Nuclear Receptor Co-repressor Is Required to Maintain Proliferation of Normal Intestinal Epithelial Cells in Culture and Down-modulates the Expression of Pigment Epithelium-derived Factor*[S]

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Stem cells of the gut epithelium constantly produce precursors that progressively undergo a succession of molecular changes resulting in growth arrest and commitment to a specific differentiation program. Few transcriptional repressors have been identified that maintain the normal intestinal epithelial cell (IEC) proliferation state. Herein, we show that the nuclear receptor co-repressor (NCoR1) is differentially expressed during the proliferation-to-differentiation IEC transition. Silencing of NCoR1 expression in proliferating cells of crypt origin resulted in a rapid growth arrest without associated cell death. A genechip profiling analysis identified several candidate genes to be up-regulated in NCoR1-deficient IEC. Pigment epithelium-derived factor (PEDF, also known as serpinf1), a suspected tumor suppressor gene that plays a key role in the inhibition of epithelial tissue growth, was significantly up-regulated in these cells. Chromatin immunoprecipitation experiments showed that the PEDF gene promoter was occupied by NCoR1 in proliferating epithelial cells. Multiple retinoid X receptor (RXR) heterodimers interacting sites of the PEDF promoter were confirmed to interact with RXR and retinoid acid receptor (RAR). Cotransfection assays showed that RXR and RAR were able to transactivate the PEDF promoter and that NCoR1 was repressing this effect. Finally, forced expression of PEDF in IEC resulted in a slower rate of proliferation. These observations suggest that NCoR1 expression is required to maintain IEC in a proliferative state and identify PEDF as a novel transcriptional target for NCoR1 repressive action.

The intestinal epithelium consists of a cell monolayer organized in crypts and villi. This epithelium is under constant and rapid renewal, which is assured by constant division of the stem cells located at the base of the crypts (1). The descendant progenitor cells are progressively instructed to differentiate to exert their functional role during their journey along the villus compartment (2). The proliferation-to-differentiation transition of single progenitor cell is tightly regulated by morphogens, growth factors and hormones that impact on intracellular signaling pathways. Molecular alterations of specific components from these different classes of molecules are suspected to be important during the development of intestinal cancer (3).

In vivo studies have demonstrated the role of steroid hormones and ligands during intestinal epithelial development and homeostasis (4). For example, the thyroid hormone exerts a positive effect on gut mucosal maturation (5) and enterocyte differentiation (6, 7). Members of the nuclear hormone receptor superfamily are activated by metabolically transformed lipids that are absorbed by intestinal epithelial cells (IEC)[4] before interacting with their receptors and associated gene targets (8). Peroxisome proliferator-activated receptors (PPARs) are well-described examples of lipophilic ligand binding transcription factors that can influence intestinal epithelial proliferation, differentiation (9, 10), and inflammation (11). These nuclear receptors can repress gene transcription via the formation of a well defined co-repressor protein complex (12). One major player of that repression activity is the nuclear receptor co-repressor (NCoR1) that was originally identified for its potential to repress genes via physical interaction with the thyroid hormone receptor (13). However, there is now growing evidence that NCoR1 can repress transcription through interaction with several other classes of transcriptional activators including activator protein (AP)-1 and NF-κB (14, 15). One additional important class of nuclear receptors that are recruiting NCoR1 repressive action is the retinoid receptors (16). These receptors are composed of either RAR-RXR heterodimers or RXR-RXR homodimers and can repress transcription in a ligand-independent manner. In absence of co-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1.

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[4] The abbreviations used are: IEC, intestinal epithelial cells; AP-1, activator protein-1; β2-mic, β2-microglobulin; ChIP, chromatin immunoprecipitation; eGFP, enhanced green fluorescence protein; HDAC, histone deacetylase; NCoR1, nuclear receptor co-repressor; PEDF, pigment epithelium-derived factor; PPAR, peroxisome proliferator-activated receptors; PBGD, porphobilinogen deaminase; qRT-PCR, quantitative reverse-transcription polymerase chain reaction; RAR, retinoid acid receptor; RXR, retinoid X receptor; SMRT, silencing mediator of retinoid and thyroid receptors; EMSA, electrophoretic mobility shift assay.
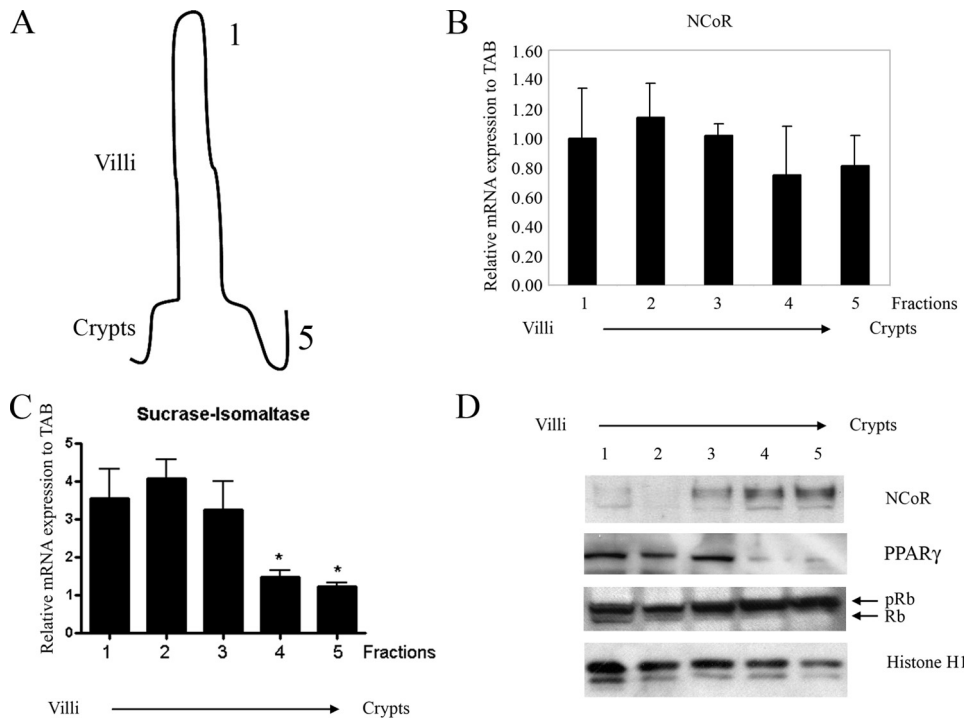
FIGURE 1. **NCoR1 protein is down-modulated along the villus-to-crypt intestinal axis.** *A*, schematic of the intestinal epithelial fractions isolated along the villus-to-crypt axis by the Weiser method. Total RNA from isolated epithelial fractions ($n = 3$) was used to monitor mRNA levels of the NCoR1 (*B*) and sucrase-isomaltase (*C*) genes. *D*, protein extracts were isolated from isolated epithelial fractions and subjected to Western blot analyses with specific antibodies for the detection of NCoR1, PPARγ, and Rb. Specific detection of histone H1 was done to control for protein loading. *, $p < 0.05$.

repressors, these receptors are involved in the differentiation of various epithelia (17, 18).

Ncor1 gene deletion in mice resulted in an impairment of neural stem cell proliferation and spontaneous differentiation into astrocytes (19). Other reports have recently suggested an antiproliferative role for NCoR1 in hepatocytes (20) and thyroid tumor cells (21). Thus, NCoR1 may influence cell proliferation by affecting different gene targets in specific cellular contexts.

Because many NCoR1-interacting partners are crucial to the regulation of many IEC functions, we sought to evaluate the functional role of NCoR1 in this specific context. We provide here the evidence that NCoR1 nuclear expression is associated with proliferative and non-differentiated epithelial cells and that NCoR1 silencing by RNA interference causes cells to growth arrest. The pigment epithelial-derived factor (PEDF), a 50-kDa member of the serine protease inhibitor family, was further identified as a transcriptional target for NCoR1 repressive action during this process. Ectopic expression of PEDF in IEC reduced the proliferation rate, an observation that was consistent with the tumor suppressor properties of this regulator in epithelial tissues (22).

EXPERIMENTAL PROCEDURES

Cell Culture—Caco-2/15 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. IEC-6 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum and 0.1 units/ml insulin. In both cases, the media were supple- mented with 4.5g/liter D-glucose, 25 mM HEPES, 50 units/ml penicillin, and 50 μg/ml streptomycin. Cells were kept subconfluent for 5–10 passages. All cell lines were grown at 37 °C in 5% CO_2. Cell proliferation assays were manually performed using a hemacytometer in the presence of 0.4% trypan blue to account for cell viability. Inhibition of proteasome activity was performed with supplementation of 50 μM MG132 into the cell culture medium (Sigma-Aldrich).

Cell Fractionation Along the Crypt-villus Axis—Animal experimentation was approved by the Institutional Animal Research Review Committee in conformity with the Canadian Council on Animal Care. CD-1 wild-type mice obtained from Charles River Laboratories (Wilmington, MA) were sacrificed and the jejunum harvested, inverted onto polyethylene tubing, ligatured at both extremities, and washed with KRB buffer, pH 7.5, as described previously (23). Segments were then incubated under agitation in ice-cold isolation buffer (2.5 mM EDTA, 0.25 mM NaCl) for 2-min intervals. After each interval, cell suspensions were centrifuged at $400 \times g$ for 5 min. Pellets were then washed with ice-cold KRB buffer and lysed for either nuclear protein or total RNA isolation (23).

RNA Analysis—Total RNA was isolated from cultured cells as described previously (23). Reverse transcription reactions were carried out at 42 °C for 1 h in the presence of 1 μg of RNA, 40 milliunits of poly-oligo(dT)$_{12-18}$ (Amersham Biosciences, Baie d'Urfé, QC) and 40 units of reverse transcriptase (Roche Molecular Biochemicals). PCR reactions were performed in a total volume of 25 μl with 1 μl of the RT reaction, 1 unit of TaqDNA polymerase (New England Biolabs, Pickering, ON), and 100 ng of each specific oligonucleotide. Real-time PCR was performed using a Lightcycler apparatus (Roche Molecular Biochemicals) as described previously (23). Experiments were run and analyzed using the Lightcycler software 4.0 (relative quantification monocolor) according to the manufacturer's instructions (Roche Molecular Biochemicals). Double-stranded DNA amplification during PCR was monitored using SYBR Green I (QuantiTect SYBR Green PCR Kit; Qiagen, Valencia, CA) and PCR amplification programs designed as described in the QuantiTect SYBR Green PCR Handbook (Qiagen). A serial dilution of a calibrator sample was used for the standard curve for each gene analyzed, which was then used to correct for the differences in the efficiency of the PCR. Primers sequences are available upon request.

Western Blotting and Antibodies—Cell protein fractionation (cytosol, membranes and organelles, nucleus, and cytoskele-
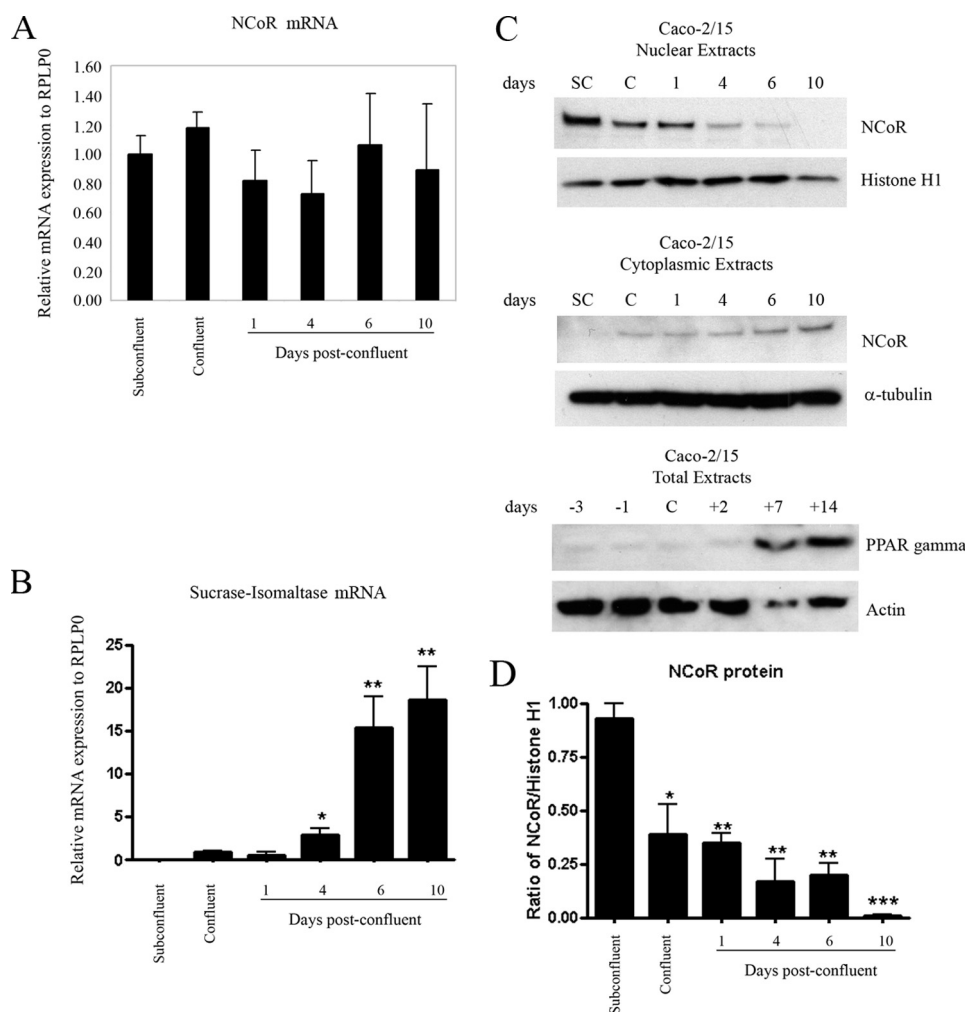
FIGURE 2. **NCoR1 protein is down-modulated during the proliferation-to-differentiation transition of Caco-2/15 cells in culture.** Total RNA from Caco-2/15 cells at different stages of confluence was used to monitor mRNA levels of the NCoR (*A*) and sucrase-isomaltase (*B*) genes (*n* = 3). *C*, subcellular fractions of Caco-2/15 cells were harvested at different stages of confluence. Western blots were performed with NCoR1, histone H1, and α-tubulin antibodies. Total extracts of Caco-2/15 cells were subjected to Western blot with PPARγ antibody. Specific detection of actin was done to control for protein loading. *D*, nuclear NCoR1 protein levels were quantified and calibrated with the histone H1 protein level (*n* = 3). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ton) was performed with the ProteoExtract subcellular proteome extraction kit according to the manufacturer's instructions (Calbiochem, EMD Biosciences, San Diego, CA). 5 μg of protein extract was analyzed by 3–8% Tris acetate NuPAGE (Invitrogen, Burlington, ON) and transferred onto a polyvinylidene difluoride membrane (Roche Molecular Biochemicals). Western blot was performed as described previously (23). NCoR1 and SMRTe antibodies were purchased from Millipore (Upstate). Actin, histone H1, and PPARγ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Plasmid Construction and Cell Infection—Three different sets of shRNA oligonucleotides were designed for rat NCoR1 targeting according to Ambion guidelines (technical bulletin 506) using the siRNA sequences tacttgccttacttcttca (1), aaaccaaagctgatcaaca (2), or gcagaactacttaggaact (3) with ttcaagaga as the loop sequence (see Fig. 3*A*). The oligonucleotide-annealed products were subcloned into pLenti6-U6 (23) between the BamHI and XhoI sites, giving rise to pLenti6-shNCoR. Lentiviruses were produced and used for cell infection according

to Invitrogen recommendations (ViraPower lentiviral expression system instruction manual). The cDNA of rat PEDF was PCR-amplified from IEC-6 total RNA and subcloned into BamH1 and EcoRI restriction sites of the retroviral vector pBabe-puro. The HEK 293T cell line was used for transfection with Lipofectamine 2000 (Invitrogen) and both the retroviral DNA construct and helper amphotropic DNA as described previously (24). Subconfluent IEC-6 cells were infected with either an empty vector (control) or PEDF recombinant viruses in the presence of 2 μg/ml of polybrene (Sigma-Aldrich). Two days after infection, cells were selected with 2 μg/ml puromycin (Sigma-Aldrich).

Microarray Screening and Data Analysis—Probes for the microarray analysis were generated from isolated RNA obtained 2 days after RNAi-dependent shutdown of NcoR1 expression. Affymetrix GeneChip® Rat Genome 230 2.0 arrays were screened with the generated probes via the microarray platform of McGill University and Génome Québec Innovation Center as described previously (25). To test for changes in signal intensity, compiled data (RMA analysis) were screened using the software available on the microarray platform website. Genes were then filtered for up- or down-regulation of expression of a minimum of 2-fold and a minimal magnitude change of 200 fluorescence units between control and NCoR1 knockdown cells.

Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitation assays (ChIP) were performed using the protocol from the ChIP assay kit (Upstate, VA). Subconfluent and confluent Caco-2/15 cells were incubated with 1% formaldehyde for 10 min at 37 °C for DNA cross-link. Chromatin was immunoprecipitated with rabbit IgG (Santa Cruz Biotechnology) or with NCoR1 antibody (catalog 06–892; Millipore, Upstate, VA). One percent of the lysate was kept to control the amount of DNA used for immunoprecipitation (Input). Immunoprecipitated DNA was purified and diluted 1:20 before PCR amplification. The following sequences were used for PEDF promoter amplification; −878 up: 5′-gaggcaggagaacctcttga-3′; −529 down: 5′-cgagaccccgtctcaaaaa-3′; −411 up: 5′-gccaacacacctgggtaattt; and −81 down: 5′-cacacatttgcacaccttcc-3′.

EMSA—Electrophoretic mobility shift assays (EMSA) were performed essentially as described previously (26, 27) with
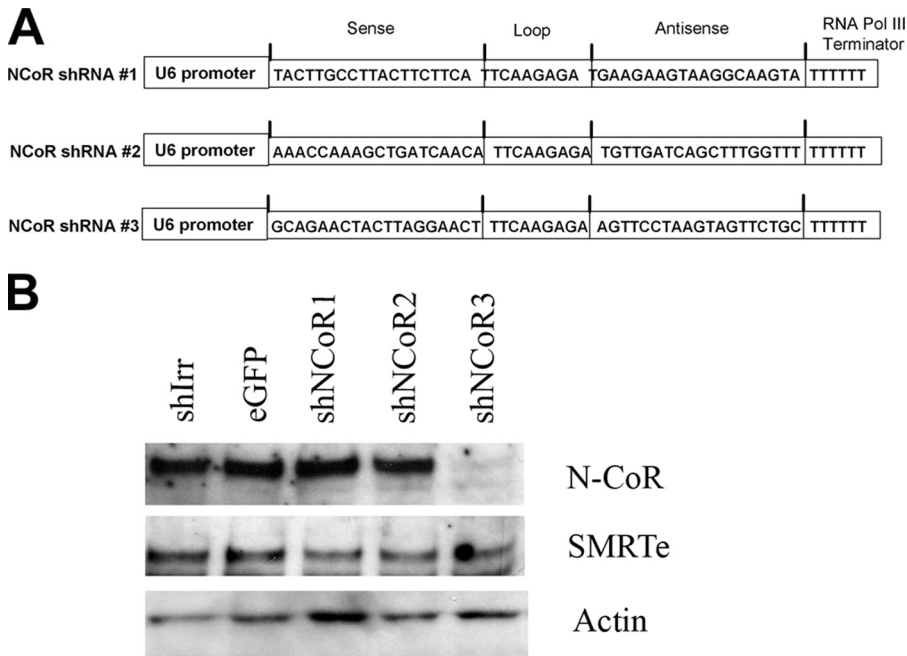
A

B

FIGURE 3. **NCoR1 is efficiently down-modulated in IEC-6 cells by RNA interference.** *A*, NCoR1 shRNA sequences (1, 2, and 3) were designed to construct lentiviral-shRNA vectors. *B*, Western blot analysis was performed with NCoR1, SMRTe, and actin polyclonal antibodies on total cell lysates obtained from populations of infected cells with lentiviruses that contained eGFP, shRNA NCoR 1, shRNA NCoR 2, shRNA NCoR 3, or shRNA irrelevant sequence (*Irr*).

some modifications. The reactions were performed in a volume of 24 μl of binding buffer D (10 m$_M$ Hepes pH 7.9, 10% glycerol, 0.1 m$_M$ EDTA, and 0.25 m$_M$ phenylmethylsulfonyl fluoride) containing 4 μg of nuclear protein extracts from 293T cells transfected or not with RXRα (Open Biosystems, MHS1010-98051588) and RARα (Open Biosystems, MHS1010-97228051) expression vectors, 50 m$_M$ KCl, 50 ng of poly(dI-dC), and 25,000 cpm of ^{32}P-labeled DNA probes for 10 min. For the supershift analysis, 600 ng of RXRα (D20SC-553) or RARα (C20SC-551) antibodies (Santa Cruz Biotechnology) were added, and the binding reactions were pursued for 10 min at room temperature. Retarded complexes were then separated on a 5% polyacrylamide gel at 4 °C for 4 h, dried for 1 h at 80 °C, and exposed overnight on a Molecular Imager FX screen (Bio-Rad). The running buffer used was a Tris-glycine 0.5× buffer (0.2 $_M$ glycine, 0.025 $_M$ Tris, and 1 m$_M$ EDTA). The DNA probes consisted of double-stranded oligonucleotides of 8 potential RXR binding sites within the promoter region of the human *PEDF* gene (Fig. 6).

Cotransfections and Luciferase Assays—The human *PEDF* gene promoter was amplified by PCR from purified genomic DNA isolated from human fetal colon. The primers used from the amplification included positions −995 to +1 relatively to the transcriptional initiation site. The PCR product was subcloned in the pGL3basic luciferase reporter vector (Promega). Integrity of the subcloned PCR product was confirmed by sequence analysis. 293T cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Cells at 50% confluence wre incubated with 0.2 μg of luciferase reporter, 0.1 μg of RARα, and/or RXRα expression vectors, 0.2 μg of NCoR1 expression vector, 0.018 μg of the pRL SV40 *Renilla* luciferase vector (Promega), and a constant total DNA amount of 0.8 μg per transfected well in the presence of 2 μl of Lipofectamine 2000/100 μl of OptiMEM. The medium was replaced with Dulbecco's modified Eagle's medium containing 10% fetal bovine serum after an incubation of 4 h. The luciferase and *Renilla* activities were determined 48 h after the transfection using the dual luciferase assay kit (Promega Biotech). Each experiment was repeated three times with five replicates.

Statistical Analysis—All data were expressed as mean ± S.E. Groups were compared using the Student's *t* test (GraphPad Prism 4, GraphPad Software, San Diego). Statistical significance was defined as $p < .05$.

RESULTS

NCoR1 Protein Profile of Expression Is Restricted to Non-differentiated Proliferating IEC—To investigate the profile of NCoR1 expression during epithelial cell differentiation, populations of epithelial cells from the mouse small intestinal mucosa were progressively isolated along the villus-to-crypt axis using a modified Weiser procedure (23) (Fig. 1*A*). Total RNA was isolated from these epithelial cell populations, and NCoR1 gene transcript levels were assessed by real-time RT-PCR analyses. No significant change in NCoR1 mRNA expression was detected along the villus-to-crypt axis (Fig. 1*B*) in contrast to the induction of the sucrase-isomaltase gene transcript in the villus epithelial cell fractions, a specific marker of intestinal epithelial cell differentiation (Fig. 1*C*). Because it has been previously reported that NCoR1 regulation of expression could be dependent on post-transcriptional mechanisms in other systems (28), we next evaluated the NCoR1 protein profile of expression in intestinal epithelial cell fractions that were progressively harvested from the villus-to-crypt axis. NCoR1 protein was detectable in mouse crypt IEC fractions and disappeared in the most upper villus-associated epithelial fractions (Fig. 1*D*). The decrease of NCoR1 protein expression correlated with the detection of faster migrating dephosphorylated forms of the Rb protein that are normally associated with non-cycling G1-arrested cells (29) and the induction of the PPARγ isoform, an inhibitor of IEC proliferation (9, 10) (Fig. 1*D*). We next undertook to explore the pattern of NCoR1 expression during the proliferation-to-differentiation transition of pure intestinal epithelial cells in culture. The Caco-2/15 cell line that spontaneously differentiates into enterocytes upon reaching confluence was chosen because it has been extensively used as a model of IEC differentiation (30, 31). Again, no significant change in NCoR1 gene transcript expression was detected during the proliferation-to-differentiation transition of Caco-2/15 cells in culture (Fig. 2*A*). The
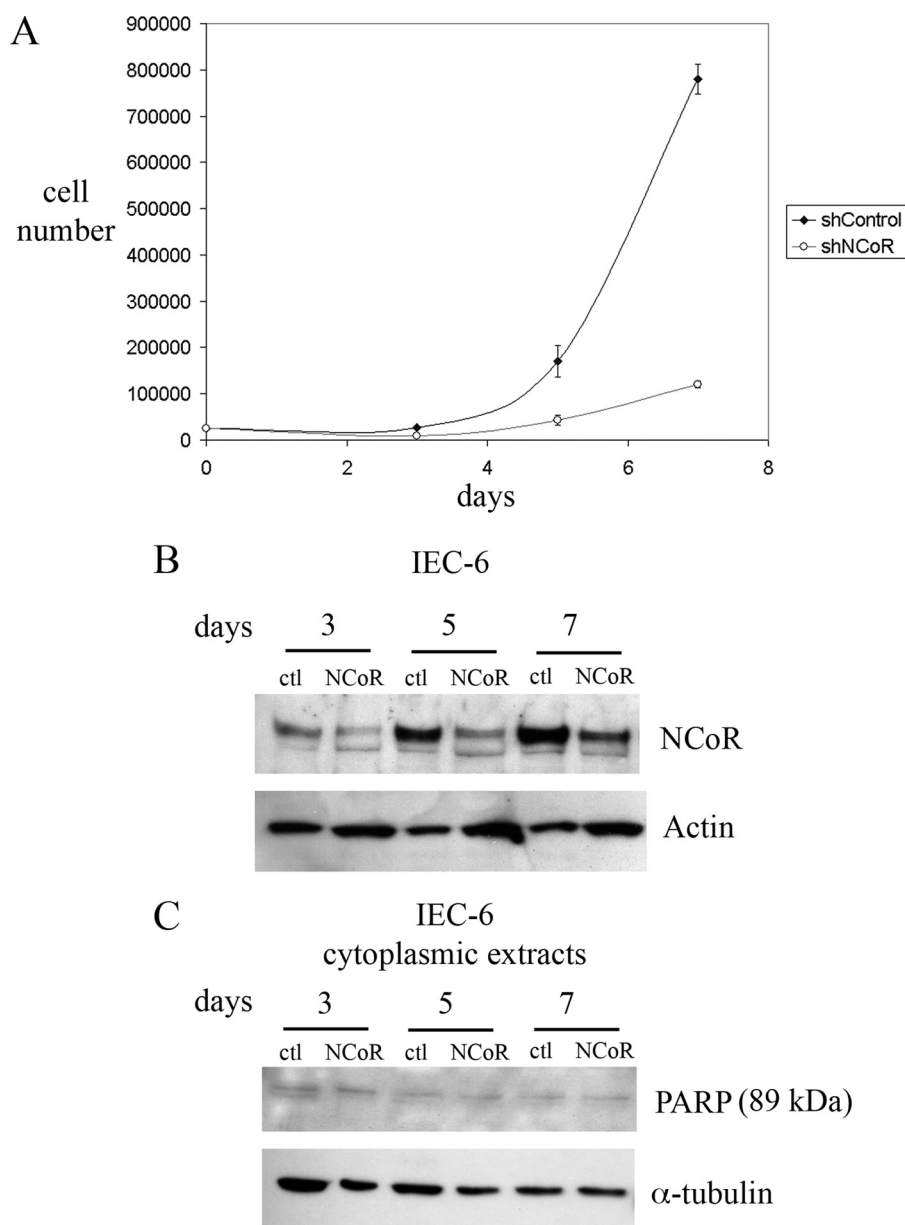
FIGURE 4. **Knockdown of NCoR1 reduces IEC-6 cell proliferation.** *A*, IEC-6 cells were infected with shNCoR 3 (*NCoR*) and control (*ctl*) shRNA lentiviruses, and cells were subsequently counted at different days (representative of three independent experiments). Protein extracts were isolated at several days during the kinetics and subjected to Western blot analysis with the use of NCoR1 (*B*) and PARP (*C*) antibodies. Actin and α-tubulin antibodies were also used to control for protein loading.

Down-modulation of NCoR1 in Intestinal Epithelial Crypt Cells Reduces Cellular Proliferation— The functional relationship between NCoR1 protein expression and maintenance of cellular proliferation was next investigated. The non-transformed IEC-6 cell line was further used because it has been described to phenotypically correspond to intestinal epithelial crypt cells (32) and has been extensively used as a model to study normal and non-cancerous IEC proliferation. We sought to directly neutralize NCoR1 expression by an RNA interference approach. In general, IEC-6 cell transfection with an enhanced green fluorescence protein (eGFP) expression vector resulted in poor efficiency of detection. However, stable infection of a lentivirus-eGFP into IEC-6/Cdx2 cells consistently resulted in over 90% of positive eGFP cells among the cell population. We thus generated lentiviral constructs that contained shRNA sequences under the control of a U6 promoter that were predicted to target the rat Ncor1 mRNA (Fig. 3*A*). Three independent shRNA NCoR1 lentiviruses were tested to down-regulate NCoR1 synthesis. A Western blot was performed with total extracts obtained from short term infected IEC-6 cell populations. The NCoR1 protein level was efficiently decreased in IEC-6 cells infected with the shRNA lentivirus 3 as opposed to cells infected with an eGFP lentivirus, an irrelevant shRNA lentivirus and two other NCoR1-specific shRNA constructs (Fig. 3*B*). The silencing mediator of retinoid and thyroid receptors (SMRT), a functionally related family member of NCoR1 (33), was not influenced under these specific conditions. The specific shRNA/NCoR3 construct was further utilized to efficiently interfere with NCoR1 expression. Stable IEC-6 cell populations were first generated with either NCoR1 shRNA or control shRNA lentiviruses. Long term antibiotic resistance selection of these recombinant cells consistently resulted in poor cell recovery of the NCoR1 shRNA populations. In addition, these cells progressively overcame the loss of NCoR1 protein expression (data not shown). A short term strategy was thus chosen to investigate whether the loss of NCoR1 could impact on cell proliferation and/or cell death. IEC-6 cells were infected with either control or shRNA/NCoR#3 lentiviruses and distributed into 6-well tissue culture

sucrase-isomaltase gene transcript was strongly increased during this process (Fig. 2*B*) as has extensively been reported in the past (30, 31). We then investigated the NCoR1 protein profile during cell growth arrest and differentiation. Nuclear and cytosolic protein fractions were harvested at different Caco-2/15 cell confluence stages and subjected to Western blot analysis. Nuclear NCoR1 protein levels significantly decreased during the proliferation-to-differentiation transition of Caco-2/15 cells in culture, a pattern that was not observed for cytosolic NCoR1 protein levels (Fig. 2, *C* and *D*). In contrast, PPARγ protein was strongly induced in postconfluent-differentiated cells (Fig. 2*C*). Taken together, these observations indicate that NCoR1 protein expression was associated with proliferating, non-differentiated IEC.
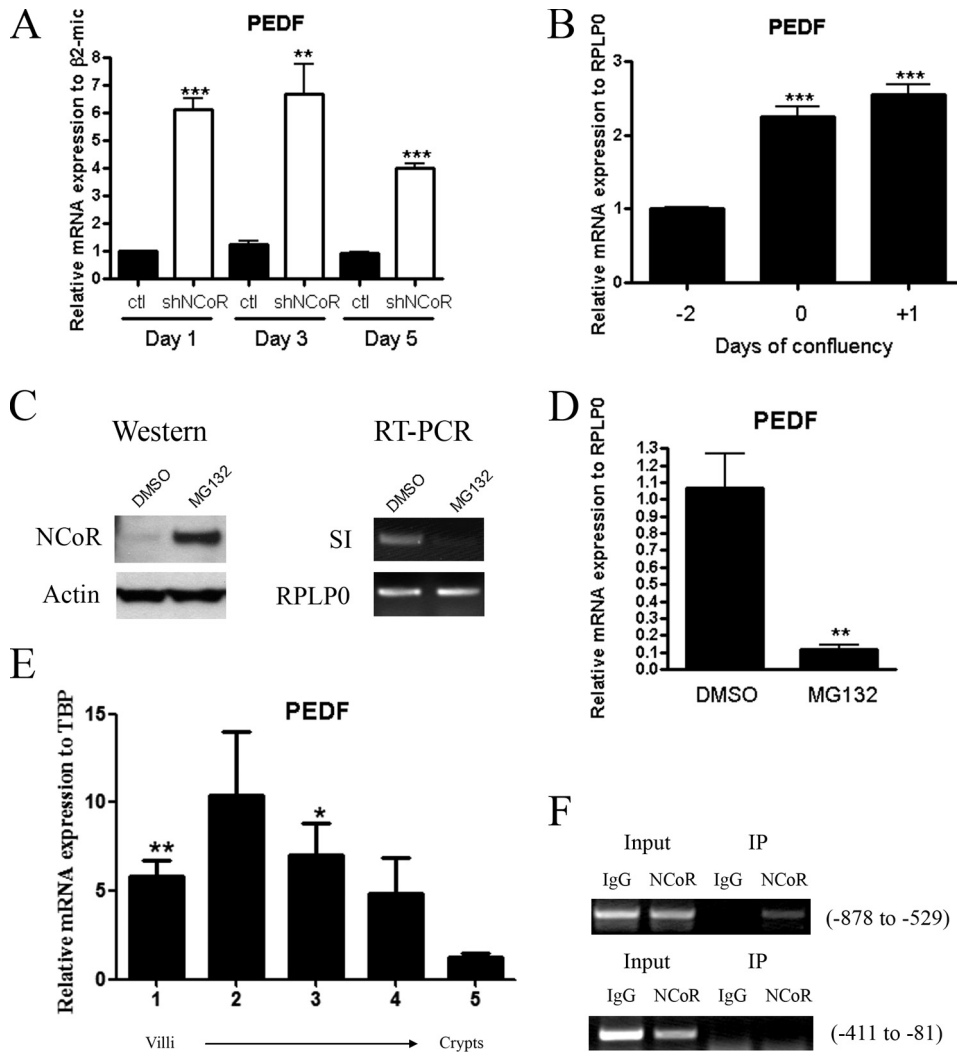
FIGURE 5. **PEDF is a novel target gene for NCoR1 repressive action.** *A*, total RNA was isolated from shNCoR 3 and control shRNA cells at different days following lentiviral infection and real time RT-PCR was performed ($n = 3$). *B*, total RNA was isolated from Caco-2/15 cells before (-2) during (0) and after reaching confluence and real time RT-PCR was performed ($n = 3$). *C*, total protein and RNA were isolated from newly confluent Caco-2/15 cells supplemented for 24 h with vehicle (DMSO) or 50 μM MG132. Western blot analysis was performed with NCoR1 and actin polyclonal antibodies. RT-PCR analysis was performed with sucrase-isomaltase (*SI*) and RPLP0-specific primers. Representative results of three independent experiments are illustrated. *D*, total RNA isolated as described in *C* was used to monitor for PEDF mRNA expression. *E*, total RNA from intestinal epithelial fractions isolated along the villus-to-crypt axis ($n = 3$) was used to monitor mRNA levels of the *PEDF* gene. *F*, chromatin immunoprecipitation assays were performed with subconfluent Caco-2/15 cell chromatin. Chromatin was immunoprecipitated with the NCoR1 antibody or with rabbit IgG as a negative control. Purified immunoprecipitated chromatin was subjected to PCR amplification of the *PEDF* gene. 10% of the chromatin extract (*Input*) was also amplified by PCR to determine the amount of DNA prior to immunoprecipitation. A representative result of three independent experiments is illustrated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

to identify significant changes at the protein level in absence of NCoR1 (data not shown). To better characterize the nature of the molecular changes occurring during the silencing of NCoR1 expression and subsequent reduction of crypt IEC proliferation, a gene expression profiling was thus performed. IEC-6 cells were freshly infected with NCoR1 or control shRNA lentiviruses and total RNA was harvested 3 days following infection. The Gene-Chip® Rat Genome 230 2.0 Arrays (Affymetrix) containing more than 30,000 transcripts from the rat genome were used to screen for mRNA expression variations after *Ncor1* was down-modulated. This analysis identified 395 gene targets predicted to be modulated by more than 2.5-fold (supplemental Table S1). Of these, *PEDF*, also known as serpinF1 or Dmrs91, was predicted to be induced more than 7-fold in cells silenced for NCoR1 expression. This target was further focused upon because of its potent and well documented role in cellular growth inhibition (22). Total RNA was isolated at different days following IEC-6 cell lentiviral infection, and the level of PEDF mRNA expression was evaluated by real-time RT-PCR. Expression of *PEDF* was significantly induced more than 6-fold in cell populations that were silenced for NCoR1 as compared with controls (Fig. 5*A*). In addition, *PEDF* mRNA expression was significantly increased during the proliferation-to-differentiation transition of Caco-2/15 cells (Fig. 5*B*). NCoR1 protein stability is well documented to be dependent on the proteasome

plates. An inhibition of cell growth was rapidly observed for shRNA/NCoR3 cells that persisted for several days when compared with control infected cells (Fig. 4*A*). Western blot confirmed that NCoR1 expression was reduced in these cell populations during the kinetics (Fig. 4*B*). No significant increase in cell death was observed between control and NCoR1 knockdown cells as determined by the trypan blue exclusion test or by detection of the cytoplasmic specific 89-kDa cleaved form (34) of polyadenosine diphosphate-ribose polymerase (PARP) (Fig. 4*C*).

The Tumor Suppressor PEDF Gene Is a Novel Transcriptional Target for NCoR1—An initial screen for variation of crucial cell cycle regulators such as p21, p27, and cyclin D was unsuccessful

(28). The effect of the peptide-aldehyde MG132, a potent inhibitor of the 20 S proteasome, was next tested on the stability of the NCoR1 protein in newly confluent Caco-2/15 cells. The NCoR1 protein level was strongly stabilized in the presence of MG132 as compared with vehicle (DMSO)-treated cells (Fig. 5*C*). The sucrase-isomaltase gene transcript that normally becomes detectable at confluency (30, 31) was reduced more than 70 times in cells supplemented with MG132 (Fig. 5*C*). In addition, *PEDF* gene transcript was significantly reduced more than 90% under these conditions (Fig. 5*D*). To quantify the level of *Pedf* gene transcript along the crypt-villus intestinal axis, qRT-PCR was performed with total RNA extracts from isolated population of cells that were used to monitor NCoR1 expres-
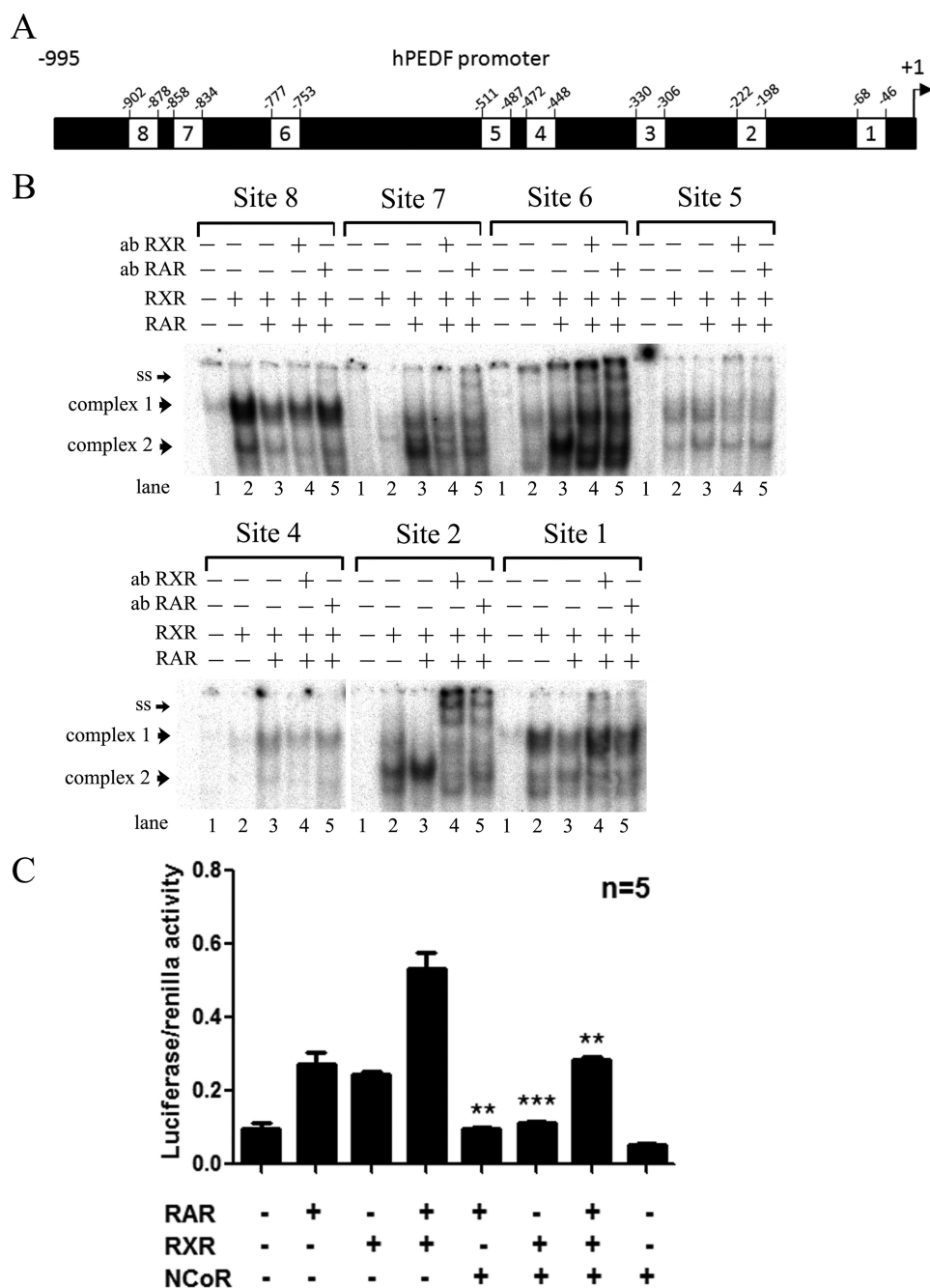
FIGURE 6. **RAR and RXR are interacting with several DNA elements of the *PEDF* gene and are mediating the transcriptional repressive effect of NCoR1.** *A*, schematic representation of the *PEDF* gene with its predicted binding sites for RXR heterodimers. *B*, nuclear protein extracts from transfected 293T cells (empty vector, RXRα, and RARα) were used for EMSA with labeled oligonucleotides. The two major retarded complexes are indicated by *arrows*. Supershift (*ss*) experiments were performed by including specific RXRα or RARα antibodies. *C*, 293T cells were transiently co-transfected with 0 or 0.1 μg of the RXRα or RARα expression vectors, 0.2 μg of NCoR1 expression vector and 0.2 μg of the pGL3basic/*PEDF* luciferase reporter construct. The pcDNA3.1 plasmid was used as an empty control vector to calibrate for the addition of the expression vectors. Cells were harvested after transfection and analyzed for luciferase activity. Data were normalized with *Renilla* values. Results obtained in five replicates are representative of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

cell chromatin was cross-linked and immunoprecipitated with the NCoR1 antibody. PCR amplification of the human *PEDF* promoter was performed on the immunoprecipitated-purified chromatin (Fig. 5*E*). NCoR1 consistently interacted with a specific region located between -878 and -529 in the *PEDF* 5′-flanking region of the promoter (Fig. 5*F*), whereas it was unefficient to interact with a more proximal region of the promoter under these conditions (Fig. 5*F*).

NCoR1 Represses the PEDF Promoter via RAR and RXR Transcription Factors—NCoR1 can repress transcription in being recruited by specific transcription factors such as nuclear receptors. To further explore how NCoR1 was able to repress the *PEDF* gene, a computer analysis was performed of 1 kb of the human *PEDF* gene. This analysis identified 8 putative RXR heterodimer binding elements (Fig. 6*A*). We then evaluated the capacity of RXR and RAR to interact with these different potential binding sites of the *PEDF* gene. EMSA was performed using double-stranded ^{32}P-labeled probes that corresponded to the predicted interacting sites. Nuclear extracts isolated from 293T cells transfected or not with RXRα and RARα expression vectors were used for the assays. When nuclear extracts containing RXRα were used, two major shift complexes were observed for most of the sites tested (complexes 1 and 2 in *lane 2* of each site, Fig. 6*B*). The intensity of complex 2 was increased for sites 1, 2, 4, 5, 6, and 7 when nuclear extracts containing both RXRα and RARα were used (complex 2 in *lane 3* of these sites, Fig. 6*B*). This complex was partially supershifted (*SS*, Fig. 6*B*) when specific RXRα or RARα antibodies were included in the binding reactions (*lanes 4* and *5* of these sites,

Fig. 6*B*). These observations suggested that 6 of 8 predicted sites could be recognized by RXR/RAR heterodimers *in vitro*. We then tested whether RXR and RAR could mediate NCoR1 transcriptional repression of the *PEDF* gene. Cotransfection experiments with individual RARα or RXRα expression vectors produced a 2.5-fold activation while combined RARα/RXRα

sion (see Fig. 1*A*). *Pedf* gene transcript was significantly induced in the villus epithelial cell fractions (Fig. 5*E*), a profile that coincided with the reduction of NCoR1 protein expression (see Fig. 1*D*). To further evaluate whether the *PEDF* gene could be a direct target of NCoR1 transcriptional repressive action, ChIP experiments were next performed. Subconfluent Caco-2/15
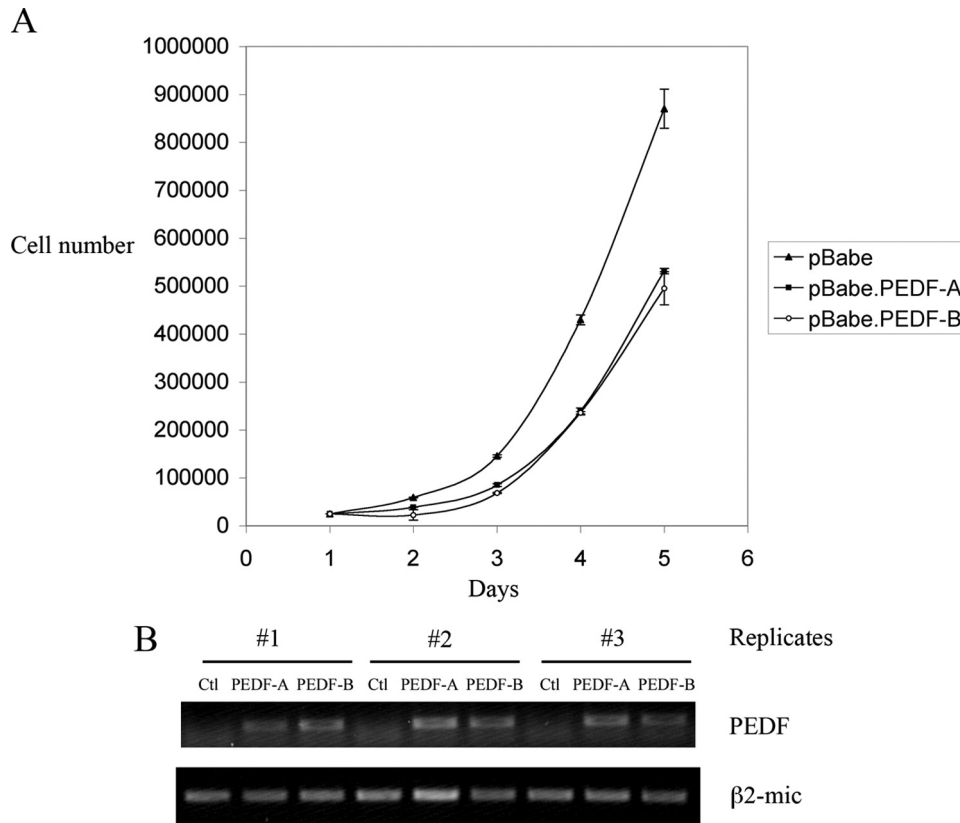
A

B

FIGURE 7. **PEDF overexpression reduces IEC-6 cell proliferation.** *A*, IEC-6 cells were infected with two independent PCR-amplified clones of rat PEDF cDNA (pBabe PEDF-A and -B) and empty vector control (pBabe) retroviruses, and cells were subsequently counted at different days (representative of three independent experiments). *B*, total RNA was isolated from three independent replicates of PEDF-A and -B or empty vector control (*ctl*) infected cells and subjected to RT-PCR for the detection of rat PEDF mRNA. β2-Microglobulin mRNA (β2-mic) was amplified as a control for RNA integrity.

resulted in a 5.3-fold activation of the *PEDF* promoter/luciferase construct (Fig. 6*C*). The addition of a NCoR1 expression vector in the cotransfection assay resulted in a complete abolition of the RARα- or RXRα-dependent activation and a 2-fold reduction of the RARα/RXRα-dependent activation of the *PEDF* promoter/luciferase construct (Fig. 6*C*).

Forced Expression of PEDF Reduces Intestinal Epithelial Crypt Cell Proliferation—The effect of PEDF on IEC proliferation was next investigated. IEC-6 cells were infected with either control or two independent rPEDF retroviruses and distributed into 6-well tissue culture plates. An inhibition of cell growth was rapidly observed in cell populations that contained the rPEDF constructs when compared with control cells (Fig. 7*A*). RT-PCR confirmed that exogenous rPEDF was indeed produced in these cell populations (Fig. 7*B*). As observed for NCoR1 knockdown IEC-6 cells, no significant increase in cell death was observed between control and PEDF-overexpressing cells as determined by the trypan blue exclusion test (data not shown).

DISCUSSION

NCoR1 is a crucial component of a multiprotein transcriptional complex well recognized to exert transcriptional repression on multiple genes in many different cellular systems (12, 33). Recently, specific roles for NCoR1 in the regulation of cell proliferation and differentiation have emerged (19, 21, 35). Our

study has provided two major and novel findings: 1) NCoR1 was crucial to maintaining intestinal epithelial crypt cell growth in culture; 2) PDEF, a putative tumor suppressor gene in stromal vasculature and epithelial systems (22), was unrepressed upon the knockdown of NCoR1 expression and caused intestinal epithelial crypt cells to reduce proliferation.

IEC fate determination is thought to be initiated by a cascade of molecular events that ultimately lead to an irreversible stop of cellular proliferation and terminal differentiation (36, 37). As depicted in many cellular systems, specific gene repression mechanisms have been associated with cell lineage specification and maintenance of cell growth properties (19, 38–41). The concept that a general transcriptional repression state can prevent IEC differentiation has been recently explored in the mouse fetal intestinal epithelium (42). This study showed that class I histone deacetylases (HDACs) are highly expressed in proliferative cells and decline in expression with the activation of differentiation. Sustained expression of class I HDACs led to a delay in the expression of certain differentiation genes whereas inhibition of class I HDACs caused premature cytodifferentiation and expression of these same genes (42). Surprisingly, the effect of neutralizing HDAC activities on IEC proliferation was not specifically addressed in this study. To our knowledge, our report is the first to functionally investigate the role of transcriptional repression on normal IEC growth.

A preliminary screen of several known critical regulators of the cell cycle was not successful in identifying candidate genes that might be affected by the loss of nuclear NCoR1 during the reduction of IEC proliferation (data not shown). In addition, no intestinal epithelial differentiation-associated genes were found to be induced following abrogation of NCoR1 expression. This was not surprising because cultured intestinal epithelial crypt cells obviously require the action of crucial intestinal epithelial transcriptional activators such as Cdx2, Hnf1α, and Hnf4α (43–45) that were not induced following the removal of NCoR1 expression (data not shown). A careful analysis of the target genes susceptible to being modulated by the absence of NCoR1 as predicted from the gene-profiling analysis confirmed that the loss of NCoR1 did not impact on differentiation in this particular context. However, an important target gene was identified from this analysis. Indeed, the *PEDF* gene transcript was confirmed to be significantly increased in cells that had lost NCoR1 and subsequently reduced proliferation.

PEDF is a member of the serine protease inhibitor family identified nearly 20 years ago for its ability to potently differentiate retinoblastoma cells in culture (46). It was later discovered that PEDF also had a powerful anti-angiogenic activity (47). More recently, PEDF-deficient mice convincingly demonstrated that this factor could act as a tumor suppressor in specific tissues such as the prostate and pancreas (22). *PEDF* gene therapy was shown to be effective in reducing human pancreatic tumor growth (48) and osteosarcoma (49) in mice. A similar role was also reported for human ovarian cancer cells in culture (50). Because we have provided evidence that PEDF is expressed in IEC and can negatively impact on IEC proliferation in culture, it is obvious that the putative role of this molecule in anticancer in general will have to be thoroughly investigated in the context of gut homeostasis and disease in the near future.

How does NCoR1 exactly maintain proliferation of IEC? It is tempting to speculate that a transcriptionally competent NCoR1 complex could constitutively act through *PEDF* gene repression thereby preventing the autocrine negative effect of PEDF on proliferation in the IEC context. Very little is known of the molecular mechanisms responsible for mediating the PEDF anti-proliferative effect on cells (51). One single report from the literature suggests that PEDF could suppress fibroblast cell cycle progression through a blockage into the G(0) state (52). The promoter region of PEDF was shown in our study to interact with NCoR1. Several nuclear receptors have been reported to mediate NCoR1 repressive transcriptional action on various gene promoters (53). PPARγ is one important NCoR1-recruiting protein but its pattern of expression was opposite to NCoR1 during Caco2 cells differentiation (Fig. 2). We identified several RAR/RXR DNA elements that could be responsible for the NCoR1-dependent repression of the *PEDF* gene. This observation is in accordance to the reported role of vitamin A and retinoic acid on PEDF expression in retinal epithelial cells (54, 55). Our findings lead us to conclude that NCoR1 can maintain IEC proliferation with efficient repression of the *PEDF* gene. A complete understanding of how PEDF can influence cell division will have to be resolved. These future investigations will directly impact on stem cell biology (56) and will contribute to a better understanding of how the intestinal epithelium can constantly regenerate throughout the life of an individual.

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