Functional Impact of Collagens on the Activity Directed by the Promoter of the α5 Integrin Subunit Gene in Corneal Epithelial Cells

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Submitted: January 31, 2015
Accepted: August 17, 2015

Citation: Lake J, Zaniolo K, Gingras M-È, Couture C, Salesse C, Guérin SL. Functional impact of collagens on the activity directed by the promoter of the α5 integrin subunit gene in corneal epithelial cells. *Invest Ophthalmol Vis Sci*. 2015;56:6217–6232. DOI:10.1167/iovs.15-16587

Purpose. The early step of corneal wound healing is characterized by the massive production of fibronectin (FN), whose secretion is progressively replaced by collagens from the basal membrane as wound healing proceeds. Here, we examined whether expression of the gene encoding the α5 subunit from the FN-binding integrin α5β1 changes as corneal epithelial cells (CECs) are cultured in the presence of collagen type I (CI) or type IV (CIV).

Methods. Responsiveness of the α5 gene toward collagen was determined by transfection of α5 promoter/chloramphenicol acetyltransferase (CAT) plasmids into rabbit and human CECs cultured on BSA or collagens. Electrophoretic mobility shift assays and Western blots were used to monitor the transcription factors required for basal α5 gene transcription in the presence of collagens. Gene profiling on microarrays was used to determine the impact of collagens on the patterns of genes expressed by CECs.

Results. All collagen types repressed the full-length α5/CAT promoter activity in confluent CECs. A moderate increase was observed in subconfluent rabbit CECs grown on CIV but not on CI. These collagen-dependent regulatory influences also correlated with alterations in the transcription factors Sp1/Sp3, NFI, and AP-1 that ensure α5 gene basal transcription. Microarray analyses revealed that CI more profoundly altered the pattern of genes expressed by human CECs than CIV.

Conclusions. Collagens considerably suppressed α5 gene expression in CECs, suggesting that during wound healing, they may interfere with the influence FN exerts on CECs by altering their adhesive and migratory properties through a mechanism involving a reduction in α5 gene expression.

Keywords: integrin, collagen, promoter, corneal epithelium, wound healing

The corneal epithelium is exposed to the external environment and serves as the frontal barrier of the anterior segment of the eye. The integrity and smoothness of the corneal epithelium is absolutely required for appropriate light refraction and the maintenance of a good visual acuity.[1] Once damaged, corneal epithelial cells (CECs) must raise a rapid healing response in order to preserve these properties. Cell–cell and cell–matrix interactions play important roles in the maintenance of the stratified structure of the corneal epithelium.[2] Corneal wound healing is primarily regulated by growth factors, cytokines, and components from the extracellular matrix (ECM).[3–6] The corneal epithelial basement membrane (BM) is a specialized ECM constituted of a variety of different collagen types, entactin, and heparin sulfate proteoglycan[7–10] that separates epithelial cells from the corneal stroma (reviewed in Refs. 5 and 11) and whose composition changes depending on whether it is located beneath the epithelial cells from the limbus or from those of the central cornea. Indeed, the BM from the corneal limbus, which is also the niche of the corneal stem cells, is enriched with collagen IV α1(IV) and α2(IV) chains but has a reduced amount of α3(IV) chain. Besides collagens, the limbal BM also contains various laminin chains (α2–α5, β1–β3, γ1–γ3) and tenascin C. Conversely, the BM from the central cornea is enriched in α3(IV) and α4(IV) collagen IV, collagen V, and collagen XII.[9,12,13] Other components, such as type IV collagen α5 and α6 chains, collagen types VII, XV, XVII, and XVIII, laminin-111, laminin-332, laminin chains α3, β3, and γ2, as well as fibronectin, have been found to be part of the BM from the entire ocular surface epithelia.[12,13] The particularly distinctive composition of the limbal BM, which is clearly different from that of the central cornea, was suggested to constitute a specialized microenvironment whose specific function is to maintain the corneal stem cells in a quiescent, undifferentiated state, thereby preserving their proliferative abilities.

Corneal injury is known to cause a rapid healing response that may transitorily alter the composition of the BM's constituents to facilitate adhesion and migration of the epithelial cells, a process particularly important in order to ensure proper repair of the damaged cornea.[4,14] For instance, small wounds

(less than 1.5 mm) have been shown to completely heal within 24 hours in a mouse debridement model without significant loss of the basement membrane protein. On the other hand, larger wounds required 48 hours for complete closure of the damaged area and were also characterized by partial to complete loss of the typical BM constituents.[15,16] Besides the massive secretion of a temporary matrix mostly enriched with fibronectin (FN) that occurs early during corneal wound healing, collagen type 1 (CI), collagen type 4 (CIV), and laminin (LM) are also subjected to profound alterations during the entire repair process.[7,8,10] Indeed, they temporarily disappear during the early steps of the wounding process until the denuded area is completely covered and then sequentially reappear beneath the newly produced epithelium as the FN staining progressively diminishes in the BM.[8,17,18] Depending on the depth of the wound, the migrating epithelial cells may also become in contact with different types of collagens. For instance, as nonpenetrating injuries leave the BM intact, the migrating epithelial cells will be exposed to CIV, a major component of this membrane.[19] However, much deeper, penetrating injuries also damage the BM and expose the migrating cells to CI that is abundantly present in the corneal stroma.[20] Consequently, the adhesive and migrating properties of the migrating epithelial cells may differ depending on whether they are exposed to CI (in deeper injury) or CIV (in superficial injury).

The rapid changes in the composition of the ECM occurring during the wounding process are also accompanied by similar changes in the expression of a few integrin subunits at the cell surface of CECs, which have been reported to normally express the integrin subunits α2, α3, α4, α5, α6, αv, α9, β1, β4, and β5.[21,22] Integrins, a large family of transmembrane receptors that mediate signaling between the ECM and the cell,[23–25] are made up of an α and a β subunit picked out among the 18 α and 8 β integrin subunits that can heterodimerize into one of the 24 integrin receptors reported to date.[23,24,26,27] Only the integrin subunits α5, α6, α9, αv, β4, and β6 have been firmly documented to seek their expression altered during the rapid changes in the composition of the ECM that occur during the corneal wounding process.[28–31] The FN-binding integrin α5β1 plays a major role in corneal wound healing by promoting epithelial cell adhesion and migration over this remodeled ECM.[32,33]

For several years, we investigated how the ECM components FN and LM may alter the expression of the integrin subunits α5 and α6 at the gene promoter level. We demonstrated that FN positively influences the activity directed by the human *α5* and *α6* integrin genes in primary cultures of rabbit corneal epithelial cells (RCECs) by improving both the expression and DNA binding of transcription factors (TFs), such as Sp1 and AP-1, that are required in order to ensure proper transcription of the *α5* gene.[34–36] Conversely, LM was found to suppress expression of both α5 and α6 integrin subunits by reducing the nuclear concentration of the TFs Sp1 and AP-1 and by increasing the expression of members from the NFI family, among which some are strong repressors of the *α5* and *α6* genes.[37,38] The chronological events that are typical of ECM remodeling, meaning the early appearance of FN that is then progressively replaced by collagens and laminins, also suggest that the need for corneal epithelial cells to express the FN-binding integrin α5β1 may also change as wound healing proceeds. Indeed, besides FN, expression of the *α5* gene may respond to the collagens present in the BM of the wounded cornea through a signal transduction cascade involving binding of collagens to their corresponding integrin receptors.

In the present study, we therefore investigated whether collagens alter the expression of the *α5* integrin subunit gene in CECs despite that α5β1 is an FN but not a collagen-binding integrin.

Methods

All experiments described in this study were conducted in 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. This 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 Matrix Production

Human CECs (HCECs) were isolated from the limbal area of normal eyes of a 52-year-old donor (for transfection, Western blot, or electrophoretic mobility shift assay [EMSA] analysis) or 37-, 44-, 45-, 52-, and 61-year-old donors (for gene expression profiling, quantitative PCR [PCR], and Western blot analyses), and obtained from the Banque d'Yeux Nationale of the Centre Universitaire d'Ophtalmologie (CHU de Québec, QC, Canada), following a procedure that was previously described.[39,40] Human CECs were primary cultured with a feeder layer of irradiated murine Swiss-3T3 (i3T3) fibroblasts (ATCC, Rockville, MD, USA) as previously reported.[40] Rabbit CECs were obtained from the entire corneal surface (central and limbal areas) of freshly dissected corneas from pathogen-free albinos rabbits obtained from a local slaughterhouse and grown in supplemented hormonal epithelial medium (SHEM) as previously described.[38,41] All cells were grown under 5% CO_2 at 37°C, except for HCECs that were maintained under 8% CO_2, and culture medium was changed after 2 to 3 days. When indicated, tissue culture plates were coated as previously described[36,38,42] either with BSA or collagen (types I, III, VI; Sigma, Oakville, ON, Canada) at concentrations ranging from 2 to 200 μg/cm^2 (as specified in the figure legends) before they were seeded with CECs.

Indirect Immunofluorescence

Indirect immunofluorescence assays were performed on tissue cultured cells (HCECs) grown on glass coverslips coated either with BSA, CI, or CIV and fixed with acetone (10 minutes at −20°C). Cells were incubated for 45 minutes with a primary antibody directed against human integrins α1 (Orb18042, Biorbyt, San Francisco, CA, USA), α2 (NBP1-96715, Novus Bio, Oakville, ON, Canada), α10 (PAC096Hu01, Cloud-Clone Corp, Houston, TX, USA), and α11 (ESAP13588, Elabsciences, Burlington, ON, Canada) at an optimal dilution of 1:200 in PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4) containing 1% BSA (the antibodies directed against the α1, α10, and α11 subunits were produced in rabbit, whereas the α2 antibody was produced in mouse). Samples were washed four times with PBS before addition of the secondary antibody (rabbit or mouse anti-mouse IgG [H+L] conjugated with Alexa-fluor 488; 1:400, Molecular Probes, Burlington, ON, Canada) and further incubation for 30 minutes. Isotypic nonimmune antibodies (either normal rabbit [1:100; Santa Cruz Biotechnology, Dallas, TX, USA] or normal mouse anti-IgG [1:100; DAKO, Burlington, ON, Canada]) were also used as negative controls. Cell nuclei were also labeled with Hoechst reagent 33258 (1:100; Sigma) following immunofluorescence staining. Tissue samples were then observed with an epifluorescence microscope (Zeiss Imager.Z2; Zeiss Canada Ltd., North York, ON, Canada). They were photographed with a numeric CCD camera (AxioCam MRm; Zeiss Canada Ltd).

Plasmids and Oligonucleotides

The plasmids –954α5-CAT, –178α5-CAT, –132α5-CAT, –92α5-CAT, and –42α5-CAT that bear the *chloramphenicol acetyltransferase* (*CAT*) reporter gene fused to DNA fragments from the human α5 gene upstream regulatory sequence extending up to 5′ positions –954, –178, –132, –92, and –42 have been previously described.[43,44] The plasmid PXGH5, which bears a secreted version of the human growth hormone (hGH), is a kind gift of David D. Moore (Department of Molecular and Cell Biology, Baylor College of Medicine, Houston, TX, USA). The double-stranded oligonucleotides bearing the DNA-binding sites for Sp1, NFI, AP-1, and PAX6 have all been described previously.[35] Their DNA sequence is listed in Supplementary Table S1.

Transfections and CAT Assays

The –954α5-CAT, –178α5-CAT, –132α5-CAT, –92α5-CAT, and –42α5-CAT recombinant plasmids were transiently transfected into RCECs grown to subconfluence (60% coverage of the culture plate) or complete confluence (100% coverage of the culture plate) in six-well tissue culture plates coated with different types of collagens (CI, CIII, CIV, and CVI) or 2% BSA (as a negative control) using the polycationic detergent Lipofectamine (Life Technologies, Burlington, ON, Canada) following a procedure we previously described[36] according to the manufacturer's recommendations. Each Lipofectamine-transfected well received 1 μg test plasmid and 0.5 μg hGH-encoding plasmid PXGH5. Rabbit CECs were harvested 48 hours following transfection. Human CECs can only be transfected with a good efficiency by electroporation (Neon Transfection System; Invitrogen) and not by any of the other procedures in use in our laboratory (including lipofection or transfection through the formation of a calcium phosphate precipitate). We therefore used this procedure to transfect HCECs with the –954α5-CAT and –132α5-CAT recombinant plasmids prior to the addition of i3T3 feeder cells to the culture plates. Transfected HCECs were then grown to subconfluence (SC60%) or complete confluence (C100%) in six-well tissue culture plates coated with either collagens (CI or CIV) or 2% BSA (as a negative control). For each triplicate, 1.2×10^6 HCECs were resuspended in 300 μL appropriate resuspension buffer (included with the Neon transfection kits) and to which 45 μg α5-CAT test plasmid and 15 μg PXGH5 were added. The optimal electroporation parameters selected were the following: 100 μL Neon Tip, 4×10^5 cells per well, Electrolytic Buffer E^2, pulse voltage of 1150 volts, pulse width of 30 ms, and pulse number of 2. Human CECs were harvested 48 hours following electroporation or until the desired coverage of the culture plate was achieved. The CAT activities were determined and normalized to the amount of hGH secreted into the culture media and assayed using a kit for quantitative measurement of hGH (Medicorp, Montréal, QC, Canada).[45,46] Each CAT value corresponded to the mean of at least three separate transfections done in triplicate.

Nuclear Extracts and EMSAs

Nuclear extracts were prepared from RCECs grown to subconfluence (70% coverage) and to confluence (100% coverage) on culture dishes coated either with CIV or 2% BSA and also from HCECs grown to confluence (100% coverage) on culture dishes coated either with collagens (CI or CIV) or 2% BSA as previously reported.[36,47] Electrophoretic mobility shift assays were conducted as described[35] by incubating 7.5 μg nuclear proteins from RCECs and 5.0 (for Sp1) or 10.0 μg (for AP-1, NFI, and PAX6) nuclear proteins from HCECs with ^{32}P-end-labeled, double-stranded oligonucleotides bearing the binding sites for the transcription factors Sp1, NFI, AP-1, or PAX6. Formation of DNA/protein complexes was then monitored by gel electrophoresis on native polyacrylamide gels run against Tris-glycine buffer.[48] Gels were dried and autoradiographed at –80°C for 6 hours to reveal the position of the shifted DNA–protein complexes generated.[34–38,49]

Western Blots

Western blots were conducted as previously described[36] using 10 (HCECs) or 30 μg (RCECs) from each nuclear protein extract, and the membranes were blotted with the following primary antibodies (all from Santa Cruz Biotechnology): rabbit polyclonal antibodies against Sp1 (diluted 1:2000), Sp3 (1:2000), NFI (1:2000), c-jun (1:2000), JunD (1:2000), FosB (1:2000), c-Fos (1:2000), Fra1 (1:2000), Fra2 (1:2000), PAX6 (1:2000), or mouse monoclonal antibodies against JunB (1:2000) and actin (CLT 9001, 1:40,000). For detection of the α5 integrin subunit in HCECs grown to subconfluence (SC60%) and postconfluence for 5 days (PC-5d) on collagens or 2% BSA, a monoclonal antibody (final concentration of 10 μg/mL) directed against the human integrin subunit α5 (P1D6, Chemicon, Temecula, CA, USA) was selected as the primary antibody. The blots were then incubated with a 1:1000 dilution of a peroxidase-conjugated AffiniPure Goat secondary antibody against either mouse or rabbit IgG (Jackson ImmunoResearch Laboratories, Baltimore, MD, USA), and the labeling was revealed using a Western blot Detection Kit (Thermo Scientific, Rockford, IL, USA) as previously described.[34,38]

Gene Expression Profiling

Total RNA was isolated from HCECs grown to 100% confluence on either 2% BSA or collagen (CI or CIV) using the RNeasy Mini Kit (QIAGEN, Toronto, ON, Canada). Biological replicates were as follows: for the experiment on BSA, total RNA was obtained from two different preparations of HCECs cultured from two different donors (44 and 61 years old); for the experiment with CI, total RNA was isolated from two preparations of HCECs cultured from two different donors (52 and 61 years old); for the experiment with CIV, total RNA was isolated from two preparations of HCECs cultured from two different donors (44 and 45 years old). Cyanine 3-CTP labeled cRNA targets were prepared from 25 ng of total RNA, using the Agilent One-Color Microarray-Based Gene Expression Analysis kit (Agilent Technologies). Then 600 ng cRNA was incubated on a G4851A SurePrint G3 Human GE 8 × 60K array slide (60 000 probes; Agilent Technologies). Slides were then hybridized (Agilent protocol), washed, and scanned on an Agilent SureScan Scanner according to the manufacturer's instructions. Data were finally analyzed using the ArrayStar V4.1 (DNASTAR, Madison, WI, USA) software for scatterplots and generation of the heat maps of selected genes of interest. All data generated from the arrays were also analyzed by robust multiarray analysis (RMA) for background correction of the raw values. They were then transformed in Log2 base and quantile normalized before a linear model was fitted to the normalized data to obtain an expression measure for each probe set on each array. All microarray data presented in this study comply with the Minimum Information About a Microarray Experiment (MIAME) requirements. The gene expression data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/, in the public domain) and are accessible through GEO Series accession number GSE65421.

Semiquantitative RT-PCR Assays

Total RNA was isolated from RCECs and HCECs at the indicated cell densities using the TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH, USA), reverse-transcribed, and used for PCR amplification of the human α5 and 18S ribosomal RNA. The DNA sequence of both the 5′ and 3′ template primers for human α5 are listed in Supplementary Table S1. The oligonucleotide primers used for the amplification of the 18S ribosomal RNA were provided in the Quantum RNA 18S Internal Standards kit (Ambion, Inc., Austin, TX, USA). Taq polymerase (Pharmacia-LKB) was selected for PCR amplification. Cycle parameters were the same for all primers used (denaturation 94°C, 30 seconds; annealing 60°C, 30 seconds; extension 72°C, 30 seconds) with an identical number of cycles (26, 28, 30, 32, 34, and 36 cycles) for both sets of primers. The PCR-amplified DNAs were fractionated on a 10% polyacrylamide gel, and their position was revealed by ethidium bromide staining. The gel photograph was scanned using a Visage 110S Bioimage analyzer (Millipore, Bedford, MA, USA) to quantify the alterations in the amount of the α5 and 18S PCR-amplified fragments at the various cell culture conditions selected.

Quantitative PCR

Quantity and quality of total RNA from HCECs grown to 60% subconfluence and to postconfluence for 5 days on either 2% BSA or collagens (CI or CIV) were assessed using an Agilent Technologies 2100 bioanalyzer and RNA 6000 Nano LabChip kit (Agilent Technologies). Reverse transcription was performed using random hexamer primers following the manufacturer's protocol for synthesis of the first-strand cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, CA, USA). Equal amounts of cDNA were run in quadruplicate and amplified in a 20-μL reaction containing 10 μL 2× Brillant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies), 250 nM upstream and downstream primers, and 10 ng cDNA target. No-template controls were also used as recommended. The mixture was incubated at 95°C for 3 minutes and then cycled at 95°C for 10 seconds and at 60°C for 20 seconds, 35 times, using the QIAGEN Rotor-Gene Q real-time cycler. Amplification efficiencies were validated and normalized to the GAPDH mRNA transcript, and quantity of target genes was calculated according to a standard curve. Primers were designed using Primer3 (v.0.4.0) and are listed in Supplementary Table S1.

Statistical Analyses

Statistical analysis of the data was performed using a 1-way ANOVA, and Dunnett's posttest was applied to analyze the significant variations. All global tests were statistically significant at a 95% confidence interval, and significant variations were analyzed in relation to the control (*$P < 0.05$) (Prism 6.0; GraphPad Software, La Jolla, CA, USA). Differences were considered to be statistically significant at $P < 0.05$. All data are also expressed as mean ± SD.

Results

Collagens Exert Different Regulatory Influences on the Human α5 Gene Promoter in RCECs

As the corneal BM is made of several types of collagens,[6] we examined whether some of them would influence the activity directed by the α5 promoter in vitro. Rabbit CECs were therefore seeded on tissue culture plates coated either with BSA (as a negative control) or with various types of collagens (CI, CIII, CIV, and CVI; 3 μg/cm^2) and grown to confluence (100% coverage of the culture plate). They were then transfected with the recombinant construct −132α5-CAT that bears only the basal α5 promoter.[35,36] As shown in Figure 1A, all collagen types suppressed the activity directed by the α5 promoter in confluent cultures of RCECs, with both CI and CIV being the most effective at repressing α5 promoter activity (4- and 5-fold repression, respectively). Because CI, but mostly CIV, is predominantly expressed during corneal wound healing,[8,10,50,51] we therefore conducted all the remaining experiments using these latter types of collagens. To more precisely delineate the area from the α5 promoter that mediates the negative regulatory influence of collagens, RCECs were seeded on CIV (which proved to be the most potent suppressor of α5 promoter activity; Fig. 1A) and grown to confluence prior to their transfection with recombinant constructs bearing 5′ deletions from the α5 promoter. Transfection of −954α5-CAT in RCECs grown on CIV yielded CAT activities that were more than 6-fold lower than those obtained with cells grown on BSA (Fig. 1B). Deletion of the α5 promoter down to position −132 (in −132α5-CAT) did not significantly alter the repressive influence of CIV. However, CIV caused only a 2.2-fold repression when the α5 promoter was deleted to position −92 (in −92α5-CAT). Further deletion of the α5 promoter segment from position −92 to −42 (in −42α5-CAT) entirely abolished CIV-mediated repression in RCECs, indicating that the −132/−42 area is sufficient to ensure full responsiveness of the α5 promoter toward CIV. We next examined the response of the α5 basal promoter toward different concentrations of CIV by transfecting RCECs seeded on tissue culture plates coated either with BSA (negative control) or with increasing concentrations of CIV (2–50 μg/cm^2) and grown to confluence (near 100% coverage of the culture plate) prior to their transfection with the recombinant construct −132α5-CAT. As shown in Figure 1C, as little as 2 μg/cm^2 was sufficient to repress by approximately 5-fold the activity directed by the α5 promoter. Increasing the concentration of CIV up to 50 μg/cm^2 did not further raise the repression of the α5 promoter. Conversely, increasing the concentration of CI caused a dramatic repression in the activity directed by the α5 promoter that reached a plateau at 100 μg/cm^2 (for a near 170-fold repression; Fig. 1D). We therefore conclude that collagens negatively regulate the activity of the α5 gene promoter in RCECs grown to confluence via a segment from the α5 basal promoter located between positions −132 to −42.

We then evaluated whether the cell density reached by RCECs may alter the cell's response toward both CI and CIV. Rabbit CECs were therefore seeded on tissue culture plates coated either with BSA (negative control) or with CI and CIV and grown to varying cell densities (subconfluent [SC] and confluent [C], corresponding to 60% and 100% coverage of the culture plates, respectively). They were then transfected with either the −132α5/CAT construct that bears only the α5 basal promoter or the −954α5/CAT plasmid that extend beyond the basal promoter to include 5′ flanking sequences up to position −954 relative to the α5 mRNA start site. Consistent with the data from Figure 1, transfections of −132α5/CAT in confluent RCECs grown on either CI (CI_C) or CIV (CIV_C) caused a 5- to 6-fold reduction in α5 activity compared with BSA (Fig. 2A). When transfected in subconfluent RCECs, a 7-fold repression was again observed for CI (CI_{SC}), but CAT activity increased by more than 2-fold when cells were seeded on CIV (CIV_{SC}). Similar results were also observed when the −954α5/CAT construct was transfected in both confluent and subconfluent RCECs (Fig. 2B). The RT-PCR analyses confirmed the increased α5 gene transcription when RCECs are grown to subcon-

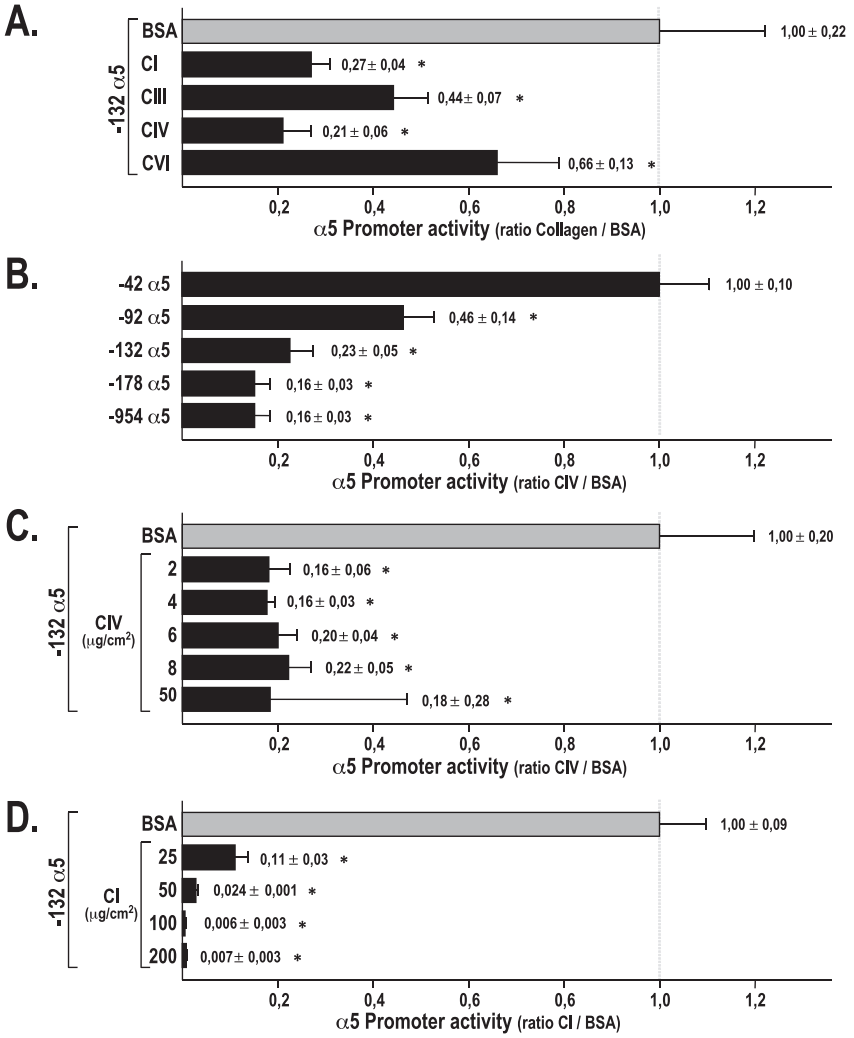

FIGURE 1. Activity of the α5 promoter in RCECs grown on culture plates coated with collagens. (**A**) The plasmid −132α5 was transfected into confluent RCECs seeded on tissue-culture plates coated with 2% BSA (used as a negative control) or on collagen types I, III, IV, and VI (CI, CIII, CIV, and CVI, respectively). Values are expressed as the ratio of CAT activities from cells grown with collagen over that from cells grown on BSA. (**B**) Recombinant plasmids bearing 5′ deletions of the α5 promoter were transfected in confluent RCECs grown on CIV (3 μg/cm^2). The CAT activities were normalized as in (**A**). (**C**) Rabbit CECs were seeded on culture plates coated with varying concentrations of CIV (2–50 μg/cm^2) and transfected at confluence with the plasmid −132α5. The CAT activities were normalized as in (**A**). (**D**) Rabbit CECs were seeded on culture plates containing increasing concentrations of CI (25–200 μg/cm^2) and transfected at confluence with the plasmid −132α5. For all panels, *asterisks* indicate CAT activities considered to be statistically significant relative to those measured in cells grown on BSA (1-way ANOVA, Dunnett's posttest; $P < 0.05$). SD is also indicated.

fluence (4-fold increase on normalization to the 18S mRNA) and its repression when they reach confluence (10-fold reduction on normalization to the 18S mRNA; Fig. 2C). CIV therefore has the ability to either repress or activate α5 promoter activity in a cell density–dependent manner. CIV has thus been selected for the remaining studies and is compared with the influences of CI.

Collagen IV Alters Both Expression and DNA Binding of the Transcription Factors That Regulate Expression of the α5 Gene

As we previously demonstrated that transcription directed by the α5 promoter was essentially dictated by interaction of the transcription factors NFI (a strong repressor of α5 gene transcription), AP-1 (a strong activator of α5 gene expression), and Sp1/Sp3 (weak activators of α5 gene transcription) with the α5 basal promoter,[34–36] we next examined whether the expression and DNA-binding properties of these TFs might be altered by CIV. The EMSA experiments were therefore conducted using nuclear extracts from RCECs grown to both subconfluence and confluence in the presence of BSA (negative control) or CIV. As shown in Figure 3A, culturing RCECs at subconfluence in the presence of CIV clearly improved the DNA-binding capacity of Sp1 (compare lanes 2 and 3), NFI (compare lanes 5 and 6), and AP-1 (compare lanes 8 and 9) toward their respective high affinity target site (used as the labeled probe in these experiments; Fig. 3A, left). These increases in Sp1, NFI, and AP-1 DNA binding also correlate with corresponding increases at the protein level, as revealed by Western blot analyses (Fig. 3B, left). Conversely, CIV caused a dramatic change in the intensity of the Sp1, Sp3, and AP-1 DNA–protein complexes and an almost complete disappearance of NFI binding when RCECs were grown to confluence (Fig. 3A, right), which is exactly the opposite effect that FN exerts on both Sp1 and NFI in RCECs.[42] This result is

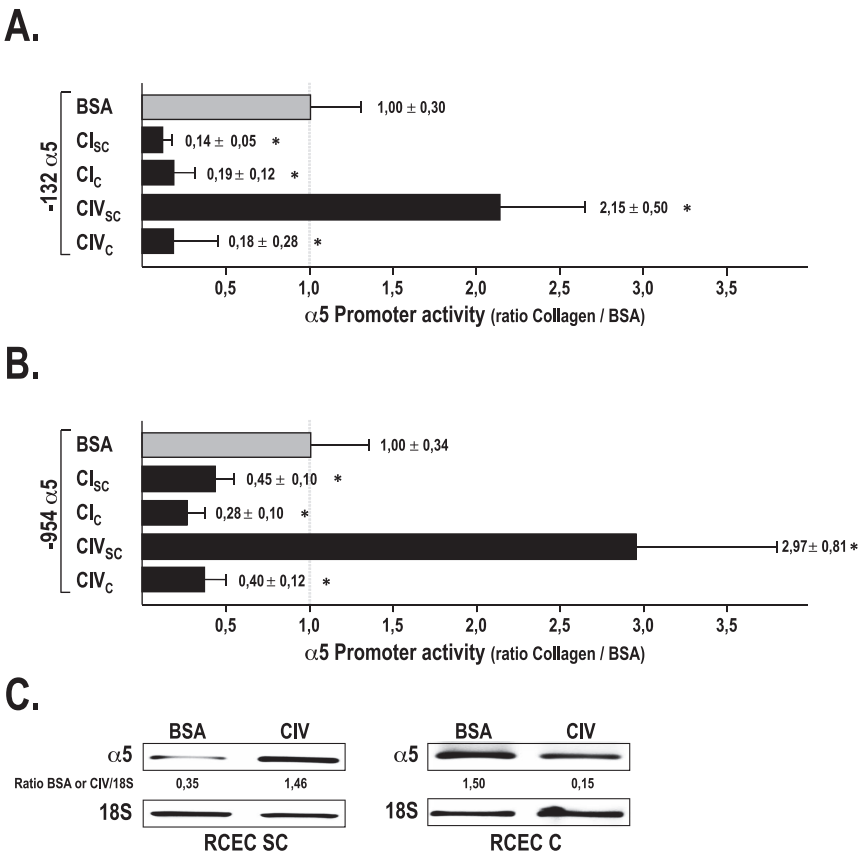

Figure 2. Influence of collagens on α5 promoter activity in RCECs grown to varying cell densities. (**A**, **B**) Rabbit CECs were seeded on culture plates containing CI (75 μg/cm^2) or CIV (50 μg/cm^2) and transfected at both subconfluence (SC) and confluence (C) with the plasmids −132α5 (**A**) and −954α5 (**B**). The CAT activities are expressed as the ratio of cells grown on collagen over that from cells grown on BSA. *Asterisks* indicate CAT activities from transfected RCECs grown on either CI or CIV that are statistically different from those measured in transfected cells grown on BSA (1-way ANOVA, Dunnett's posttest; $P < 0.05$). (**C**) Total RNAs extracted from RCECs grown on BSA or on CIV-coated culture plates were reverse-transcribed and PCR amplified using synthetic, oligonucleotide primers specific to both the α5 and 18S ribosomal RNAs. Values shown correspond to the α5 signal intensity normalized to that of 18S RNA.

consistent with the disappearance of Sp1 and most of the AP-1–constituting subunits at the protein level (Fig. 3B, right). On the other hand, the total amount of NFI proteins increased in RCECs grown to confluence on CIV, suggesting that NFI proteins lack their ability to bind their DNA target sites, probably due to change in their posttranslational modification status. We recently reported that DNA binding of NFI was entirely dependent on its phosphorylation,[52] which suggests that the lack of NFI binding observed when RCECs are grown to confluence on CIV may also rely on the lack of proper phosphorylation of this transcription factor.

Influence of Collagens Is Different in HCECs

Because HCECs show characteristics that distinguish them from RCECs (such as the need for coculture with a feeder layer), we therefore evaluated whether both CI and CIV would exert influences in HCECs similar to those observed in RCECs. To that purpose, HCECs were cultured on CI or CIV to either subconfluence (SC) or complete confluence (C) and then transfected with the α5 promoter bearing constructs −132α5/CAT and −954α5/CAT. As shown in Figure 4A, CI had little influence, if any, on the activity directed by the α5 promoter contained on the −132α5/CAT plasmid in either subconfluent (CI$_{SC}$) or confluent (CI$_C$) HCECs. Conversely, CIV caused a significant increase in the CAT activity in a cell density–independent manner (2.5- and 2.2-fold increase in subconfluent [CIV$_{SC}$] and confluent [CIV$_C$] HCECs compared with BSA, respectively). When the −132α5/CAT construct was substituted by the −954α5/CAT plasmid, repression of α5 promoter activity was observed using either CI or CIV at any cell density (Fig. 4B). Quantitative PCR analyses further supported repression of the endogenous α5 gene transcription by both CI and CIV relative to BSA in HCECs grown to sub- or postconfluence (Fig. 4C). These CI- and CIV-dependent repressions of endogenous α5 gene transcription also translated into corresponding reductions in the amount of α5 integrin subunit at the protein level (reduction ranging from 2.5- to 15.5-fold on normalization to actin levels), as revealed by Western blot analyses (Fig. 4D). The reduced α5 promoter activities observed in HCECs grown on collagens also correlate with reduced DNA-binding interaction of Sp1 (Fig. 5A, compare lanes 2 and 3) and NFI (Fig. 5A, compare lanes 6 and 7) by CI and AP-1 by CIV (Fig. 5A, compare lanes 10 and 12). Interestingly, DNA binding of PAX-6, a transcription factor expressed in the cornea[53] that has been shown to participate to α5 gene transcription,[54] is also reduced by CIV (Fig. 5A, compare lanes 14 with 16). However, very little change in the nuclear content of these transcription factors was observed by Western blot, with the exception of a weak decrease in the expression of the AP-1 subunits c-Fos and FosB in cells grown on CI and c-Fos and Fra2 in cells grown on CIV (Fig. 5B). Therefore, the reduced binding of these TFs to their respective target sites is more likely to be related to changes in their

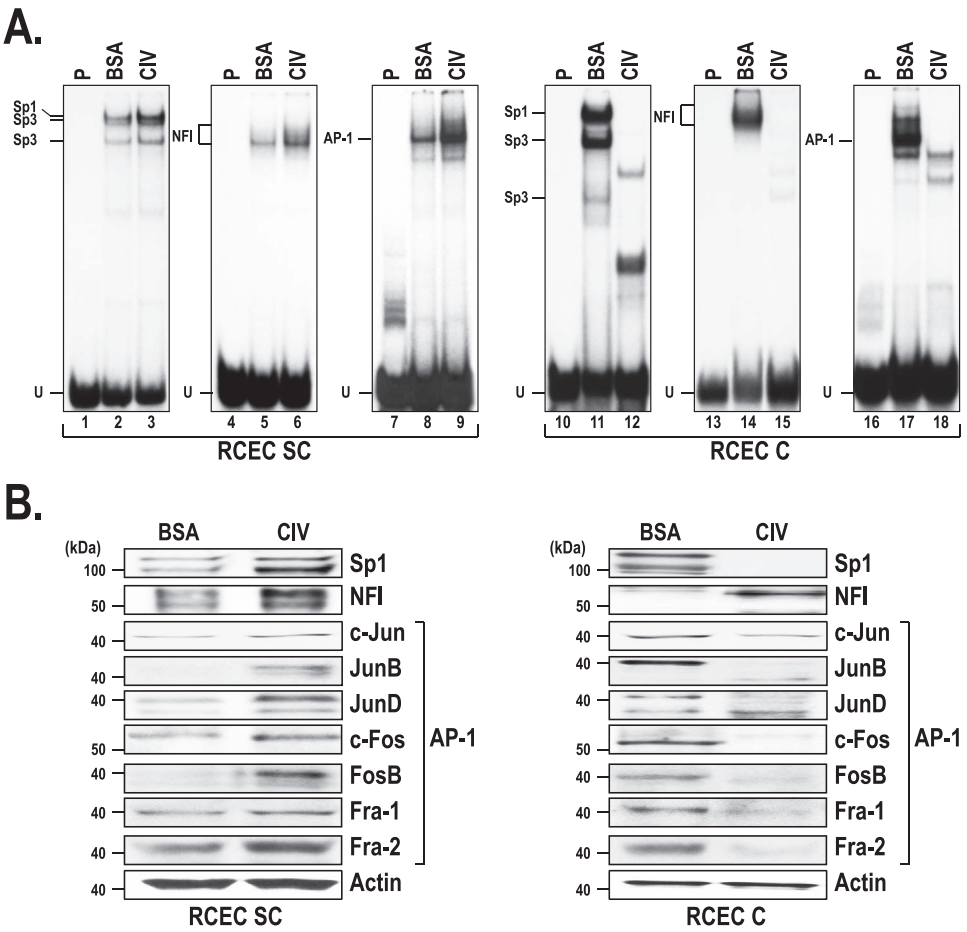

FIGURE 3. Influence of CIV on the expression and DNA binding properties of Sp1, NFI, and AP-1. (**A**) Nuclear proteins (7.5 μg) from both subconfluent (SC) and confluent (C) RCECs grown on BSA or on CIV-coated culture plates (3 μg/cm^2) were incubated with 5′-end labeled oligonucleotides bearing the high affinity binding site for Sp1, AP-1, and NFI. Formation of the DNA–protein complexes was then monitored by EMSA. The position of the Sp1/Sp3, AP-1, and NFI complexes is indicated. U, unbound fraction of the probe; P, labeled probe with no added proteins. (**B**) Approximately 30 μg nuclear proteins from the extracts used in (**A**) were Western blotted with antibodies against the TFs Sp1, Sp3, NFI, JunB, c-Jun, JunD, FosB, c-Fos, Fra-1, and Fra-2. Actin expression was also monitored as a normalization control. The position of the nearest molecular mass markers is indicated.

posttranslational status, as previously reported,[49,52] rather than to corresponding changes in their protein concentration. In addition, α5 gene promoter repression in HCECs (Fig. 4) was clearly of a lesser amplitude than that observed in RCECs (Fig. 2), which is also consistent with the less massive alterations in the expression of the transcription factors that drive expression of α5 gene transcription in HCECs (Fig. 5) compared with that observed in RCECs (Fig. 3).

The Gene Signature of HCECs Is Influenced by Collagens

We next conducted gene expression profiling on a microarray using total RNA prepared from HCECs grown to confluence on either CI or CIV, and we compared their pattern of expressed genes with that of HCECs grown on BSA. Scatterplot analysis revealed moderate changes in the pattern of genes expressed by HCECs grown on either CI or CIV relative to those grown on BSA as indicated by the minor variations noted in the slope of the regression curves (R^2 = 0.97 and 0.97 for HCECs grown on CI [Fig. 6A, left] and CIV [Fig. 6A, right], respectively).

A heat map for all the genes showing a 2-fold or more expression variation unique to HCECs grown on CI and CIV compared with when they are grown solely on BSA was then generated (Fig. 6B). A total of 3252 genes fitted into that category of differentially regulated genes when HCECs are grown on CI, which is consistent with the clearly different pattern obtained on the heat map when cells grown on CI are compared with those grown on BSA (Fig. 6B). Conversely, the pattern obtained for HCECs grown on CIV is very similar to that of cells grown on BSA, as there are only 349 genes whose expression is deregulated by more than 2-fold between these two conditions (Figs. 6A, 6B). Of all these genes, 138 are commonly deregulated by both CI and CIV, which also correspond to ~40% of all those deregulated in HCECs grown on CIV (Fig. 6C).

We next examined the data files from the microarrays to sort out genes, beside α5, whose expression is the most deregulated in HCECs grown on CI and CIV. The filters from the Arraystar program were set to restrict the search to only the 55 most deregulated genes in HCECs grown either on CI or CIV relative to their expression level in HCECs grown on BSA. Of the 55 genes identified as deregulated in HCECs grown under each culture condition, 13 were similarly influenced (all repressed) by both CI and CIV (identified in red in Fig. 6D; Supplementary Table S2). We then randomly selected candidate genes among those that are repressed by CI and/or CIV

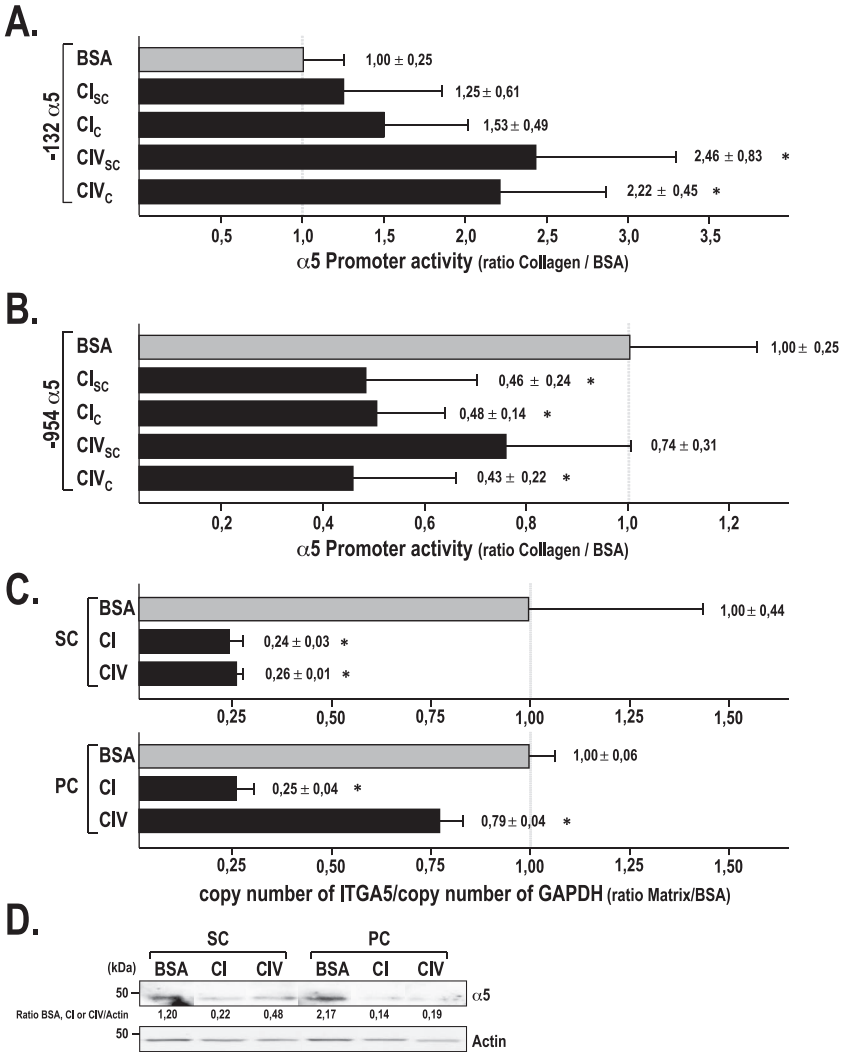

Figure 4. Activity of the α5 promoter in HCECs grown to varying cell densities on collagen-coated culture plates. (**A**, **B**) Human CECs were seeded on culture plates containing CI (75 μg/cm^2) or CIV (50 μg/cm^2) and transfected at both subconfluence (SC) and confluence (C) with the plasmids −132α5 (**A**) and −954α5 (**B**). CAT activities are expressed as the ratio of cells grown on collagen over that from cells grown on BSA. *Asterisks* indicate CAT activities from transfected HCECs grown on either CI or CIV that are statistically different from those measured in transfected cells grown on BSA (1-way ANOVA, Dunnett's posttest; $P < 0.05$). (**C**) Quantitative PCR analysis of α5 gene expression conducted on total RNA extracted from subconfluent (SC) or postconfluent (PC) HCECs grown on BSA or on either CI- or CIV-coated culture plates. Data were normalized to GAPDH, and their statistical relevance was analyzed by 1-way ANOVA (Dunnett's posttest; $P < 0.05$). (**D**) Western blots conducted on total proteins extracted from HCECs grown to subconfluence (SC) or postconfluence (PC) on BSA or either CI- or CIV-coated culture plates using antibodies against the α5 integrin subunit and actin (loading control). Signal intensities were determined by densitometric analysis and are presented as the ratio of α5 protein over that of actin.

(*IGFBP6*, *ALOX15B*, *TNNT1*, *ALDH1A1*, *TMEM91*, *ALDH3A1*, *TXLNG*, *FOS*, and *FOSB*) among the 55 genes shown in Figure 6D and validated their expression by qPCR. As shown in Figure 6E, analysis of the expression behavior for each of these randomly selected genes perfectly matched that observed by gene profiling on microarrays (Fig. 6D). As AP-1 is a key regulator of α5 gene expression[35] and both *FOS* (that encodes the AP-1 subunit c-Fos) and *FOSB* (that encodes the AP-1 subunit FosB) were among the 55 genes that are the most deregulated when HCECs are grown on CI (c-Fos, but not FosB, is also down-regulated to a lesser level on CIV [Supplementary Table S2]), we also examined whether these alterations also translate into similar changes at the protein level by Western blot. As is shown in Figure 6F, and consistent with the data from the microarray and Western blots from Figure 5B, expression of the c-Fos protein is considerably reduced (by 64%) in HCECs grown on CI and moderately reduced (by 27%) when they are grown on CIV, relative to their level in HCECs grown on BSA. Similarly, expression of FosB is reduced by 73% in HCECs grown on CI but not when they are grown on CIV, a result also consistent with the microarrays (Fig. 6D) and Western blot analyses presented in Figure 5B.

Expression of the Collagen-Binding Integrins Changes in HCECs Grown on CI and CIV

Cells can adhere to the various collagens primarily through the integrins α1β1, α2β1, α10β1, or α11β1 (reviewed in Ref. 55). We therefore examined which of these integrins are expressed by HCECs and whether their expression is influenced by CI or CIV. Human CECs cultured on CIV had morphologic characteristics similar to those of HCECs grown

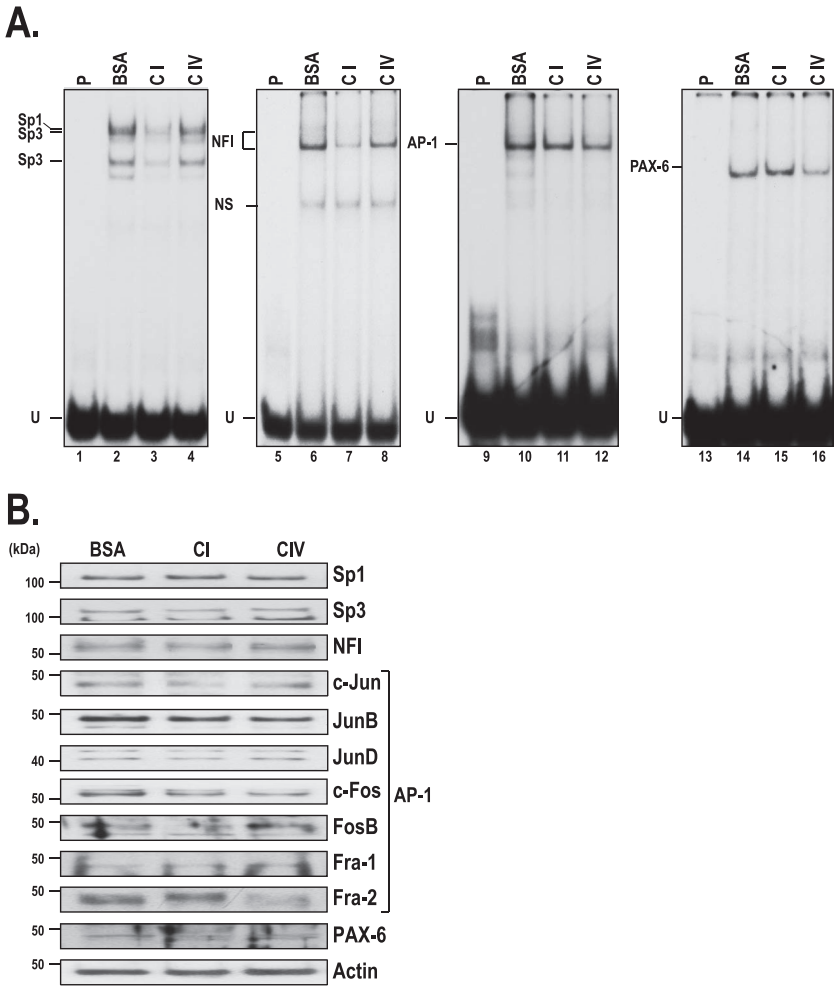

Figure 5. Influence of CI and CIV on the expression and DNA binding properties of Sp1, NFI, AP-1, and PAX-6 in HCECs. (**A**) Nuclear proteins (5 or 10 μg) from confluent HCECs grown on culture plates containing CI (75 μg/cm^2) or CIV (50 μg/cm^2) were incubated with 5′-end labeled oligonucleotides bearing the high affinity binding site for Sp1, AP-1, NFI, and PAX-6. Formation of the DNA–protein complexes was then monitored by EMSA as described in Figure 3. NS, nonspecific complex. (**B**) Approximately 10 μg nuclear proteins from the extracts used in (**A**) were Western blotted with antibodies against the TFs Sp1, Sp3, NFI, and PAX-6 and the AP-1 subunits JunB, c-Jun, JunD, FosB, c-Fos, Fra-1, and Fra-2. Actin expression was also monitored as a normalization control. The position of the nearest molecular mass markers is indicated.

on BSA, with a large proportion of very small, highly proliferative cells (Fig. 7A). Conversely, many cells presented a round morphology when HCECs were grown on CI-coated culture plates, indicating clearly that they were detaching from the coated surface. In addition, there were fewer small, less differentiated, and highly proliferative cells on CI than on CIV. Examination of the microarray data indicates that expression of the *α1* integrin subunit gene is barely detectable in HCECs, whereas moderate levels are observed for the *α2*, *α10*, and *α11* genes (Fig. 7B). Interestingly, transcription of these integrin subunit genes was significantly reduced when HCECs were maintained on CI but not when grown on CIV. However, only the reduced expression noted for the *α10* gene on CI could be validated by qPCR (Fig. 7C). All four integrin subunits were found to be present at the cell surface of HCECs, as shown by indirect immunofluorescence analyses, although both α2 and α11 were clearly the predominant forms (Fig. 7D). In addition, although expression of these integrin subunits may be subjected to alterations at the mRNA level when HCECs are grown on CI or CIV, no such alterations are obvious at the protein level.

Discussion

Corneal wound healing involves the integrated actions of multiple growth factors, cytokines and proteases produced by epithelial cells, stromal keratocytes, inflammatory cells, and lacrimal gland cells. The remodeling of the corneal architecture that takes place after injury also requires proteins from the ECM, whose numerous interactions with specific cell surface integrin receptors trigger very critical signal transduction pathways that regulate various cellular responses during that mechanism. Alterations in the expression of some of the components from the ECM during the wound healing process can lead to corneal scarring or alter corneal transparency, which would ultimately cause a reduction of the visual acuity.[56] The α5β1 integrin is a major participant in corneal wound healing as it allows the attachment and migration of the epithelial cells to FN, whose secretion is dramatically increased during the early steps of that process.[32,57] Transcription of the gene encoding the α5 subunit from the α5β1 integrin has been demonstrated to be positively regulated by FN.[36] Besides FN, both CI and CIV are also subjected to profound alterations during the repair process of injured cornea.[7,8,10] In this study, we verified whether primary culturing of both RCECs and

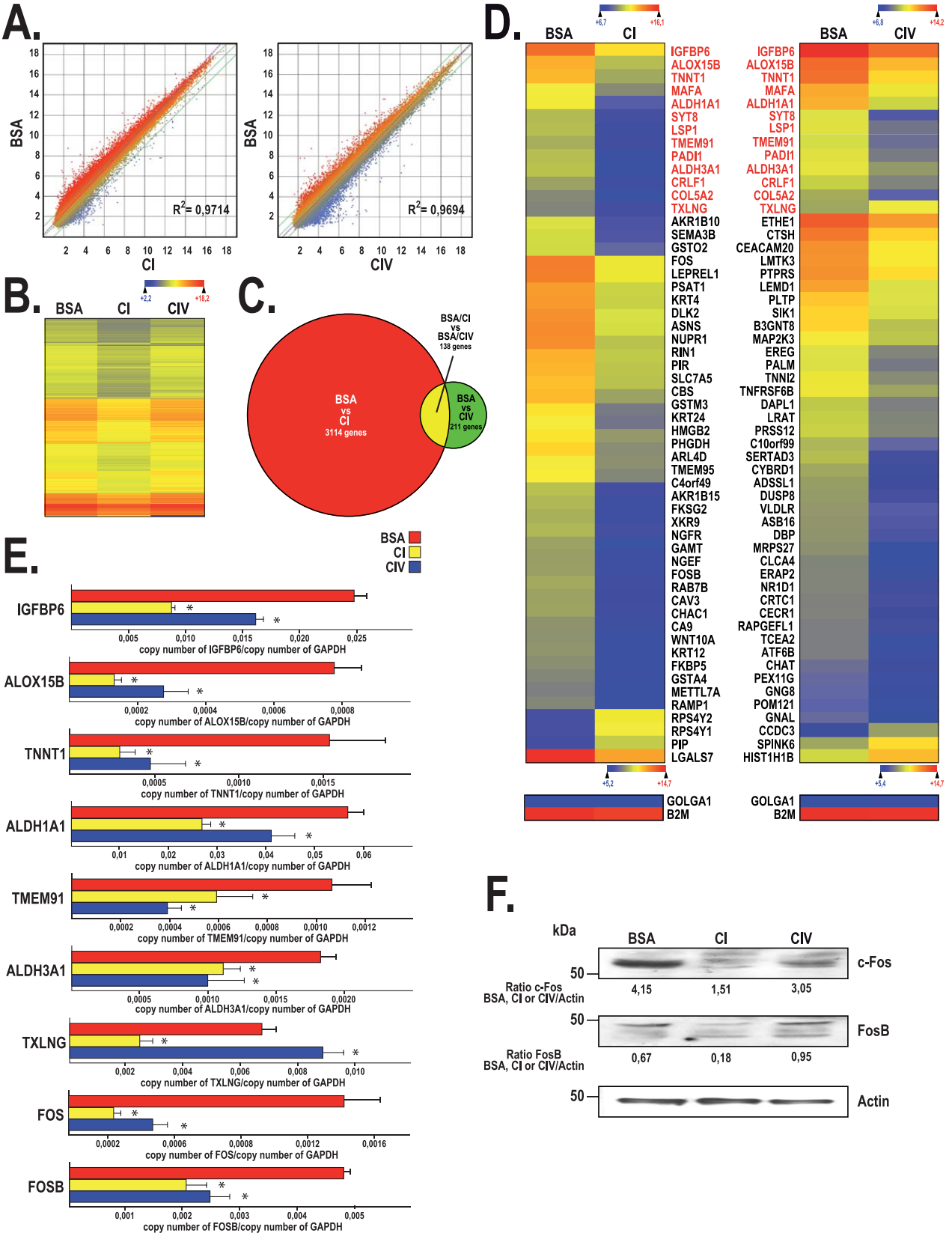

Figure 6. Microarray analysis of gene expression patterns in HCECs grown on collagens I and IV. (**A**) Scatterplots of $\log_2$ of signal intensity from 60,000 different targets covering the entire human transcriptome of HCECs grown on BSA (*y*-axis) plotted against HCECs grown on CI (*x*-axis; *left*) or CIV (*x*-axis; *right*) at confluence. (**B**) Heat map representation of genes whose expression is differentially regulated by at least 2-fold in HCECs grown on BSA against cells grown on collagen (CI or CIV). The *color scale* used to display the log2 expression level values is determined by the hierarchical clustering algorithm of the Euclidian metric distance between genes. Genes indicated in *dark blue* correspond to those whose expression is very low, whereas highly expressed genes are shown in *orange/red*. (**C**) Venn diagram that depicts the number of deregulated genes in

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HCECs grown on CI (*red circle*) or CIV (*green circle*) relative to cells grown on BSA. Deregulated genes common to both culture conditions (CI and CIV) are also indicated (in *yellow*). (**D**) Heat map representation of the 55 most deregulated genes expressed by HCECs grown at confluence on collagen (CI and CIV) relative to their levels in HCECs grown on BSA. Deregulated genes common to both culture conditions (CI and CIV) are indicated in *red*. (**E**) Quantitative PCR analysis of randomly selected genes whose expression has been found to be altered by CI and/or CIV by microarray. Data were normalized to GAPDH and their statistical relevance analyzed by 1-way ANOVA (Dunnett's posttest; $P < 0.05$). (**F**) Western blots conducted on total proteins extracted from HCECs grown on BSA or either CI or CIV culture plates using antibodies against the proteins c-Fos, FosB, and actin (loading control). Signal intensities were determined by densitometric analysis and are presented as the ratio of the target protein over that of actin.

HCECs on collagen-coated tissue culture plates influences expression of the α5 integrin subunit gene despite that the α5β1 integrin is not a collagen-binding receptor.

Comparing the results between RCECs and HCECs grown on collagen-coated culture plates was well justified as the histologic origin of both types of CECs is still a matter of debate. Indeed, most of the epithelial cells from the central area of human corneas are derived and continuously replaced by the epithelial stem cells from the limbal epithelial crypts, a special niche at the peripheral edge of the cornea.[58–60] Upon request, such as during wound healing, these cells will undergo asymmetric division and give rise to transient amplifying cells (TACs). The TACs then differentiate into mature HCECs that lose their ability to proliferate.[61,62] However, this scenario is far from being that simple with RCECs. Indeed, animal studies have shown that there are only approximately 100 progenitor cells that give rise to CECs.[63] A recent study by Haddad and Faria-e-Sousa[64] suggested that rabbit corneal epithelial stem cells reside in the corneal basal layer rather than in the basal layer of the limbal area and that it is this cell population rather than those from the limbus that have the task of renewing the rabbit corneal epithelium. Their observations are consistent with the fact that HCECs from the central cornea cannot be expanded efficiently in culture even when grown in the presence of a feeder layer,[65,66] unlike those from the central area of rabbit corneas (RCECs) that are easily cultured without any such feeder cells.[67,68] It can also explain why the amplitude of the α5/CAT promoter response toward collagens differs between RCECs and HCECs. In addition, the difference in the response toward collagen noted between rabbit and human CECs may also rely on the fact that RCECs may express a pattern of collagen-binding integrins quite different from that of HCECs. Although unlikely, HCECs from human donors of different ages or with varying postmortem delays before they are put in culture may yield results that differ slightly when they are grown on collagen. Despite this, our results demonstrated that both CI and CIV frequently acted negatively on the expression directed by the α5 promoter in CECs especially when they reach confluence.

Our results demonstrate that the negative regulatory influence exerted by collagens in RCECs but not in HCECs is determined by the α5 promoter sequences located between positions −132 and −42 relative to the α5 mRNA start site. Interestingly, this DNA region, which is required to ensure basal transcription of that gene, is also the same that we previously reported to bind the transcription factors Sp1/Sp3, AP-1, and NFI.[35] It is also this particular sequence that confers responsiveness of the α5 gene promoter toward FN.[36] In RCECs grown on CIV (that causes a more severe repression of α5 promoter activity), both the expression and DNA binding of these transcription factors were dramatically reduced when they reached cell confluence. Conversely, the significant increase in α5 promoter activity observed when RCECs are cultured to subconfluence on CIV also correlates with the increased DNA binding and expression of these transcription factors. Interestingly, a study by Takahra et al.[69] reported that gels of collagen type I increased DNA binding of both Sp1 and AP-1 to the basal promoter of the *MMP-9* gene in subconfluent rat hepatic stellate cells (HSCs), as revealed by EMSA analyses. These observations further validate our hypothesis that collagens impact on the expression of the α5 gene by altering the expression and/or DNA-binding properties of the transcription factors required to ensure expression of that gene during the progression of wound healing. Although we could not observe a similar repression by the basal α5 promoter (contained in the −132α5-CAT construct) when transfections were conducted in HCECs (Fig. 4A), both the longer α5 promoter bearing construct −954α5-CAT (2.3-fold repression; Fig. 4B) and the endogenous α5 gene (4.2-fold repression; Fig. 4C) were consistently repressed in these cells when grown on either CI or CIV, providing evidence that further upstream located negative regulatory elements are required to repress α5 gene transcription in HCECs.

Suppression of α5 gene expression has been reported to severely reduce cell adhesion and migration of both normal and cancer cells.[70–72] Consequently, the reduced expression of α5 when both RCECs and HCECs are cultured on collagen would then be expected to reduce the adhesion/migration properties of these cells. Indeed, attachment of fibroblasts to polymerized collagen was demonstrated to promote the formation of a β1 integrin–protein phosphatase 2A (PP2A)–tuberous sclerosis complex 2 (TSC2) complex that represses S6K1 kinase activity and leads to repression of the G_1/S cell cycle transition phase, thereby suppressing fibroblast proliferation.[73] More recently, culturing highly aggressive human breast cancer cells in three-dimensional collagen type I was reported to suppress their proliferative properties by delaying S phase entry from G1 phase of the cell cycle and increasing the proportion of cells in G0.[74] Upon corneal injury, it is the massive secretion of FN that characterizes the very first changes occurring in the ECM, while in the meantime, CI and CIV temporarily disappear from the BM.[8,17,18] This first step, which has been shown to require the interaction of α5β1 with this temporary FN-enriched ECM, promotes cell adhesion and migration of CECs in order to completely cover the denuded corneal surface. Once completely covered, FN expression returns to a normal level, whereas both CI and CIV reappear beneath the newly produced epithelium.[8,17,18] One can then speculate that a particularly important function of the corneal BM collagens would consist to inform CECs that cell migration is no longer required, allowing them to differentiate vertically into suprabasal epithelial cells. However, more in-depth analyses will be required to validate this hypothesis.

Microarray analyses revealed that CI obviously altered a much greater number of genes (3252) than CIV (349) in HCECs, a difference that is also highlighted by the much divergent pattern of genes whose expression is deregulated by more than 2-fold in HCECs grown on CI, whereas cells grown on CIV have a pattern closer to HCECs grown on BSA (Fig. 6B). Interestingly, the genes encoding the AP-1 subunits c-Fos and FosB are among the 55 genes whose expression is the most deregulated when HCECs are grown on CI (Fig. 6D; Supplementary Table S2), a result that is consistent with a reduction of both these proteins in Western blot (Figs. 5B, 6F). Of the transcription factors that participate in basal transcrip-

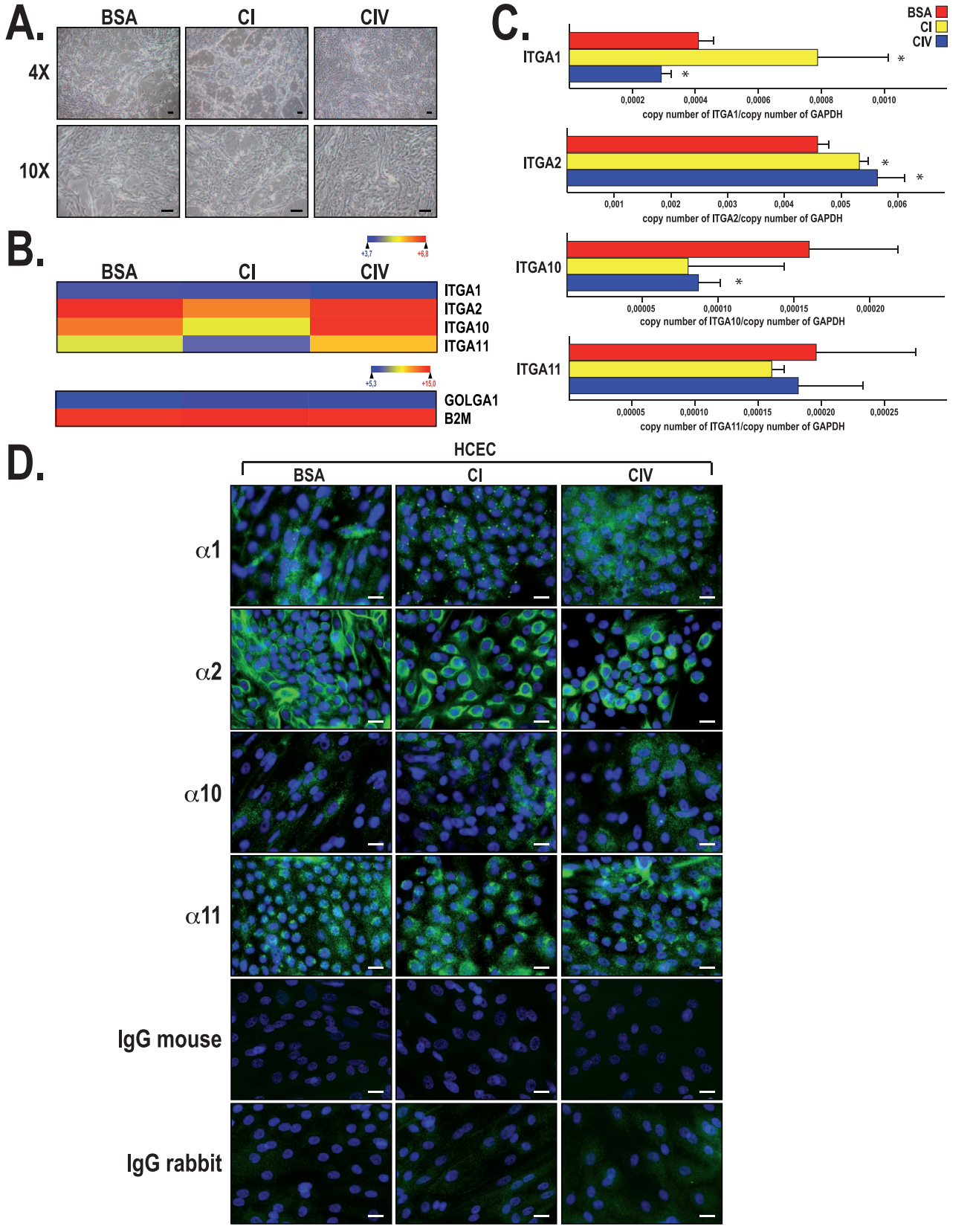

Figure 7. Collagen-binding integrins expressed by HCECs grown on collagen I and IV. (**A**) Phase contrast micrographs of HCECs grown on BSA or on either CI or CIV (magnification: 4× or 10×; *Scale bar*: 20 μM). (**B**) Heat map representation of all α integrin subunit genes reported to bind collagens and expressed by HCECs grown on BSA against cells grown on either CI or CIV. (**C**) Quantitative PCR analysis of α1, α2, α10, and α11 integrin mRNA transcript in HCECs grown on BSA (control) or on either CI or CIV. Data were normalized to GAPDH and their statistical relevance analyzed by 1-way ANOVA (Dunnett's posttest; $P < 0.05$). (**D**) Immunofluorescence analysis of α1, α2, α10, and α11 integrin expression (in *green*) in HCECs grown on BSA or on either CI or CIV. Isotypic nonimmune antibodies (either rabbit or mouse anti-IgG) were also used as negative controls. Nuclei were counterstained with Hoechst 33258 reagent and appear in *blue*. *Scale bar*: 20 μM.

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tion of the α5 gene, AP-1 was found to be the most important positively acting regulator of that gene.[35] Although not considered statistically significant as its expression level did not reach the more than 2-fold variation in gene expression settled as the lower limit, there is, however, an increased expression tendency for the gene encoding NFIA (1.5-fold increase), one of four members from the NFI family that have been reported to act as strong negative regulators of α5 gene expression.[35]

Of the 55 genes identified as the most deregulated when HCECs are grown on CI and CIV, 13 (*IGFBP6*, *ALOX15B*, *TNNT1*, *MAFA*, *ALDH1A1*, *ALDH3A1*, *SYT8*, *LSP1*, *TMEM91*, *PADI1*, *CRLF1*, *COL5A2*, and *TXLNG*) are commonly deregulated (all repressed, with the exception of *TXLNG*) by both types of collagens, of which a few may be of importance in the wound healing process. For instance, aldehyde dehydrogenase (ALDH) is a family of enzymes that comprises 19 proteins in human that are involved in maintaining cellular homeostasis by metabolizing reactive aldehydes. They modulate several cellular functions such as proliferation, differentiation, survival, and cellular response to oxidative stress. The protein encoded by the *ALDH 1 family, member A1* gene (*ALDH1A1*), which also belongs to the corneal crystallins family, also helps maintaining the transparency and refractory aspects of the cornea.[75] In addition, the protein encoded by the *aldehyde dehydrogenase 3A1* (*ALDH3A1*) gene, reported to be expressed in the cornea, was suggested to contribute to the protection of the cornea against UV-induced oxidative damage.[76] Elevated levels of ALDH1A1 and ALDH3A1 are associated with poor clinical outcome and chemoresistance in a wide variety of human malignancies,[77–79] suggesting that the product of these genes may contribute to tumor growth. Therefore, down-regulating *ALDH1A1* and *ALDH3A1* gene expression by a molecular mechanism involving intracellular signaling by CI and CIV, as reported in this study, might be required to restrict the growth ability of HCECs and prompt them to differentiate into suprabasal cells. Also, the gene coding for arachidonate 15-lipoxygenase (*ALOX15B*) has been shown to be expressed by human corneal epithelial cells.[80] The 15-LOX-2 protein is a nonheme, iron-containing lipid peroxidizing enzyme that dioxygenates arachidonic acid; 15-LOX2 has a very restricted expression pattern limited to prostate, lung, skin, and cornea.[81,82] Because of this tissue-restricted pattern of expression, it has been proposed that 15-LOX2 may play a role in the normal development of these tissues, and any abnormality in its expression/function could contribute to their progression toward tumorigenesis. Therefore, signaling through collagens may also participate in the fine-tuned expression of the *ALOX15B* gene. Interestingly, the *MAFA* gene, whose expression is similarly corepressed by both CI and CIV in microarray analysis, encodes for a bZip transcription factor (mafA) that can induce sustained proliferation of postmitotic quail neuroretinal cells.[83] MafA was reported as sufficient to induce lens-specific crystallin expression in the head ectoderm of the chick.[84–86] As of today, no study ever reported the expression of MafA in the cornea. Therefore, one of the many functions of CI and CIV might be to maintain low levels of expression of the *MAFA* gene in the cornea, as it is not intended to be expressed in that tissue.

The varying influences of CI and CIV on the pattern of genes expressed by HCECs observed in the present study may depend on outside-in signaling through the use of different integrin-mediated signal transduction pathways. Our results demonstrated clearly that the predominant collagen-binding integrins expressed by HCECs are α2β1 and α11β1, although they also express both the α1β1 and α10β1 integrins to low levels. Collagen Type IV has been reported to trigger activation of the mitogen-activated protein kinases (MAPK) pathway through its interaction with the α1β1 integrin.[87] In addition, culturing human colon cancer GEO cells on CIV was also demonstrated to activate the focal adhesion kinase (FAK)/extracellular signal-regulated kinase (ERK)/μ-calpain pathway through interaction with the α2β1 integrin, a signaling route thought to be particularly important for tumor cell motility.[88] Furthermore, culturing LNCaP prostate cancer cells on CI led to activation of an intracellular signalization route, the FAK/src/paxillin/Rac/c-Jun N-terminal kinase (JNK) transduction pathway,[89] which is very distinctive from that mentioned above for CIV, thus further supporting the possibility that different types of collagens may also activate different transduction pathways. In avian embryonic corneal epithelia, actin reorganization in response to exposition to collagen type I was found to be influenced by integrin-dependent signalization through the (phosphoinositide kinase-3) PI3K and MAPK/mitogen-activated kinase kinase (MEK)/ERK pathways.[90] Interestingly, expression of the α11β1 integrin has been shown to be positively regulated by a TGF-β2 signaling pathway when cardiac fibroblasts are cultured on glycated collagen. This TGF-β2–dependent increase in expression is apparently mediated by a regulatory mechanism involving the positive action of the transcription factors Sp1 and Smad3.[91] Transforming growth factor-β (especially the TGF-β1 isoform) is well known to be of importance during corneal wound healing,[92] as are cytokines[93] that often have opposite effects to those of the TGF-β isoforms.[92,94] Furthermore, both cytokines and growth factors have recently been shown to considerably improve immune tolerance after corneal transplantation.[95,96]

Taken together, these results demonstrate that, along with FN, the changes occurring in the ECM's secretion of collagens have an impact on the pattern of genes that also comprises the α5 integrin subunit gene, which become deregulated in response to corneal wound healing. Further studies using pharmacologic inhibitors of key modulators from the major signal transduction pathways or blocking antibodies directed against the collagen-binding integrins expressed by HCECs will be needed to precisely determine which signal transduction cascades become activated when these cells are grown on either CI and CIV.

Acknowledgments

The authors thank Patrick Carrier (CUO-Recherche, Hôpital du Saint-Sacrement, Centre de recherche du CHU de Québec, Québec, QC, Canada) for help with the primary culture of HCECs and Caroline Diorio (CHU de Québec, Hôpital du Saint-Sacrement, Québec, QC, Canada) for contributions to the statistical analysis of the data.

Supported by a grant from the Natural Sciences and Engineering Research Council of Canada (SLG 138624-2012). JL and MEG were supported by studentships from the Canadian Institutes of Health Research. CC was supported by a studentship from the Fonds de Recherche du Québec-Santé (FRQS). The Banque d'yeux Nationale is partly supported by the Réseau de Recherche en Santé de la Vision from the FRQS.

Disclosure: **J. Lake**, None; **K. Zaniolo**, None; **M.-È. Gingras**, None; **C. Couture**, None; **C. Salesse**, None; **S.L. Guérin**, None

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