml) and NOR1 mRNA (B) and protein expression (C) were analyzed at the indicated time points. (D) Densitometric analysis of NOR1 protein expression in (C). (E-G) HUVEC were stimulated with vehicle (PBS) or the indicated doses of TNFα. NOR1 mRNA (E) and protein (F) expression were analyzed after 2 hours or 6 hours, respectively. (G) Densitometric analysis of NOR1 protein expression in (F). NOR1 mRNA expression levels were normalized to hTBP and presented as mean ± SEM fold increase over vehicle-treated cells (*$P < 0.05$ vs. vehicle). Densitometric analysis was performed on three independent experiments. Cohybridization for GAPDH was performed to assess equal loading. (H) NOR1+/+ and NOR1-/- MAEC were incubated with TNFα (5 ng/ml) and mRNA expression of NOR1, Nur77 and Nurr1 was analyzed by real-time RT-PCR. Experiments were repeated at least three times in duplicate with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*$P < 0.05$ vs. vehicle).

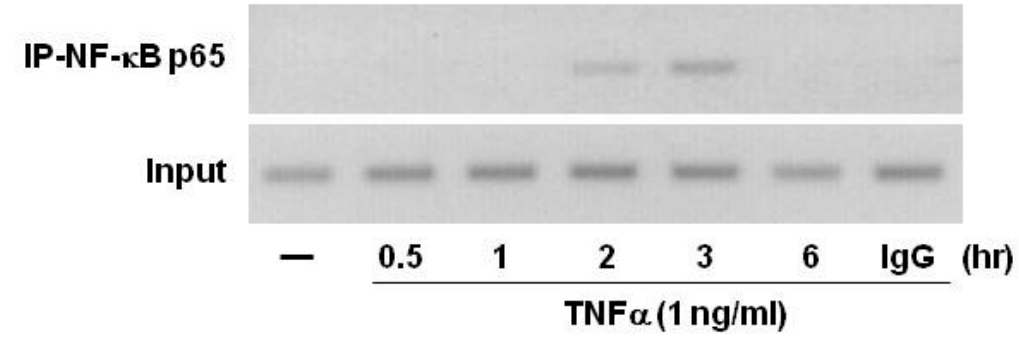
A




B
NF-κB
NF-κB
pNOR1-4.0kb
GGGATTAGCC
GGAGTTTCC
-595
-586
-198
-190
+Luc
pNOR1-1.7kb-WT
GGGATTAGCC
GGAGTTTCC
+Luc
pNOR1-1.7kb-Mut
ATGATTAGAA
ATAGTTTAA
+Luc


C
Relative luciferase activity (fold increase)
0
0.5
1
1.5
2
2.5
pNOR1-4.0kb
Vehicle
TNFα
*
pNOR1-1.7kb-WT
Vehicle
TNFα
*
pNOR1-1.7kb-Mut
Vehicle
TNFα
#

D


IP-NF-κB p65
Input
—
0.5
1
2
3
6
IgG
(hr)
TNFα (1 ng/ml)

E

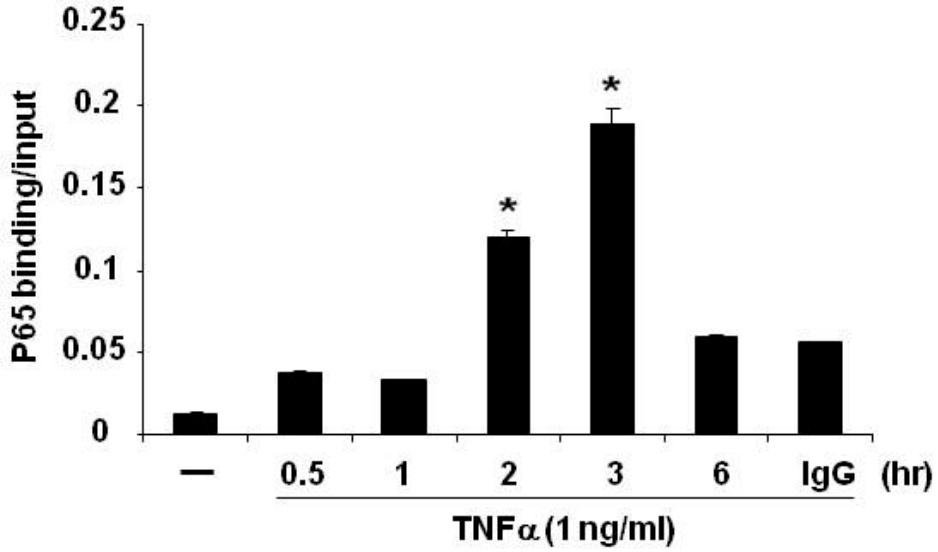
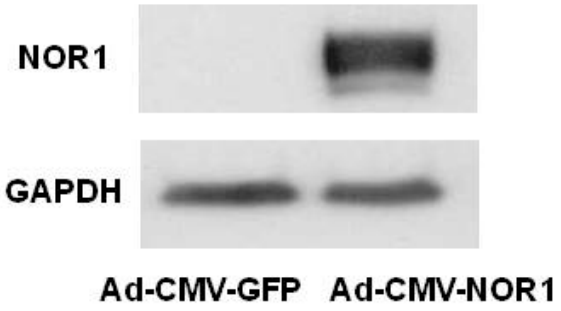

P65 binding/input
0.25
0.2
0.15
0.1
0.05
0
*
*
—
0.5
1
2
3
6
IgG
(hr)
TNFα (1 ng/ml)

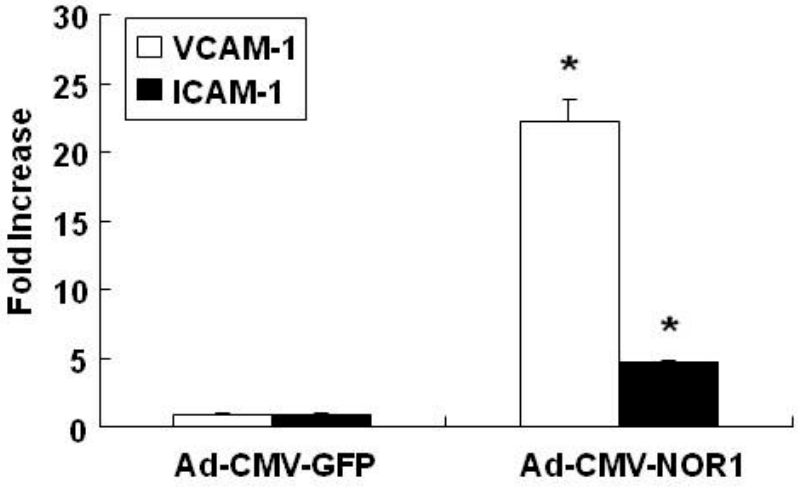
Figure 2.3. NOR1 expression in endothelial cells is mediated through NF-κB-dependent signaling pathways

(A) HUVEC were infected with 25 PFU Ad-CMV-IκB(DN) or Ad-CMV-GFP and stimulated with vehicle (PBS) or TNFα (1 ng/ml) for 2 hours. NOR1 mRNA expression was analyzed and normalized to hTBP. Data are presented as mean ± SEM fold increase over Ad-CMV-GFP-infected cells treated with vehicle (*$p < 0.05$ vs. vehicle; #$p < 0.05$ vs. Ad-CMV-GFP). (B) Schematic structure of the human 4.0 kb and 1.7 kb NOR1 promoter constructs. (C) HAEC were transfected with a luciferase reporter construct driven by the indicated NOR1 promoter, stimulated with vehicle (PBS) or TNFα (10 ng/ml), and analyzed for luciferase activities. Data are presented as mean ± SEM from three independently performed experiments (*$p < 0.05$ vs. vehicle; #$p < 0.05$ vs. pNOR1-1.7kb-WT). (D-E) HUVEC were stimulated with TNFα (1 ng/ml) and harvested at the indicated time point for ChIP assays. Chromatin complexes were immunoprecipitated with an antibody against NF-κB p65 or species-matched IgG. PCR products were amplified using primers covering the -595 bp to -586 bp NF-κB binding site. (D) The autoradiograms are representative of three independently performed experiments. (E) Densitometric analysis of NF-κB p65 binding normalized to input from three independently performed experiments.

A


NOR1
GAPDH
Ad-CMV-GFP
Ad-CMV-NOR1

B


30
25
20
15
10
5
0
Fold Increase
VCAM-1
ICAM-1
*
*
Ad-CMV-GFP
Ad-CMV-NOR1

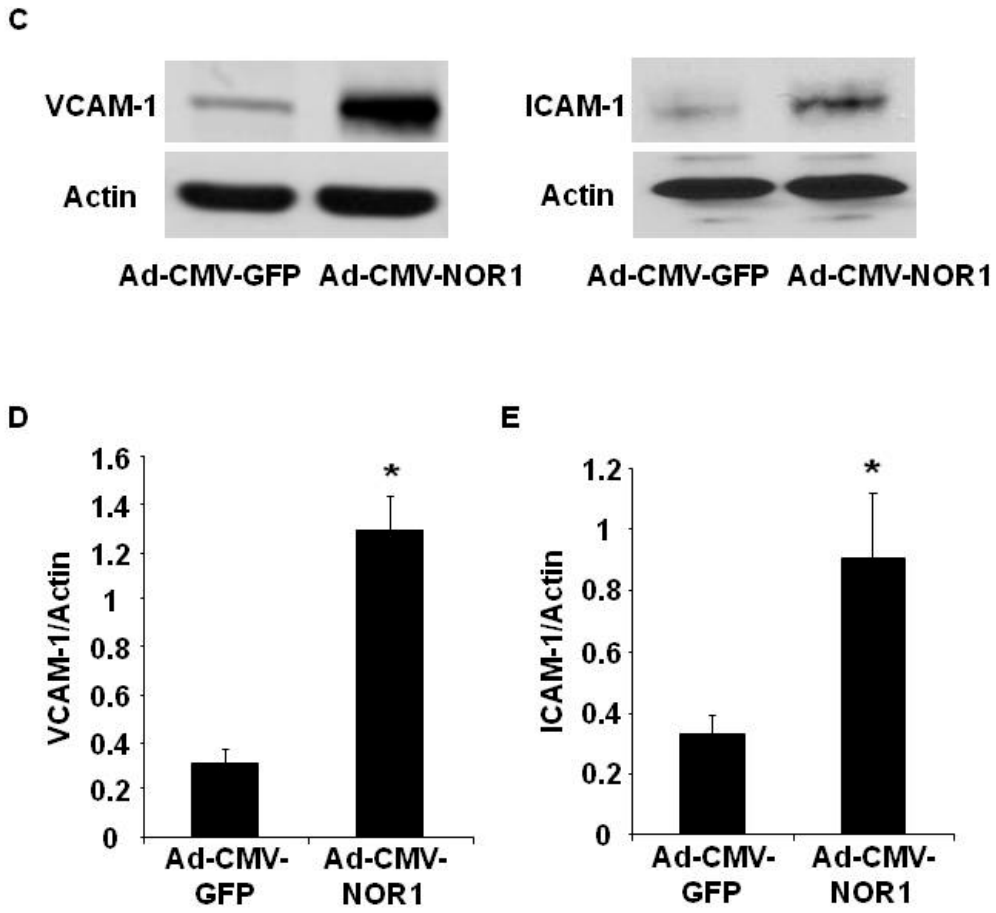
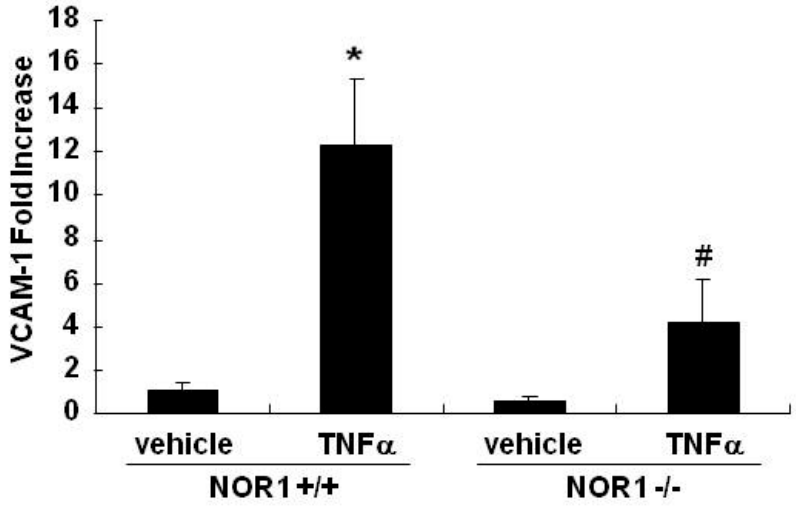


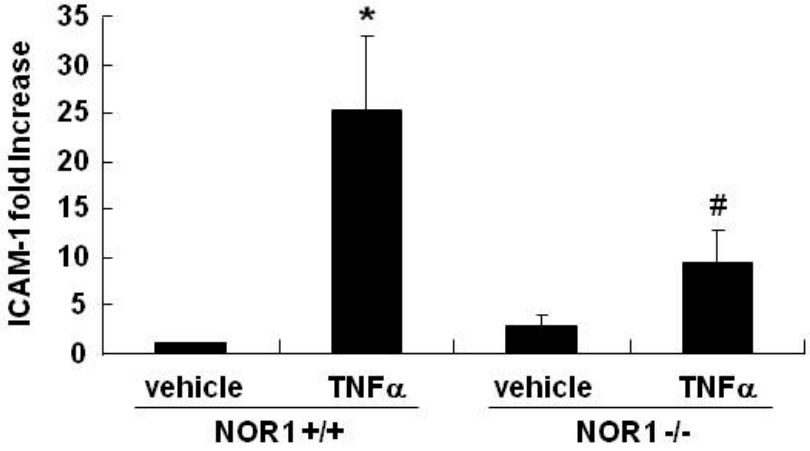
Figure 2.4. NOR1 induces VCAM-1 and ICAM-1 expression in endothelial cells

(A-E) HUVEC were infected with 50 PFU Ad-CMV- NOR1 or Ad-CMV-GFP. (A) Western Blotting confirmed NOR1 over-expression after transduction. (B) VCAM-1 and ICAM-1 mRNA expression was determined by real-time RT-PCR. Experiments were performed at least three times in triplicate. Data were calculated by normalizing NOR1 values to hTBP values and presented as mean ± SEM fold induction over Ad-CMV-GFP infected cells (*$p < 0.05$ vs. Ad-CMV-GFP). (C-E) VCAM-1 and ICAM-1 protein expression was analyzed by Western blotting. Cohybridization for actin was performed to assess equal loading. (C) Representative autoradiograms and densitometric analysis (D and E) of three independently performed experiments.

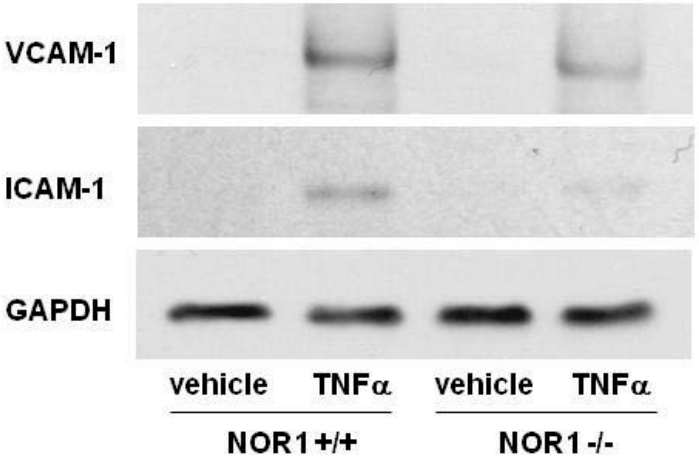
A


VCAM-1 Fold Increase
18
16
14
12
10
8
6
4
2
0
*
#
vehicle
TNFα
vehicle
TNFα
NOR1 +/+
NOR1 -/-

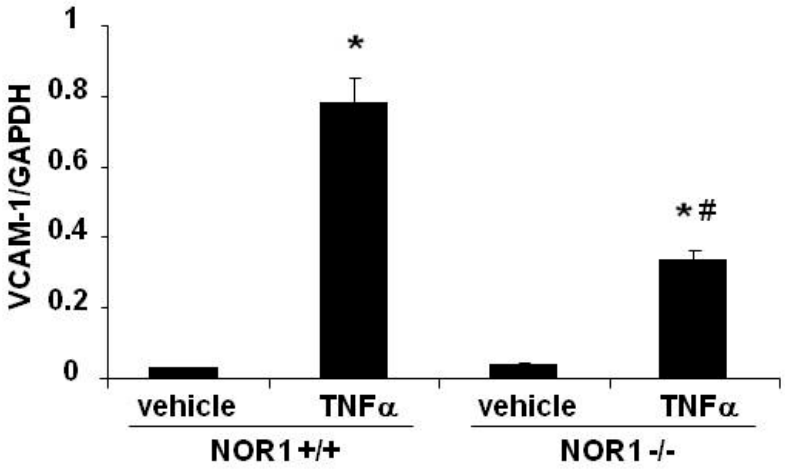
B


ICAM-1 fold Increase
35
30
25
20
15
10
5
0
*
#
vehicle
TNFα
vehicle
TNFα
NOR1 +/+
NOR1 -/-

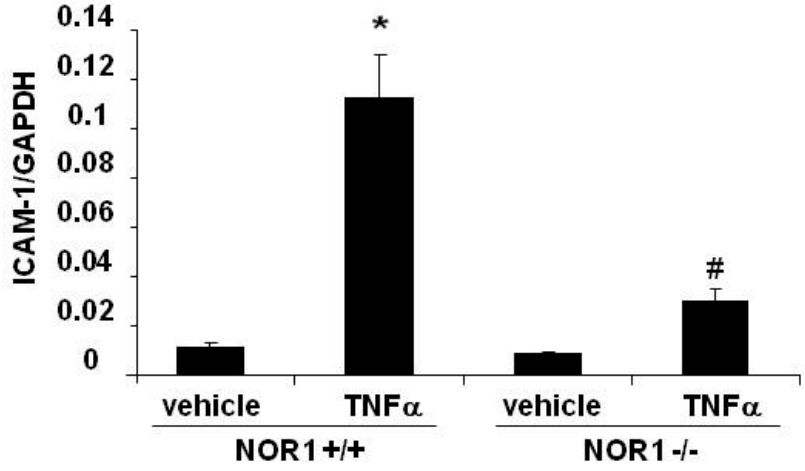
C

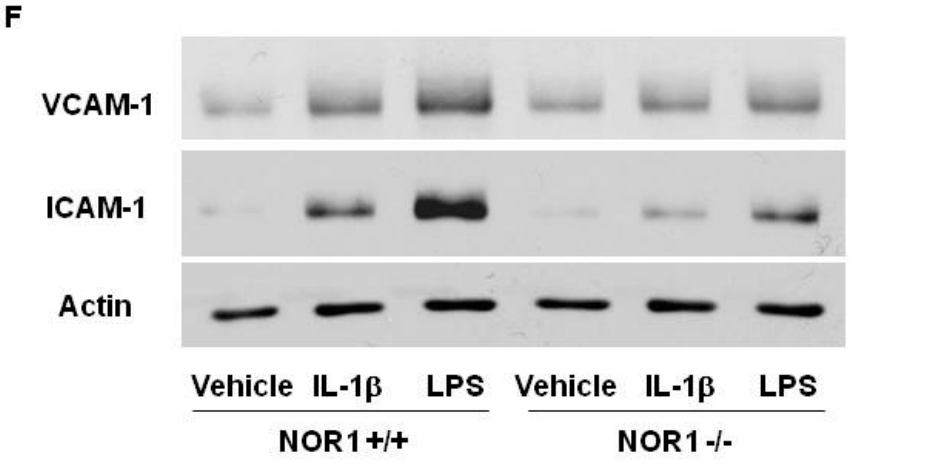

VCAM-1
ICAM-1
GAPDH
vehicle
TNFα
vehicle
TNFα
NOR1+/+
NOR1-/-

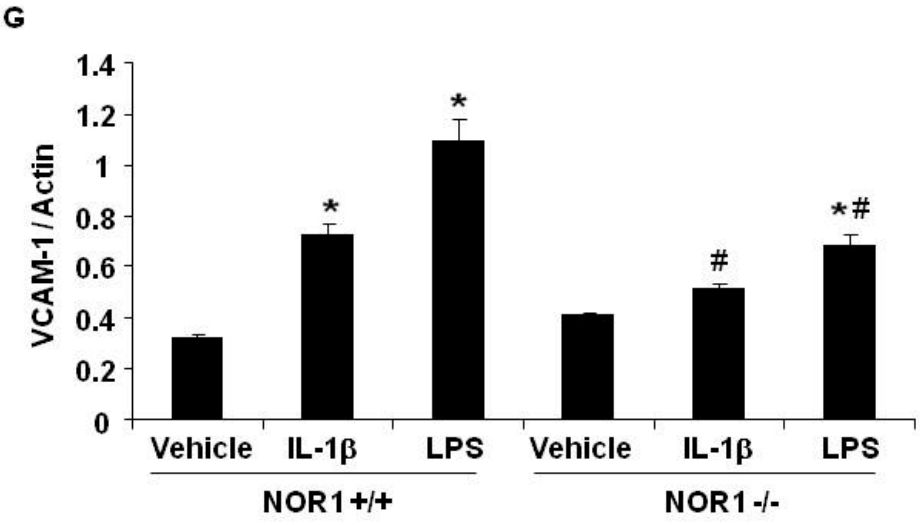
D


VCAM-1/GAPDH
1
0.8
0.6
0.4
0.2
0
*
*#
vehicle
TNFα
vehicle
TNFα
NOR1+/+
NOR1-/-

E


ICAM-1/GAPDH
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
*
#
vehicle
TNFα
vehicle
TNFα
NOR1+/+
NOR1-/-


F
VCAM-1
ICAM-1
Actin
Vehicle
IL-1β
LPS
Vehicle
IL-1β
LPS
NOR1+/+
NOR1-/-


G
VCAM-1 / Actin
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*#
#
Vehicle
IL-1β
LPS
Vehicle
IL-1β
LPS
NOR1+/+
NOR1-/-

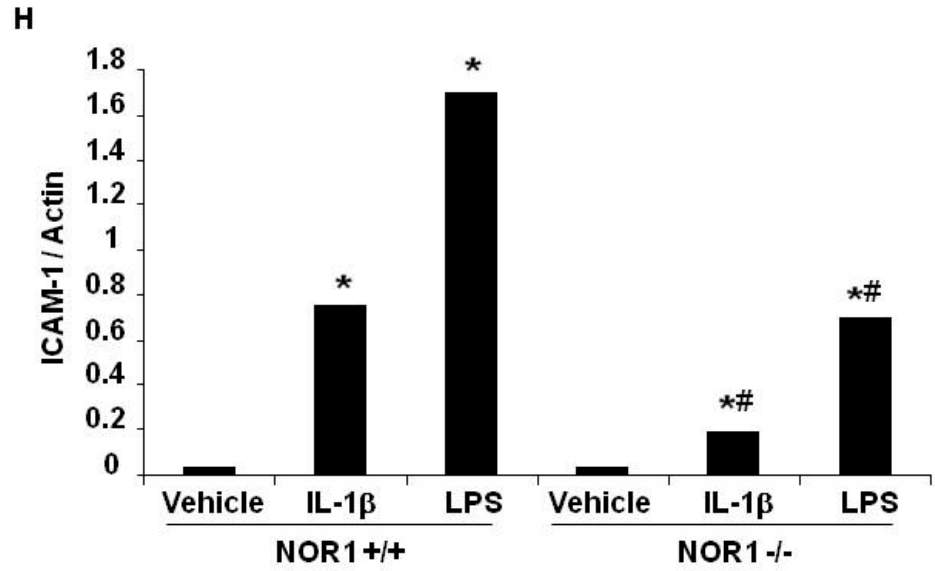
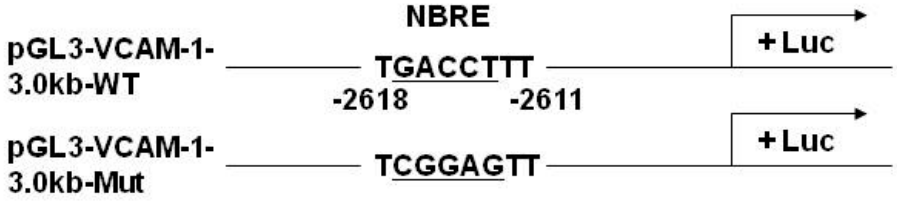

H
ICAM-1 / Actin
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
*#
*#
Vehicle
IL-1β
LPS
Vehicle
IL-1β
LPS
NOR1+/+
NOR1-/-

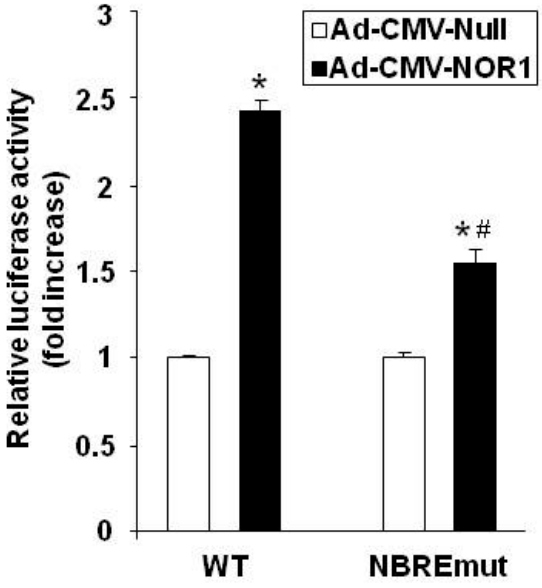
Figure 2.5. NOR1 is required for VCAM-1 and ICAM-1 expression in endothelial cells

(A-E) NOR1+/+ and NOR1-/- MAEC were stimulated with vehicle or TNFα (1 ng/ml) for 6 hours. VCAM-1 and ICAM-1 mRNA (A and B) and protein (C-E) expression were analyzed by real-time RT-PCR and Western blotting, respectively. (C) The autoradiograms are representative of three independent experiments. (D and E) Densitometric analysis of VCAM-1 and ICAM-1 protein expression after Western blotting. All experiments were repeated at least three times in duplicate with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*$P < 0.05$ vs. vehicle, # $P < 0.05$ vs. NOR1+/+ cells). (F-H) NOR1+/+ and NOR1-/- MAEC were stimulated with vehicle, IL-1β (1 ng/ml), or LPS (50 ng/ml) for 6 hours. VCAM-1 and ICAM-1 protein expression were analyzed by Western blotting. Cohybridization for actin was performed to assess equal loading. (F) Autoradiograms are representative of three different experiments. (G and H) Densitometric quantification of VCAM-1 and ICAM-1 protein expression from three experiments with different cell preparations. Results are presented as mean ± SEM fold increase over vehicle-treated wildtype cells (*$P < 0.05$ vs. vehicle, # $P < 0.05$ vs. NOR1+/+ cells).

A


NBRE
pGL3-VCAM-1-3.0kb-WT
TGACCTTT
-2618
-2611
+Luc
pGL3-VCAM-1-3.0kb-Mut
TCGGAGTT
+Luc

B


Ad-CMV-Null
Ad-CMV-NOR1
Relative luciferase activity (fold increase)
3
2.5
2
1.5
1
0.5
0
*
*#
WT
NBREmut

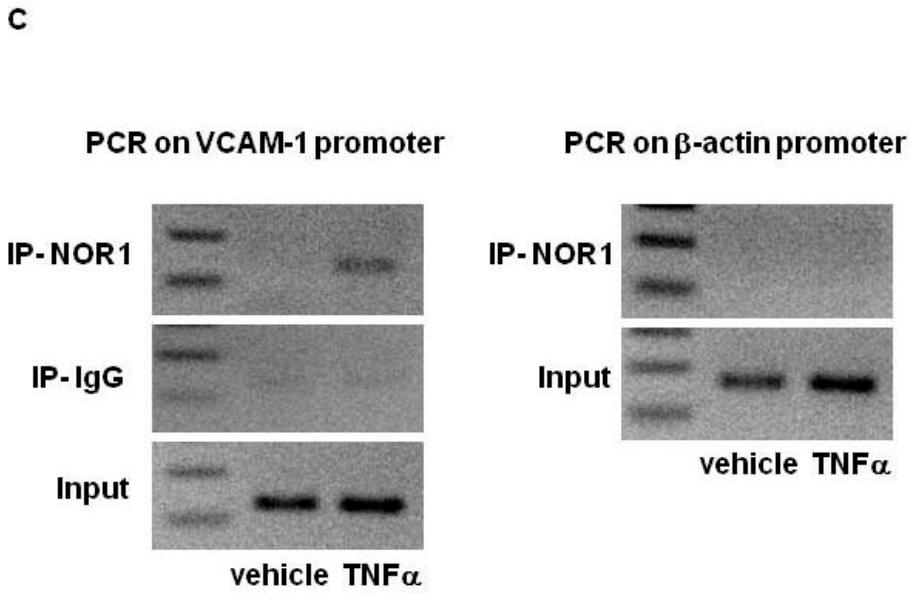
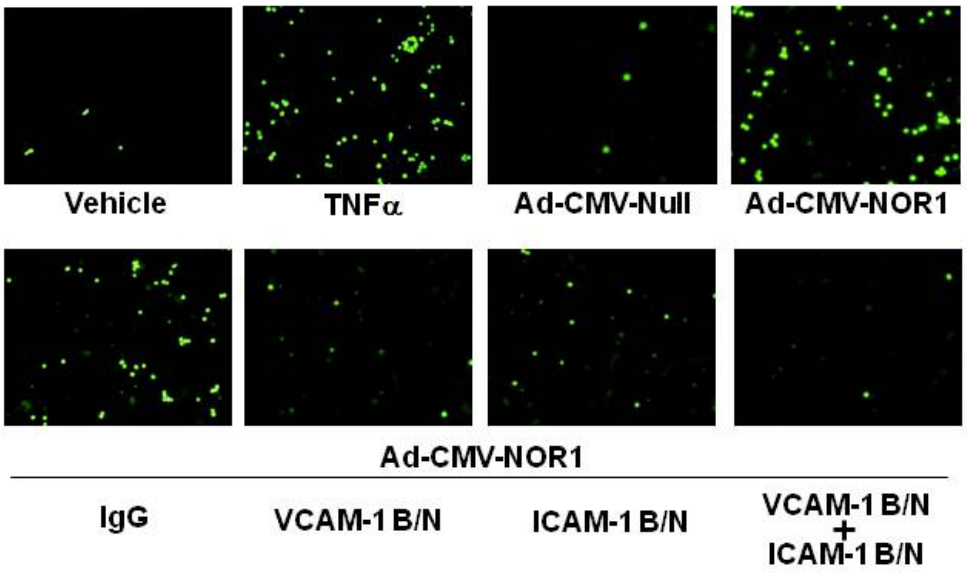


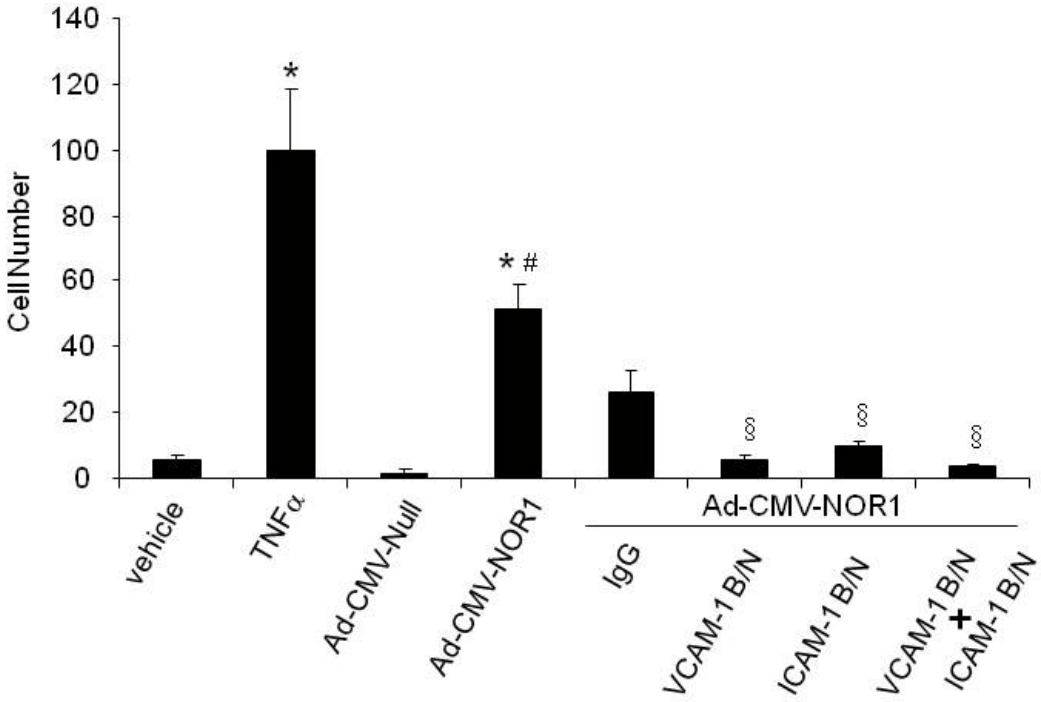
Figure 2.6. NOR1 *trans*-activates the VCAM-1 promoter by binding to an NBRE consensus site

(A) Schematic structure of the human 3.0 kb VCAM-1 promoter and the NBRE consensus site at -2618 bp. (B) HUVEC were infected with 50 PFU Ad-CMV-Null or Ad-CMV-NOR1 for 6 hours and recovered for 24 hours. Infected cells were transiently transfected with luciferase reporter constructs driven by the VCAM-1 wildtype promoter or the similar promoter bearing a mutation in the NBRE site. Luciferase activities were analyzed after 48 hours. Data are presented as mean ± SEM fold induction from three independently performed experiments (*$p < 0.05$ vs. empty vector; #$p < 0.05$ vs. pVCAM-1-WT). (C) HUVEC were stimulated with vehicle (PBS) or TNFα (1 ng/ml) for ChIP assays. PCR for an unrelated promoter fragment in the β-actin promoter served as control for specificity. The autoradiograms are representative of three independently performed experiments.

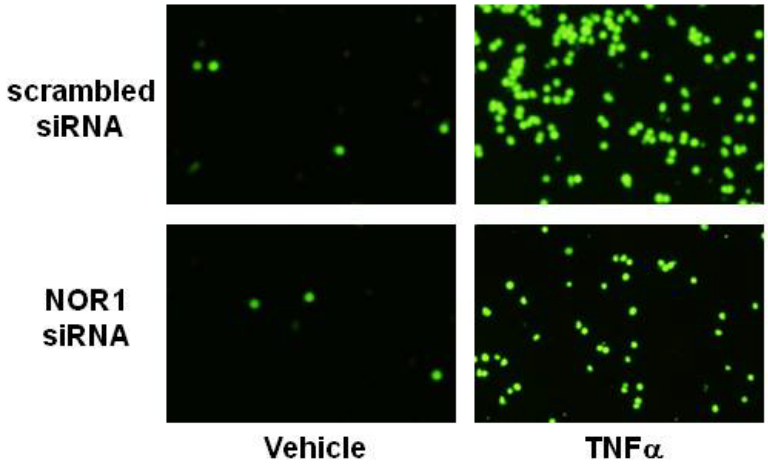
A


Vehicle
TNFα
Ad-CMV-Null
Ad-CMV-NOR1
Ad-CMV-NOR1
IgG
VCAM-1 B/N
ICAM-1 B/N
VCAM-1 B/N
+
ICAM-1 B/N

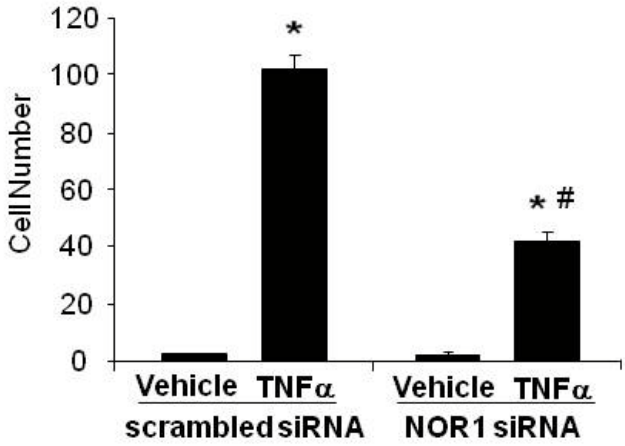
B

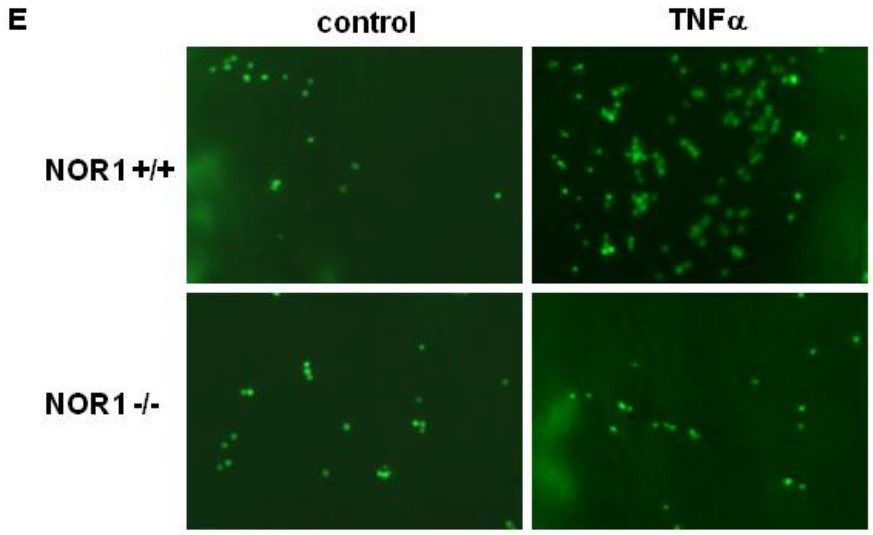

Cell Number
140
120
100
80
60
40
20
0
*
* #
§
§
§
Ad-CMV-NOR1
vehicle
TNFα
Ad-CMV-Null
Ad-CMV-NOR1
IgG
VCAM-1 B/N
ICAM-1 B/N
VCAM-1 B/N
+
ICAM-1 B/N

C


scrambled siRNA
NOR1 siRNA
Vehicle
TNFα

D


Cell Number
120
100
80
60
40
20
0
*
* #
Vehicle
TNFα
scrambled siRNA
Vehicle
TNFα
NOR1 siRNA


E
control
TNFα
NOR1+/+
NOR1-/-

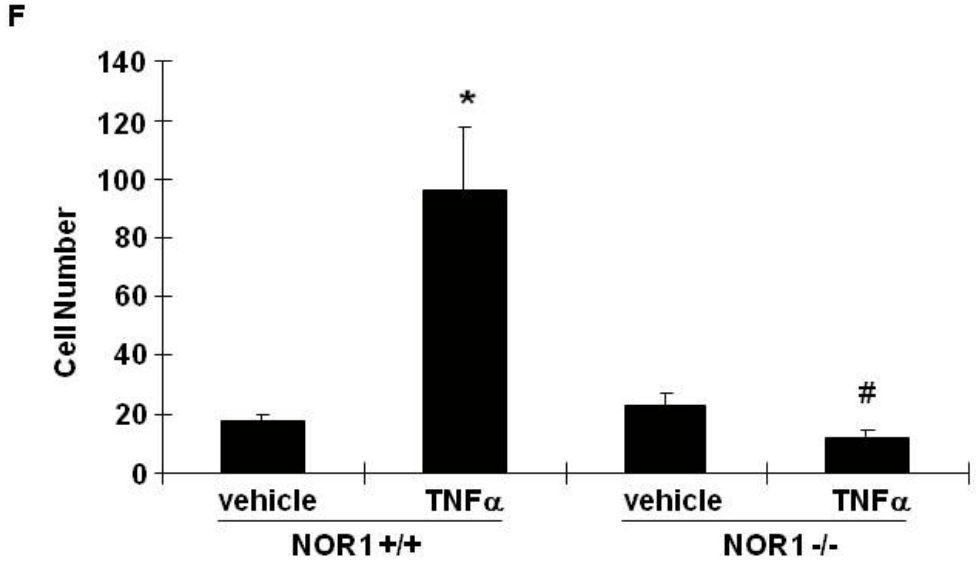
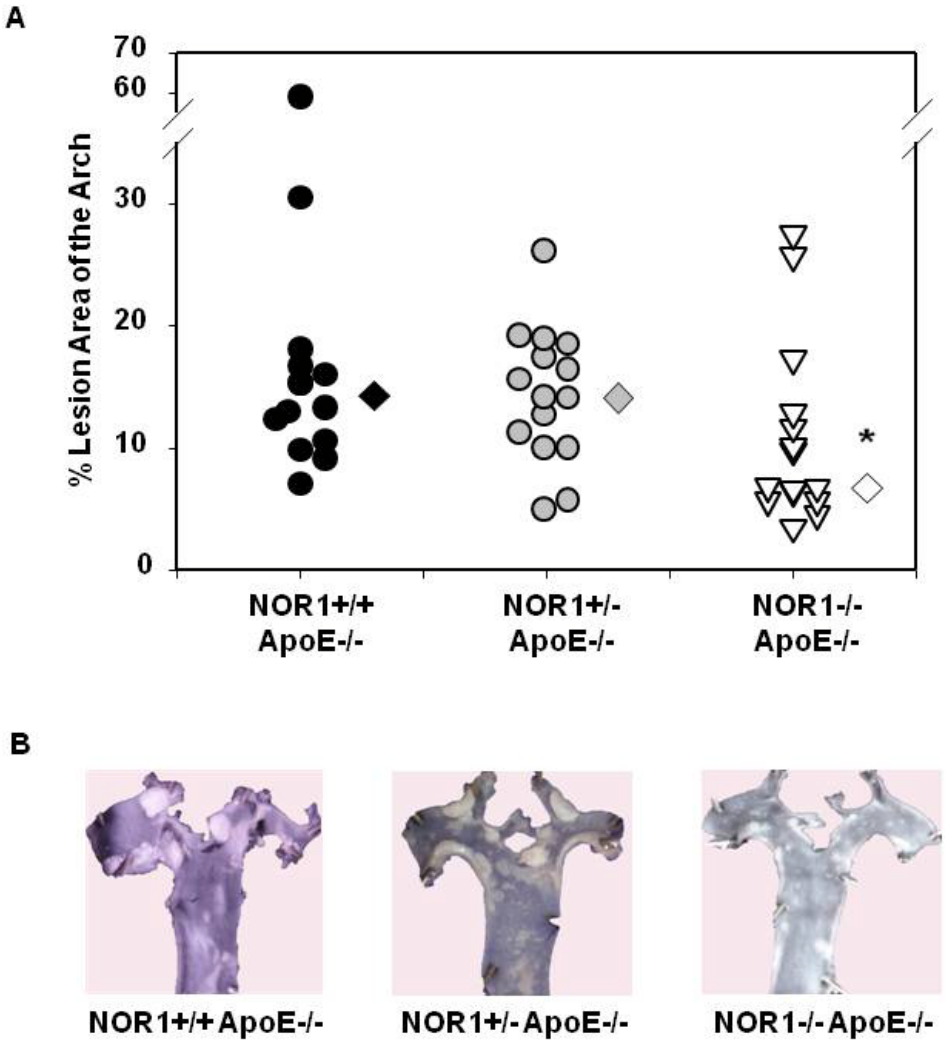
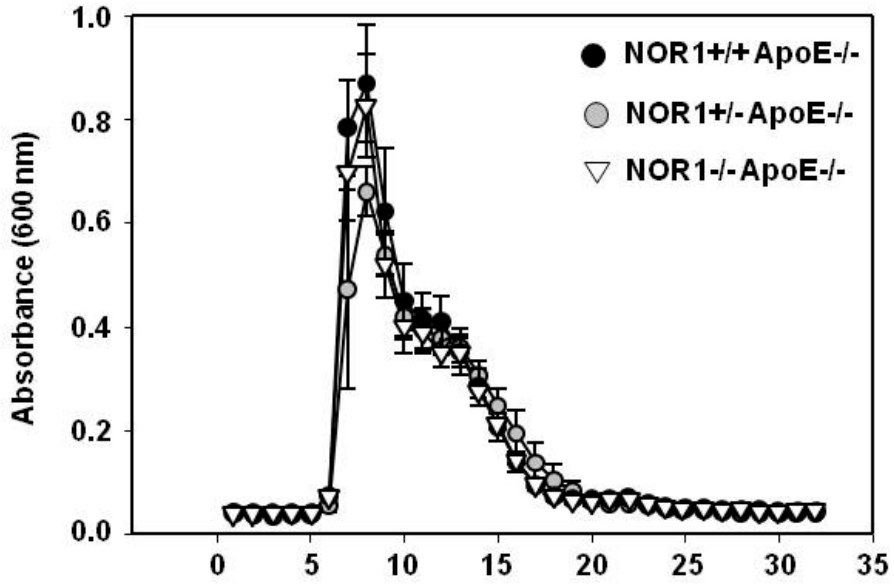

F
Cell Number
140
120
100
80
60
40
20
0
*
#
vehicle
TNFα
vehicle
TNFα
NOR1+/+
NOR1-/-

Figure 2.7. NOR1 mediates monocyte adhesion by regulating VCAM-1 and ICAM-1 expression

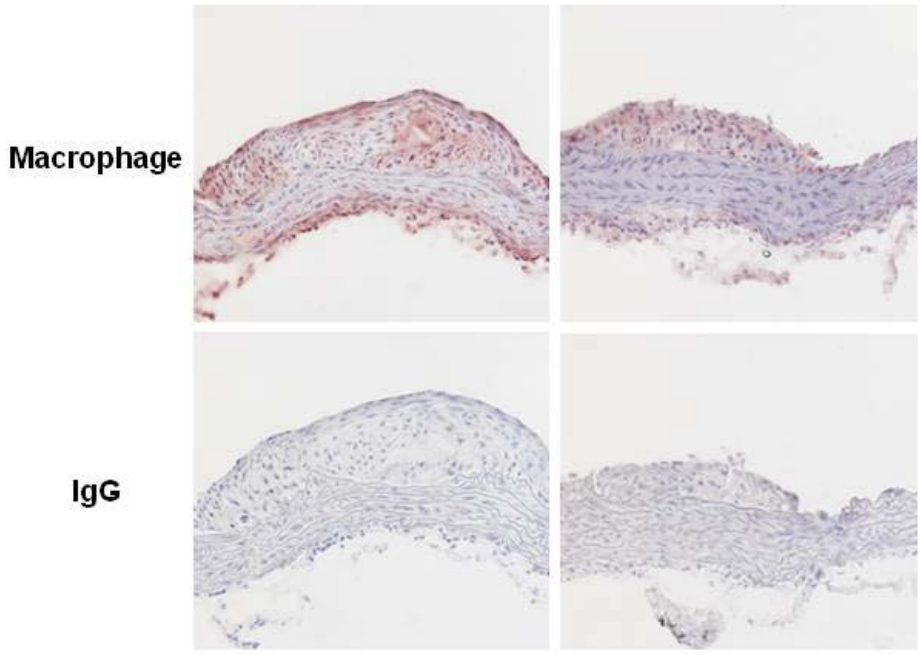
(A) Upper panel: HUVEC were infected with 50 PFU Ad-CMV-NOR1 or Ad-CMV-Null, and fluorescently labeled THP-1 monocytes were added onto HUVEC monolayers. TNFα-treated (1 ng/ml) HUVEC were employed as a positive control. Lower panel: Ad-CMV-NOR1-infected cells were pre-incubated with VCAM-1 and ICAM-1 blocking antibodies (B/N) or control IgG. After 1 hour, fluorescently labeled THP-1 monocytes were added onto HUVEC monolayers. (B) Quantification is presented as mean ± SEM from three independently performed experiments in duplicate (*$p < 0.05$ vs. vehicle or Ad-CMV-Null, # $p < 0.05$ vs. TNFα, § $p < 0.05$ vs. IgG). (C and D) siRNA-mediated knock-down of NOR1 expression decreased monocyte adhesion. (C) HUVEC were transfected with scrambled or NOR1 siRNA, stimulated with TNFα (1 ng/ml), and analyzed for THP-1 monocyte adhesion. Representative images showing monocyte adhesion. (D) Quantification is presented as mean ± SEM from three independently performed experiments in duplicates (*$P < 0.05$ vs. vehicle, # $P < 0.05$ vs. scrambled siRNA). (E and F) Aortae were isolated from NOR1+/+ and NOR1-/- mice and TNFα-induced monocyte adhesion was analyzed. (E) Representative sections demonstrating adhesion of monocytes. (F) Quantification of adhesion from three independent experiments performed in duplicate using different aortic preparations. Data are expressed as mean ± SEM (*$P < 0.05$ vs. vehicle, # $P < 0.05$ vs. NOR1+/+ cells).


A
70
60
30
20
10
0
% Lesion Area of the Arch
*
NOR1+/+
ApoE-/-
NOR1+/-
ApoE-/-
NOR1-/-
ApoE-/-
B
NOR1+/+ ApoE-/-
NOR1+/- ApoE-/-
NOR1-/- ApoE-/-

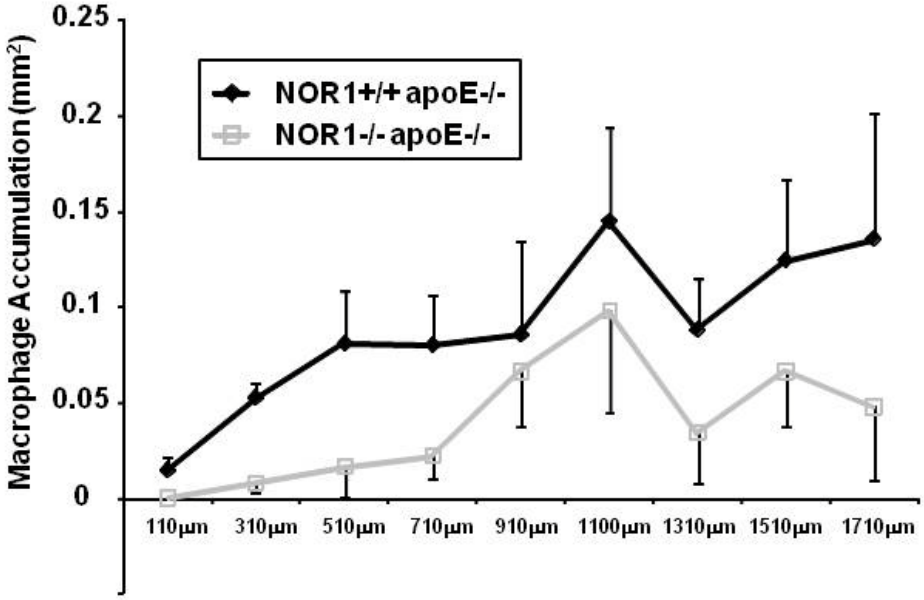
C


Absorbance (600 nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
5
10
15
20
25
30
35
NOR1+/+ ApoE-/-
NOR1+/- ApoE-/-
NOR1-/- ApoE-/-

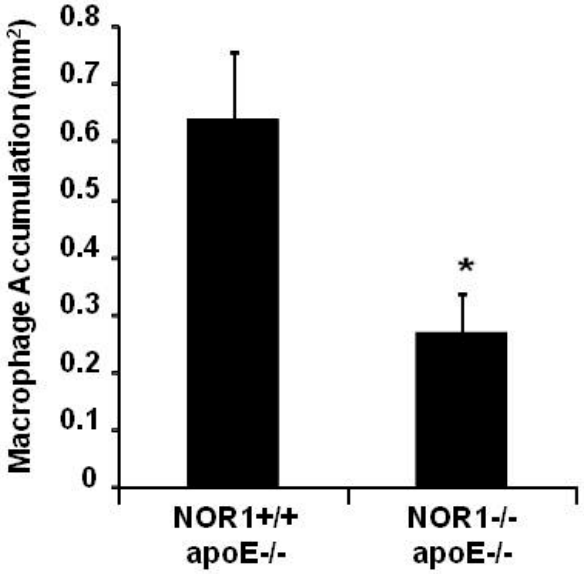
D


NOR1+/+apoE-/-
NOR1-/-apoE-/-
Macrophage
IgG

E


Macrophage Accumulation (mm²)
0.25
0.2
0.15
0.1
0.05
0
NOR1+/+ apoE-/-
NOR1-/- apoE-/-
110μm
310μm
510μm
710μm
910μm
1100μm
1310μm
1510μm
1710μm

F


Macrophage Accumulation (mm²)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
NOR1+/+ apoE-/-
NOR1-/- apoE-/-

G

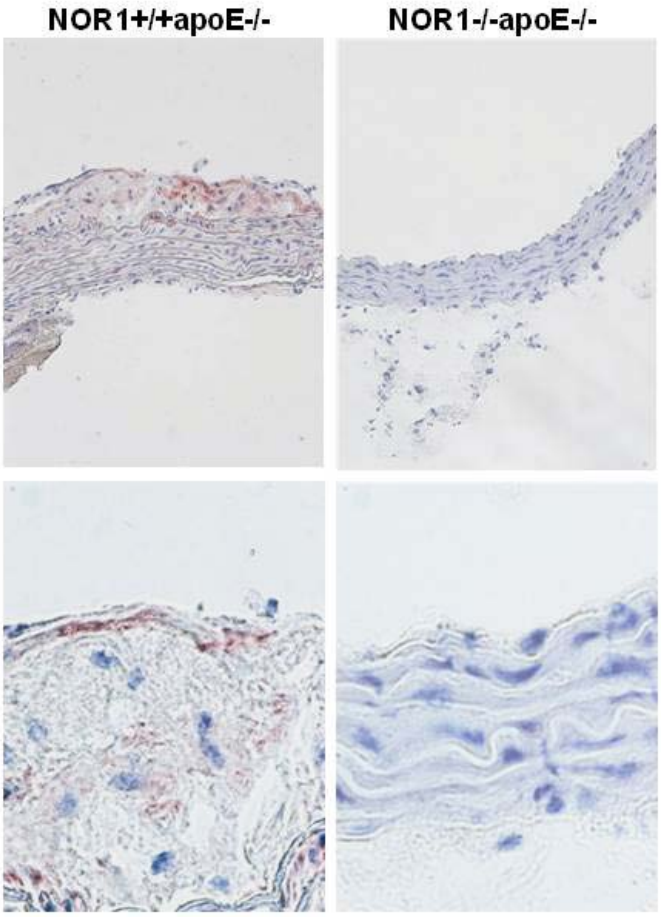


Figure 2.8. NOR1 deficiency decreases atherosclerosis in apoE-/- mice

(A) Atherosclerotic lesion size was measured on aortic arches from female NOR1+/+apoE-/- (n=14), NOR1+/-apoE-/- (n=15) and NOR1-/-apoE-/- (n=15) mice. Circles and triangles represent individual mice; diamonds represent medians ($P < 0.05$ between group, $^*P < 0.05$ vs. NOR1+/+apoE-/-). (B) Representative aortic arches from each genotype. (C) Lipoprotein cholesterol distributions. Values represent the mean cholesterol content of each fraction (± SEM). (D-G) Serial sections from a 2-mm segment beginning at the lesser curvature of the aortic arch were collected from NOR1+/+apoE-/- and NOR1-/-apoE-/- mice. Sections of atherosclerotic lesions were immunostained using antisera against macrophages or VCAM-1. (D) Representative sections for macrophage staining (top) or IgG (bottom) (objective magnification ×20). (E) Macrophage content was quantified in nine sections 200 μm apart using computer-assisted image analysis. Data are presented as mean area in mm^2 ± SEM. Statistical analysis was performed using factorial ANOVA test. No significant difference between single segments of the arch in NOR1+/+apoE-/- and NOR1-/-apoE-/- mice was detected. (F) Macrophage accumulation was quantified in the entire aortic arch from NOR1+/+apoE-/- and NOR1-/-apoE-/- mice ($^*P < 0.05$ vs. NOR1+/+apoE-/-). (G) Representative sections for VCAM-1 staining (objective magnification ×20 and x100).

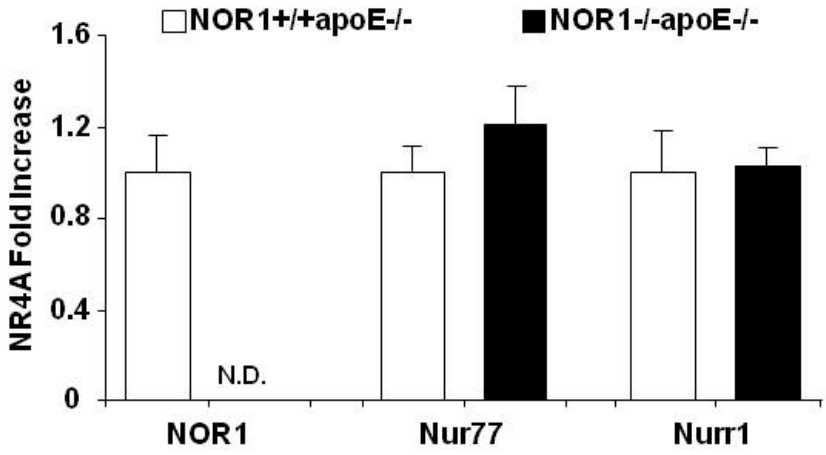


Figure 2.9. The expression of NR4A nuclear receptors in mouse aortic arteries in response to high-fat diet feeding

NOR1+/+apoE-/- (n=5) and NOR1-/-apoE-/- (n=5) mice were fed a diet enriched in saturated fat for 2 weeks. Aortae were collected and mRNA expression of NOR1, Nur77 and Nurr1 was analyzed by real-time RT-PCR. Results are presented as mean ± SEM fold increase over NOR1+/+apoE-/- mice. Note, no NOR1 transcript was detected in NOR1-/-apoE-/- mice (N.D., not detectable).

Chapter three: Epigenetic regulation of the NR4A orphan nuclear receptor NOR1 by histone deacetylase inhibition

This part is in manuscript preparation for submission.

3.1. Synopsis

Many nuclear receptors function as key transcriptional regulators of gene expression in cardiovascular diseases. While most nuclear receptors are ligand-dependent transcription factors, members of the NR4A subfamily function as immediate/early response genes whose transcriptional activity is dependent on their expression level or their posttranslational modification. In our previous studies, we established that the neuron-derived orphan receptor 1 (NOR1) of the NR4A subfamily serves a mitogenic role and promotes neointima formation. In smooth muscle cells, NOR1 is induced through phosphorylation of the cAMP response element binding protein (CREB) and subsequent binding of phosphorylated CREB to the NOR1 promoter. In the current study, we extend these observations and demonstrate that NOR1 expression is enhanced by inhibition of histone deacetylation. Pre-treatment of smooth muscle cells with the histone deacetylase (HDAC) inhibitor scriptaid potentiated and sustained PDGF-induced NOR1 mRNA and protein expression. Conversely, specific small interfering RNA-mediated knockdown of HDAC3 augmented PDGF-induced NOR1 mRNA expression. Scriptaid increased NOR1 promoter activity in reporter assays without affecting NOR1 transcript stability. This increased NOR1 transcription was associated with enhanced CREB phosphorylation by HDAC inhibition. Chromatin immunoprecipitation assays further confirmed that HDAC inhibition increased histone acetylation at CREB binding sites in the NOR1 promoter region and results in increased binding of phosphorylated CREB to its consensus sites. Finally, we demonstrate that HDAC inhibition also increases NOR1 protein half-life. Collectively, these data suggest that HDAC inhibition increases NOR1 expression at multiple levels, including gene transcription and protein stability. These findings may have

important implications for understanding the epigenetic mechanisms orchestrating the expression of this mitogenic transcription factor.

3.2. Introduction

Epigenetic traits are considered as chromosome modifications that regulate gene transcription without altering DNA sequences[30, 42]. These modifications can be translated into signals dictating gene activation/repression[30, 42]. One factor involved in epigenetic regulation is the chemical modification of histone tails, including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation, all of which regulate the degree of gene expression[33, 34]. The acetylation status of chromatin is determined by the dynamic opposing functions of histone acetyl-transferases (HAT) and histone deacetylases (HDAC). HAT add acetyl-groups to histones and create a hyperacetylated chromatin environment which is associated with gene activation[33, 34]. HDAC remove acetyl-groups resulting in a condensed chromatin structure which is associated with gene repression[33, 34]. Additionally, HAT and HDAC also add/remove acetyl-groups to/from the lysine residues of non-histone proteins[38]. To date, eighteen human HDAC have been identified and they are grouped into four classes based on their sequence homology to yeast HDAC: Class I (HDAC 1, 2, 3 and 8), Class II (HDAC 4, 5, 6, 7, 9 and 10), Class III (SIRT 1-7), and Class IV (HDAC11)[38, 145]. HDAC inhibitors (HDACi) are chemical HDAC modulators which interact with HDAC and inhibit their activity with distinct potency and specificity[38, 145-147]. Initially studied as anti-cancer agents, HDACi have been shown to promote cell differentiation, growth arrest, apoptosis and senescence in cancer cells[38, 145, 148]. Further studies revealed that HDACi have pleiotropic effects on other cell types. HDACi have been shown to inhibit vascular smooth muscle cell (SMC) proliferation and prevent mechanically induced SMC migration[149, 150]. Additional evidence suggests an antithrombotic role of HDACi in suppressing tissue factor expression in endothelial cells and monocytes[151]. HDACi have also been shown to block inflammatory cytokine production in macrophages and dendritic

cells[152, 153]. Additionally, treatment with HDACi enhanced the pool of beta cells and protected beta cells from cytokine induced cell death[154, 155].

Nuclear receptors (NR) function as key transcriptional regulators of gene expression. They share structurally conserved domains including N-terminal *trans*-activation domain, central DNA binding domain and C-terminal ligand binding domain. In addition to ligand-activated NR such as peroxisome proliferator-activated receptors (PPAR) and liver X receptors (LXR), a group of orphan NR has been demonstrated to function as ligand-independent NR. The primary mechanism by which NR regulate gene expression is through recognizing and binding to their consensus response elements on target promoters. The transcription complex initiated by NR binding further requires HAT/HDAC activities to activate/silence gene transcription. cAMP response element binding protein (CREB)-binding protein (CBP) and its homolog p300 are well characterized transcription co-activators and their HAT activities are essential for NR-dependent trans-activation[156, 157]. Nuclear receptor co-repressors (NCoR) have been shown to interact with HDAC3 to silence NR target gene expression in the absence of ligand binding[158]. In addition, the expression level of NR is also controlled by epigenetic regulation. For instance, DNA hypermethylation within the promoter region plays a fundamental role in silencing the expression of estrogen receptor alpha (ERα) and androgen receptor (AR)[159]. Furthermore, the acetylation status of NR is also regulated by HAT and HDAC, which influence NR ligand sensitivity and transcriptional activity[159].

The neuron-derived orphan receptor 1 (NOR1) belongs to the ligand-independent NR4A subfamily, a group of nuclear receptors which have been demonstrated to function as immediate/early response genes. Transcriptional activity of the NR4A NR is dependent on their expression level and post-translational modifications. Members of the NR4A subfamily, including Nur77 (NR4A1), Nurr1 (NR4A2) and NOR1 (NR4A3), can be induced by a wide range of physiological/pathological signals in different tissues[52, 160].

This induction often involves *de novo* RNA synthesis through transcriptional activation of the NR4A promoters. In addition, NR4A orphan receptors are also subjected to phosphorylation, sumoylation and acetylation modifications, which could affect their transcriptional activity[63, 68, 161]. We have previously demonstrated that NOR1 plays a mitogenic role in vascular SMC, and NOR1 deficiency suppresses SMC proliferation and decreases neointima formation[61, 94, 95]. A key biological event regulating mitogen-induced NOR1 expression is CREB phosphorylation and the recruitment of phospho-CREB to the consensus cAMP response elements (CRE) in the NOR1 promoter[94, 106, 162]. However, it remains unknown whether phospho-CREB modifies the chromatin architecture at the NOR1 promoter. In this study, we demonstrate that HDAC inhibition and platelet-derived growth factor (PDGF) synergistically up-regulate NOR1 expression in SMC through modifying the histone acetylation status of chromatin in the NOR1 promoter.

3.3. Materials and Methods

Cell culture

Rat aortic smooth muscle cells (RASMC) were purchased from Lonza and maintained in DMEM supplemented with 10 % FBS. RASMC were starved in 0.01 % FBS DMEM for 48 hours when they were 70 % confluent. Quiescent cells were pretreated with Scriptaid (Sigma) (2 μg/ml) for 30 min and stimulated with rat PDGF-BB (R & D Systems) (25 ng/ml) for indicated time points. For mRNA stability assays, actinomycin D (Sigma) (10 μg/ml) was added to cell cultures to inhibit mRNA transcription. For protein stability assays, cycloheximide (Sigma) (10 μg/ml) was added to cell cultures to inhibit protein synthesis.

Quantitative real-time RT-PCR

Total RNA was isolated using TRIzol® (Invitrogen) and reverse transcribed into cDNA using SuperScript II (Invitrogen). RNA expression levels of target genes were

quantified using an iQ^{TM} SYBR Green Supermix (BioRad). Primers used in this study are listed: NOR1, forward: 5'-CAGCAGCTGCGAACTCAA-3'; reverse: 5'-CGGTGTAGAAGGCGGAGA-3'; HDAC1, forward: 5'-CCATCAAAGGACATGCCAAGT-3'; reverse: 5'-CGAGCGACATTACGGATGGT-3'; HDAC2, forward: 5'-CGGCAAGAAGAAAGTGTGCTACT-3'; reverse: 5'-ATGAGTCATCCGGATCCTATGG-3'; HDAC3, forward: 5'-GCACCCGCATCGAGAATC-3'; reverse: 5'-TGGACACTGGGTGCATGGT-3'; RPL13A, forward: 5'GTACGCTGTGAGGGCATCAA-3'; reverse: 5'-CTCGAGACGGGTTGGTGTTC-3'. PCR reactions were performed on an iCycler (BioRad) using the following PCR cycles: 1 cycle of 95 °C 10 min; 40 cycles of 95 °C 30 sec, 55 °C 30 sec, 72 °C 30 sec; 1 cycle of a final extension at 72°C for 10 min. Each sample was analyzed in triplicate and normalized to expression values of the house-keeping gene RPL13A. Data were calculated using the $2^{-\Delta\Delta CT}$ method[137].

Western blotting

Western blotting was performed as previously described using antibodies against NOR1 (R & D Systems), phospho-Ser133 CREB (Cell Signaling), CREB (Cell Signaling) and GAPDH (FL 335) (Santa Cruz)[94, 163].

Plasmids, transient transfections and luciferase Assay

The NOR1 promoter constructs have previously been described[94]. RASMC were transiently transfected with 2 μg NOR1 promoter constructs for 6 hours using lipofectamine (Invitrogen) in OPTI-MEM. Following transfection, cells were starved in 0.4% FBS DMEM and stimulated with scriptaid (2 μg/ml) for overnight. Luciferase activities were analyzed using a Dual Luciferase Reporter Assay (Promega). Transfection efficiency was normalized to renilla luciferase activities generated by cotransfection of pRL-null-renilla.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using the MAGnify^{TM} Chromatin Immunoprecipitation System (Invitrogen) according to the manufacturer's instructions. Briefly, quiescent RASMC were pre-treated with scriptaid (2 μg/ml) for 30 min and stimulated with PDGF (25 ng/ml) for indicated time points. Sheared chromatin was immunoprecipitated using antibodies against phospho-Ser133 CREB (Cell Signaling), AcH3K9 (Cell Signaling). Target DNA product was amplified by PCR using primer pairs covering the three CRE consensus sequences between -79 and -46 in the NOR1 promoter: forward, 5'-ACACACACCCTCGCACAC-3'; reverse, 5'-TCGGCACGTCATTTATGC-3'.

siRNA-mediated HDAC1-3 knockdown

siRNA experiments were performed using the SMARTpool technology (Dharmacon RNA Technologies), which provides a mix of four different proprietary siRNAs specific for the target gene. RASMC were transfected with HDAC1, HDAC2, HDAC3 or scrambled siRNA (50 nM) using Lipofectamine^{TM} RNAiMAX (Invitrogen) for 6 hours. Following transfection, cells were recovered in growth media overnight and starved in 0.01 % FBS DMEM for 48 hours, which was followed by stimulation with PDGF (25 ng/ml) for 6 hours.

Statistical analysis

Results were represented as means ± SEM. Unpaired Student's t-test was utilized to compare the means between two independent groups on a single variable. One-way or Two-way ANOVA was used to compare groups. P values < 0.05 were considered to be statistically significant.

3.4. Results

NOR1 expression is enhanced by HDAC inhibitor Scriptaid in vascular SMC

To investigate the epigenetic regulation of NOR1 expression by histone acetylation, we employed the HDACi Scriptaid. Scriptaid is a hydroxamic acid-containing HDACi and has been reported to increase histone acetylation with low cytotoxicity[164]. Pre-treatment with Scriptaid (2 μg/ml) potentiated NOR1 mRNA and protein expression in response to PDGF (25 ng/ml) in vascular SMC. This effect was more apparent at the later time point after PDGF stimulation (Figure 3.1A and B). Scriptaid alone also induced NOR1 mRNA expression in quiescent vascular SMC, while control vehicle (DMSO) did not have any effect, which indicates that HDAC activity is required to suppress NOR1 expression in the quiescent SMC (Figure 3.1C). However, Scriptaid treatment without mitogenic stimulation only induced modest NOR1 expression compared to co-stimulation with PDGF (Figure 3.1A and C). Collectively, these experiments suggest that cooperation between mitogenic stimulation and hyperacetylation is important for maximal and sustained NOR1 expression in vascular SMC.

siRNA-mediated knockdown of HDAC3 expression increases PDGF-induced NOR1 mRNA expression

Scriptaid functions as a pan-HDAC inhibitor, primarily targeting to Class I and Class II HDACs. In order to investigate which HDAC regulates NOR1 mRNA expression, vascular SMC were transiently transfected with siRNA against HDAC1, HDAC2 and HDAC3 followed by PDGF stimulation. siRNA mediated knockdown of HDAC1-3 was confirmed by quantitative RT-PCR (Figure 3.2A). Acute knockdown of HDAC3 significantly enhanced NOR1 mRNA expression at the basal level and after PDGF stimulation, while HDAC1 or HDAC2 knockdown had no effect on NOR1 mRNA expression (Figure 3.2B). Therefore, these experiments indicate that the HDACi Scriptaid increases NOR1 mRNA expression through the regulation of HDAC3.

Scriptaid increases NOR1 promoter activity without affecting NOR1 transcript stability

mRNA accumulation is generally due to transcription activation, transcript stabilization or both. To understand the mechanism by which NOR1 mRNA is increased by HDAC inhibition, NOR1 promoter activity was first analyzed. We performed transient transfection assays with reporter constructs driven by a 1.7kb NOR1 promoter and demonstrated that Scriptaid increased NOR1 promoter activity in vascular SMC (Figure 3.3A). In order to analyze the effect of Scriptaid on NOR1 mRNA stability, vascular SMC were pre-treated with Scriptaid or DMSO as control and followed by PDGF stimulation for 2 hours to induce NOR1 mRNA accumulation. Actinomycin D (10 μg/ml) was then added to cell cultures to inhibit mRNA transcription. As depicted in Figure 3.3B, Scriptaid treatment did not have any effect on NOR1 mRNA stability. In summary, these two experiments demonstrate that Scriptaid increases NOR1 mRNA expression by activating NOR1 transcription.

Scriptaid enhances CREB phosphorylation and the recruitment of phospho-CREB to its binding sites in the NOR1 promoter

We next continued to investigate the mechanisms by which Scriptaid activates NOR1 transcription. We have previously demonstrated that CREB phosphorylation and the recruitment of phospho-CREB to the NOR1 promoter are required for NOR1 transcription activation in response to PDGF[94]. Therefore, CREB phosphorylation was analyzed. Interestingly, Scriptaid alone rapidly induced CREB phosphorylation within 30 min pre-treatment, although this effect was modest compared to PDGF stimulation (Figure 3.4A). As expected, PDGF-induced CREB phosphorylation was strongly enhanced by Scriptaid at each time point of this experiment (Figure 3.4A), which may account for the augmented NOR1 mRNA expression with scriptaid pre-treatment (Figure 3.1A).

HDAC inhibition has been demonstrated to increase histone acetylation which is associated with chromatin decondensation and gene activation[34, 38]. To test this possibility, histone acetylation in the NOR1 promoter region that contains the CRE sites was analyzed by ChIP assays. Increased H3-lysine9 acetylation (AcH3K9) has been shown to correlate with gene activation[33]. Analyses with antibodies against AcH3K9 demonstrated that Scriptaid alone increased histone acetylation at the CRE binding sites in the NOR1 promoter (Figure 3.4B). This increased histone acetylation caused a corresponding increase in the binding of phospho-CREB to the same region in the NOR1 promoter (Figure 3.4C). As expected, Scriptaid pre-treatment further enhanced PDGF-induced histone H3 acetylation and phospho-CREB recruitment at the CRE sites in the NOR1 promoter (Figure 3.4B and C). Collectively, these findings suggest that HDAC inhibition by itself can increase phospho-CREB binding to the NOR1 promoter; however, upstream signaling induced by mitogenic stimulation is required for further chromatin decondensation and phospho-CREB enrichment, which ensures maximal NOR1 transcription.

HDAC inhibitor Scriptaid increases NOR1 protein stability

Post-translational modification of NR by acetylation and deacetylation has been demonstrated to regulate NR stability, ligand binding sensitivity and transcription activity[159, 161, 165, 166]. Here we demonstrate that pre-treatment with HDACi Scriptaid increases NOR1 protein half-life in response to PDGF stimulation (Figure 3.5A and B). Collectively, HDACi Scriptaid enhances NOR1 expression at both the transcriptional and post-translational levels.

3.5. Discussion

The complex initiated by the interaction of NR with co-activators/co-repressors also involves histone modifier proteins facilitating gene activation and silencing[167]. In

addition to regulating chromatin dynamics, NR themselves are also subjected to the control by histone modifier proteins[159]. The transcriptional activity of NOR1 is primarily determined at the expression level and by post-translational modifications[160]. NOR1 has previously been demonstrated to mediate mitogenic signaling in vascular SMC and its deficiency suppresses SMC proliferation[93-95]. In the present study, we demonstrate that NOR1 is induced, although modestly, by HDACi Scriptaid in vascular SMC. HDAC inhibition and mitogenic stimuli have a synergistic effect on activating NOR1 transcription by increasing CREB phosphorylation and the recruitment of phospho-CREB to the NOR1 promoter. Finally, we demonstrate that NOR1 protein stability is increased by HDAC inhibition. Therefore, our findings provide an additional layer of regulation by which mitogens regulate NOR1 expression.

The normal medial SMC proliferate at a very low rate[28]. However, in response to mitogenic signals, SMC switch to a proliferative phenotype contributing to the development and progression of vascular diseases, such as atherosclerosis/restenosis[28, 168]. NOR1 has previously been demonstrated to be induced by PDGF in quiescent SMC, and its deficiency reduces SMC proliferation and neointima formation in a murine model of endothelium denudation[94]. In the present study, HDAC inhibition induces NOR1 expression in quiescent SMC, suggesting that HDAC activity is required to prevent this mitogenic transcription factor from inappropriate activation in the normal vasculature. This effect was confirmed by transient transfection assays in which Scriptaid treatment increased NOR1 promoter activity in SMC. (Fig. 3A). Contrary to our findings, cAMP-activated NOR1 expression in PC12 cells has been reported to be suppressed by TSA, an HDAC inhibitor sharing a similar structure with Scriptaid, which might be due to the different cell line or stimulus used in that study[169]. In support of our findings, another NR4A member Nur77 has been reported to be induced by TSA[169]. In thymocytes, HDAC7 suppresses Nur77 expression which is increased by treatment with pan-HDAC

inhibitors[170]. Additionally, HDAC1 has been documented to be recruited to the Nurr1 promoter to repress Nurr1 expression in unstimulated cells[171]. Collectively, these findings suggest that HDAC may serve as a general regulator of NR4A nuclear receptors to ensure their appropriate expression. Furthermore, it is worth noting that Scriptaid induced NOR1 expression is much lower than co-stimulation with PDGF, linking the extracellular signals and chromatin modification in inducing NOR1 expression in SMC.

Previous studies point to a critical role of CREB activation in inducing NOR1 expression in vascular SMC[94], hepatocytes[69] and adipocytes[77], demonstrating NOR1 as a CREB-responsive gene. The interaction of HDAC1/2 with phosphatase 1 (PP1) has been implicated in repressing cAMP-activated CREB-responsive gene expression by cooperatively mediating CREB dephosphorylation and histone deacetylation in the target promoters[171]. To test which HDAC is involved in controlling NOR1 promoter activation, we employed siRNA technology to knockdown HDAC1, HDAC2 or HDAC3 in SMC. Surprisingly, HDAC1 or HDAC2 knockdown did not affect PDGF-induced NOR1 mRNA expression. By contrast, HDAC3 knockdown results in more than a 2-fold increase in NOR1 expression at both the basal level and after PDGF stimulation. These two studies suggest that the specificity of HDAC recruitment to a CREB target gene is influenced by the local chromatin structure within the promoter region. In support of our observation, HDAC6 has been shown to be recruited to the promoter of glucocorticoid receptor (GR) and repress GR expression by uncoupling the CREB responses[172].

As previously mentioned, NOR1 is a CREB responsive gene[69, 77, 94]. We next analyzed CREB phosphorylation and found that Scriptaid alone increased CREB phosphorylation and co-stimulation with PDGF further enhanced CREB phosphorylation at each time point examined in the Figure 3.4A. This observation is consistent with previous findings that HDACi TSA sustained CREB phosphorylation [173]. However, TSA failed to increase CREB phosphorylation and gene transcription over the early time

points in that study, suggesting that cAMP activation is capable of inducing the maximal amount of CREB phosphorylation. Therefore, these distinct effects of HDAC inhibition in promoting CREB target gene transcription indicate that PDGF alone is insufficient to lead to maximal CREB phosphorylation and gene activation. Our observation confirmed previous findings that growth factors or stress signals are less effective in promoting CREB target gene transcription compared to cAMP agonist[174, 175].

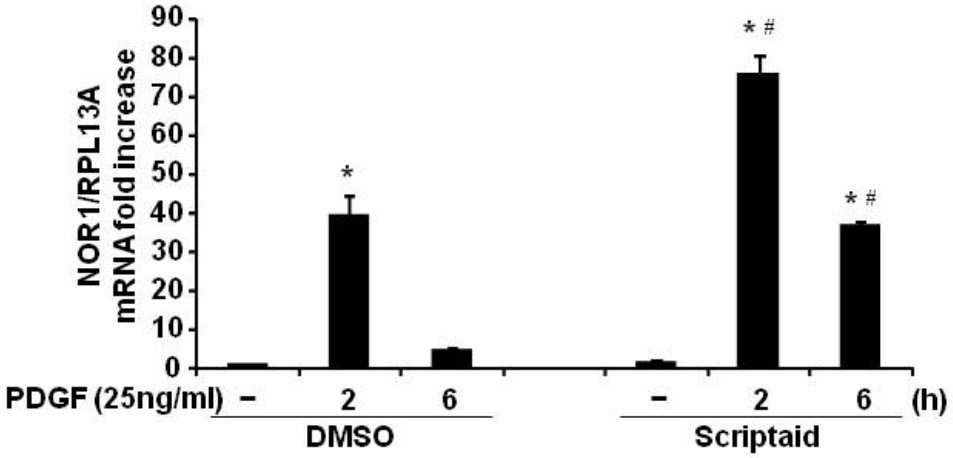
HDAC inhibition has been demonstrated to increase histone acetylation which is associated with gene activation[34, 38]. In ChIP assays, HDACi scriptaid increased histone acetylation at the CRE sites within the NOR1 promoter, resulting in increased recruitment of phospho-CREB (Figure 4B and 4C). Scriptaid alone increased histone H3 acetylation and phospho-CREB recruitment to levels comparable to PDGF stimulation alone. However, PDGF co-stimulation after Scriptaid treatment only modestly increased histone acetylation, which indicates that HDAC inhibition alone maximally loosened chromatin. By contrast, phospho-CREB recruitment was highly increased by PDGF co-stimulation after scriptaid treatment. This indicates that although upstream signaling did not further relax chromatin structure, it is necessary for the enrichment of phospho-CREB availability in the nucleus. These experiments reinforce the necessity of upstream signaling for maximal CREB phosphorylation, recruitment and NOR1 transcription.

HDAC substrates include a large amount of non-histone proteins and acetylation of these non-histone proteins has been shown to modulate their functionality and protein stability[38]. Many nuclear receptors are subjected to acetylation, which regulates their stability, ligand sensitivity and *trans*-activation ability[159]. Finally, NOR1 stability was demonstrated to be enhanced by scriptaid, indicating NOR1 as a HDAC substrate.

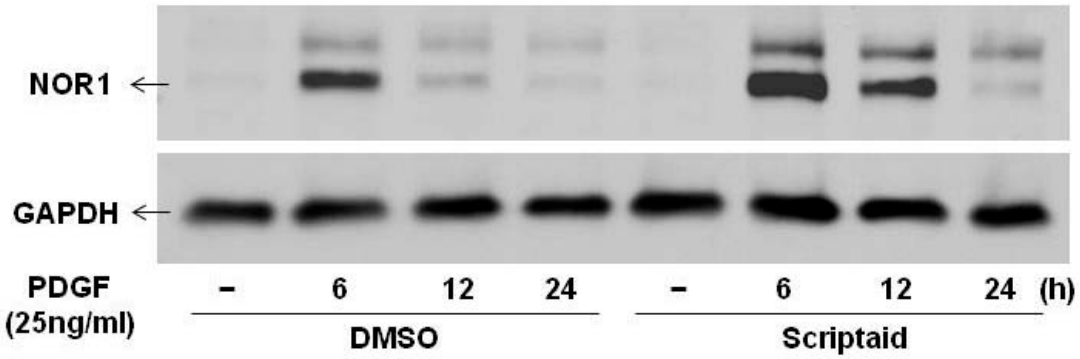
In summary, our findings suggest that HDAC inhibition increases NOR1 expression at multiple levels, including gene transcription and protein stability. HDACi have been suggested to suppress smooth muscle cell proliferation and migration[149, 150], serving as

potential therapeutic targets for treating vascular diseases driven by SMC proliferation. NOR1 has previously been demonstrated as a mitogenic transcription factor promoting SMC cell proliferation and survival[160]. Therefore, our studies may provide a potential target for further sensitizing smooth muscle cells to HDAC inhibition.

A


NOR1/RPL13A mRNA fold increase
90
80
70
60
50
40
30
20
10
0
*
* #
* #
PDGF (25ng/ml) – 2 6 – 2 6 (h)
DMSO
Scriptaid

B


NOR1
GAPDH
PDGF (25ng/ml) – 6 12 24 – 6 12 24 (h)
DMSO
Scriptaid

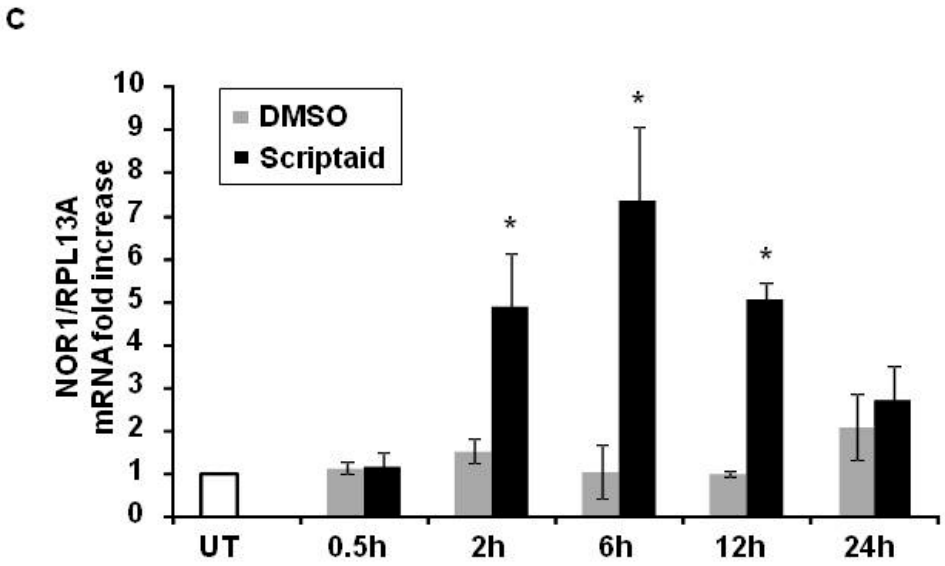
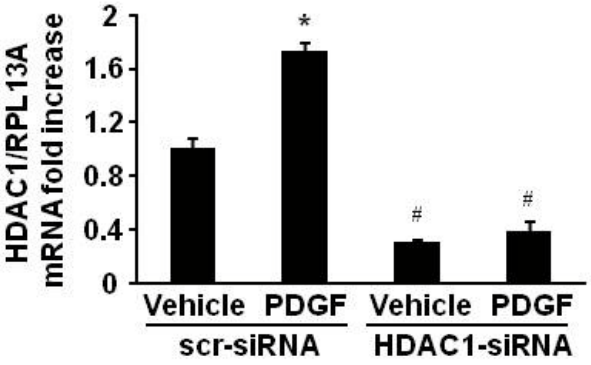
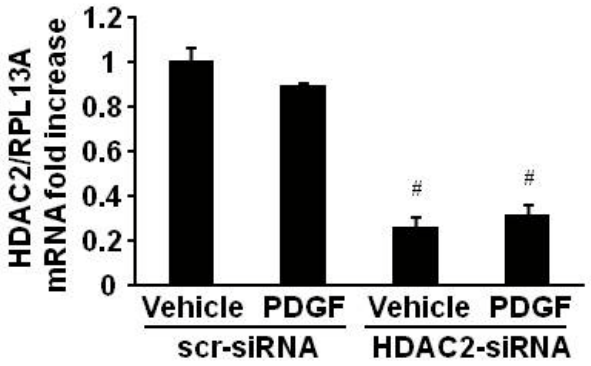


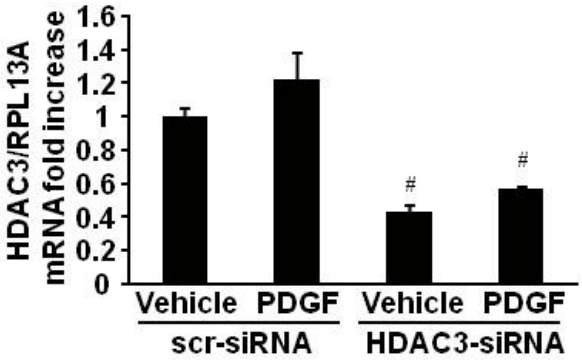
Figure 3.1. NOR1 expression is enhanced by HDACi Scriptaid in VSMC

(A-B) Quiescent RASMC were pretreated with Scriptaid (2 μg/ml) or DMSO for 30 min, and subsequently stimulated with PDGF (25 ng/ml). NOR1 mRNA (A) and protein expression (B) were analyzed at the indicated time points. mRNA expression was normalized to RPL13A and expressed as mean ± SEM fold increase over DMSO-untreated cells (*P < 0.05 vs. untreated; #P < 0.05 vs. DMSO). Cohybridization for GAPDH was performed to assess equal loading. The autoradiograms are representative of three independently performed experiments. (C) Quiescent RASMC were stimulated with Scriptaid (2 μg/ml) or DMSO for 0.5, 2, 6, 12 and 24 hr for NOR1 mRNA analyses. mRNA expression was normalized to RPL13A and expressed as mean ± SEM fold increase over untreated (UT) cells (*P < 0.05 vs. UT). Experiments were repeated at least three times in duplicate with different cell preparations.

A


HDAC1/RPL13A mRNA fold increase
2
1.6
1.2
0.8
0.4
0
*
#
#
Vehicle
PDGF
Vehicle
PDGF
scr-siRNA
HDAC1-siRNA


HDAC2/RPL13A mRNA fold increase
1.2
1
0.8
0.6
0.4
0.2
0
#
#
Vehicle
PDGF
Vehicle
PDGF
scr-siRNA
HDAC2-siRNA


HDAC3/RPL13A mRNA fold increase
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
#
#
Vehicle
PDGF
Vehicle
PDGF
scr-siRNA
HDAC3-siRNA

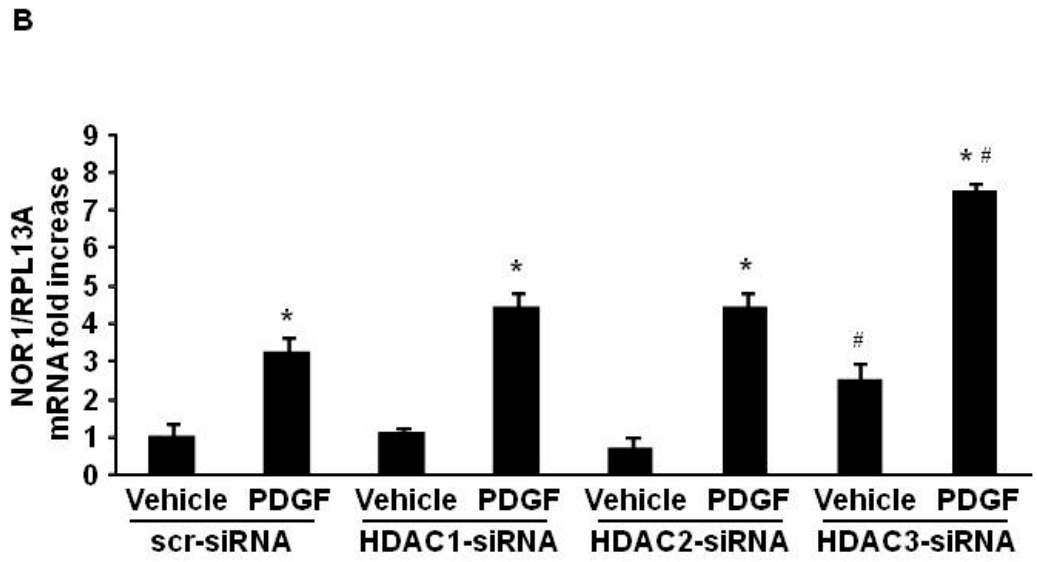
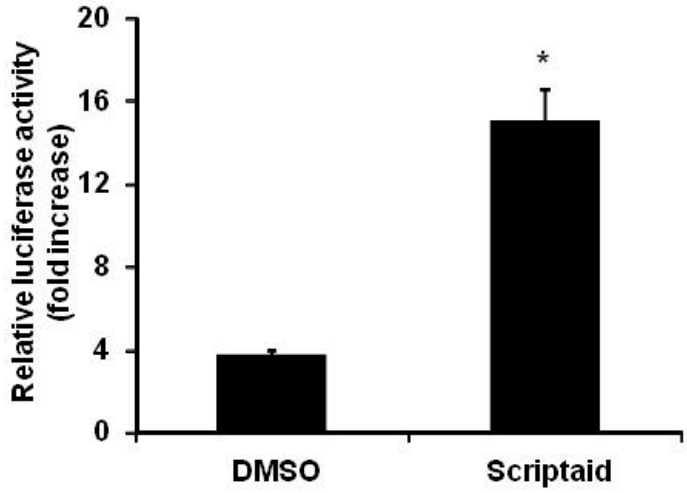


Figure 3.2. siRNA-mediated knockdown of HDAC3 expression increases PDGF-induced NOR1 mRNA expression

(A-B) RASMC were transiently transfected with HDAC1, HDAC2, HDAC3 or scrambled (scr) siRNA (50 nM) for 6 hours, and recovered in growth media overnight. Transfected cells were starved in 0.01% FBS DMEM for 48 hours and stimulated with PDGF (25 ng/ml) or vehicle (PBS) for 6 hours for mRNA analyses. The expression of HDAC1, HDAC2, HDAC3 and NOR1 was normalized to RPL13A and expressed as mean ± SEM fold increase over scr-siRNA-transfected vehicle-treated cells (*$P < 0.05$ vs. vehicle, #$p < 0.05$ vs. scr-siRNA).

A


Relative luciferase activity (fold increase)
20
16
12
8
4
0
*
DMSO
Scriptaid

B

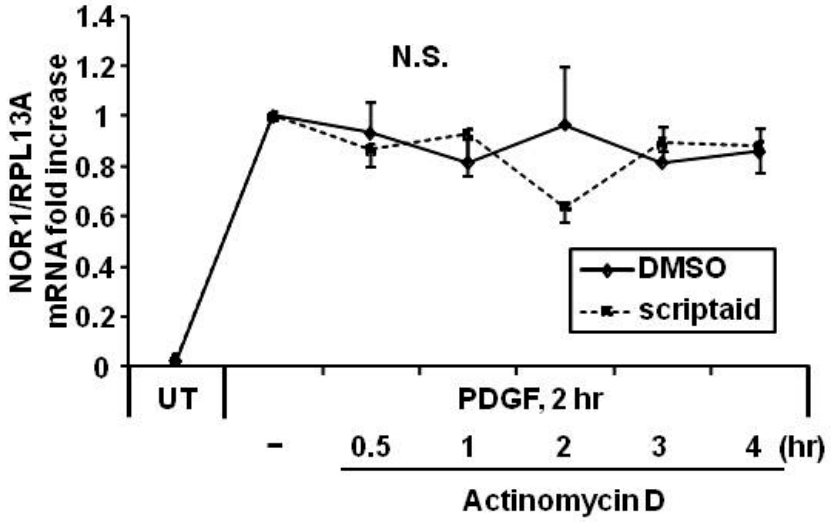

NOR1/RPL13A mRNA fold increase
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
DMSO
scriptaid
UT
PDGF, 2 hr
–
0.5
1
2
3
4
(hr)
Actinomycin D

Figure 3.3. Scriptaid increases NOR1 promoter activity without affecting NOR1 transcript stability

(A) RASMC were transiently transfected with a luciferase reporter construct (2 μg) driven by a 1.7kb NOR1 promoter and stimulated with DMSO or Scriptaid (2 μg/ml) overnight. Protein lysate was collected and analyzed for luciferase activities. Data were normalized to renilla luciferase activities and presented as mean ± SEM from three independently performed experiments (*$p < 0.05$ vs. DMSO). (B) Quiescent RASMC were pretreated with Scriptaid (2 μg/ml) or DMSO for 30 min and subsequently stimulated with PDGF (25 ng/ml) for 2 hours. Actinomycin D (10 μg/ml) was added to inhibit transcription and mRNA was collected at the indicated time points. NOR1 mRNA expression was normalized to RPL13A from three independent experiments. Data are expressed as mean ± SEM fold increase over the samples stimulated with PDGF for 2 hr. N.S. indicates that no statistical significance was detected between DMSO and Scriptaid treatments.

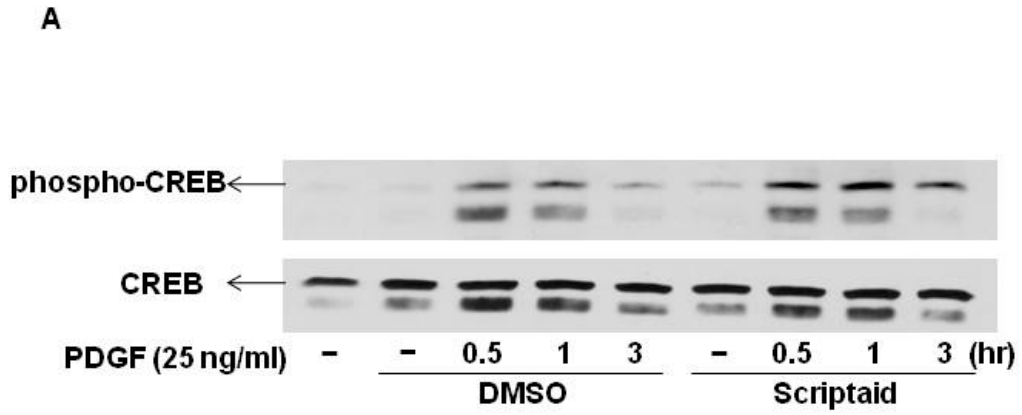
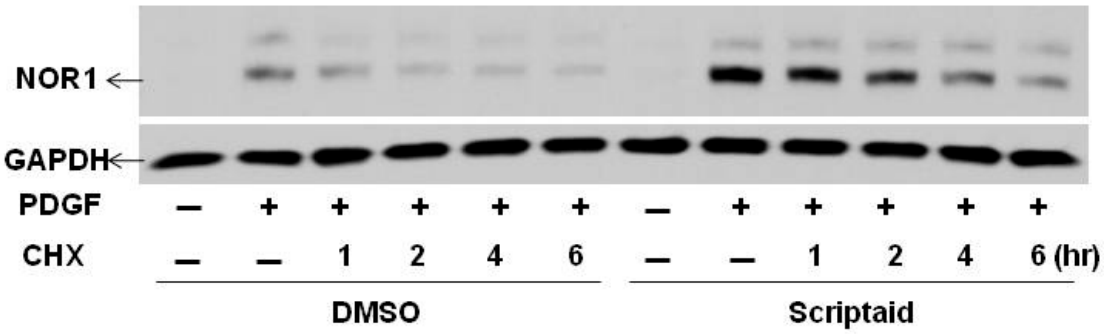

A
phospho-CREB
CREB
PDGF (25 ng/ml) - - 0.5 1 3 - 0.5 1 3 (hr)
DMSO
Scriptaid
B
IP-AcH3
Input
100bp
100bp
PDGF (25ng/ml) 0 1h 0 1h IgG
DMSO
Scriptaid
C
IP-
phospho-CREB
Input
100bp
100bp
PDGF (25ng/ml) 0 1h 0 1h IgG
DMSO
Scriptaid

Figure 3.4. Scriptaid enhances CREB phosphorylation and the recruitment of phospho-CREB to its binding sites in the NOR1 promoter

(A) Quiescent RASMC were pretreated with Scriptaid (2 μg/ml) or DMSO for 30 min and subsequently stimulated with PDGF (25 ng/ml) for 0.5, 1 and 3 hr for protein analyses. Cohybridization for total CREB was performed to assess equal loading. The autoradiograms are representative of three independently performed experiments. (B-C) Quiescent RASMC were pretreated with Scriptaid (2 μg/ml) or DMSO for 30 min and subsequently stimulated with PDGF (25 ng/ml) for 1 hr for ChIP assays. Chromatin complexes were immunoprecipitated with antibodies against phospho-S133 CREB, AcH3K9, and species-matched IgG. PCR products were amplified using primers covering the CRE sites from -79 bp to -46 bp in the NOR1 promoter. The agarose gels shown are representative of three independently performed experiments.

A


NOR1
GAPDH
PDGF – + + + + + – + + + + +
CHX – – 1 2 4 6 – – 1 2 4 6 (hr)
DMSO
Scriptaid

B

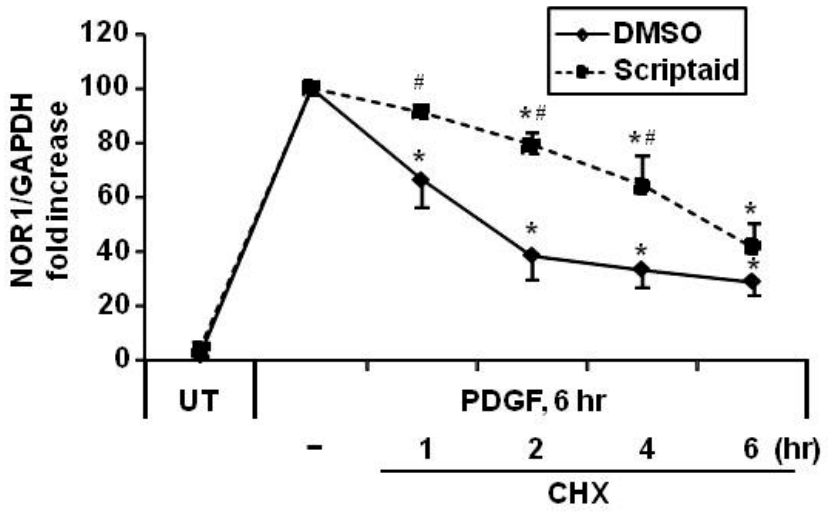

DMSO
Scriptaid
NOR1/GAPDH fold increase
120
100
80
60
40
20
0
UT
PDGF, 6 hr
– 1 2 4 6 (hr)
CHX

Figure 3.5. HDAC inhibitor Scriptaid increases NOR1 protein stability

(A-B) Quiescent RASMC were pretreated with Scriptaid (2 μg/ml) or DMSO for 30 min and subsequently stimulated with PDGF (25 ng/ml) for 6 hours. Cycloheximide (CHX, 10 μg/ml) was added to inhibit protein synthesis and protein was collected at the indicated time points. (A) The autoradiograms are representative of three independently performed experiments. Cohybridization for GAPDH was performed to assess equal loading. (B) Densitometric quantification of NOR1 expression was performed from three independent experiments and normalized to GAPDH expression. Results are expressed as mean ± SEM fold increase over samples treated with PDGF for 6 hr. (*$p < 0.05$ vs. PDGF 6 hr, # $p < 0.05$ vs. DMSO).

Chapter four: General discussion

4.1. Discussion

4.1.1. Summary

The primary goal of this study is to understand the role of the NR4A3 orphan nuclear receptor (NR) NOR1 in the development of atherosclerosis. We demonstrate that NOR1 is highly expressed in human atherosclerotic lesions, while NOR1 expression is absent in the normal vasculature. In advanced atherosclerotic lesions, NOR1 expression co-localizes with endothelial and sub-endothelial cells. Following experiments performed in this study focus on the regulation of NOR1 expression in endothelial cells and smooth muscle cells, and the significance of NOR1 in the development of atherosclerosis. In endothelial cells we demonstrate that NOR1 expression is rapidly and potently induced by several pro-inflammatory factors through an NF-κB dependent signaling pathway. Functional analyses point to a previously unrecognized role of NOR1 in promoting monocyte adhesion by increasing VCAM-1 and ICAM-1 expression in endothelial cells. Consistently, NOR1 deficiency decreased atherosclerosis formation in a hypercholesterolemia mouse model. Quantitative macrophage staining reveals reduced macrophage accumulation in the NOR1-deficient atherosclerotic lesions. In smooth muscle cells (SMC), NOR1 expression is induced by mitogenic stimulation, and this effect is further enhanced by HDAC inhibition. Mechanistic studies demonstrate that HDAC inhibition increases NOR1 expression by promoting NOR1 mRNA transcription and protein stability. In summary, this study has demonstrated that NOR1 acts as an atherogenic transcriptional regulator promoting monocyte-endothelial cell adhesion, and has provided mechanisms for the epigenetic control of mitogen-induced NOR1 expression in SMC. In the following discussion of my dissertation, I will further discuss the role of NOR1 in vascular diseases, focusing on the concepts not mentioned in previous chapters as well as the relationship to its NR4A siblings Nur77 and Nurr1.

Finally, I will discuss some of my own understandings regarding the significance of NRs in the prevention of cardiovascular diseases.

4.1.2. The role of NOR1 in endothelial cells

Activation of several NR has been reported in endothelial cells[176-185]. PPARα and PPARγ are the best studied NRs in endothelial cells and PPAR activation has been implicated in endothelial cell activation, proliferation and survival[176-181, 183]. In addition to ligand-activated nuclear receptors[177, 186], an increasing number of studies have pointed to the significance of orphan NR, including COUP-TFII[185], retinoic acid receptor (RAR)-related orphan nuclear receptor α (RORα) [182] and NR4A NR[160], in regulating endothelial cell proliferation and inflammation.

In Chapter two, we demonstrate in endothelial cells that NOR1 is induced by inflammatory cytokines and enhances monocyte adhesion by increasing VCAM-1 and ICAM-1 expression. We performed transient transfection assays and ChIP assays showing that NOR1 activates VCAM-1 transcription by directly binding to the NBRE site in the NOR1 promoter. However, mutations introduced into the NBRE site did not completely abolish the VCAM-1 promoter activity induced by NOR1 over-expression (Figure 2.6B), indicating that other transcriptional regulators may be involved in VCAM-1 *trans*-activation.

Several studies have revealed that NR also function as co-regulators modulating gene expression through interacting with other transcription factors[187, 188]. Both NF-κB and AP-1 are critical transcription factors regulating VCAM-1 expression during endothelial activation/dysfunction[189-191]. Therefore, it is intriguing to understand whether NOR1 promotes NF-κB/AP-1-dependent transcription activation. Preliminary experiments were performed in endothelial cells by transiently transfecting luciferase reporter constructs driven by multiple NF-κB/AP-1 response elements. Interestingly,

NOR1 over-expression increased the promoter activity of plasmids driven by NF-κB response elements, while no induction was observed on plasmids driven by AP-1 response elements (Figure 4.1A and B). NF-κB proteins are ubiquitously expressed in many cell types and they are sequestered in the cytoplasm by binding to the inhibitor of NF-κB (IκB) in unstimulated cells. NF-κB is activated by a series of phosphorylation reactions which ultimately lead to IκB degradation and release of NF-κB to the nucleus[192]. We first analyzed whether NOR1 expression induces NF-κB nuclear translocation in endothelial cells. As described in Figure 4.1C, NOR1 over-expression exhibited no apparent effect on NF-κB nuclear enrichment when evaluated by p65 immunostaining. Another possibility relies on the direct interaction between NOR1 and NF-κB. Immunoprecipitation (IP) assays demonstrated an interaction between NOR1 and NF-κB p65 (Figure 4.1D), thus indicating that NOR1 may interact with NF-κB in the nucleus and stabilize the binding of NF-κB to its promoters. Future experiments need to be performed to demonstrate the co-recruitment of NOR1 to the NF-κB binding sites in the VCAM-1 promoter. In support of our observation, AP-1 transcription factors c-Fos and c-Jun have been shown to interact with NF-κB and enhance VCAM-1 *trans*-activation in endothelial cells[193].

By contrast, other NR, such as PPARγ, estrogen receptor (ER), and glucocorticoid receptor (GR), have been implicated in the repression of VCAM-1 expression in endothelial cells by suppressing NF-κB/AP-1 dependent *trans*-activation[194, 195]. Nur77, an NR4A orphan NR sharing high protein sequence homology with NOR1, has recently been shown to repress VCAM-1 and ICAM-1 expression in endothelial cells by increasing the expression of NF-κB inhibitor IκBα[104]. This study suggests a novel mechanism that NR repress adhesion molecule expression by interfering with NF-κB upstream signaling pathway. However, we did not observe any evidence of increased IκBα expression by NOR1 in our study (data not shown). These seemingly opposing

findings suggest that Nur77 and NOR1 may interact with different co-factors to regulate target gene expression.

In addition, PPARα has been shown to interfere with AP-1/NF-κB-dependent IL-6 transcription by physically interacting with c-Jun and p65 in vascular SMC[196]. Activation of Liver X receptors (LXR) suppresses matrix metalloproteinase (MMP)-9 expression through antagonizing the NF-κB signaling pathway in macrophages[197]. Recently, NR4A nuclear receptor Nurr1 was shown to dock to NF-κB-p65 resulting in NF-κB clearance and *trans*-repression of inflammatory gene expression in microglia and astrocytes[60]. Therefore, the involvement of NR in NF-κB transcription complexes likely serves as a general mechanism, by which NR regulate transcriptional responses to inflammatory signaling. While most studies point to a NR-mediated *trans*-repression mechanism, our findings have provided evidence that NF-κB target gene expression could be modulated through a *trans*-activation mechanism.

4.1.3. The role of NOR1 in vascular smooth muscle cells

Atherosclerosis progression is associated with SMC migration into the arterial intima. Intimal SMC are characterized by an accelerated proliferation rate and greater synthetic capability, while normal medial SMC exhibit a contractile phenotype maintaining a quiescent state. HDAC inhibitors (HDACi) have been used in clinical trials as novel anticancer agents due to their beneficial effect on promoting cell differentiation, growth arrest, and apoptosis of tumor cells[145, 148]. In addition, recent discovery has shown that HDACi exhibit beneficial effects in vascular cells. HDAC inhibition represses vascular SMC proliferation and prevents mechanically induced vascular SMC migration[149, 150, 164]. Consistent with these observations, our preliminary data show that HDACi Scriptaid increases apoptosis in quiescent SMC (Figure 4.2).

We have previously demonstrated that NOR1 plays a critical role in promoting SMC

survival and mitogen-induced proliferation[95]. In Chapter three, we demonstrate that HDACi Scriptaid and PDGF synergistically increase NOR1 expression in SMC. Therefore, Scriptaid-induced NOR1 expression may impair the efficacy of HDAC inhibition and attenuate HDACi-induced SMC apoptosis/growth arrest. In fact, HDACi resistance has been previously investigated and the underlying mechanisms involve up-regulation of antioxidant pathways and increased expression of anti-apoptotic proteins[148, 198]. Experiments in which Scriptaid-induced apoptosis is compared between NOR1 wildtype and deficient SMC may provide some insight on the role of NOR1 in HDACi resistance. Silencing or suppressing NOR1 expression may increase SMC sensitivity to HDACi induced apoptosis/growth arrest, hence potentiating the efficacy of HDACi for treating vascular diseases primarily driven by SMC proliferation.

Statins, which are used in clinical trials for treating cardiovascular diseases by reducing plasma LDL-cholesterol levels, have shown some promise by reducing LDL/hypercholesterolemia-induced NOR1 expression in SMC and aortae[107]. More interestingly, statins have recently been demonstrated as novel HDAC inhibitors[199]. Computational modeling and immunoprecipitation assays provided evidence suggesting that the carboxylic acid moiety of statins directly targets the catalytic site of HDAC thereby inhibiting the activities of HDAC1-3. These findings challenged our observations in Chapter three and raised the question of how statins inhibit HDAC activity yet also repress NOR1 expression. One possible explanation resides in the disrupted CREB phosphorylation by statins. Although statins can increase histone acetylation creating looser chromatin structure, the lack of phospho-CREB may account for the repression of NOR1 expression. This is reminiscent of our observation that HDAC inhibition alone only induces NOR1 expression modestly, while maximal NOR1 expression relies on the abundance of phospho-CREB. These seemingly contradictory findings emphasize the importance and necessity to investigate the epigenetic control of NOR1 expression.

4.1.4. The role of NOR1 in atherosclerosis development

The expression of NR4A nuclear receptors has been implicated in vascular diseases including atherosclerosis, restenosis and vascular remodeling[160, 200]. *In vivo* studies show that Nur77 or Nurr1 over-expression in SMC decreases lesion formation in a carotid artery ligation model by restricting SMC proliferation[91, 201]. This repressive effect is potentiated by 6-mercaptopurine, an antineoplastic agent mediating the activation of NR4A nuclear receptors, in a mouse model of cuff-induced neointima formation[202]. Nur77 over-expression in SMC also protects against vascular outward remodeling by reducing macrophage accumulation and MMP expression[203]. Additionally, NR4A receptors have been shown to repress the expression of inflammatory cytokines and lipid loading in macrophages[97]. Together, these studies point to a protective role of Nur77 and Nurr1 in reducing vascular SMC proliferation and macrophage inflammation.

Conversely, opposing regulatory effects of NR4A receptors have been suggested by other studies. Nur77 over-expression increases inflammatory signaling in macrophages and endothelial cells[99, 116]. In addition, NOR1 promotes SMC survival and proliferation and its deficiency reduces neointima formation in a murine endothelium denudation model[95]. Nonetheless, direct evidence is still lacking with regard to the role of NR4A receptors in atherosclerosis development.

Based on our findings in Chapters two and three, NOR1 expression in endothelial cells increases monocyte adhesion and mediates mitogen-induced cell proliferation in smooth muscle cells. Hence, we hypothesized that NOR1 functions as an atherogenic transcriptional regulator in the vascular wall. To investigate our hypothesis, NOR1-/-apoE-/- mice were generated to create a hypercholesterolemia background to study the role of NOR1 in atherosclerosis development. As expected, NOR1 deficiency decreases atherosclerosis formation accompanied by reduced macrophage accumulation in the sub-endothelial space.

However, it is worth noting that NOR1 deficiency only partially blocked atherosclerosis formation. Although macrophage accumulation is reduced in NOR1 deficient lesions, significant amount of macrophages are still readily detectable in the arterial intima. One possible explanation may reside in the excessive leukocytosis in NOR1 deficient mice (Figure 4.3A). The leukocytosis in NOR1-deficient mice is primarily due to increased monocyte, lymphocyte, and granulocyte numbers in the absence of changes in red blood cell counts. Studies have demonstrated that monocyte recruitment is mediated by different adhesion molecules on the endothelial cell surface[7]. As proposed in this study, NOR1 deficiency reduces monocyte adhesion via VCAM-1 and ICAM-1. However, the reduction of monocyte adhesion could be impaired due to increased monocyte availability in the blood. In addition, a large amount of evidence supports the presence of T lymphocytes and granulocytes in the atherosclerotic lesions, which contributes to atherosclerosis development and progression[17, 18, 204, 205]. Increased lymphocyte and granulocyte numbers may also account for the residual lesions seen in the NOR1-deficient mice.

Our preliminary experiments also showed that NOR1-deficient leukocytes isolated from the peritoneal cavity after thioglycollate injection are more susceptible to apoptosis compared to NOR1 wildtype peritoneal cells (Figure 4.3B). This indicates that NOR1-deficient leukocytes are defective in clearing exotoxins or surviving immune insults. Recently, the concept inflammation resolution has drawn increasing attention and has provided new insights in the treatment of atherosclerosis[23]. One key step in resolving inflammation in atherosclerotic plaques is efferocytosis of apoptotic inflammatory cells[23]. The major effectors of efferocytosis include macrophages and dendritic cells[23]. It is very likely that NOR1-deficent macrophages/dendritic cells are less efficient in clearing apoptotic/dead inflammatory cells in the vascular wall, which will ultimately result in the accumulation of apoptotic macrophages and aggravation of inflammatory responses. For

that reason, it would be helpful to analyze the efferecytosis ability of NOR1-deficient monoyctes/macrophages and compare their viability in response to proatherogenic stimuli.

In order to understand whether NOR1 deficiency results in increased macrophage accumulation, several approaches may be considered. One experiment is bone marrow transplantation, in which NOR1-deficient and wildtype bone marrow cells are transplanted to lethally irradiated apoE-/- mice. Based on our hypothesis, mice transplanted with NOR1-deficient bone marrow cells will develop more atherosclerosis than mice transplanted with NOR1-wildtype bone marrow. However, bone marrow cells are highly heterogeneous, consisting of hematopoietic stem cells and progenitor cells at various differentiation stages. As an alternative, mice with tissue-specific NOR1 knockdown in the monocyte-macrophage lineage will serve as a better strategy. Furthermore, mice with specific NOR1 knockdown in endothelial cells will confirm our previous findings that NOR1 deficiency decreases atherosclerosis formation by blocking monocyte adhesion. In summary, these studies will provide a better understanding of the mechanisms by which NOR1 promotes atherosclerosis.

4.1.5. Implications for the prevention of cardiovascular diseases

Numerous studies have been performed in order to provide potential targets for the prevention and treatment of cardiovascular diseases. Many nuclear receptors have shown beneficial effects in the vasculature. The activation of PPARα by synthetic agonists reduces cardiovascular disease by improving lipid homeostasis[206]. PPARγ, which was originally used for increasing insulin sensitivity, also reduces a wide range of cardiovascular risk factors, including inflammatory cytokines, adipokines and free fatty acids[207]. LXR are key transcriptional regulators controlling cholesterol metabolism and promoting reverse cholesterol transport in macrophages[208]. *In vivo* studies have shown that activation of LXR plays a protective role in atherosclerosis development in mouse

models[208-211]. In addition, estrogen receptors and glucocorticoid receptors have shown some vasoprotective effects by repressing inflammatory signaling[212, 213].

The activation/repression of NR by the binding of small molecules has proved advantageous for the treatment of cardiovascular diseases. In this study, we demonstrate that decreased NOR1 expression in the vasculature protects against atherosclerosis development[163]. However, the lack of a typical ligand-binding pocket in the NOR1 protein considerably limits the possibility of using small molecules to suppress NOR1 transcriptional activity[51]. In addition, induced NOR1 expression in other tissues has been shown to be indispensible for maintaining normal physiological functions[69, 77, 78, 89]. In response to starvation, NOR1 increases gluconeogenesis in the liver[69]. In the skeletal muscle, NOR1 enhances oxidative metabolism[77] and glucose uptake[78]. NOR1 also plays a critical role in regulating thermogenesis in the adipose tissue[89]. Furthermore, our preliminary data showed that NOR1 deficiency resulted in an increase in the body weight when NOR1+/+ and NOR1-/- mice were fed a chow diet (data not shown). Therefore, unless tissue specific modulators are developed, NOR1 suppression by antagonists may result in unpredictable side effects on the normal processes of metabolism.

These two challenges raise the question of the implications of our studies on the role of NOR1 in atherosclerosis development. NOR1 can be induced by a variety of cardiovascular risk factors, such as hypercholesterolemia and inflammatory cytokines[160], and plays an atherogenic role in the vascular wall. Therefore, a tight control on these risk factors may reduce the possibility and magnitude of NOR1 induction and protect against atherosclerosis. For example, fat constitutes an important component in our daily diet. Dietary fat has a great impact on the plasma lipoprotein profile, which is a key factor in mediating atherosclerosis development[214]. Long-chain saturated fatty acids (LC-SFA), but not long-chain un-saturated fatty acids (LC-USFA) or short-chain fatty acids (SC-FA), have been shown to activate NF-κB and promote inflammatory gene expression in endothelial cells[215, 216]. Our studies demonstrate NOR1 as an NF-κB target gene in

endothelial cells, indicating that NOR1 is likely to be induced by LC-SFA[163]. In smooth muscle cells, NOR1 can be induced by low density lipoprotein (LDL), a key risk factor in the onset of atherosclerosis[106]. By contrast, USFAs tend to improve the lipoprotein profile by decreasing LDL cholesterol levels[214]. Omega-3 fatty acids have been shown to repress endothelial activation by reducing NF-κB activation[217]. Collectively, these studies indicate that limiting the amount of LC-SFAs in the diet may provide beneficial effects on reducing NOR1 expression in endothelial cells and smooth muscle cells. A diet with a higher percentage of USFA will be favorable in reducing cardiovascular risk by improving plasma lipoprotein profiles and thereby suppressing NOR1 expression in the vascular wall.

4.2. Perspectives

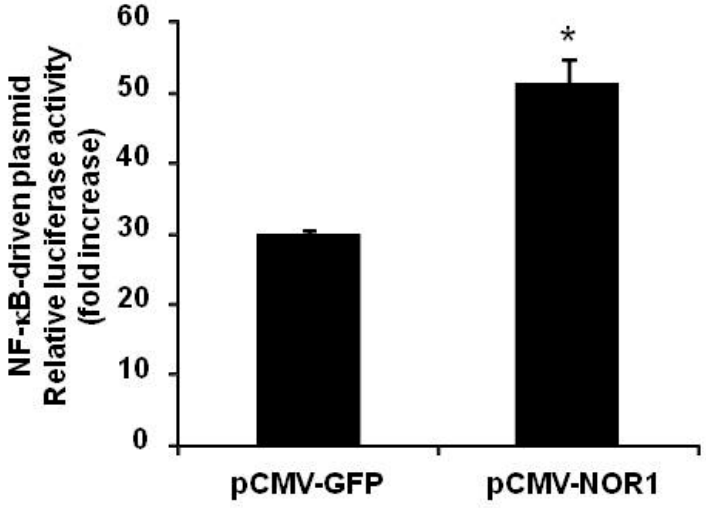
As investigated and discussed in this study, the ligand-independent NR4A3 orphan nuclear receptor NOR1 functions as an immediate early response gene. All cell types participating in vascular diseases rapidly induce NOR1 expression in response to arterial injury. Our *in vivo* studies using a murine hypercholesterolemia model demonstrate NOR1 as an atherogenic transcriptional regulator. Nevertheless, several key questions still need to be answered. All the NR4A nuclear receptors bind to the same NBRE site, which is indicative of functional redundancy among these NR4A members. However, deficiency of any NR4A nuclear receptor is associated with an apparent phenotype (NOR1 deficiency decreases atherosclerosis[163], Nur77 deficiency impairs insulin sensitivity[76], Nurr1 deficiency results in neonatal lethality[218]), demonstrating that each NR4A member has specific functions which cannot be compensated by the other two siblings. In addition, an intriguing question is what are the distinct roles of NOR1 in endothelial cells and monocytes/macrophages with relevance to regulating atherosclerosis development? Another important question for future investigation is how to reconcile the distinct biological effects of NOR1 and Nur77/Nurr1 in vascular cells and the development of

atherosclerosis? In order to answer these questions, a key emphasis of future research is to identify NR4A target genes and define the genome-wide pattern of NR4A chromatin occupancy. In addition, advances in understanding NR4A posttranslational modifications and co-activator/co-repressor recruitment will help establish detailed transcriptional networks by which NR4A receptors control vascular gene expression programs. Continued investigation of these questions will provide new insights into how these NR4A receptors participate in the processes of normal metabolism and the development of cardiovascular diseases.

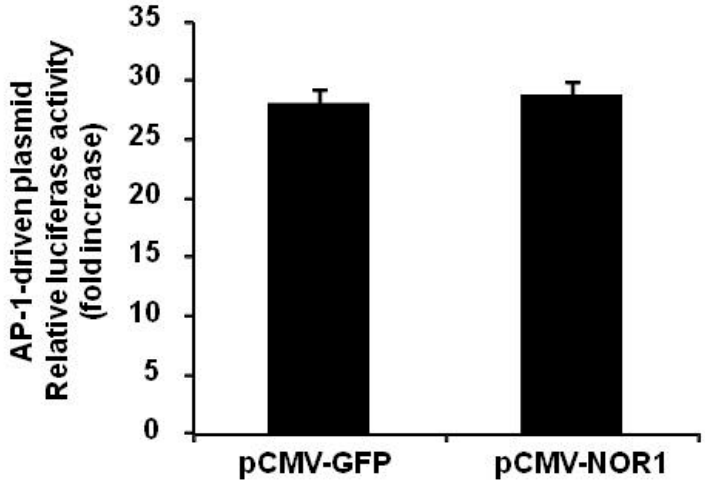
4.3. Conclusion

In this study, we have performed extensive studies and made substantial progress in understanding the regulation of NOR1 expression in endothelial cells and smooth muscle cells. We further identified VCAM-1 as a *bona fide* NOR1 target gene in endothelial cells. *In vivo* studies using a murine model of hypercholesterolemia provide initial evidence characterizing NOR1 as an atherogenic transcriptional regulator. Increased NOR1 expression in response to HDAC inhibition suggests NOR1 may contribute to HDACi resistance in smooth muscle cells. Therefore, manipulation of NOR1 expression may provide some insight into therapeutic treatment for vascular diseases such as atherosclerosis and restenosis.

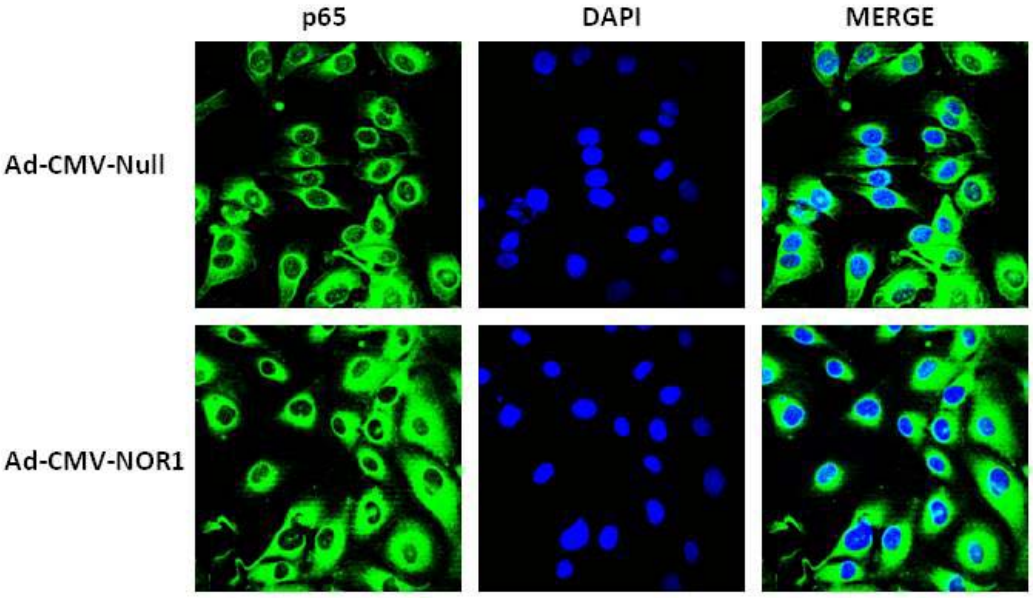
A


NF-κB-driven plasmid
Relative luciferase activity
(fold increase)
60
50
40
30
20
10
0
*
pCMV-GFP
pCMV-NOR1

B


AP-1-driven plasmid
Relative luciferase activity
(fold increase)
35
30
25
20
15
10
5
0
pCMV-GFP
pCMV-NOR1

C


p65
DAPI
MERGE
Ad-CMV-Null
Ad-CMV-NOR1

D

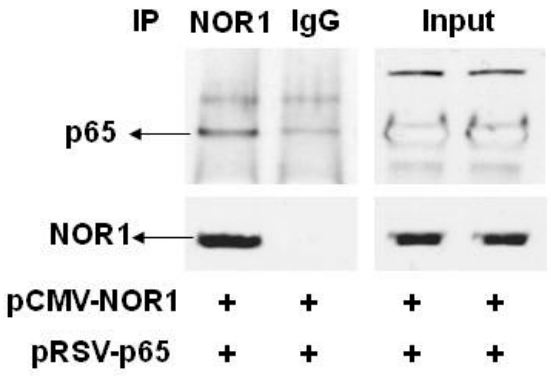

IP
NOR1
IgG
Input
p65
NOR1
pCMV-NOR1 + + + +
pRSV-p65 + + + +

Figure 4.1. NOR1 increases NF-κB-dependent transcription activation by interacting with NF-κB p65 without affecting p65 translocation

(A-B) HUVEC were transiently transfected with luciferase reporter constructs (0.5 μg) driven by multiple NF-κB/AP-1 response elements. Cells were co-transfected with NOR1 or GFP expression vector (0.5 μg) and recovered overnight following transfection. Protein lysate was collected and analyzed for luciferase activities. Data were normalized to renilla luciferase activities and presented as mean ± SEM (*$p < 0.05$ vs. pCMV-GFP). (C) HUVEC were infected with 50 PFU Ad-CMV-NOR1 or Ad-CMV-Null as control. Infected cells were immunostained for NF-κB p65. Images were acquired using confocal microscopy (objective magnification x200). Stainings for p65 and DAPI were merged into one image as indicated. (D) HEK293 cells were transiently transfected with NOR1 and p65 over-expression vectors (2 μg) and recovered for 24 hours. Cell lysates were collected and immunoprecipitated with an antibody against p65 (5 μg). Precipitates were subjected to SDS–PAGE and immunoblotted with indicated antibodies.

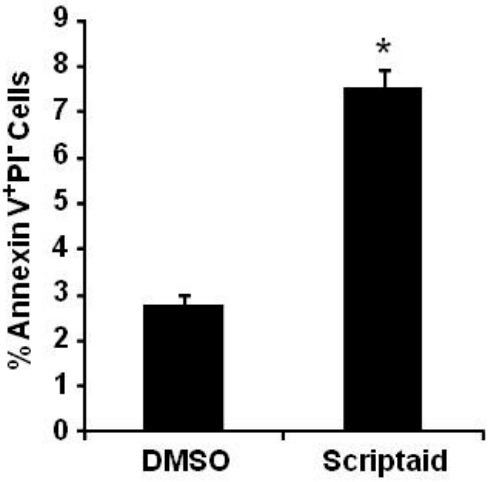
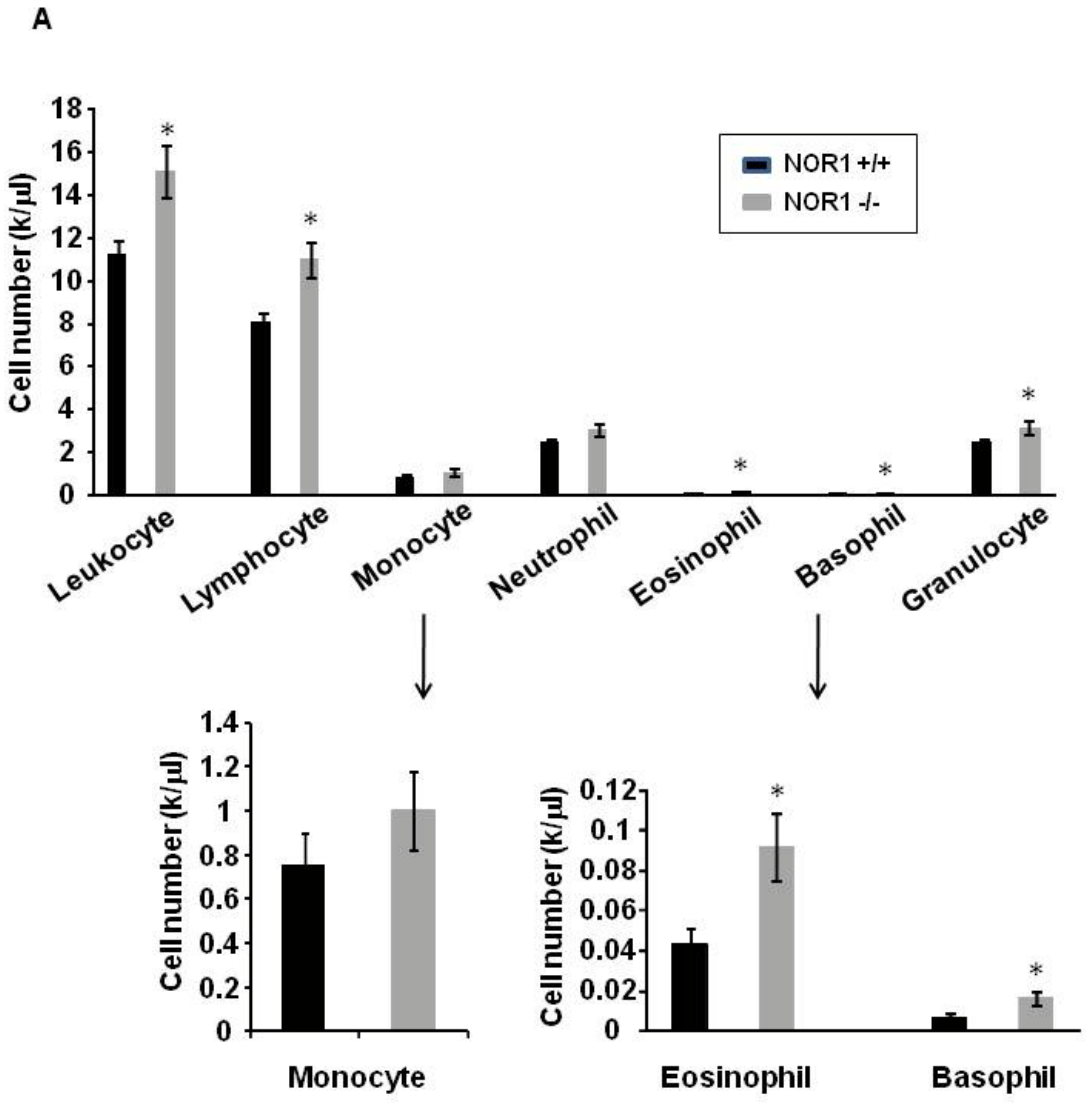


Figure 4.2. HDAC inhibition increases smooth muscle cell apoptosis

Quiescent human coronary smooth muscle cells were stimulated with Scriptaid (2 μg/ml) or DMSO for 2 days. Cells were then collected and subjected to annexin V and propidium iodide (PI) staining and apoptosis was quantified using fluorescence activated cell sorting (FACS). Data are presented as mean ± SEM (*$p < 0.05$ vs. DMSO).


A
Cell number (k/μl)
NOR1 +/+
NOR1 -/-
Leukocyte
Lymphocyte
Monocyte
Neutrophil
Eosinophil
Basophil
Granulocyte
Monocyte
Eosinophil
Basophil

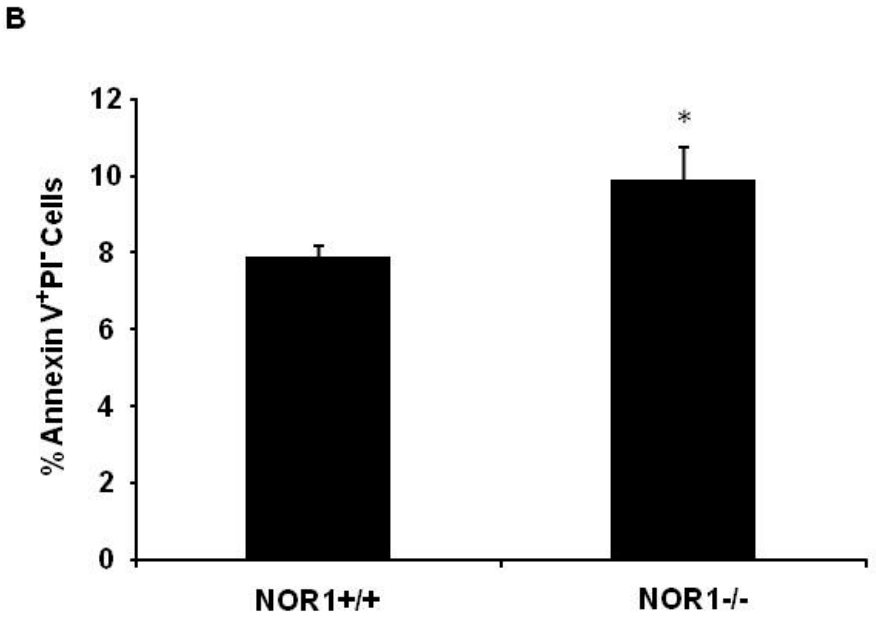


Figure 4.3. NOR1 deficiency increases leukocytosis and leukocyte apoptosis in the peritoneal cavity

(A) 6 NOR1+/+ and 6 NOR1-/- mice (8-12 weeks old) were used for this study. Blood was drawn from the mouse retro-orbital sinus and blood cell count was assessed using a Hemavet cell counter. Data were presented as mean ± SEM (*$p < 0.05$ vs. NOR1+/+). (B) 6 NOR1+/+ and 6 NOR1-/- mice (8-12 weeks old) were injected intraperitoneally with 3 ml thioglycollate media (3 %). 3 days after injection, peritoneal cells were isolated in 6 ml PBS by peritoneal lavage and subjected to annexin V and PI staining. Apoptosis was analyzed using fluorescence activated cell sorting (FACS). Data are presented as mean ± SEM (*$p < 0.05$ vs. NOR1+/+).

Appendices

Appendix A. Methods

1. Whole cell lysate collection and Western blotting

(1) Whole cell lysate collection

- Perform each step on ice
- Rinse the cell monolayer twice with ice-cold phosphate buffered saline (PBS)
- Remove the remaining PBS completely
- Add 100 μl/ 10 cm cell culture plate of 1x ice-cold lysis buffer (Cell Signaling) containing 1 % protease inhibitor cocktail (Sigma)
- Collect cells in a 1.5 ml eppendorf tube using cell lifters
- Homogenize by vortexing and sitting in ice for 10 min
- Centrifuge at 13,000 rpm, 4 °C for 15 min
- Transfer the supernatant to a fresh tube, and proceed to the next step

(2) Protein concentration measurement

- Measure protein concentration using the Bio-Rad DC Protein Assay Reagents Package containing Reagents A, B and S
- Prepare dilutions of Bovine Serum Albumin (BSA) Standard (2 mg/ml) in 1.5 ml eppendorf tubes as follows:

Dilution	2 mg/ml BSA	Distilled H_2O
S1, 0 mg/ml		25 μl
S2, 0.16 mg/ml	2 μl	23 μl
S3, 0.32 mg/ml	4 μl	21 μl
S4, 0.8 mg/ml	10 μl	15 μl
S5, 1.0 mg/ml	12.5 μl	12.5 μl
S6, 1.2 mg/ml	15 μl	10 μl

- Prepare protein dilutions by adding 2.5 μl protein to 10 μl distilled H_2O

- Prepare dye reagent by diluting 25 µl Reagent S in 1 ml Reagent A
- Pipette 5 µl standards (S0-S6) and sample dilutions in duplicate to a 96 well plate
- Pipette 25 µl dye reagent into each well
- Pipette 200 µl Reagent B into each well
- Measure absorbance at 595 nm using a microplate reader

(3) Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

- 1x Electrode buffer

Tris Base	3.03 g
Glycine	14.4 g
SDS	1 g
Distilled H2O	to 1 L

- 1x Transfer Buffer

Tris Base	4.64 g
Glycine	2.32 g
Methanol	160 ml
10 % SDS	3 ml
Distilled H2O	640 ml
Total	800 ml

- 10x TBS

Tris Base	24.2 g
NaCl	80 g
Adjust pH to 7.6 with HCl	~ 10 ml
Distilled H2O	To 1 L

- 1x TBST

10x TBS	100 ml
Tween-20	1 ml (0.1 %)
Distilled H2O	To 1 L

- Separating Gel, 7.5 %, 15 mm (for 2 gels)

Distilled H2O	6.8 ml
1.5 M Tris-HCl, pH 8.8	3.5 ml
10 % SDS	140 µl
30 % Acrylamide/Bis	3.5 ml
10 % Ammonium Persulfate (APS)	100 µl
TEMED	20 µl
Total	14 ml

- Stacking Gel, 15 mm (for 3 gels)

Distilled H2O	5.5 ml
0.5 M Tris-HCl, pH 6.8	2.25 ml
10 % SDS	90 µl
30 % Acrylamide/Bis	1.2 ml
10 % Ammonium Persulfate (APS)	60 µl
TEMED	10 µl
Total	9 ml

- Load 40 µg protein/ well
- Run the gel at 50 V for 1 hour and switch to 100 V for 1.5 hours
- Transfer proteins onto the nitrocellulose membrane (GE Healthcare Life Sciences)
- Transfer proteins at 100 V for 2 hours

(4) Western blotting

- Block the membrane in 5 % fat-free milk/ TBST buffer at room temperature (RT) for 1 hour
- Add primary antibody (1:1000 v/v dilution) and incubate the membrane on shaker at 4 °C overnight
- Rinse the membrane with 1x TBST for 5 min on shaker
- Repeat the last step twice with fresh TBST

- Bind secondary antibody (1:2000 v/v dilution) in 5 % milk/ TBST buffer at RT for 1.5 hours
- Rinse the membrane with 1x TBST for 5 min on shaker
- Repeat the last step 4 times with fresh TBST
- Visualize target proteins with autoradiography using Amersham ECL™ Western Blotting Detection Reagents (GE Healthcare Life Sciences)

2. RNA isolation and Quantitative reverse transcription polymerase chain reaction (RT-PCR)

(1) RNA isolation and RNA concentration measurement

- Rinse the cell monolayer with PBS and remove the remaining PBS completely
- Add 1ml Trizol (Invitrgen)/ 10 cm cell culture plate
- Homogenize cells by pipetting up and down for a few times and transfer the cell lysate to a fresh 1.5 ml tube
- Isolate RNA according to the manufacture's instruction
- Resuspend RNA in diethyl pyrocarbonate (DEPC) treated water
- Incubate in a water bath at 55°C for 10 min
- Measure RNA concentration using an NanoDrop Spectrophotometer

(2) RT-PCR

- Synthesize first-strand cDNA using SuperScript™ II RT (Invitrogen)
- Set up a 20-μL reaction volume on ice using 1 μg total RNA as follows:

Random primers (500 ng/ml)	1 μl
dNTP mixture (10 mM each)	1 μl
RNA	1 μg
Sterile distilled H2O	To 13 μl

- Heat the mixture to 65°C for 5 min and quick chill on ice. Collect the contents of the tube by brief centrifugation and add:

5x First-Strand Buffer	4 µl
0.1 M DTT	2 µl

- Mix contents gently and incubate the mixture at RT for 2 min
- Add 1 µl SuperScript™ II RT and mix by pipetting gently up and down
- Perform RCP reactions on a thermocycler using the following PCR cycles:

25 °C	10 min
42 °C	50 min
70 °C	15 min

- cDNA can be stored at 4 °C for a short period or at -80 °C for long term storage

(3) Realtime-PCR

- Dilute cDNA by 1:50 in sterile distilled water
- Set up a 25-µL reaction volume on ice using 10 µl diluted cDNA as follows:

Diluted cDNA	10 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
iQ™ SYBR Green Supermix (BioRad)	12.5 µl
Sterile distilled water	1.5 µl

- Perform RCP reactions on an iCycler (Bio-Rad) using the following PCR cycles:

Cycle 1 (1x)	95 °C	10 min
Cycle 2 (40x)	95 °C	30 sec
	55 °C	30 sec
	72 °C	30 sec
Cycle 3 (1x)	72 °C	10 min
Cycle 4 (1x)	10 °C	forever

(4) Primers used for realtime-PCR in this study

Name	Sequence
human NOR1	F : 5’-GGGCTTTTTCAAGAGAACAGTG-3’ R: 5’-ATCTCTGGGTGTTGAGTCTGTT-3’
human VCAM-1	F: 5’-TGAGGGGACCAATTCCAC-3’ R: 5’-ATTCACGAGGCCACCACT-3’
human ICAM-1	F: 5’-ACCGTGAATGTGCTCTCC-3’ R: 5’-GGCTTGTGTGTTCGGTTT-3’
murine VCAM-1	F: 5’-TCAAAGAAAGGGAGACTG-3’ R: 5’-GCTGGAGAACTTCATTATC-3’
murine ICAM-1	F: 5’-AGATCACATTCACGGTGCTG-3’ R: 5’-CTTCAGAGGCAGGAAACAGG-3’
human TBP	F: 5’-GGAGAGTTCTGGGATTGTACCGC-3’ R: 5’-ATATTCGGCGTTTCGGGCAC-3’
murine TFIIB	F: 5’-CTCTCCCAAGAGTCACATGTCC-3’ R: 5’-CAATAACTCGGTCCCCTACAAC -3’
murine NOR1	F: 5’-GGCCGCAGCTGCACTCAGTC -3’ R: 5’-GCGGAGGGAAGGTCAGCGTG -3’
murine Nur77	F: 5’-TTGATGTTCCCGCCTTTG-3’ R: 5’-GGTAGCCATGTGCTCCTTC-3’
murine Nurr1	F: 5’-TCACCTCCGGTGAGTCTGATC-3’ R: 5’-TGCTGGATATGTTGGGTATCATCT-3’
rat NOR1	F: 5’-CAGCAGCTGCGAACTCAA-3’ R: 5’-CGGTGTAGAAGGCGGAGA-3’
rat RPL13A	F: 5’GTACGCTGTGAGGGCATCAA-3’ R: 5’-CTCGAGACGGGTTGGTGTTC-3’
rat HDAC1	F: 5’-CCATCAAAGGACATGCCAAGT-3’ R: 5’-CGAGCGACATTACGGATGGT-3’
rat HDAC2	F: 5’-CGGCAAGAAGAAAGTGTGCTACT-3’ R: 5’-ATGAGTCATCCGGATCCTATGG-3’
rat HDAC3	F: 5’-GCACCCGCATCGAGAATC-3’ R: 5’-TGGACACTGGGTGCATGGT-3’

3. Immunostaining of OCT-embedded frozen sections

- Heat slides in heated distilled water for 2 minutes and dry slides at RT
- Fix tissues in chilled acetone at -20 °C for 1 min
- Incubate in 0.5 % Triton X-100/ PBS at 40 °C for 10 min
- Incubate in Redusol at 40 °C for 2 min
- Incubate in automation buffer at RT for 1 min
- Rinse slides with automation buffer 4 times at RT
- Incubate in DAKO Blocking Reagent at 40 °C for 5 min
- Rinse with automation buffer twice at RT
- Dilute primary antibody in Dako Antibody Diluent (typically 1: 100 dilution)
- Bind primary antibody at 40 °C for 15 min
- Rinse slides with automation buffer 5 times at RT
- Dilute biotinylated secondary antibody in Dako Antibody Diluent (typically 1: 200 dilution)
- Bind secondary antibody at 40 °C for 15 min
- Rinse with automation buffer 5 times at RT
- Amplify antigen-antibody interaction using the Avidin-Biotin Complex (ABC) (Vector Laboratories) at 40 °C for 10 min
- Rinse with automation buffer 5 times at RT
- Visualize signals by chromogen red 3-amino-9-ethylcarbazole (AEC) (Biomeda Corp) at 40 °C for 10 min
- Repeat the last step if necessary
- Rinse slides with distilled water twice at RT
- Flush slides with hematoxylin for nuclei visualization
- Mount slides in glycerol with a coverglass

4. Transient transfection assays

(1) Transient transfection of human endothelial cells using promofectin-HUVEC (PromoKine)

- Seed cells at 15x 10^4 / well in 6-well plates 24 hours before experiment
- Dilute 4 μl promoter constructs (0.5 μg/ml) for each transfection in 25 μl Opti-MEM® I Reduced Serum Medium (Opti-MEM) (Invitrogen), and mix well by vortexing for 10 sec
- Dilute 4 μl promofectin for each transfection in 25 μl Opti-MEM, and mix well by vortexing for 10 sec
- Mix DNA and promofectin dilutions and vortex the mixture for 10 sec
- Incubate the mixture at RT for 20 min
- While the mixture is incubating, wash the endothelial cells once with PBS and add 2 ml/ well fresh Opti-MEM
- Add 50 μl/ well of the promofectin / DNA solution to the cells and shake the plate gently
- Incubate at 37 °C for 3 hours
- Wash the cells three times with PBS and add 2 ml/ well fresh growth media

(2) Transient transfection of rat aortic smooth muscle cells using lipofectamine 2000 (Invitrogen)

- Seed cells at 20x 10^4 / well in 6-well cell culture plates 24 hours before experiment
- Dilute 4 μl promoter constructs (0.5 μg/ml) for each transfection in 250 μl Opti-MEM, and mix well by vortexing for 10 sec
- Dilute 6 μl lipofectamine 2000 for each transfection in 250 μl Opti-MEM, mix well by vortexing for 10 sec and incubate at RT for 5 min
- Mix DNA and lipofectamine 2000 dilutions and vortex the mixture for 10 sec
- Incubate the mixture at RT for 20 min
- While the mixture is incubating, add 2 ml/ well 5 % FBS DMEM without antibiotics

- Add the lipofectamine / DNA solution to the cells and shake the plate gently
- Incubate at 37 °C for 7 hours
- Wash the cells three times with PBS and add 2 ml/ well fresh growth media

5. Isolation of mouse aortic endothelial cells (MAEC)

(1) Cultrex® Basement Membrane Extract (BME) (R & D Systems)

- Thaw BME at 2 – 8 °C on ice in a refrigerator overnight
- Mix by slowly pipetting the solution up and down and be careful not to introduce air bubbles
- Pipette 50 μl/cm^2 (500 μl/ 35 mm dish) onto the growth surface
- Place coated object at 37 °C for 30 minutes and coated objects are ready for use

(2) Isolation of endothelial cells

- Perfuse intracardially through the aorta using PBS and remove the ascending aorta
- Remove adventitial tissue under a dissecting microscope
- Cut the aorta into 3 μm long segments and rinse the segments with PBS
- Place the segments on BME gel
- Add 2 ml/ well of the endothelial cell growth media containing low-glucose DMEM supplemented with 15 % FBS, 180 μg/ml heparin and 20 μg/ml endothelial cell growth supplement
- Change media every 2 days

(3) MAEC extraction and cell culture

- Thaw Dispase in water bath and equilibrate to 37 °C
- Remove medium from cell culture
- Add 0.2 ml/cm^2 Dispase to dish using sterile technique (e.g. 2 ml/ 35 mm dish, 6 ml/ 60 mm dish, 16 ml/ 100 mm dish)
- Incubate at 37 °C for 2 hours to insure complete dissolution of the gel
- Pipette mixture up and down to disperse cell suspension
- Transfer mixture to sterile centrifuge tube

- Stop action of Dispase by dilution or chelation of Ca^{++} and Mg^{++} using 5 - 10 mM EDTA
- Centrifuge cells at 1000 rpm, RT for 5 min and wash several times with PBS
- Plate cells onto gelatin-coated dishes for 2 days in growth media containing D-valine to eliminate possible fibroblast contamination
- Return endothelial cells after 2 days to growth medium without D-valine and grow cells to confluence
- Test the purity of mouse endothelial cell cultures at passage 2 using either von Willebrand Factor staining or di-acetylated LDL uptake
- Perform subsequent passages with Trypsin-EDTA

6. Adhesion assay

- Seed endothelial cells in the wells of a 6-well plate 24 hours before experiment
- Maintain monocytes in suspension in 10 % FBS DMEM growth media
- Fluorescently label monocytes (1x 10^7/ ml) in growth media containing 5 μg/ml Calcein-AM at 37 °C for 30 min
- Pellet cells by centrifugation at 1000 rpm, RT for 5 min
- Wash cell three times with PBS
- Resuspend fluorescently labeled monocytes in endothelial cell growth media at 1x 10^6 cells/ ml
- Transfer 1 ml/ well monocyte dilutions onto endothelial cell monolayers
- Bind at 37 °C for 30 min
- Wash off unbound monocytes with PBS
- Acquire images using fluorescent microscopy

Appendix B. Additional data

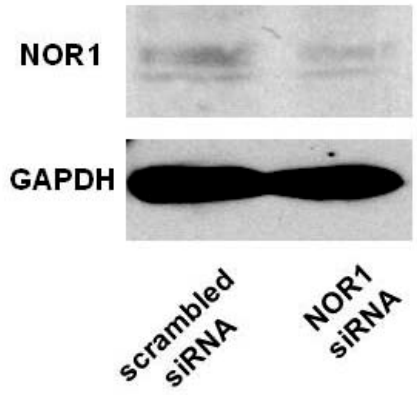


Figure I. siRNA-mediated knock-down of NOR1 expression in endothelial cells

Western Blotting for NOR1 expression in endothelial cells transfected with scrambled or NOR1 siRNA (50 nM) and treated with TNFα (1 ng/ml) for 6 h.

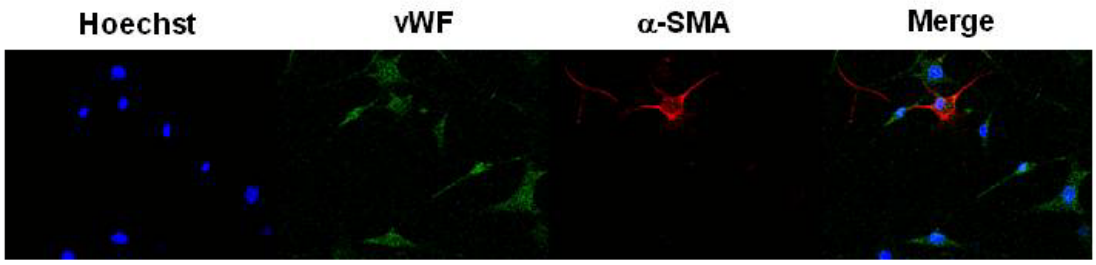


Figure II. The purity of mouse aortic endothelial cells (MAEC) at passage 2

MAEC were cultured on coverglasses and immunostained for von Willebrand Factor (vWF) and smooth muscle α-actin (α-SMA) to identify endothelial cells and smooth muscle cells. Images were acquired using confocal microscopy (objective magnification x60). Stainings for vWF, α-SMA and Hoechst were merged into one image as indicated. The purity of MAEC at passage 2 was greater than 85 %.

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Vita

Yue Zhao

Date and place of birth: 07/12/1983, Tianjin, China PR

Education

Bachelor of Science, 09/2002 ~ 07/2006

Major in Biotechnology

School of Life Science and Biotechnology, Shanghai Jiao Tong University, China

Scholastic and professional honors

2010 ~ 2011, Dissertation Year Fellowship

2010, Keystone Symposia Scholarship

2008 ~ 2010, American Heart Association Pre-doctoral Fellowship (average priority score: 1.1; percentile rank: 1.25)

2007, Kentucky Opportunity Fellowship

2005, Excellent Academic Scholarship, Class C (granted to top 10% students of the department)

2004, Excellent Academic Scholarship, Class B (granted to top 6% students of the department)

2003, Exceptional Student of Shanghai Jiao Tong University (granted to top 10% students in the school)

2003, Excellent Academic Scholarship, Class C (granted to top 10% students of the department)

Professional publications

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1) Deficiency of the NR4A Orphan Nuclear Receptor NOR1 Decreases Monocyte Adhesion and Atherosclerosis. **Zhao Y**, Howatt DA, Gizard F, Nomiyama T, Findeisen HM, Heywood EB, Jones KL, Conneely OM, Daugherty A, Bruemmer D. *Circ Res. 2010 Aug 20;107(4):501-11.*
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ABSTRACTS:

1) Epigenetic Regulation of the NR4A Orphan Nuclear Receptor NOR1 by Histone Deacetylase Inhibition. **Zhao Y**, Nomiyama T, Findeisen H, Hua Q, Heywood EB, Cohn D, Jones KL, Bruemmer D. *2011 Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference;* Poster.
2) Deficiency of the NR4A Orphan Nuclear Receptor NOR1 Increases Bone Marrow Cell Proliferation. **Zhao Y**, Nomiyama T, Findeisen H, Hua Q, Heywood EB, Cohn D, Jones KL, Bruemmer D. *2011 Arteriosclerosis, Thrombosis and Vascular Biology Annual Conference;* Poster.
3) The NR4A orphan nuclear receptor NOR1 mediates monocyte adhesion and atherosclerosis formation in mice. **Zhao Y**, Gizard F, Findeisen H, Heywood EB,

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