Transcriptional Regulation of the Human α6 Integrin Gene by the Transcription Factor NFI during Corneal Wound Healing

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Purpose. Wound healing of the corneal epithelium is highly influenced by regulation of integrin gene expression. A recent study demonstrated that laminin (LM), a major constituent of the extracellular matrix (ECM), reduces expression of the human α6 integrin subunit gene by altering the properties of the transcription factor (TF) Sp1. In this work, a target site was identified for the TF nuclear factor I (NFI) on the human α6 gene, and its regulatory influence was characterized in corneal epithelial cells.

Methods. Plasmids bearing the α6 promoter fused to the CAT gene were transfected into human (HCECs) and rabbit (RCECs) corneal epithelial cells grown on LM. The DNA-binding site for NFI in the α6 promoter was identified by DNase I footprinting. Expression and DNA binding of NFI was monitored by Western blot, RT-PCR, and electrophoretic mobility shift assays (EMSAs), and its function was investigated through RNAi and NFI overexpression assays.

Results. All NFI isoforms were found to be expressed in HCECs and RCECs. Transfection analyses revealed that NFI is a repressor of α6 expression in both types of cells. LM increases expression of NFI, whereas inhibition of each NFI isoform increases promoter activity suggesting that NFI is a key repressor of α6 transcription. In addition, the negative influence of NFI appears to be potentiated by the degradation of Sp1 when cells are grown on LM.

Conclusions. Repression of α6 expression therefore contributes to the final steps of corneal wound healing by both reducing proliferation and allowing attachment of the epithelium to the basal membrane. (*Invest Ophthalmol Vis Sci.* 2008; 49:3758–3767) DOI:10.1167/iovs.08-1913

The corneal epithelium is composed of well-organized and structured epithelial cells.[1,2] When a wound occurs, the epithelial cells are triggered to restore proper visual acuity.[3,4] This process is promoted by the extracellular matrix (ECM), which constitutes the basal membrane (BM) of the epithelium.[5,6] The early events of the wounding process are characterized by massive secretion of fibronectin (FN), which is believed to serve as a temporary matrix for the attachment and migration of the basal epithelial cells that border the injured area.[7] FN levels peak between 3 and 12 hours in the BM after corneal injury and start disappearing 1 week thereafter.[5,8] As FN staining progressively diminishes, secretion of LM, also a major component of the BM, increases to reach maximum expression 1 week after corneal damage.[5] LM-1 (recently reclassified as LM-111), LM-5 (recently reclassified as LM-332), and LM-10 (recently reclassified as LM-511)[9] are the major LM isoforms present in the corneal BM.[10,11] Although the primary function of both FN and LM is to ensure cell–matrix attachment, interaction with their corresponding transmembrane integrin receptors also controls cell shape, gene expression, cell migration and proliferation, and programmed cell death.

Integrins function as transmembrane heterodimers consisting of individual α and β chains. They constitute adhesion receptors that link the ECM to the cytoplasm components to mediate the transfer of information through diverse signaling pathways[12,13] (reviewed in Ref. 14). In the corneal epithelium, the expression of 12 heterodimers have been reported: α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α6β4, α9β1, αvβ1, αvβ3, αvβ5, and αvβ6 (reviewed in Refs. 15, 16). The integrin subunit α6, which was first described as forming an α6β1 complex, can also partner with the β4 integrin subunit.[17,18] Both the α6β1 and α6β4 integrins have been recognized as LM receptors, but anchorage of the basal corneal epithelial cells to the corneal BM is a process ensured by the α6β4 integrin.[19–21] Although the involvement of α6β4 in hemidesmosome (HD) organization[17,22] has dominated the study of this integrin, recent studies have demonstrated its critical role in the migration of epithelial and carcinoma cells, where the primary signaling event triggered by α6β4 appears to be the activation of the PI3-K pathway, which has profound consequences on the migration, invasion, and survival of carcinoma cells.[23–25] Mice lacking either α6 or β4 integrins die at birth; their skin separates at the dermal–epidermal junction because of the lack of HD.[26,27] Patients who carry mutations in *ITGA6* or *ITGB4* (the genes encoding the α6 and β4 integrin chains, respectively) also develop epidermolysis bullosa, a severe skin disease.[28,29]

It is now clearly established that the integrin levels fluctuates greatly during corneal wound healing. Such fluctuations as occur during cell cycle progression are mediated by transcription factors (TFs) that bind the promoters of these integrin's genes to regulate their transcription.[16,30] Many TFs have been

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reported specifically to regulate integrin's gene expression during corneal wound healing, such as Pax6, Sp1, and Sp3 for the integrins subunit α4, α5, and α6.[31–34] NFI is of particular interest in that target sites for this TF have been shown to be present on the promoter of many integrin genes, including α6[31] (see Ref. 16 for complete review of all known TFs on integrin's regulation), although none of them has been fully characterized. The NFI family includes four genes in vertebrates: NFIA, -B, -C, and -X. The mRNA transcript of each NFI gene can be differentially spliced and may encode NFI proteins that differ in their ability to either activate or repress transcription by interacting with other cofactors via their C-terminal catalytic domain.[35–38] The first 200 amino acids from the N-terminal region of NFI are rigorously conserved in all four isoforms and act as a DNA-binding domain that can bind the 5′-TGGA/C(N)$_5$GCCAA-3′ consensus element often located in close proximity of binding sites for other transcription factors in gene promoter regulatory regions.[39–42]

In a previous study, we investigated the positive regulatory influence exerted by the TFs Sp1 and Sp3 on the transcriptional regulation of the α6 gene.[31] The α6 promoter was found to be downregulated when RCECs were grown on LM-coated culture plates. The reduced α6 promoter activity was shown to rely at least in part on a reduced level of expression of both Sp1 and Sp3 in corneal epithelial cells (both rabbit corneal epithelial cells [RCECs] and human corneal epithelial cells [HCECs]) when grown on LM. Because a potential NFI consensus binding sequence was identified close to the promoter proximal Sp1 site from the human α6 gene and that NFI has been reported to compete against Sp1,[43,44] we investigated further whether NFI also contributes to the LM-mediated downregulation of the α6 gene. In the present study, we found evidence that NFI binds to the α6 promoter and represses its activity in corneal epithelial cells. This repressive influence of NFI was found to be potentiated by LM in HCECs but not RCECs. Most of all, NFI repressed α6 transcription without competing with Sp1, as both proteins were capable of interacting simultaneously with their respective target sites in the basal promoter from the α6 gene. NFI may therefore contribute to the α6-mediated growth arrest of the basal epithelial cells when LM is present during the final steps of the corneal wound-healing process.

Methods

All experiments described in this article were conducted in voluntary compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the Laval University Animal Care and Use Committee. The study was also conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were approved by the institution's Committee for the Protection of Human Subjects.

Cell Culture and Media

HCECs were isolated from the limbal area of normal eyes from donors 44, 62, 64, and 78 years of age, obtained through the Eye Bank from the CHUL (Centre Hospitalier Universitaire Laval) Research Center, according to a procedure previously described.[30,45] RCECs were obtained from the central area of freshly dissected rabbit corneas and grown into supplemented SHEM, as recently described.[31,46] Laminin-coating of tissue culture plates has been detailed elsewhere.[31]

Plasmids and Oligonucleotides

The recombinant plasmid bearing the α6 promoter sequence from −181 to +76 bp relative to the transcription initiation start (TIS) site fused to the CAT reporter gene (referred as α6-181) was constructed as previously described.[31] The α6-181/mSp1p recombinant construct that bears mutations in the promoter proximal Sp1 site has been described previously.[31]

The double-stranded oligonucleotides used for the competition assays in the EMSAs contained the following DNA sequences: The high-affinity binding sites for the TFs NFI (NFI; 5′-TTATTTTGGATTGAAGCCAATATGAG-3′)[47] and Sp1 (5′-GATCATATCTGCGGGGCGGGGCAGACACAG-3′)[48]; the NFI site from the α6 promoter (from position −37 to +3) (α6NFI; 5′-CGTCCTCGTCACTTGATAAAACGCCTGCGAGTCTCCAGAG-3′).

DNase I Footprinting

DNase I footprinting was performed as described previously,[31] with increasing amounts of a carboxymethyl (CM)-Sepharose–enriched preparation of rat liver NFI.[49] The probe used for the assay consisted of a 257-bp *Hin*dIII/*Xba*I DNA fragment (and digested from the recombinant plasmid α6-181[31]) spanning the α6 promoter sequence from positions −181 to +76 and 5′ end labeled at its *Xba*I site.

Transient Transfections and CAT Assays

Both HCECs and RCECs were grown to near confluence (80% coverage of the culture plate) into six-well (35 mm) tissue culture plates and transiently transfected using polycationic detergent (Lipofectamine; Invitrogen-Gibco, Grand Island, NY) according to a procedure we previously described.[31] Each transfected plate received 1 μg of the α6-CAT test plasmid and 0.5 μg of the human growth hormone (hGH) encoding plasmid pXGH5. Levels of CAT activity for all transfected cells were determined and normalized to the amount of hGH secreted into the culture medium and assayed using a kit for quantitative measurement of hGH (Immunocorp, Montréal, Québec, Canada). The value presented for each test plasmid transfected corresponds to the mean of at least three separate transfections performed in triplicate. To be considered significant, each value had to be at least three times over the background level caused by the reaction buffer used (usually corresponding to 0.15% chloramphenicol conversion). Student's *t*-test was performed for comparison of the groups. Differences were considered to be statically significant at $P < 0.05$. All data are expressed as the mean ± SD. The pCH NFI constructs were kindly provided by Richard M. Gronostajski (Case Western Reserve University, Cleveland, OH).

RNAi Assays

Negative control (ID 4611, 4613, 4615) and predesigned siRNA duplexes (Silencer) against NFIA (id 115,686, 115,687, 144,076), NFIB (id 115688, 115686, 115690), NFIC (ID 215174, 21573), NFIX (ID 115298, 115297, 3296), and Sp1 (ID 36737, 36912, 116546) were purchased from Ambion, Inc. (Austin, TX) and used according to the manufacturer's specifications. Briefly, 250 ng total siRNA directed against these mRNAs was combined and transfected in triplicate into HCECs cultured to subconfluence onto 39-mm tissue culture dishes (25×10^4 cells per dish at start) by lipofection (Lipofectamine; Invitrogen-Gibco), along with 1 μg of the plasmid construct α6-181. The cells were harvested 24 hours after the addition of fresh medium and processed as mentioned earlier for the CAT assay.[38]

Nuclear Extracts, EMSAs, and SDS-PAGE/Western Blot

Crude nuclear extracts were prepared and EMSAs conducted as detailed previously.[9,31,33] Briefly, EMSAs were conducted by incubating either crude nuclear extracts (5 μg) or CM-Sepharose–enriched NFI (either 0.5 or 1 μL)[50] in the presence of either the high-affinity NFI or the α6NFI oligomer as labeled probes. For the co-binding experiment, both the enriched NFI and a recombinant preparation of Sp1 (GST-Sp1-8xHis protein; kindly provided by Claude Labrie, Oncology and Molecular Endocrinology Research Center, CHUL Research Center)[51] were used either individually or in combination. When indicated, unlabeled oligomers were added as competitors (100- and 250-fold molar excesses). DNA–protein complexes were then separated by gel

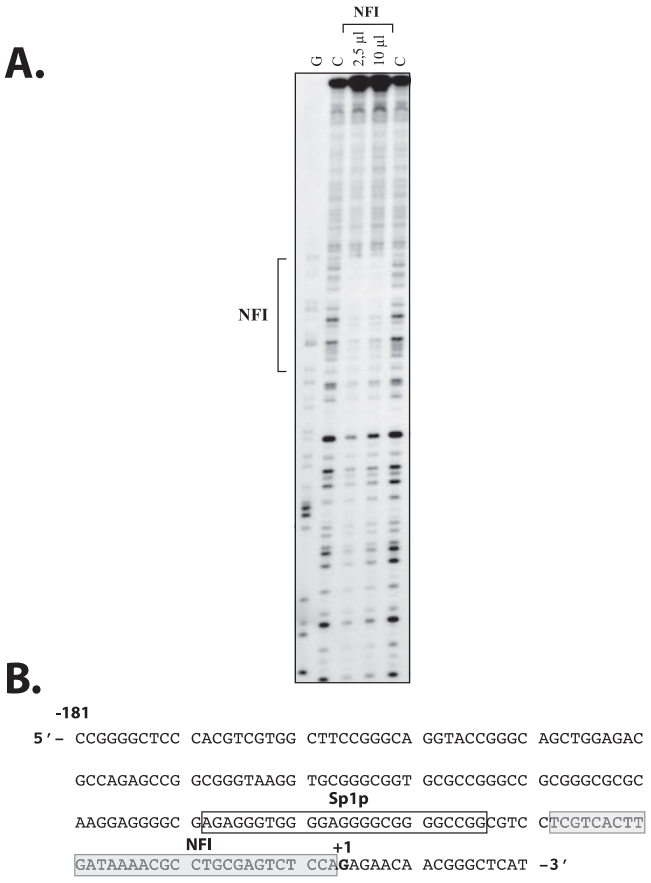

FIGURE 1. DNase I footprinting of NFI on the α6 gene promoter. (**A**) A labeled probe bearing the α6 promoter from positions −181 to +76 was incubated with 2.5 and 10 μg nuclear proteins from a CM-Sepharose–enriched preparation of rat liver NFI and subjected to DNase I digestion. The position of the NFI protected site (NFI) is indicated. G, Maxam and Gilbert G sequencing ladder; C, labeled probe subjected to DNase I digestion but without nuclear proteins. (**B**) Positioning of both the footprinted NFI site and the Sp1 proximal (Sp1p) target site along the α6 promoter relative to the TIS (+1).

electrophoresis through 10% native polyacrylamide gels run against Tris-glycine buffer.[52] Supershift experiments were conducted by incubating 5 μg nuclear proteins from RCECs in the presence of 200 ng, 500 ng, or no polyclonal antibodies raised against NFI (SC-5567) or Sp1 (SC-59; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). SDS-PAGE and Western blot protocols have been described elsewhere.[31]

Semiquantitative RT-PCR

Total RNA was isolated with the (RNA Easy Kit) according to the manufacturer's protocol (Molecular Research Center Inc., Cincinnati, OH) from HCECs grown on BSA or LM-coated culture plates or from HCECs transfected with the siRNA (as a control; Silencer; Ambion), the Sp1 siRNA, or a combination of the NFIB, -C and -X siRNAs. Ten micrograms of each RNA sample was then used to create cDNAs (Superscript II Transcriptase kit; Invitrogen-Gibco) and was purified as recently detailed.[31,32] PCR, visualization, and analysis were conducted with DNA polymerase (Platinum *Taq*; [Invitrogen, Carlsbad, CA]), as previously detailed.[31]

The following primer sets were used: α6 (PCR product of 210 bp) forward 5′-CAAGATGGCTACCCAGATAT-3′ and reverse 5′-CTGAATCTGAGAGGGAACCA-3′; glyceraldehyde-3-phosphate (GAPDH; PCR product of 220 bp) forward 5′-ATGCAACGGATTTGGTCGTAT-3′ and reverse 5′-TCTCGCTCCTGGAAGATGGTG-3′[53]; β-actin (ACTB; PCR product of 494 bp) forward 5′-CCTGGACTTCGAGCAAGAGAG-3′ and reverse 5′-CTAACGCAACTAAGTCATAGTCCG-3′[54]; β-2-microglobulin (B2M, PCR product 86 bp) forward 5′-TGCTGTCTCCATGTTTGATGTATCT-3′ and reverse 5′-TCTCTGCTCCCCACCTCTAAGT-3′[55]; NFIA (PCR product of 300 bp) forward 5′-ACGCTGAAAGAATTTGTCCAACT-3′ and reverse 5′-GGGGTCAGGTGGTCTGTCT-3′; NFIB (PCR product of 149 bp) forward 5′-TATTCGCCAGGAGTATCGAGAG-3′ and reverse 5′-CTAGATCCAGACGCCAGACTT-3′; NFIC (PCR product of 205 bp) forward 5′-TACCTGGCCTACTTCGTGC-3′ and reverse 5′-AGTTGGGTCCTGTTCCAGTCA-3′; NFIX (PCR product of 275 bp) forward 5′-GAAGCCCGAGATCAAGCAGAA-3′ and reverse 5′-GAGGCGACTTGTAGAGCCG-3′; and Sp1 (PCR product of 241 bp) forward 5′-ATTGAGTCACCCAATGAGAACAG and reverse 5′-CAGCCACAACATACTGCCC-3′. To ensure that PCR saturation was not reached, we performed 23, 25, 27, 29, 31, and 34 cycles.

RESULTS

In Vitro Footprinting of the NFI Binding Site on the α6 Promoter

As previous works conducted in our laboratory using chromatin immunoprecipitation (ChIP) assays showed evidence of the binding of NFI to the α6 promoter in HCECs cultured either on BSA- or LM-coated culture plates,[31] we conducted DNase I footprinting on this area to locate the NFI site precisely. A 257-bp 5′ end-labeled DNA fragment spanning the entire α6 basal promoter from position −181 to +76 was incubated with increasing amounts of a CM-Sepharose–enriched preparation of rat liver NFI. A single target site for NFI was identified along the α6 promoter sequence, between positions −32 and −1 relative to the TIS (Figs. 1A, 1B). Although it did not exactly match the known consensus NFI binding sequence, the formation of a DNase I-protected site was a clear indication that NFI interacted directly with this area of the α6 promoter (Fig. 1B).

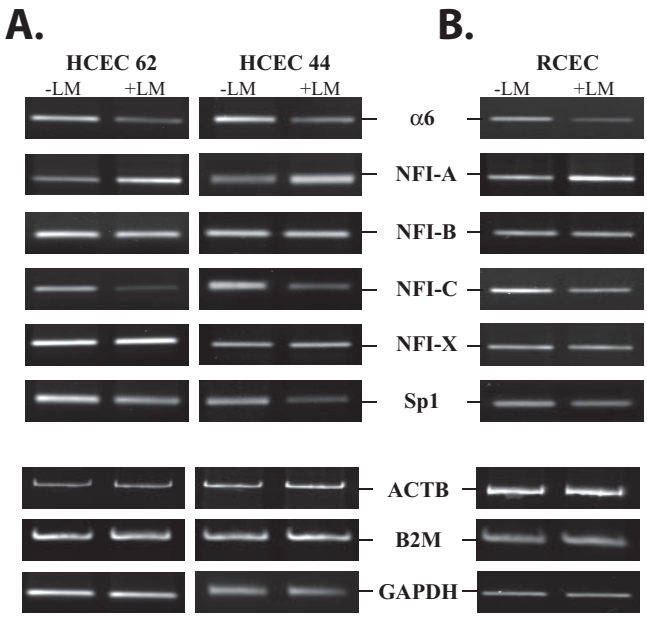

FIGURE 2. Expression of the mRNA transcripts corresponding to each of the NFI isoforms in HCECs and RCECs. (**A**) RT-PCR analysis of the α6; NFIA, -B, -C, -X; Sp1; ACTB; B2M; and GAPDH transcripts on total RNAs extracted from primary cultures of HCECs isolated from the corneas of two donors of age 44 (HCEC44) and 62 (HCEC62) years and grown on culture plates coated with either BSA (−LM) or LM (+LM). (**B**) Same as in (**A**) except that the RT-PCR was conducted on total RNAs isolated from RCECs grown either on BSA- or on LM-coated culture plates.

Effect of Laminin on the Transcription of the NFI Genes

To determine which of the four NFI proteins (A, B, C, or X) are expressed in HCECs and RCECs and whether LM alters their expression, we conducted semiquantitative RT-PCR analyses. PCR amplification of all products remained linear from 23 to 29 cycles of amplification (data not shown). RT-PCR analysis showed that all NFI isoforms were transcribed in HCECs grown on BSA (−LM; two primary cultures were used: HCEC44 and HCEC62, isolated from the eyes of 44- and 62-year-old donors, respectively; Fig. 2A). However, substantial variations in mRNA levels were observed relative to the level observed for the housekeeping genes that are used for normalization of the data (for instance, GAPDH, ACTB, and B2M[55]), when both cell lines were grown on LM-coated culture plates. Indeed, NFIA mRNA increased considerably, whereas expression of NFIC decreased when HCECs were grown on LM. However, expression of both NFIB and NFIX remained unchanged under this culture condition. As previously reported, mRNA levels for both the α6 and Sp1 transcripts were markedly decreased in the presence of LM. Identical results (increased expression of NFIA and decreased expression of both NFIC and α6) were also observed in RCECs (Fig. 2B).

Effect of LM on Expression and DNA Binding of NFI

Because of the difficulty in obtaining the human corneas from which HCECs are cultured, and as the LM-dependent changes in the expression of the NFI mRNA transcripts were the same between HCECs and RCECs, we performed the subsequent analyses with RCECs. To determine whether the changes in NFI mRNA expression would also translate into corresponding alterations in the amount of NFI proteins, Western blot analyses were first conducted using nuclear extracts from RCECs that have been grown either on BSA- or on LM-coated culture plates. With a polyclonal Ab directed against the N-terminal DNA-binding domain shared by all four NFI isoforms (i.e., it indiscriminately recognizes all four NFI proteins), we noted an important increase in the amount of a slow-migrating NFI isoform (Fig. 3A, +) whereas other, faster migrating isoforms disappeared (Fig. 3A, −) when cells were grown in the presence of LM (+LM; Fig. 3A). As expected from previous work,[31] we noted the reduced expression of Sp1 under the same conditions, in control experiments. We then examined the binding capability of NFI by EMSA using a double-stranded oligonucleotide bearing a high-affinity binding site for NFI as the labeled probe. Binding of NFI substantially increased in the presence of LM in comparison to NFI derived from nuclear extracts of RCECs grown on BSA (Fig. 3B, compare lane 3 with lane 2 and lane 10 with lane 9). Of interest, the DNA–protein complex yielded by the LM-derived extract had a slightly faster migration pattern suggesting that, when cells are grown in the presence of LM (+LM), this NFI isoform may be subjected to posttranslational modifications that are different from those in cells grown on BSA (−LM). The identity of the NFI complex was further validated by EMSAs, as addition of a polyclonal Ab directed against NFI almost entirely supershifted (SC on Fig. 3B) the NFI DNA–protein complex on the gel (−LM: lanes 4 and 5; +LM: lanes 11 and 12). Furthermore, only the oligonucleotide bearing the high-affinity binding site for NFI (−LM: lanes 6 and 7; +LM: lanes 13 and 14), but not that for Sp1

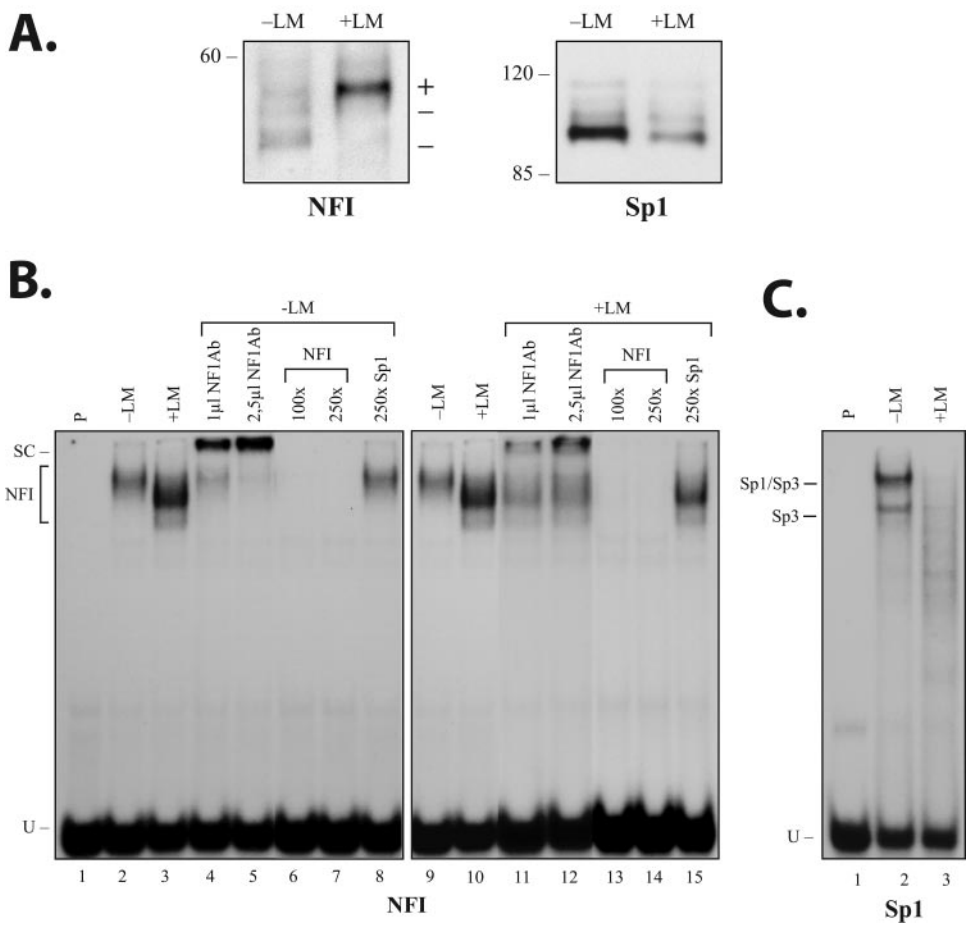

FIGURE 3. Influence of LM on the DNA binding and expression of NFI. (**A**) Western blot analysis of the NFI and Sp1 proteins conducted on nuclear extracts isolated from RCECs grown on culture plates coated with either BSA (−LM) or LM (+LM). The position of the nearest molecular weight markers (60, 85, and 120 kDa) is indicated. (**B**) Crude nuclear proteins (5 μg) isolated from RCECs grown either on BSA (−LM; *lanes 2*, *4-8*, and *9*) or on LM-coated culture plates (+LM; *lanes 3*, *10-15*) were incubated with the high-affinity NFI binding site (NFI). Formation of DNA–protein complexes was then monitored by EMSA. Supershift analysis with a polyclonal Ab (either 1 or 2.5 μL) directed against NFI (*lanes 4*, *5* and *11*, *12*), as well as competition experiments with unlabeled oligonucleotides (bearing either the NFI [*lanes 6*, *7*, and *13*, *14*] or Sp1 [*lanes 8*, *15*] target site) added at a 100- or 250-fold molar excess are also presented. NFI, DNA–protein complex corresponding to the recognition of the labeled probe by NFI; SC, supershifted complex yielded by the binding of the NFI Ab to the NFI-labeled probe DNA–protein complex; P, labeled probe alone; U, unbound fraction of the probe. (**C**) EMSA analysis of Sp1 binding conducted on the extracts from (**B**) using the Sp1 oligonucleotide as the labeled probe. The position of the Sp1 and Sp3 complexes is shown along with that of the free probe (U).

(−LM: lane 8; +LM: lane 15), could compete for the formation of the NFI complex, therefore establishing that the DNA–protein complex seen on the gel indeed corresponds to the recognition of the labeled probe by NFI. Incubation of the same protein extracts with the Sp1-labeled probe resulted in a reduced formation of both the typical Sp1/Sp3 DNA–protein complexes when cells were grown on LM (Fig. 3C, lane 3), a result consistent with the reduced Sp1 level observed in Western blot analysis (Fig. 3A).

EMSAs were then conducted by substituting the labeled probe bearing the consensus NFI site with a similar oligomer bearing the DNA sequence of the NFI site footprinted on Figure 1 along the α6 promoter (α6NFI) using the same RCEC extracts grown with (+LM) or without (−LM) LM (Fig. 4). An NFI complex of weak intensity was detected with the protein extract from RCECs grown without LM (lane 2). However, formation of this DNA–protein complex markedly increased in the nuclear extract from cells grown on LM (lane 3). Formation of this complex was almost entirely prevented by the addition of a 100-fold molar excess of the oligomer bearing the consensus sequence for NFI (lane 4) but not by the Sp1 oligomer (lane 5), thereby establishing the specificity for the formation of this complex. The identity of NFI as being the protein component of the DNA–protein complex observed in Figure 4B was further demonstrated by supershift analyses. Indeed, the addition of the NFI Ab to the reaction mixture reduced formation of the fast-migrating complex (NFI) and resulted in the formation of a new complex (SC) with a lower electrophoretic mobility that resulted from the binding of the Ab to the NFI protein component from the NFI/α6NFI complex (lane 6).

The NFI target site from the α6 promoter differs from the prototypical sequence by two nucleotides over the entire 15 bp that constitute this site (Fig. 4A). However, and based on the binding site predictor algorithm developed by Roulet et al.,[41] NFI was expected to bind the α6 NFI site with an affinity near 57%. To assess the affinity of NFI toward both the α6 and the prototypical NFI target sites, we incubated a CM-Sepharose–enriched preparation of NFI with the high-affinity NFI binding site labeled probe in the presence of various concentrations (50- to 800-fold molar excesses) of either an unlabeled DNA fragment from the α6 promoter (positions −84 to +76), which also bears the NFI site footprinted in Figure 1 (α6-84/+76) or the NFI oligomer. As shown in Figure 4C, the unlabeled α6-84/+76 fragment was almost as efficient as the prototypical NFI-bearing oligonucleotide in competing for the formation of the NFI complex in EMSA. Densitometric measurement of the band intensities indicated that the affinity of NFI toward the α6 NFI site contained on the α6-84/+76 was approximately 80% of that obtained for the prototypical NFI site. This result provided evidence that NFI binds to the α6-degenerated NFI site with an affinity very near that obtained with the prototypical NFI site. We therefore conclude that NFI possesses the ability to bind the NFI site from the α6 promoter efficiently and that both its expression and DNA-binding properties can be modulated by the presence of LM.

Sp1 and NFI Binding to the α6 Basal Promoter In Vitro

Because the NFI site identified in this study is located near the promoter proximal Sp1 site, we then examined whether both transcription factors can interact simultaneously with their respective target site or whether they compete with each other for the availability of their DNA-binding site in the α6 promoter. For that purpose, EMSAs were conducted with the 160-bp DNA fragment covering the entire α6 promoter from position −84 to +76 (α6-84/+76) used as the labeled probe, therefore including both the NFI and proximal Sp1 sites. As Figure 5 indicates, incubating the α6-84/+76 labeled probe with either Sp1 (lanes 2 and 3) or NFI (lanes 4 and 5) yielded

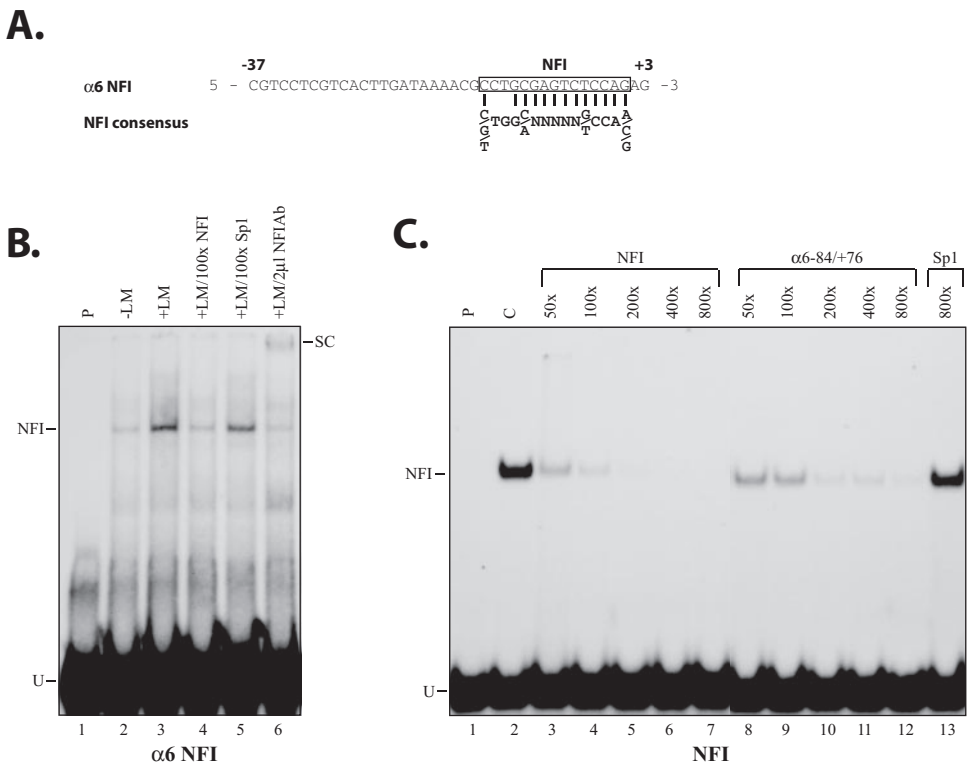

FIGURE 4. Binding capability of NFI over the α6 promoter sequence. (**A**) DNA sequence of the NFI binding site identified in the α6 promoter and used as a probe (α6NFI) in the EMSA. (**B**) Approximately 10 μg proteins from the nuclear extracts used in Figure 3 and prepared from cells grown with (+LM) or without (−LM) LM were incubated with the α6NFI-labeled probe, either alone (*lanes 2*, *3*) or in the presence of unlabeled NFI (*lane 4*) or Sp1 (*lane 5*) oligonucleotides as competitors (100-fold molar excesses were used). Formation of the NFI DNA–protein complex was then monitored by EMSA. When indicated, 2 μL of the NFI Ab was added to the reaction mixture (*lane 6*). SC, super-shifted complex yielded by the binding of the NFI Ab to the NFI/labeled probe DNA–protein complex; P, labeled probe alone; U, unbound fraction of the probe. (**C**) The labeled probe bearing the high-affinity NFI site was incubated with a CM-Sepharose–enriched preparation of NFI, alone (*lane 2*) or in the presence of increasing concentrations (50- to 800-fold molar excesses) of the unlabeled high-affinity NFI oligonucleotide (NFI; *lanes 3* to *7*) or of a DNA fragment from the α6 promoter that also bears the NFI site footprinted in Figure 1 (α6-84/+76; *lanes 8–12*). The unlabeled Sp1 oligonucleotide (800-fold excess) was also used as a negative control in this competition experiment (*lane 13*).

the appropriate DNA–protein complexes corresponding to these TFs in EMSA. No individual Sp1 or NFI complex was observed when Sp1 and NFI were added together (lane 6) but a new, very intense complex with a much lower electrophoretic mobility appeared on the gel (NFI/Sp1) suggesting that Sp1 and NFI are both sequestered together in this new complex, a clear indication that these TFs do not compete with each other for the availability of their respective α6 target sites.

RNAi Suppression of NFI on α6 Gene Expression

Evidence that NFI functionally represses α6 promoter activity was further examined through the suppression of the endogenous NFI transcripts by RNAi (Fig. 6). The recombinant construct α6-181 was cotransfected with a pool of siRNAs directed against NFIA, -B, -C, and -X, as well as against Sp1 which has been used as a control in these experiments. Cotransfection with NFIB, -C, and -X siRNAs considerably released repression over the α6 promoter construct (2.7×, 2.4×, and 1.7× increase, respectively), suggesting that all three isoforms function as transcriptional repressors of α6 gene expression. Of note, cotransfection with the NFIA siRNAs had no noticeable effect, therefore suggesting that either NFIA is not a repressor of α6 or that its nuclear concentration is much too low in HCECs to have any influence on the transcription of the α6 gene. Silencing Sp1 by RNAi decreased the expression of the α6 promoter by approximately 32%, further supporting a role for Sp1 as a transcriptional activator of α6 gene expression. As promoter analyses may occasionally differ from those conducted on the endogenous transcript, we therefore examined whether silencing NFI would cause the same effect on the expression of the α6 gene from HCECs. As shown on Figure 6B, silencing NFIB, -C, and -X simultaneously in HCECs through RNAi considerably increased the expression of the endogenous α6 gene, whereas silencing Sp1 entirely abolished it. The efficiency of NFI and Sp1 siRNA knockdown was also investigated by monitoring the DNA-binding capability for their corresponding consensus sequence by EMSAs (Fig. 6C). As expected, DNA binding of both NFI (lane 3) and Sp1 (lane 7) was severely reduced in cells transfected with the NFI and Sp1 siRNAs, respectively. This effect was highly specific, as binding of NFI remained unaffected by the Sp1 siRNAs (lane 4) as that of Sp1 toward the NFI siRNAs (lane 8).

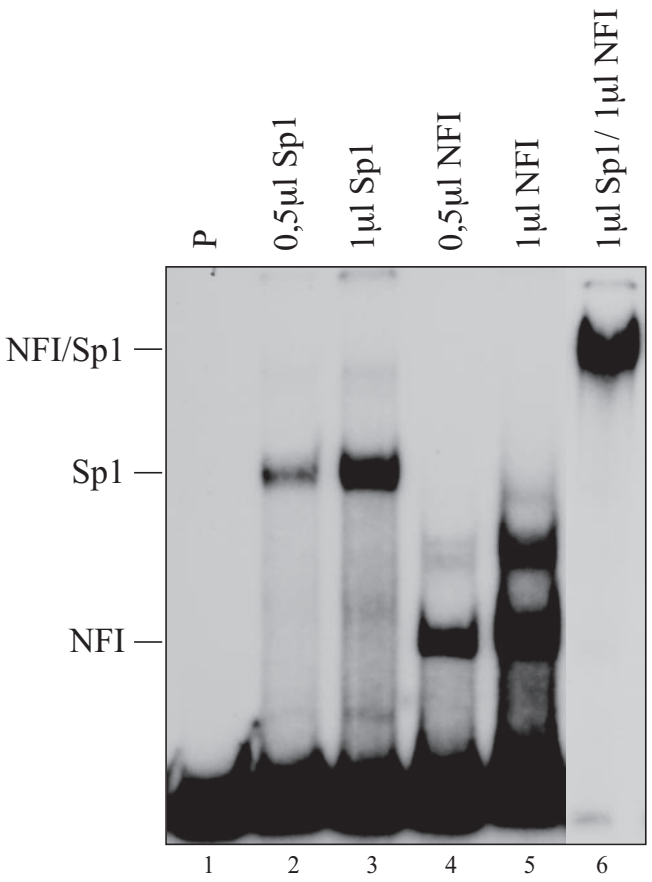

FIGURE 5. Cobinding analysis of Sp1 and NFI on the α6 basal promoter. The α6-84/+76 fragment from the α6 promoter was 5′ end-labeled and incubated with enriched NFI (0.5 or 1 μL) or a recombinant preparation of Sp1 (0.5 or 1 μL), either individually (in *lanes 2*, and *3* for Sp1; *4* and *5* for NFI) or in combination (*lane 6*). Formation of the DNA–protein complexes was then examined by EMSA as in Figure 4. P, labeled probe alone.

Influence of NFI Overexpression α6 Gene Transcription

To examine further the negative regulatory influence of NFI on the α6 promoter, the α6-181 recombinant construct was cotransfected along with expression plasmids encoding high levels of each of the NFI isoforms into HCECs or RCECs grown either on BSA or on LM. Transfections revealed that all four NFI isoforms could strongly repress (ranging from 3- to 15-fold repression) the activity directed by the α6-181 construct when compared with cells transfected with an empty vector (+EV) as a control (Fig. 7A, filled columns), irrespective of whether cells were of human (HCECs; Fig. 7A) or rabbit (RCECs; Fig. 7A) origin. These results also confirmed that NFIA acts as a repressor of α6 transcription and therefore suggest that the lack of any regulatory influence observed with the NFIA siRNAs (Fig. 6A) is probably the consequence of low levels of expression of this isoform in HCECs or that NFIA possesses a much lower DNA-binding capability than does the remaining NFI isoforms toward the NFI site from the α6 promoter. A dramatic repression in the basal activity directed by the α6 promoter was also observed when HCECs were grown on LM-coated culture plates in the absence of NFI expression plasmids (which dropped by 11-fold on LM-coated culture plates; Fig. 7A, +EV). This drop in the α6 promoter activity is consistent with the substantial increase in the expression and DNA binding of NFI combined to the reduced expression of Sp1 observed above in Western blot and EMSA analyses when cells are grown on LM. Overexpression of the NFI isoforms in HCECs grown on LM (Fig. 7A, gray columns) resulted in a further repression in the activity directed by the α6-181 construct, suggesting that the α6 promoter was not yet saturated in vivo by naturally occurring NFI levels, which would allow for some residual Sp1 activity under the presence of a high repressive environment. Very much the same results were obtained in the RCECs (Fig. 7A), although the negative influence of LM was clearly less pronounced than in the HCECs.

We then transfected RCECs that had been grown either on BSA or LM with the α6-181 construct or its derivative, which carries the mutation into the proximal Sp1 site. As expected, mutating the proximal Sp1 binding site resulted in a much more reduced expression directed by the α6 promoter (Fig. 7B, +EV, gray columns). Most interesting, overexpression of the NFI isoforms in the background of the α6-181/mSp1p mutated construct led to much stronger repression of the α6 promoter (30-, 20-, 16-, and 10-fold repression with NFIA, -B, -C and –X, respectively) than in the background of the wild-type construct α6-181 (11-, 8-, 7-, and -6-fold repression with NFIA, -B, -C and -X, respectively) indicating that somehow, binding of Sp1 to the α6 promoter may attenuate repression by NFI even though neither is competing with the other for their respective target sites.

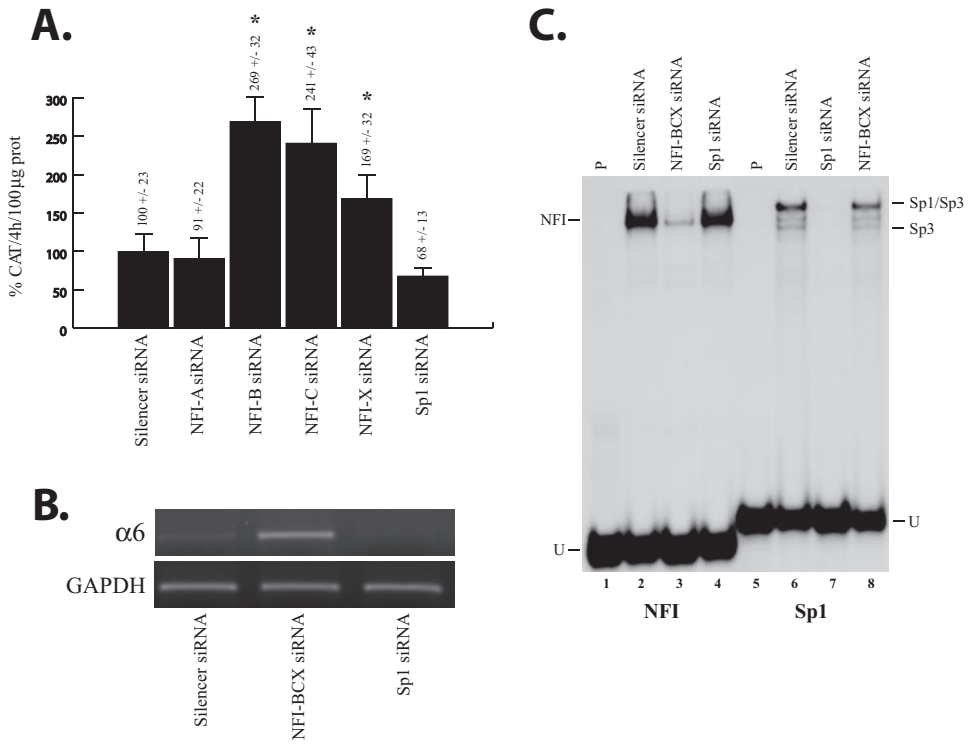

FIGURE 6. RNAi suppression of NFI expression. (**A**) The recombinant construct α6-181 was transfected in HCECs alongside siRNAs complementary to the NFIA, -B, -C, and -X and Sp1 transcripts. CAT activities were determined and normalized to secreted hGH. *CAT activities that are significantly different from those obtained with the α6-181 promoter construct transfected solely with the siRNA silencer negative control ($P < 0.05$; paired samples, *t*-test). (**B**) RT-PCR analysis of the α6 and GAPDH transcripts on total RNAs extracted from HCECs transfected either with the Sp1 siRNA or a combination of siRNAs complementary to the NFIB, -C, and -X (NFI-BCX). (**C**) RNAi assays were performed in HCECs by using a pool of siRNAs complementary to the NFI and Sp1 transcripts. Crude nuclear extracts were prepared from siRNA-transfected cells (the control silencer siRNA [*lanes 2*, *6*], a combination of the NFIB, -C and -X siRNAs [*lanes 3*, *8*], or the Sp1 siRNA [*lanes 4* and 7]) and then incubated with either the NFI (*lanes 1–4*) or Sp1 (*lane 5-8*) high-affinity–labeled probes. Formation of the NFI and Sp1 DNA–protein complexes was then monitored by EMSA as in Figure 3.

DISCUSSION

Corneal wound healing is a process that requires complex cellular interactions between the cell surface integrin receptors and the ECM to achieve efficient cell migration and differentiation. Although FN deposition can be observed as early as 3 hours after damage to the corneal epithelium, no LM can be seen beneath the leading edge of migrating epithelial cells until 24 hours after the injury.[56] FN has been shown to induce the attachment and migration of the basal epithelial cells that border the injured area and to influence the transcriptional activity of the gene encoding the integrin subunit α6,[57] ensuring early expression well before LM accumulates beneath the leading edge. The signal transduction pathway that is activated on binding of the α6 integrin to LM is thought to trigger regulatory signals distinct from those resulting from the binding of FN to its corresponding integrins. As FN expression diminishes, secretion of LM increases to reach maximal expression 1 week after corneal damage[5] by inducing HDs assembly via its α6β4 interaction to enforce stable attachment of basal cells to the basal membrane.[58,59] Evidently, HDs also need to be disassembled to allow initial cell migration and proliferation after damage to the cornea. Thus, LM is desired only at the end of the wound-healing process, to return the cells to a normal proliferative state and attachment to initiate wound closure. Repression of α6 expression contributes to this final step of the corneal repair process by both restricting proliferation and allowing attachment of the epithelium to the basal membrane. We previously demonstrated that LM reduces expression of α6 during wound healing by lowering the levels of the nuclear TFs Sp1 and Sp3, which are believed to act as strong activators of α6 gene expression during the initial FN-mediated migration/proliferation step that chiefly characterize this process.[31] In this study, we demonstrated that LM also increases the level of the transcription factor NFI that in turn binds the α6 promoter to repress its transcription. The location of the NFI site nearby the promoter-proximal Sp1 site along with the opposite regulatory influences of these TFs suggest that both may prejudice each other once they are bound to their respective target site in the α6 promoter and that the Sp1/NFI ratio must be very critical in dictating whether expression of the α6 gene will be activated or repressed, a process that is highly dependent on the orchestrated changes in the remodeling of the ECM components (such as FN or LM) during wound healing.

Although the α6 promoter bears a DNA sequence slightly different from the known consensus NFI-binding site, it clearly allows for specific NFI-binding, which in turn acts as a strong repressor of transcription. Because there are four NFI isoforms and their functional activity is poorly understood, it is difficult to understand which NFI isoforms actually bind and repress the α6 promoter in vivo. Because their DNA-binding domain is highly conserved, it is safe to state that any of these four isoforms possesses the capability to bind the α6 promoter. As NFI function is highly influenced by interaction with coactivators (no corepressor has been reported yet to interact with NFI) via their various catalytic domains,[60–64] more extended studies are necessary to understand whether any particular NFI isoform is favored in the various cellular requirements. One interesting hypothesis is that each isoform exists only to act as a redundant fail-safe factor to ensure accomplishment of key cellular functions. Because NFI has been shown to act as a critical repressor of p21 (CDKN1A) gene expression during cell cycle and senescence,[38] it is possible that genomic evolution favors such redundancy to maintain critical housekeeping gene regulation. In our model, RNAi and overexpression experiments showed that each of the four isoforms could function as a strong repressor of α6 transcription in HCECs grown over LM, which would give support to this hypothesis.

Since NFI and Sp1 nuclear levels vary inversely to one another when cells are grown on LM, we can hypothesize that regulation of α6 is mediated most probably by overall expression of these factors and their cellular availability and less likely by posttranslational modifications that may alter their binding properties or their structural integrity. Previous reports demonstrated O-glycosylation[65] and phosphorylation[37,66] of NFI

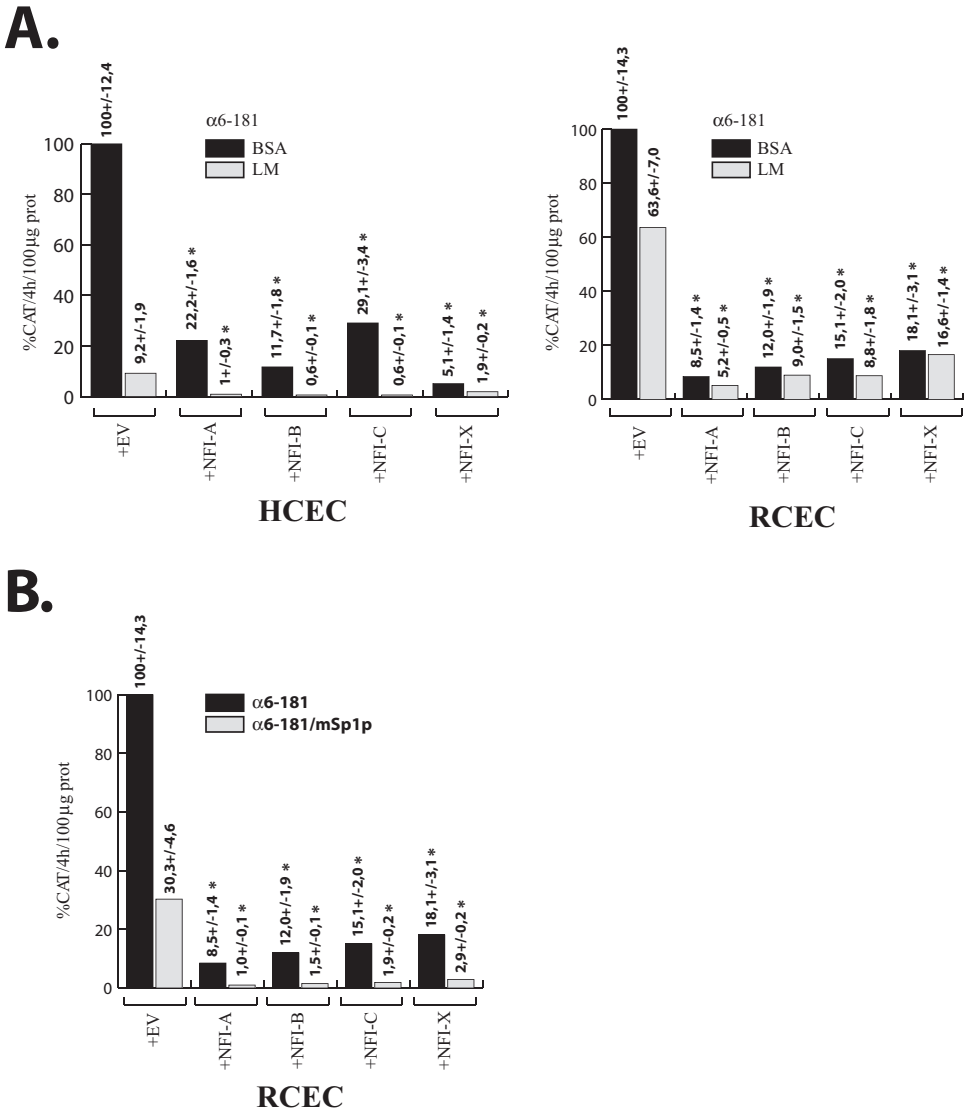

FIGURE 7. Influence of NFI overexpression on α6 promoter activity in HCECs. (**A**) The α6-181 recombinant construct (α6-181) was cotransfected in HCECs or RCECs grown on BSA- or on LM-coated culture plates along with the empty vector pCH (+EV), or with expression plasmids encoding each of the NFI isoforms (+NFIA, -B, -C, and -X). CAT activities were determined and normalized. *CAT activities that are significantly different from those obtained with α6-181 transfected solely with the empty vector pCH (+EV) ($P < 0.05$; paired samples, *t*-test). (**B**) The α6-181 construct (α6-181) or its derivative-bearing mutations into the promoter proximal Sp1 site (α6-181/mSp1p) was cotransfected in RCECs along with the empty vector pCH (+EV), or with the various NFI expression plasmids (+NFIA, -B, -C, and -X) and CAT activities determined as above. *CAT activities that are significantly different from those obtained with either the α6-181 or the α6-181/mSp1p promoter constructs transfected solely with the empty vector pCH (+EV) ($P < 0.05$; paired samples, *t*-test).

peptides. Indeed, posttranslational modifications such as glycosylation, have been reported to stimulate DNA binding of NFIB[67] in the transcriptional activation of the whey acidic protein (WAP) gene. Similarly, N-glycosylation of NFIC has been postulated to improve its potential to recruit coactivators, corepressors, or other nuclear regulatory targets in the transcriptional activity directed by the mouse *clustering* promoter.[68] Of particular interest, phosphorylation of NFIC by Jak2 has been shown to restrain this TF from proteasomal degradation.[69] Although these modifications cannot be excluded, the clear influence of LM in both increasing NFI levels (and by way of consequence, its availability) and altering its electrophoretic mobility in EMSA are most likely the keys to α6 repression under such a physiological condition. Considering the growing interest given to microRNAs (miRNA) in gene suppression and the fact that NFI-A is a target of two such miRNAs, for instance miR-223 and miR-424,[70,71] an investigate into that extent that LM alters the expression of these miRNAs in relation to α6 gene expression would surely be interesting.

Although the EMSA provided evidence that both Sp1 and NFI can bind simultaneously to their respective target sites in the α6 promoter, transfection analysis also suggested that Sp1 may restrict the negative regulatory influence of NFI, as mutation of the Sp1 site translated into a deeper repression by NFI. Direct interaction between NFIX and Sp1, which blocks the transactivating properties of the latter TF and results in the repression of PDGF-B gene transcription, has been recently documented and shown to be dependent on residues 243-416 from NFIX.[43] This raises the interesting possibility that subtle alterations in the Sp1-to-NFI ratio dictate whether transcription of the α6 gene should be enhanced or suppressed in cultured cells.

Of interest, NFIA was the only isoform that remained unaffected in the RNAi experiment, although overexpression of the NFIA cDNA resulted in a high repressive action similar to that achieved with the other NFI isoforms. Besides, NFIA was the least expressed of all four NFI transcripts when the cells were grown without LM, whereas it was transcribed to the highest level in the presence of LM. This finding suggests that initial low mRNA levels do not show much reactivity to RNAi, but when overexpressed, the repressive action of NFIA over the α6 promoter then becomes significant. One must keep in mind that overexpression experiments bring large amounts of transcripts that would not normally be observed in an in vivo environment. This raises the possibility that NFIA may not be the favored isoform in binding the α6 promoter in vivo, which would also justify the RNAi results observed.

In summary, we demonstrated that binding of NFI to the basal promoter of the human α6 gene strongly represses its

transcription, a process that is obviously mediated by increased levels of NFI when primary cultured cells (RCECs and HCECs) are grown in the presence of the ECM component laminin. The LM-induced NFI production is coordinated with a reduction in the level of Sp1 expression, a process that switches α6 transcription from activation to repression. These in vitro data therefore support the hypothesis that repression of α6 in vivo may contribute to the final steps of wound healing in the cornea.

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