THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 41, pp. 35485–35493, October 14, 2011

Transcriptional Regulation of S Phase Kinase-associated Protein 2 by NR4A Orphan Nuclear Receptor NOR1 in Vascular Smooth Muscle Cells*[S]

Received for publication, August 22, 2011 Published, JBC Papers in Press, August 25, 2011, DOI 10.1074/jbc.M111.295840

Florence Gizard[‡], Yue Zhao[‡§1], Hannes M. Findeisen[‡], Hua Qing[‡], Dianne Cohn[‡], Elizabeth B. Heywood[‡], Karrie L. Jones[‡], Takashi Nomiyama[¶], and Dennis Bruemmer[‡§2]

From the [‡]Saha Cardiovascular Research Center, University of Kentucky College of Medicine, Lexington, Kentucky 40536, the [§]Graduate Center for Nutritional Sciences, University of Kentucky College of Medicine, Lexington, Kentucky 40536, and the [¶]Department of Endocrinology and Diabetes Mellitus, School of Medicine, Fukuoka University, Fukuoka City, Fukuoka 814-0180, Japan

Background: The nuclear receptor NOR1 serves a mitogenic role; however, the mechanisms underlying this activity remain poorly understood.
Results: NOR1 induces *Skp2* transcription, leading to a decrease in p27 protein abundance.
Conclusion: *Skp2* constitutes a NOR1-regulated gene, which contributes to the mitogenic activity of this nuclear receptor.
Significance: These studies detail a novel transcriptional cascade regulating proliferation.

Members of the NR4A subgroup of the nuclear hormone receptor superfamily have emerged as key transcriptional regulators of proliferation and inflammation. NOR1 constitutes a ligand-independent transcription factor of this subgroup and induces cell proliferation; however, the transcriptional mechanisms underlying this mitogenic role remain to be defined. Here, we demonstrate that the F-box protein SKP2 (S phase kinase-associated protein 2), the substrate-specific receptor of the ubiquitin ligase responsible for the degradation of p27^{KIP1} through the proteasome pathway, constitutes a direct transcriptional target for NOR1. Mitogen-induced *Skp2* expression is silenced in vascular smooth muscle cells (VSMC) isolated from *Nor1*-deficient mice or transfected with *Nor1* siRNA. Conversely, adenovirus-mediated overexpression of NOR1 induces *Skp2* expression in VSMC and decreases protein abundance of its target p27. Transient transfection experiments establish that NOR1 transactivates the *Skp2* promoter through a nerve growth factor-induced clone B response element (NBRE). Electrophoretic mobility shift and chromatin immunoprecipitation assays further revealed that NOR1 is recruited to this NBRE site in the *Skp2* promoter in response to mitogenic stimulation. *In vivo Skp2* expression is increased during the proliferative response underlying neointima formation, and this transcriptional induction depends on the expression of NOR1. Finally, we demonstrate that overexpression of *Skp2* rescues the proliferative arrest of *Nor1*-deficient VSMC. Collectively, these results characterize *Skp2* as a novel NOR1-regulated target gene and detail a previously unrecognized transcriptional cascade regulating mitogen-induced VSMC proliferation.

Vascular smooth muscle cells (VSMC)[3] resident in the normal arterial wall are quiescent but migrate, proliferate, and secrete extracellular matrix proteins in response to cues released at sites of injury (1). Proliferative VSMC remodeling constitutes an essential component for the initiation of atherosclerosis formation as well as for complications following revascularization procedures (2, 3). With the recognition of this seminal involvement of VSMC proliferation in vascular remodeling, understanding the intrinsic mechanisms that endow VSMC with the capability to excessively proliferate may yield valuable insights into the pathogenesis of all major vascular diseases.

Growth factors released during vascular injury converge into $G_1 \rightarrow S$ cell cycle progression of the VSMC (2, 3). In late G_1 hyperphosphorylation of the retinoblastoma protein (Rb) by cyclin-dependent kinases (CDKs) releases the repression of E2F transcription factors, which results in the expression of genes required for DNA synthesis (4). The activity of CDK complexes to phosphorylate Rb is negatively regulated by cyclin-dependent kinase inhibitors (CDKIs), including p27^{KIP1} (p27) (5). Therefore, transition into S phase necessitates the ubiquitin-mediated degradation of p27 through the SCF (Skp1/Cul1/F-box) ubiquitin ligase complex (6). The rate-limiting component of this SCF complex is *Skp2* (S phase kinase-associated protein 2) (7), which is induced by growth factors and responsible for substrate recognition and degradation of p27 (8, 9). In vascular cells, overexpression of p27 inhibits neointima formation, whereas its deficiency accelerates atherosclerosis formation

* This work was supported, in whole or in part, by National Institutes of Health Grant R01HL084611 and the American Recovery and Reinvestment Act of 2009 Grant R01HL084611-04S1 (to D. B.).
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1 and Fig. 1.
[1] Supported by Fellowship Grant 0815514D from the American Heart Association.
[2] To whom correspondence should be addressed: Saha Cardiovascular Research Center, University of Kentucky College of Medicine, Wethington Health Sciences Building Room 575, 900 South Limestone Street, Lexington, KY 40536-0200. Tel.: 859-323-4933 (ext. 81418); Fax: 859-257-3646; E-mail: Dennis.Bruemmer@uky.edu.

[3] The abbreviations used are: VSMC, vascular smooth muscle cell(s); NBRE, nerve growth factor-induced clone B response element; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor.

(10, 11). Conversely, ectopic SKP2 expression decreases p27 levels in VSMC, induces VSMC proliferation, and promotes pathological neointima formation (12, 13). Collectively, these recent studies have established a key role of SKP2-dependent p27 degradation in vascular biology.

Proliferation of VSMC in response to extracellular signals released at sites of vascular injury is ultimately controlled through transcriptional mechanisms (14). Among the growth factor-responsive transcription factors that translate mitogenic cues to VSMC proliferation, members of the NR4A subfamily of orphan nuclear receptors have emerged as key regulators of vascular gene expression (15–17). This subfamily functions as immediate early response genes, which are rapidly induced by a pleiotropy of stimuli to modulate important cellular functions, including proliferation and apoptosis (15–17). NR4A receptors transactivate target promoters by binding to different variations of the canonical NGFI-B response element (NBRE) (15–17). The transcription factor NOR1 (NR4A3) of this subfamily is highly induced during proliferative vascular remodeling and required for VSMC proliferation, neointima formation, and atherosclerosis formation (18–22). Considering this evidence as well as the demonstration that the cell cycle-dependent induction of *Skp2* is regulated through transcriptional mechanisms and that *Skp2* transcript levels are increased during neointima formation (23, 24), we tested in the present study whether *Skp2* constitutes a transcriptional target for NOR1 in VSMC.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary murine aortic VSMC were isolated from wild-type *Nor1*$^{+/+}$ and littermate *Nor1*$^{-/-}$ mice as described recently (19). Murine VSMC passage three to eight were subjected to G_0/G_1 phase synchronization by serum-deprivation in 0.4% FBS (Invitrogen) for at least 36 h followed by stimulation with 10 or 20% FBS as indicated. Primary human coronary artery VSMC were obtained from Lonza (Allendale, NJ) and cultured as directed by the manufacturer. For all data shown, individual experiments were performed at least in triplicate using different preparation or lots of VSMC.

NOR1 Overexpression by Adenoviral Infection—The adenovirus overexpressing NOR1 (Ad-NOR1) was kindly provided by Dr. Tontonoz (University of California, Los Angeles, CA) (25). The adenovirus overexpressing green fluorescent protein (Ad-GFP) was used as control and purchased from Quantum Biotechnologies. For adenoviral infections, murine wild-type or human coronary artery VSMC were plated at a density of 2×10^5 cells on 60-mm or 100-mm dishes, respectively, and subjected to serum deprivation for at least 36 h. Transduction was achieved by infecting cells in starvation medium with adenoviral constructs at 25 pfu per cell. Following infection, cells were harvested after 36 h for further analysis of protein or mRNA expression.

siRNA Experiments—Human coronary artery VSMC passage three to eight were plated at a density of 2.5×10^4 cells in six-well plates and subjected to serum starvation for 48 h in basal medium supplemented with 0.4% FBS. Cells were transfected with siRNA in Opti-MEM (Invitrogen) using 12 μl of INTERFERin (Polyplus Transfection, New York, NY) and 0.31 μg of *Nor1* or scrambled control siRNA (Dharmacon, Lafayette, CO). Twelve hours after transfection, medium supplemented with 5% FBS was added, and cells were harvested after 24 h for *Skp2* mRNA expression analysis.

Transient Transfection Assays—The murine Skp2 luciferase reporter constructs were kindly provided by Dr. Nakayama (Kyushu University, Fukuoka, Japan) (23). The NBRE site in the *Skp2* promoter located between −1443 and −1436 bp from the transcription initiation site (23) was mutated from CAGAAAGGACAAAGT to CAGAAAAAACAAAGT using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The CMV expression vectors overexpressing GFP or NOR1 have been described previously (19, 20). The NBRE(8×)-luciferase reporter construct was kindly provided by Dr. Ohkura (National Cancer Center Research Institute, Tokyo, Japan) (19). Murine wild-type VSMC were cultured in six-well plates and transfected with the indicated *Skp2* reporter construct in DMEM supplemented with 5% FBS using 5 μl of Lipofectamine 2000 (Invitrogen) and 1.6 μg of total transfected plasmid DNA per well. Six hours after transfection, the medium was replaced with DMEM containing 0.5% FBS and 1% antibiotics (penicillin/streptomycin). After 24 h, cells were stimulated with 20% FBS for an additional 24 h. In transactivation assays, reporter constructs were co-transfected with CMV expression vectors overexpressing NOR1 (pCMV-NOR1) or GFP (pCMV-GFP) as control. Luciferase activities were assayed after 36 h using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Transfection efficiency was normalized to renilla luciferase activities generated by cotransfection of 10 ng/well pGL4.74[hRluc/TK] (pRL-Tk, Promega, Madison, WI).

Reverse Transcription and Quantitative PCR—Isolation of RNA and quantitative real-time RT-PCR analyses were performed as described previously (20). All primer sequences are provided in supplemental Table 1. Target mRNA expression levels were normalized to the housekeeping genes TBP (TATA box binding protein) and transcription factor TFIIB in human and murine cells, respectively. 28 S ribosomal RNA (r28S) was used as housekeeping gene in murine femoral arteries.

Western Blotting—Western blotting was performed on whole cell lysates using antibodies raised against SKP2 (ab68455, Abcam), p27 (ab7961-1, Abcam), p21/WAF1 (ab7960, Abcam), NOR1 (PP-H7833, R&D Systems), and GAPDH (sc-25778, Santa Cruz Biotechnology) as described (18, 19).

EMSA—EMSA were performed as described previously using the gel shift assay system from Promega (20). 4 μg of nuclear proteins isolated with the NE-PER^{TM} kit (Pierce) were incubated with double-stranded oligonucleotides end-labeled with [γ-^{32}P]ATP (supplemental Table 1).

ChIP Assays—Following stimulation of murine VSMC with FBS as indicated, cells were harvested for ChIP assays using the Magnify ChIP assay system from Invitrogen per the manufacturer's instructions. Chromatin was immunoprecipitated using 5 μg of an antibody raised against NOR1 (PP-H7833.00, R&D Systems), RNA polymerase II (Millipore, catalogue no. 05-623), or a mouse monoclonal control IgG (Santa Cruz Biotechnology, used as negative control). Precipitated DNA was amplified by

PCR using primer pairs encompassing the NBRE site in the murine *Skp2* promoter (supplemental Table 1). Quantification was performed by real-time PCR using the SYBR Green System (Bio-Rad).

Endothelial Denudation Injury—Guidewire endothelial denudation injuries of the right femoral artery of *Nor1*$^{+/+}$ and littermate *Nor1*$^{-/-}$ mice were performed as described previously (20). Sham surgery without endovascular injury was performed on the left side. Twelve h after injury mice were euthanized, and femoral arteries were isolated. Total RNA was extracted and analyzed for *Skp2* expression levels by real-time RT-PCR. The institutional animal care and use committee at the University of Kentucky approved all procedures on mice.

Skp2 Overexpression and Cell Proliferation Assays—The adenovirus overexpressing *Skp2* (Ad-Skp2) was purchased from Vector Biolabs. VSMC isolated from *Nor1*$^{+/+}$ and *Nor1*$^{-/-}$ mice were plated at a density of 5×10^4 cells on 60-mm dishes and infected in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin with Ad-GFP or Ad-Skp2 adenoviral constructs at 100 pfu per cell. Cell proliferation was analyzed 6 days after infection by cell counting using a hemocytometer. For all experiments, wild-type and *Nor1*-deficient VSMC of similar passages were employed. Experiments were performed in triplicates using different preparations of aortic vascular VSMC.

Statistical Analysis—Differences between different groups were assessed by one-way analysis of variance followed by Tukey tests. S.D. and S.E. were given for *in vitro* and *in vivo* assays, respectively. p values <0.05 were considered to be statistically significant.

RESULTS

NOR1 Is Required for Mitogen-induced Skp2 Expression—Previous studies from our group and other laboratories have established that NOR1 serves a mitogenic role in VSMC and promotes cell cycle progression (18–20). In an effort to identify target genes that orchestrate NOR1-mediated $G_1 \rightarrow S$ phase progression, we initially employed a profiling approach using a biased cell cycle array. These experiments suggested that mitogen-induced expression of *Skp* family members was abolished in *Nor1*-deficient VSMC (data not shown). Confirmative experiments using primary aortic VSMC isolated from *Nor1*$^{+/+}$ and *Nor1*$^{-/-}$ mice corroborated that mitogen-induced Skp2 mRNA expression was silenced in cells deficient for the *Nor1* locus (Fig. 1*A*). Similarly, stimulation of *Nor1* wild-type VSMC with FBS resulted in an induction of SKP2 protein; however, this induction of Skp2 was absent in *Nor1*-deficient VSMC (Fig. 1*B*). Finally, we confirmed decreased expression levels of cyclin D1, a previously characterized target gene of NOR1 (20), in *Nor1*-deficient VSMC (supplemental Fig. 1).

Considering that *Skp2* family members are evolutionary conserved, we next evaluated whether NOR1 is also required for the induction of *Skp2* in human cells. To test this hypothesis, we employed a siRNA approach in primary human coronary artery VSMC. *Nor1* mRNA expression analysis confirmed an ~60% knockdown of *Nor1* transcript levels following transfection of the siRNA (Fig. 1*C*). Consistent with our observation in murine VSMC, knockdown of *Nor1* prevented mitogen-induced *Skp2* mRNA expression in human cells, suggesting that the regulation of *Skp2* by NOR1 is conserved between murine and human cells (Fig. 1*D*).

Adenovirus-mediated Overexpression of NOR1 Induces Skp2 Expression—To further evaluate whether NOR1 is not only necessary but also sufficient for *Skp2* expression, we next employed a gain-of-function approach using an adenoviral construct overexpressing NOR1. As shown in Figs. 2, *A* and *B*, infection of murine aortic and human coronary artery VSMC with 25 pfu of the adenoviral vector resulted in a solid increase of NOR1 protein expression. The extent of NOR1 expression achieved using this adenoviral approach was comparable to the physiological induction of NOR1 protein expression observed in response to PDGF in our recent studies (19). In line with the data obtained using a loss-of-function approach, overexpression of NOR1 induced SKP2 protein expression in both murine and human VSMC (Fig. 2, *A* and *B*, respectively). Furthermore, as shown in Fig. 2*C*, the increase in SKP2 protein expression following NOR1 overexpression was paralleled by an increase in *Skp2* transcript levels. Consistent with the key role of SKP2 to promote ubiquitin-mediated degradation of p27 (8), NOR1 overexpression decreased p27 abundance in the absence of mitogens, suggesting that the induction of SKP2 protein expression by NOR1 is functional (Fig. 2*D*). In contrast, protein expression levels of the CDKI p21 were not affected by NOR1 overexpression (Fig. 2*E*). Collectively, these experiments characterize NOR1 as a novel positive transcriptional regulator of *Skp2* expression and downstream p27 degradation in VSMC.

NOR1 Transactivates Skp2 Promoter in VSMC—Mitogen-induced *Skp2* expression during $G_1 \rightarrow S$ phase cell cycle progression is primarily regulated through transcriptional mechanisms (23). To determine the transcriptional mechanisms underlying inducible *Skp2* expression in VSMC, we next performed transient transfection of reporter constructs driven by the 5′-deletion of the *Skp2* promoter region spanning from −2275 to +94 bp from the transcription initiation site. This promoter sequence has previously been demonstrated to confer a high level of transcriptional activity and to contain elements that mediate the cell cycle-dependent expression of *Skp2* (23). As depicted in Fig. 3*A*, mitogenic stimulation induced a 3-fold increase in the transcriptional activity of the pGL2–2275 construct. Consistent with previous observations (23), deletion of the 5′-flanking region from −2275 to −318 increased basal activity of the pGL2–318 construct, likely due to the loss of repressive elements. Furthermore, a 5′-deletion to −318 resulted in a complete loss of the responsiveness to mitogens, indicating that this sequence contains binding sites that confer the transcriptional induction of *Skp2* during VSMC proliferation.

Considering that NOR1 constitutes an early response gene required for VSMC proliferation and our observation that NOR1 is necessary and sufficient for *Skp2* transcription, we hypothesized that NOR1 may be involved in the regulation of the *Skp2* promoter. Indeed, computer-assisted analysis of the *Skp2* promoter sequence spanning from −2275 to −318 revealed the presence of a NBRE consensus motif 1443 bp upstream of the transcription start site (Fig. 3*B*). Mutation of this NBRE element in the pGL2–2275 construct prevented the

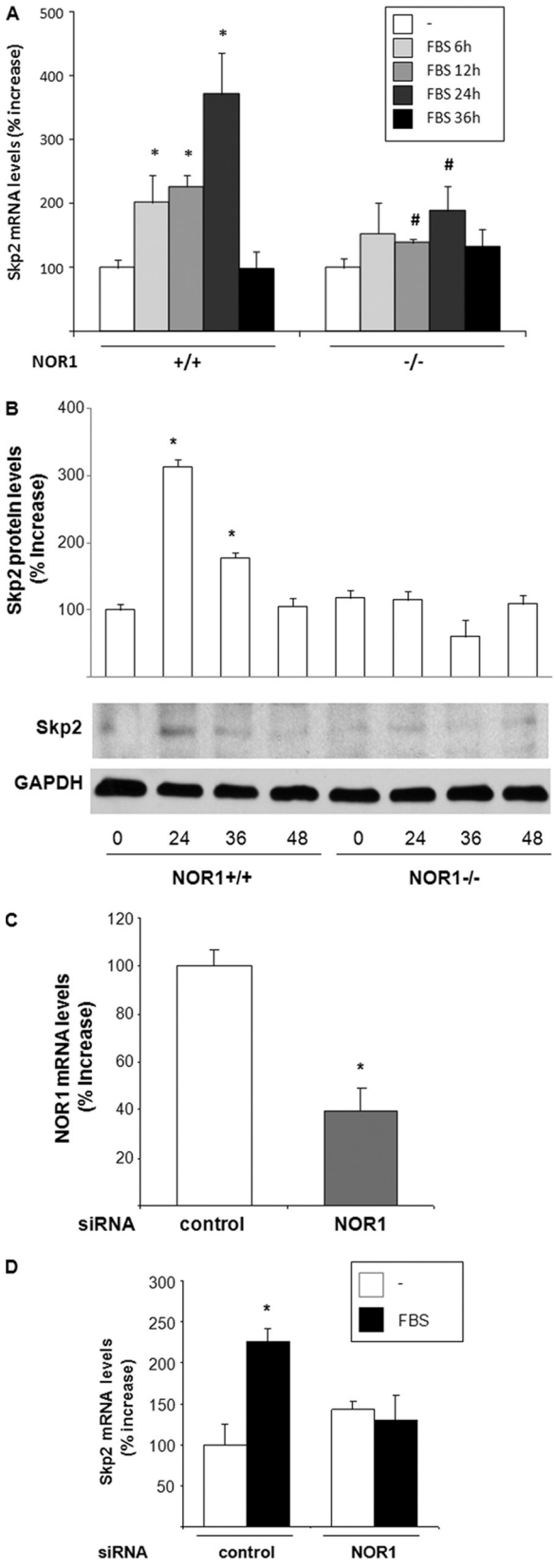


FIGURE 1. ***Nor1* deficiency prevents mitogen-induced *Skp2* expression in VSMC.** *A*, serum-deprived murine VSMC isolated from $Nor1^{+/+}$ or littermate $Nor1^{-/-}$ mice were stimulated with 10% FBS. *Skp2* mRNA expression was analyzed at the indicated time-points and normalized to transcript levels of the house-keeping gene TFIIB. Data are expressed as mean ± S.D. percent increase relative to serum-deprived wild-type cells (*, $p < 0.05$ *versus* time point 0 h; #, $p < 0.05$ *versus* wild-type; $n = 3$). *B*, serum-starved $Nor1^{+/+}$ and $Nor1^{-/-}$ VSMC were stimulated with 10% FBS and analyzed for SKP2 protein expression. Cohybridization for GAPDH was performed to control for equal protein loading. The autoradiographs shown are representative of three independently performed experiments. Densitometric quantification and normalization to GAPDH is provided in the *upper panel* (*, $p < 0.05$ *versus* time point 0 h, $n = 3$). *C* and *D*, serum-deprived human coronary artery VSMC were transfected with *Nor1* siRNA or scrambled control siRNA and stimulated with 5% FBS for 24 h. *C*, *Nor1* mRNA expression was analyzed by real-time RT-PCR in the FBS-stimulated samples to confirm knockdown (*, $p < 0.05$ *versus* scrambled control, $n = 3$). *D*, *Skp2* mRNA expression was quantified by real-time RT-PCR (*, $p < 0.05$ *versus* quiescent control, $n = 3$). Target transcript levels in *C* and *D* were normalized to mRNA levels of the housekeeping gene TBP and expressed as mean ± S.D. percent increase.

mitogen-induced increase and even slightly decreased promoter activity, compared with VSMC transfected with the wild-type pGL2–2275 reporter (Fig. 3*A*). Moreover, transfection of a reporter driven by multiple NBRE repeat elements revealed potent activation of the NBRE site in response to FBS stimulation (Fig. 3*A*). To finally confirm that NOR1 activates the *Skp2* promoter, we performed transactivation activation assays and cotransfected the *Skp2* promoter with eukaryotic expression vectors overexpressing NOR1 or GFP as control. As depicted in Fig. 3*C*, overexpression of NOR1 significantly induced the activity of the pGL2–2275 *Skp2* promoter. In contrast, this NOR1-dependent induction of the *Skp2* promoter was completely lost upon 5′ deletion to −318 bp or upon site-directed mutation of this NBRE sequence (Fig. 3*C*). These data suggest that NOR1 induces *Skp2* promoter activity through transactivation of an NBRE site located at −1443/−1436 bp in the murine *Skp2* promoter.

NOR1 Is Recruited to Canonical NBRE Consensus Site in Skp2 Promoter—To further validate that NOR1 is recruited to the *Skp2* promoter, we first tested whether transcriptional complexes form at this NBRE site in response to mitogenic stimulation. EMSA assays using oligonucleotides encompassing this NBRE site revealed the formation of a transcriptional complex, which is induced in response to mitogens (Fig. 4*A*). Furthermore, mutation of the NBRE site completely prevented complex formation, confirming the specificity of this complex. Second, ChIP assays on chromatin isolated from murine VSMC using primer pairs that cover the −1443/−1436 bp NBRE element, established that NOR1 is recruited to this endogenous NBRE site in response to mitogenic stimulation (Fig. 4*B*). This inducible NOR1 occupancy of the NBRE motif was paralleled by the recruitment of RNA polymerase II, indicating the initiation of active transcription from the gene (Fig. 4*C*). In concert with the data obtained from transient transfection assays, these experiments support the notion that NOR1 induces *Skp2* expression by binding and transactivating an NBRE site located 1443 bp upstream of the transcription initiation site.

NOR1 Is Required for Inducible Skp2 Expression during Neointima Formation in Vivo—*Nor1* and *Skp2* are both highly induced during proliferative vascular remodeling and promote neointima formation (13, 18, 20, 24). Therefore, we next tested whether the regulation of *Skp2* by NOR1 applies *in vivo* and whether inducible *Skp2* expression in the neointima depends on NOR1. To address this question, we performed endothelial denudation injuries in $Nor1^{+/+}$ and $Nor1^{-/-}$ mice. As reported previously (24), expression analysis confirmed a potent induc-

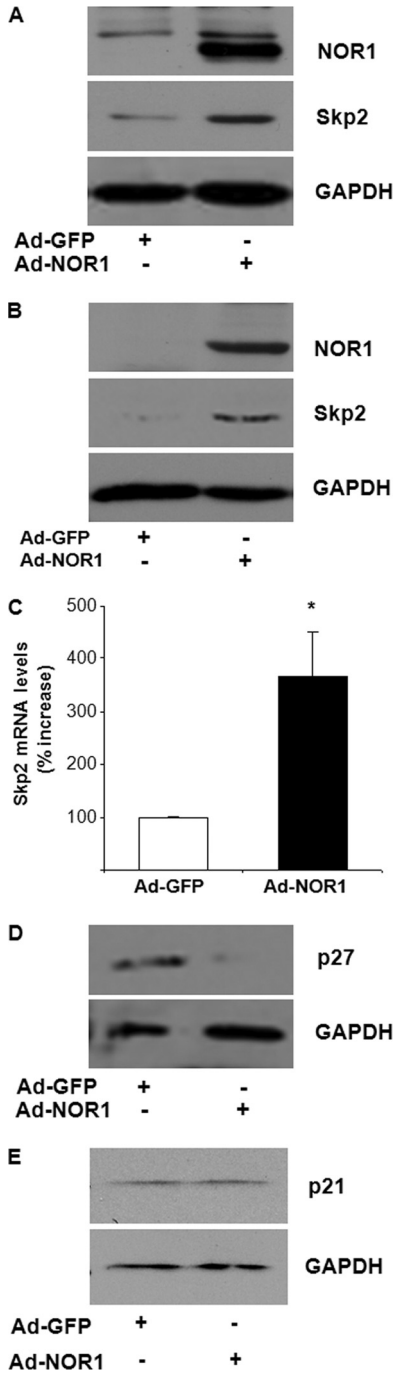


FIGURE 2. **Overexpression of NOR1 induces SKP2 protein expression in VSMC.** Serum-deprived murine aortic VSMC (*A*) or human coronary artery VSMC (*B–E*) were infected with either control Ad-GFP or Ad-NOR1 adenoviral constructs. Following infection, cells were harvested after 36 h for protein or mRNA analysis as indicated. Cohybridization for GAPDH was performed to assess equal loading. All Western blots depicted are representative of at least three independently performed experiments using different cell preparations. In *C*, *Skp2* mRNA expression was analyzed by real-time RT-PCR and normalized to transcript levels of the housekeeping gene TBP. Values are expressed as mean ± S.D. percent increase compared Ad-GFP-infected cells (*, $p < 0.05$, $n = 3$).

tion of *Skp2* transcript levels 12 h after endovascular injury of *Nor1* wild-type mice (Fig. 5). In contrast, this inducible *Skp2* mRNA expression was significantly attenuated in *Nor1*$^{-/-}$ mice. Therefore, the induction of *Skp2* expression in response to vascular injury is at least in part dependent on NOR1, pointing to a previously unrecognized transcriptional cascade governing *Skp2* expression.

Overexpression of Skp2 Rescues Mitogenic Defect of NOR1-deficient VSMC—To corroborate that the positive regulation of *Skp2* constitutes an important mechanism underlying the mitogenic effect of NOR1, we next overexpressed *Skp2* in *Nor1*-deficient VSMC. Adenovirus-mediated transduction of murine VSMC resulted in a solid ectopic expression of SKP2 protein (Fig. 6*A*). Consistent with prior experiments by Bond *et al.* (12), overexpression of SKP2 in wild-type VSMC increased cell proliferation (Fig. 6*B*). Compared with VSMC that are wild-type for the *Nor1* locus, cells from *Nor1*-deficient mice revealed a solid proliferative defect (19, 20). However, SKP2 overexpression in these *Nor1*$^{-/-}$ VSMC rescued proliferation to the level of wild-type cells infected with the control Ad-GFP. These observations support the notion that *Skp2* constitutes an important target for the mitogenic activity of NOR1.

DISCUSSION

The CDKI p27 constitutes a principal regulator of VSMC proliferation and a molecular target for a variety of clinically used approaches to limit cardiovascular complications, including rapamycin (26), HMG-CoA reductase inhibitors (27), and thiazolidinediones (28). The cellular abundance of p27 is regulated by SKP2-dependent proteasome degradation (6). However, although SKP2 has recently been implicated in the control of VSMC proliferation (12, 13, 24), the molecular mechanisms underlying mitogen-dependent *Skp2* expression in VSMC remain to be defined. In the present study, we employed loss- and gain-of-function approaches to characterize *Skp2* as a transcriptional target gene for the orphan nuclear receptor NOR1. Consistent with the well established regulation of p27 degradation by SKP2 (6), NOR1 overexpression decreased the abundance of p27. Moreover, our *in vivo* experiments establish a specific requirement of NOR1 for *Skp2* expression during neointima formation in response to vascular injury. These observations indicate that NOR1 plays a critical role in the transcriptional induction of *Skp2* and provide a previously unrecognized transcriptional cascade regulating p27 abundance and VSMC proliferation.

In proliferating VSMC, expression of the early response gene *Nor1* is rapidly induced in the G_1 phase (18, 19). Recent functional studies (18), including experiments from our group (19, 20), have demonstrated that this spatial and temporal *Nor1* expression serves a mitogenic role and stimulates neointimal VSMC proliferation by promoting Rb phosphorylation and subsequent $G_1 \rightarrow S$ cell cycle transition. Our previous characterization of cyclin D1 as a NOR1 target gene has provided initial insight into the transcriptional mechanisms underlying this mitogenic function of NOR1 (20). However, considering the mild growth disadvantage reported in cyclin D1-deficient cells (29), the profound proliferative defect of *Nor1*-deficient VSMC is unlikely attributable to a single transcriptional target gene but rather involves the regulation of a transcriptional network of genes governing cell cycle progression (19, 20). In the present studies, we extend our prior observations and identify *Skp2* as a transcriptional target gene for NOR1. Consistent with previous studies (12, 23), we observed a cell cycle-dependent

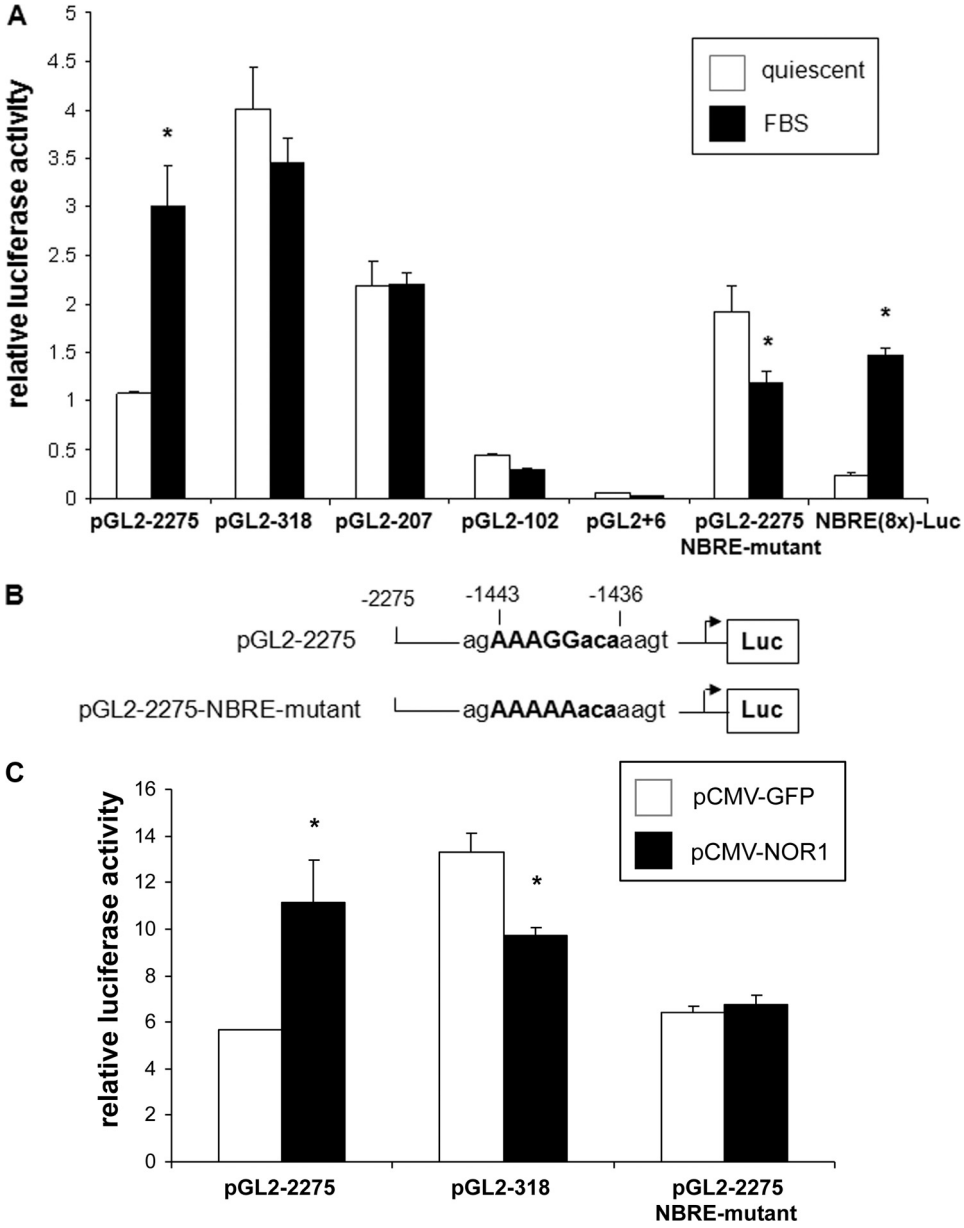
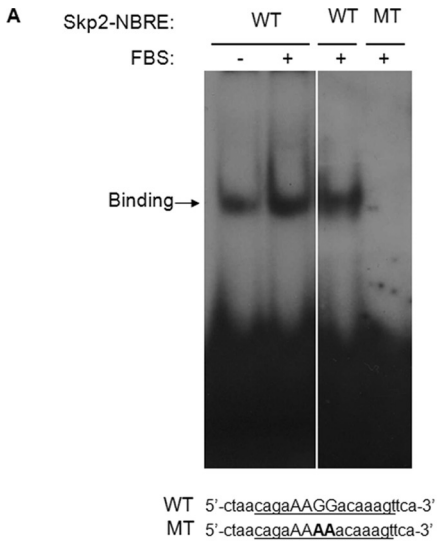
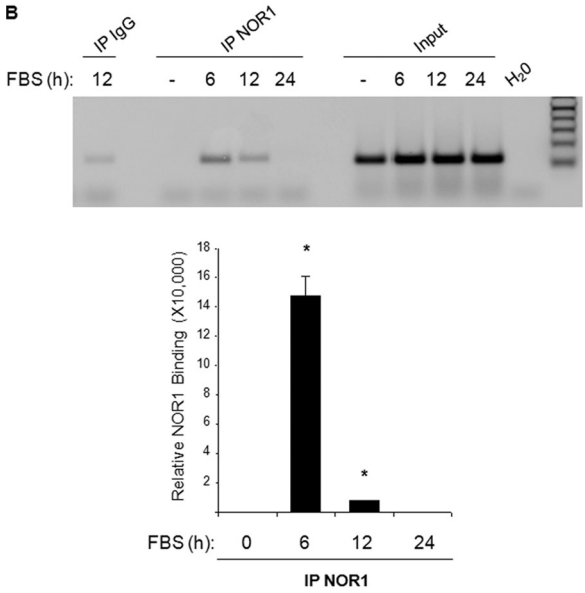


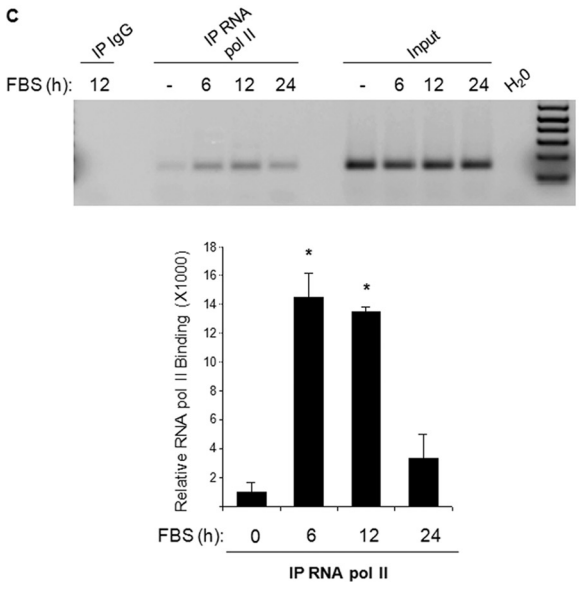
FIGURE 3. **NOR1 transactivates the proximal *Skp2* gene promoter through an NBRE site.** *A*, murine VSMC were transfected with the indicated reporter constructs driven by a 5′-deletion of the murine wild-type *Skp2* promoter (pGL2–2275, pGL2–318, pGL2–207, pGL2–102, pGL2+6), a pGL2–2275 reporter bearing a site-directed mutation of the NBRE site (pGL2–2275NBREmutant), or a reporter driven by eight NBRE elements (NBRE(8×)-luciferase (*Luc*)). Following transfection, cells were serum-deprived and stimulated with 20% FBS for 24 h. (*, $p < 0.05$ *versus* quiescent, $n = 3$). *B*, sequence of the wild-type and mutated NBRE site in the murine *Skp2* promoter. *C*, VSMC were co-transfected with the indicated *Skp2* reporter construct and CMV expression vectors overexpressing NOR1 (pCMV-NOR1) or GFP (pCMV-GFP) as control. Following transfection, cells were harvested after 36 h for the analysis of luciferase activities. (*, $p < 0.05$ *versus* quiescent, $n = 3$). Transfection efficiency was adjusted by normalizing firefly luciferase activities to *Renilla* luciferase activities generated by cotransfection with pRL-TK. Data are expressed as mean ± S.D. normalized luciferase activities.

induction of *Skp2* expression following growth factor stimulation *in vitro*. Intriguingly, *Nor1* knockdown or genetic deficiency prevented this mitogen-induced *Skp2* mRNA and protein expression in VSMC. Complementary results using adenoviral overexpression of NOR1 established that NOR1 is sufficient to induce *Skp2* expression. The importance of SKP2 for the mitogenic function of NOR1 was further validated by our observation that overexpression of SKP2 in *Nor1*-deficient VSMC rescued their altered proliferative capacity. Collectively, these data identify *Skp2* as a NOR1-regulated gene, and this positive regulation constitutes an important means to translate mitogenic stimulation and promote cell cycle progression.

The kinetic of NOR1 expression in response to growth factor stimulation of VSMC precedes the cell cycle-dependent increase in *Skp2* transcription (12, 19). Considering this evidence combined with the positive regulation of *Skp2* by NOR1, we performed an *in silico* analysis of the *Skp2* promoter to identify the *cis*-motif and potential *trans*-element regulatory interactions that are responsible for this regulation. Reporter assays and 5′-deletion of the *Skp2* promoter confirmed a solid promoter induction in response to mitogenic stimulation. NOR1 is thought to transactivate a different variation of the NBRE hexanucleotide (30) and, indeed, sequence analysis of the minimal promoter required for this







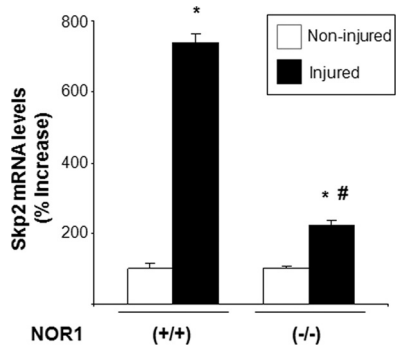


FIGURE 5. **Skp2 induction following vascular injury is attenuated in *Nor1*-deficient mice.** Guidewire-induced endothelial denudation injuries of the femoral artery were performed in $Nor1^{+/+}$ ($n = 9$) and $Nor1^{-/-}$ mice ($n = 8$). Tissues from injured or sham-operated femoral arteries were harvested 12 h after injury and analyzed for *Skp2* mRNA expression. Data are normalized to r28 S mRNA expression levels and presented as mean ± S.E. percent increase relative to sham-operated mice (*, $p < 0.05$ *versus* non-injured sham-operated mice; #, $p < 0.05$ *versus* wild-type mice).

mitogenic response identified a canonical NBRE localized −1443/−1436 bp from the transcription initiation site. Transactivation experiments confirmed the induction of the *Skp2* promoter by NOR1, an effect that was completely abolished upon site-directed mutation of the NBRE sequence. Using gel shift analysis combined with ChIP assays, we confirmed that NOR1 is recruited to this site upon mitogenic stimulation. From these experiments, we infer that an NBRE site at −1443 confers the transcriptional induction of *Skp2* by NOR1, providing a novel mechanism underlying inducible *Skp2* expression. However, the transactivating mechanisms for *Skp2* promoter activation may be stimulus- and cell-specific, and it is possible that additional transcriptional mechanisms act in concert to induce transcription of the *Skp2* gene. For example, in fibroblasts and cancer cells *Skp2* transcription has been demonstrated to involve a cell cycle-dependent activation of its promoter by GA-binding proteins or E2F transcription factors, respectively (23, 31). Although our studies clearly support a key role of a direct NBRE-dependent transactivation of the *Skp2* promoter by NOR1, indirect mechanisms may contribute to the increase in *Skp2* transcript levels following NOR1 overexpression. Considering for example the reported activation of *Skp2* transcription by E2F (31), it is possible that NOR1-depen-

FIGURE 4. **Mitogenic stimulation induces NOR1 recruitment to an NBRE site in the proximal *Skp2* promoter.** *A*, nuclear extracts were isolated from quiescent murine VSMC stimulated for 24 h with 20% FBS. DNA binding activities were analyzed by EMSA using [γ-^{32}P]ATP-end-radiolabeled WT or mutant (*MT*) oligonucleotides encompassing the murine NBRE site. The autoradiograms shown are representative of four independently performed experiments using different cell preparations. *B* and *C*, serum-deprived murine VSMC were stimulated with 20% FBS for the indicated time points. Cells were harvested for chromatin immunoprecipitation (*IP*) assays using antibodies raised against NOR1 (*B*), RNA polymerase II (*RNA pol II*; *C*), or non-immune IgG as negative control. The *upper* gels depict a representative ethidium-bromide-stained agarose gel of PCR products obtained using NOR1 (*B*) or RNA polymerase II (*C*) chromatin immunoprecipitation. The *lower* graphs depict quantification of immunoprecipitated chromatin by real-time PCR using primers pairs covering the NBRE in the proximal Skp2 promoter. Cycle threshold (C_t) values were normalized to C_t values of input samples and presented as mean ± S.D. (*, $p < 0.05$ *versus* time point 0 h, $n = 3$). Controls included PCR performed on non-precipitated genomic DNA (*Input*) or on chromatin immunoprecipitated with non-immune IgG (*IP IgG*).

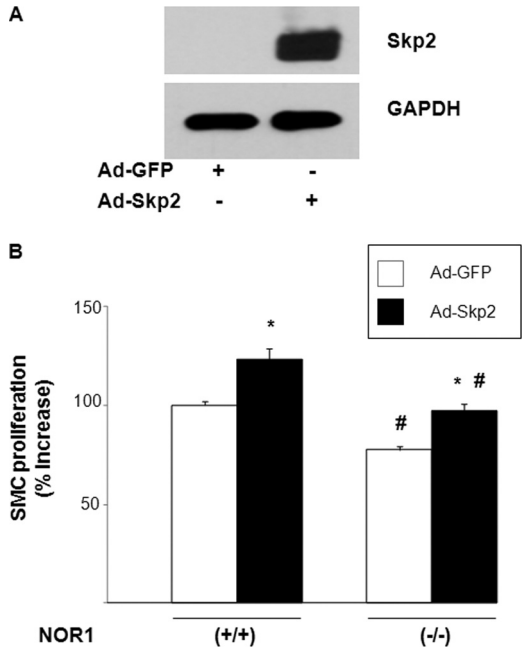


FIGURE 6. **SKP2 overexpression rescues proliferation of *Nor1*-deficient VSMC.** Mouse aortic VSMC were isolated from littermate wild-type mice and *Nor1*-deficient mice. Equal numbers of plated cells were infected with adenoviral vectors expressing SKP2 (Ad-Skp2) or GFP as control (Ad-GFP). VSMC were maintained in DMEM supplemented with 10% FBS. After 6 days, the cells were harvested, and cell proliferation was analyzed by cell counting using a hemocytometer. Cell proliferation was expressed as percent increase compared with wild-type VSMC infected with Ad-GFP and presented as mean ± S.E. (*, $p < 0.05$ *versus* Ad-GFP; #, $p < 0.05$ *versus Nor1* wild-type, $n = 3$).

dent cyclin D1 expression induces Rb phosphorylation and subsequent E2F release, resulting in an E2F-dependent activation of the *Skp2* promoter.

Our observation that NOR1 overexpression induces a decline of p27 abundance is consistent with the established activity of SKP2 to promote p27 degradation (6, 8, 9). These findings provide an important mechanistic link between the induction of NOR1-dependent *Skp2* expression in response to mitogenic signaling and downstream p27 protein abundance. Maintaining elevated p27 levels prevents neointima formation following vascular injury (10). Conversely, recent work by Wu *et al.* (24) has outlined that *Skp2* deficiency increases p27 protein levels and inhibits VSMC proliferation. Moreover, *Skp2* deficiency in mice limits aberrant proliferative vascular remodeling, whereas SKP2 overexpression enhances medial VSMC proliferation (13). Although these previous reports confirmed that SKP2-mediated degradation of p27 is necessary for VSMC proliferation, our current *in vivo* observations suggest a specific requirement of NOR1 for *Skp2* expression during proliferative vascular remodeling. In consonance with recent work, *Skp2* transcript levels were highly induced following vascular injury (24); however, this regulated *Skp2* expression was dependent on the presence of NOR1. Considering this transcriptional regulation of *Skp2* by NOR1 *in vivo*, our recent demonstration that *Nor1*-deficient mice develop less neointima in response to endovascular injury is in line with the smaller neointima formation reported previously in *Skp2*-deficient mice (20). Collectively, these findings add a third layer of regulation to the *Skp2*-p27 pathway and suggest that the early response gene NOR1 constitutes a key upstream transcriptional regulator of *Skp2* expression in VSMC.

In summary, data provided in the present study indicate that the activation of the *Skp2*-p27 pathway in response to mitogenic stimulation of VSMC and during neointima formation is mediated at least in part by NOR1. These findings provide an important transcriptional mechanism linking mitogenic signaling pathways released at sites of vascular injury to cell cycle progression and VSMC proliferation.

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