

Full length article

Enhanced wound healing of tissue-engineered human corneas through altered phosphorylation of the CREB and AKT signal transduction pathways



Camille Couture ^{a,b,c,d}, Pascale Desjardins ^{a,b,c,d}, Karine Zaniolo ^{a,b,c,†}, Lucie Germain ^{a,b,c,d}, Sylvain L. Guérin ^{a,b,c,*}

^a CUO-Recherche, Médecine Régénératrice – Centre de recherche FRQS du CHU de Québec-Université Laval, Québec, Canada

^b Centre de Recherche en Organogénèse expérimentale de l'Université Laval/LOEX, Canada

^c Département d'Ophtalmologie, Faculté de médecine, Université Laval, Québec, QC, Canada

^d Département de Chirurgie, Faculté de médecine, Université Laval, Québec, QC, Canada

ARTICLE INFO

Article history:

Received 13 November 2017

Received in revised form 5 April 2018

Accepted 10 April 2018

Available online 12 April 2018

Keywords:

Tissue-engineering

Cornea

Wound healing

CREB

AKT

ARTICLE INFO

The cornea is a transparent organ, highly specialized and unique that is continually subjected to abrasive forces and occasional mechanical or chemical trauma because of its anatomical localization. Upon injury, the extracellular matrix (ECM) rapidly changes to promote wound healing through integrin-dependent activation of specific signal transduction mediators whose contribution is to favor faster closure of the wound by altering the adhesive and migratory properties of the cells surrounding the damaged area. In this study, we exploited the human tissue-engineered cornea (hTECs) as a model to study the signal transduction pathways that participate to corneal wound healing. By exploiting both gene profiling and activated kinases arrays, we could demonstrate the occurrence of important alterations in the level of expression and activation of a few mediators from the PI3K/Akt and CREB pathways in response to the ECM remodeling taking place during wound healing of damaged hTECs. Pharmacological inhibition of CREB with C646 considerably accelerated wound closure compared to controls. This process was considerably accelerated further when both C646 and SC79, an Akt agonist, were added together to wounded hTECs. Therefore, our study demonstrate that proper corneal wound healing requires the activation of Akt together with the inhibition of CREB and that wound healing *in vitro* can be altered by the use of pharmacological inhibitors (such as C646) or agonists (such as SC79) of these mediators.

Statement of significance

Corneal wounds account for a large proportion of all visual disabilities in North America. To our knowledge, this is the first time that a tissue-engineered human cornea (hTEC) entirely produced using normal untransformed human cells is used as a biomaterial to study the signal transduction pathways that are critical to corneal wound healing. Through the use of this biomaterial, we demonstrated that human corneal epithelial cells engaged in wound healing reduce phosphorylation of the signal transduction mediator CREB while, in the mean time, they increase that of AKT. By increasing the activation of AKT together with a decrease in CREB activation, we could considerably reduce wound closure time in our punch-damaged hTECs. Considering the increasing interest given to the reconstruction of different types of tissues, we believe these results will have a strong impact on the field of tissue-engineering and biomaterials. Altering the activation status of the Akt and CREB proteins might prove to be a therapeutically interesting avenue and may also find applications in wound healing of other tissues beside the cornea, such as the skin.

© 2018 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.

* Corresponding author at: CUO-Recherche/LOEX, Hôpital du Saint-Sacrement, Centre de recherche du CHU de Québec, Québec, QC, Canada.

E-mail address: Sylvain.Guerin@fmed.ulaval.ca (S.L. Guérin).

† Deceased November 2017.

1. Introduction

Our ability to see our surrounding relies directly on the integrity of the anatomical structures composing our eyes. One of these key components is the cornea, consisting of the outer layer of the eye. Its transparency is crucial for light transmission to the retina and allows proper visual perception of our environment. However, because of its location, the cornea is subjected to chemical and mechanical injuries. Corneal wounds are often the result of abrasion caused by fingernails [1] or prolonged contact lenses wear [2]. However, chemical burns, which can extensively damage the eye surface, remain the more severe cause of corneal wounds. Burn severity depends greatly on the nature of the chemical substance [3]. Contact with the limbal area is also a key factor of the injury's severity, the prognostic of healing being poor when more than 50% of the limbal area is damaged [4]. The consequences of such corneal damages are dramatic and can lead to the complete loss of vision. Infections causing ulceration are frequent after corneal injuries [5]. If not treated rapidly and properly, the infectious pathogen can penetrate the entire cornea and cause damages to the subjacent tissues [6]. Many of these injuries will compel to corneal transplant or eye enucleation when they are untreated [7]. Furthermore, the loss of stem cells from the limbus of the damaged eye can lead to the limbal stem cell deficiency syndrome that also causes a partial or complete opacification of the cornea [8]. In the more severe cases, a complete loss of vision may arise [9]. Regardless of the cause, the faster wound closure is occurring following corneal injury, the fewer consequences will result.

Recent improvements in tissue engineering have led to the reconstruction of a functional human corneal substitute that mimics the native cornea. This tridimensional substitute is composed of both a stroma and a stratified epithelium made up of 5–7 layers of untransformed human corneal epithelial cells (HCECs) [10–12]. More recently, this 3D-model was completed by the addition of a monolayer of corneal endothelial cells [11]. This novel corneal substitute relies on the ability of fibroblasts to secrete their own extracellular matrix (ECM) when ascorbic acid is added to the culture media, described as the self-assembly approach [13]. Because it is devoid of any synthetic material, our human tissue-engineered corneas (hTECs) share many aspects with the native cornea, such as a well-organized basement membrane (BM) and a typical epithelial stratification [11,12]. Recently, this substitute has been used in our laboratory to study both the cellular and molecular processes occurring during wound healing [14]. The dynamics of wound closure were found to be very similar between our hTEC and the native cornea, as the same genes and enzymes (in this case, matrix metalloproteinases) were found to be similarly altered during this process [15]. Because of these similarities, our hTEC represents an outstanding model that we can exploit to study in detail the molecular mechanisms of corneal wound healing as a prerequisite to further studies in animals.

Corneal wound healing is a complex event involving many processes such as cell death, proliferation, migration, adhesion and differentiation [16]. During these steps, the composition of the ECM is continually modified to allow proper reepithelialization, epithelial cell migration and differentiation [17,18]. The ECM is a non-cellular network of proteins and polysaccharides on which cells adhere through cell-matrix interactions [19]. Changes in the composition of the ECM are perceived by the integrins, a family of membrane-anchored receptors that bind components of the ECM [20,21]. The downstream cascades of mediators activated by these cell-matrix interactions then lead to the transcription of genes involved in wound healing [22]. The major pathways activated by the integrins include the JAK/STAT [23], MAPK [24] and PI3K/AKT pathways [25,26]. However, in corneal wound healing,

little is known about which pathway contributes the most to the healing process.

In the present study, we used the hTEC as a model to investigate the signal transduction cascades that are activated in response to the remodelling of the ECM occurring during corneal wound healing. Alterations in the activation status of both the CREB and Akt mediators from the MAPK and PI3K/AKT pathways, respectively, were observed during corneal wound closure. Furthermore, by simultaneously reducing activation of CREB with the pharmacological inhibitor C646, and increasing that of Akt with the SC79 agonist, we considerably accelerated wound closure using wounded hTECs as a biological model.

2. Materials and methods

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki. The protocols were approved by the CHU de Québec – Université Laval hospital and Université Laval Committees for the Protection of Human Subjects.

2.1. Cell culture, production and wounding of the tissue-engineered human corneas

Human corneal epithelial cells (HCECs) were isolated from the limbal area of normal eyes (obtained from the Banque d'Yeux Nationale of the Centre Universitaire d'Ophtalmologie; CHU de Québec, Hôpital du Saint-Sacrement, Québec, QC, Canada) of 44-, 52- and 71 year-old donors as previously reported [10,27]. HCECs were cultured with a feeder layer of irradiated murine Swiss-3T3 fibroblasts (ATCC, Rockville, MD) [10] whereas human corneal fibroblasts were isolated from the stromal portion of a 26 days-old donor cornea left after dispase digestion and removal of both the endothelium and epithelium, and primary cultured as before [10,28]. All cells were grown under 8% CO₂ at 37 °C and culture medium was changed after 2–3 days [12].

The tissue-engineered, two-layers 3D human corneas that have been used in this study were produced following the self-assembly approach [11,12]. Briefly, corneal fibroblasts were seeded and cultured in fibroblast growth medium and prompted to lay down their own ECM by the further addition of 50 µg/ml ascorbic acid (Sigma, Oakville, Ont., Canada) for 35 days [12]. After peeling from the flasks, two tissue sheets were superimposed to form a reconstructed stroma and were cultured for another week so that they could adhere to each other. HCECs were then seeded on the surface of the reconstructed stroma and cultured in submerged conditions in complete epithelial cell medium supplemented with ascorbate [12,28,29]. After 7 days, reconstructed tissues were fed an EGF-free epithelial cell medium and raised at the air–liquid interface for 10 days to induce epithelial differentiation. Reconstructed partial thickness corneas produced by the self-assembly approach were then wounded using a 8-mm biopsy punch (Fig. 1A and B). After wounding, the tissue-engineered corneas were placed over two supplementary fibroblast sheets (F3 and F4 on Fig. 1B) to allow reepithelialization over a natural matrix and the culture pursued at the air–liquid interface. It is important to point out that the culture medium was provided under the fibroblast sheets, and was not in direct contact with the epithelium. When indicated, the Akt inhibitor GDC-0068 (36 nM; Selleckchem, Houston, TX, USA), the p300/CREB-binding protein (CBP) inhibitor C646 (800 nM; Selleckchem) or the Akt agonist SC79 (22 nM; Sigma-Aldrich, Oakville, Ontario, Canada) were added to hTECs (in the culture medium, underneath the fibroblast sheets) immediately after they were wounded with the biopsy punch. The specific concentration selected for each of these pharmacological compounds is based on MTS assays conducted on HCECs

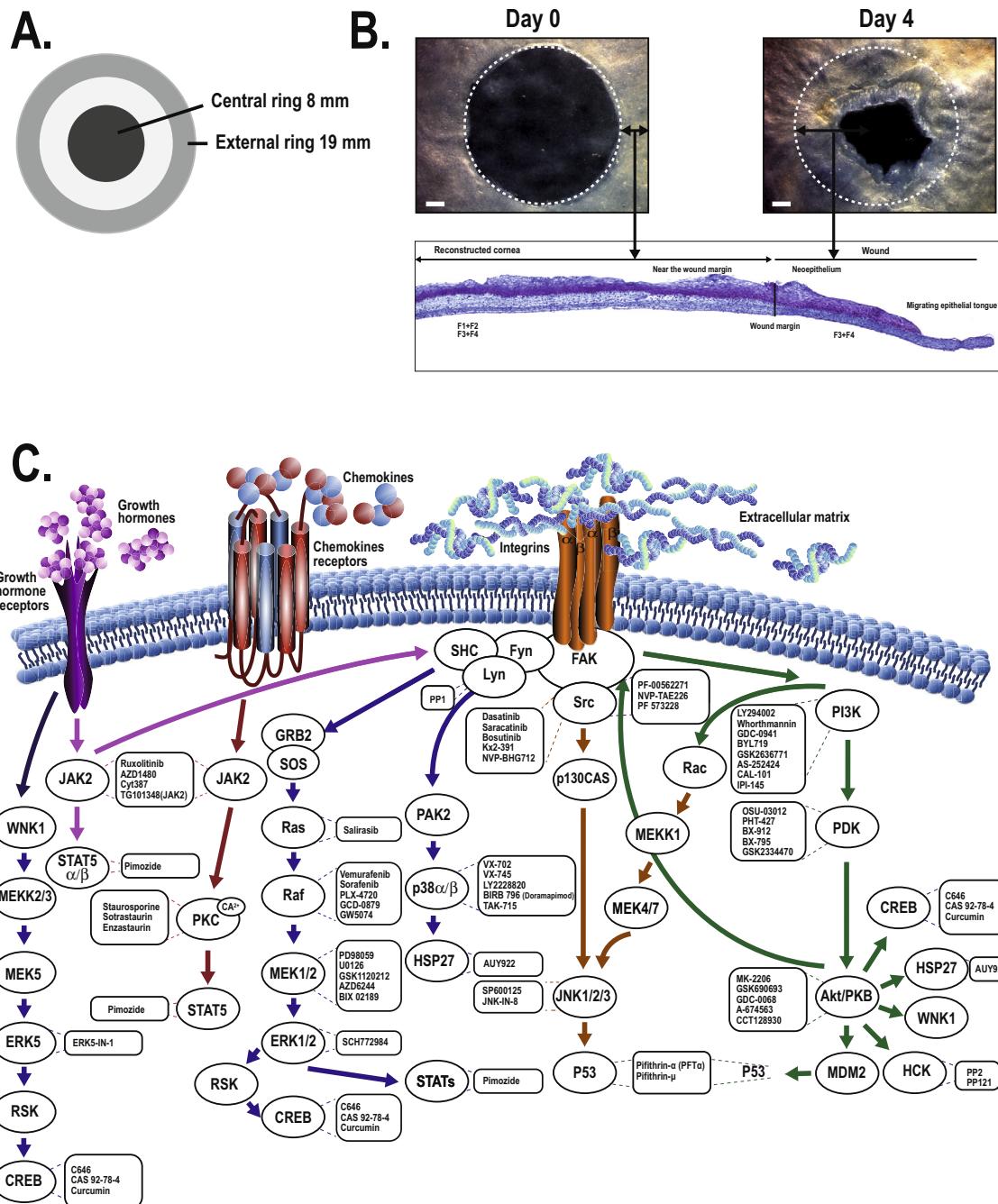


Fig. 1. Wound healing model on tissue-engineered human corneas. A: Schematic representation of the areas, from the wounded reconstructed cornea, that were recovered five days following wounding in order to prepare total RNA in view of microarray and Western blot analyses. **B:** Wound closure on hTEC produced using HCECs isolated from the eyes of a human donor. Closure of the wounded epithelia is shown at day 0 (control) and 4 days following corneal injury. Bottom panel: Composite image showing a complete view of the wounded hTEC 4 days following corneal damage (sections were stained with Masson trichrome; cells are pink and collagen is bluish). The wound margin created by the biopsy punch is indicated. F1+F2: initial fibroblast sheets present in the reconstructed cornea prior to wounding; F3+F4: supplementary fibroblast sheets added following hTEC wounding. Scale bars: 1 mm. **C:** Major signal transduction pathways activated during wound healing. The identity of the various protein mediators are indicated along with their known pharmacological inhibitors.

grown as a monolayer (see *Supplementary Methods* and *Supplementary Fig. 4*). Wound closure was then monitored macroscopically every 24 h for 4 days and photographed at 24 h intervals (Zeiss Imager.Z2 microscope (Zeiss Canada Ltd, North York, ON, Canada). All experiments were conducted in quadruplicates.

2.2. Scratch wounds assay

Corneal epithelial cells (1×10^6 cells) were plated with irradiated murine 3 T3 fibroblasts (3×10^6 cells) in 110 cm^2 plates (86

$\text{mm} \times 128 \text{ mm}$) in DH medium (Dulbecco-Vogt modification of Eagle's medium with Ham's F12 in a 3 : 1 ratio) with supplements (5% FetalClone II serum, 5 $\mu\text{g}/\text{mL}$ insulin, 0.4 $\mu\text{g}/\text{mL}$ hydrocortisone, 10 ng/mL epidermal growth factor, 10^{-10} mol/L cholera toxin, 100 $\mu\text{g}/\text{ml}$ Penicillin, and 25 $\mu\text{g}/\text{ml}$ Gentamycin). When cells reached confluence, a scratch (1.8 cm large \times 12.8 cm long) was created in the center of the plate using a cell scraper (Fisher Scientific, Asheville, USA). SC79 (22 μM) alone, C646 (800 nM) alone and a combination of both pharmacological agents (C646 + SC79) were added to fresh culture media every day until one condition was

completely healed. Cells were then harvested to proceed immediately to the nuclear protein extraction.

2.3. Nuclear extracts and EMSA (electrophoretic mobility-shift assay)

Nuclear extracts prepared from all cell types were dialyzed against DNase I buffer [50 mM KCl, 4 mM MgCl₂, 20 mM K₃PO₄ pH 7.4, 1 mM β-mercaptoethanol (Bio-Rad Laboratories, Mississauga, ON, Canada) and 20% (v/v) glycerol], as described [30]. EMASAs were then conducted as described previously [31] by incubating nuclear proteins with a 5' ³²P-end-labelled double-stranded oligonucleotide bearing the high affinity binding site for the transcription factor CREB and used as a probe in EMASAs. Briefly, 5 × 10⁴ cpm labeled probe was incubated with the amount of crude nuclear proteins specified in the figure legend in the presence of 125 ng of poly(dIdC)-(dI-dC) (Amersham Biosciences, Piscataway, NJ, USA) and 50 mM KCl in buffer D [10 mM Hepes pH 7.9, 10% v/v glycerol, 0.1 mM EDTA, 0.5 mM DTT (dithiothreitol; Sigma-Aldrich Canada, Oakville, ON, Canada) and 0.25 mM phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich Canada)]. Competition experiments in EMSA were conducted by adding unlabeled, double-stranded oligonucleotides (100- to 500-fold molar excesses) bearing either the CREB binding site or the high affinity binding sites for the transcription factors Sp1 and AP1 ([Supplementary Table 1](#)) during the assay. DNA-protein complexes were next separated by gel electrophoresis through 6% native polyacrylamide gels run against Tris-glycine buffer (50 mM Tris, 2.5 mM EDTA, 0.4 M glycine) at 4 °C. Gels were dried and autoradiographed at –80 °C. Super-shift experiments were conducted by incubating 5 µg nuclear proteins from HCECs in the presence of 2 µl of a polyclonal antibody raised against CREB (Cell Signaling Technology Inc., Danvers, MA, USA).

2.4. Western blots

Western blots were conducted as described [32] using the following primary antibodies: rabbit polyclonal antibodies against phospho-Akt (1:1000; Cell Signaling Technology Inc.; detects endogenous levels of Akt only when phosphorylated at Ser473), total Akt (1:1000; Cell Signaling Technology Inc.), phospho-ERK1/2 (1:7500; Cell Signaling Technology Inc.; detects endogenous levels of Erk1 and Erk2 when dually phosphorylated at Thr202 and Tyr204 of Erk1 (Thr185 and Tyr187 of Erk2), and singly phosphorylated at Thr202), and actin (1:40000; Santa Cruz Biotechnology, Dallas, Texas, USA), a rabbit monoclonal antibody against total CREB (1:1000; Cell Signaling Technology Inc.), mouse monoclonal antibodies against total ERK1/2 (1:7500; Cell Signaling Technology Inc.) and phospho-CREB (1:100; Cell Signaling Technology Inc.; detects endogenous levels of CREB only when phosphorylated at serine 133) as well as a peroxidase-conjugated AffiniPure Goat secondary antibody against either mouse or rabbit IgG (1:1000 dilution; Jackson ImmunoResearch Laboratories, Baltimore, PA, USA). The labeling was revealed using a Detection Kit (Amersham, Baie d'Urfé, Canada) as described [33,34].

2.5. Gene expression profiling

Total RNA was isolated from the epithelial cells isolated from the central, the internal ring, and the external ring of both wounded and unwounded (used as negative controls) hTECs using the RNeasy Mini Kit (QIAGEN, Toronto, ON, CA) and its quality determined (2100 bioanalyzer, Agilent Technologies, Mississauga, ON, Canada) as recently described [15]. Because corneal fibroblasts are less abundant (36.2 +/– 1.0%) than epithelial cells (63.9 +/– 0.9%) are in the hTECs and that they are trapped in the stromal collagen matrix and not mitotically active, they will not

significantly contribute to the total RNAs isolated as nearly all of it will originate from the epithelial cells. Labeling of Cyanine 3-CTP labeled targets, their hybridization on a G4851A SurePrint G3 Human GE 8x60K array slide (Agilent Technologies) and data acquisition and analyses were all done as recently reported [15] (GSE#113438). For statistical purpose, cell samples used for microarrays were obtained from both wounded and unwounded hTECs prepared using HCECs cultured from 3 different donors (44, 52 and 71 years old).

2.6. Human phospho-kinase profiler array

The relative levels of 43 different human phosphorylated protein kinases were determined using a membrane-based antibody array (R&D Systems, Minneapolis, MN USA) according to the manufacturer's instructions. Briefly, equal amounts (300 µg) of cell lysates prepared from either the central or the external area of wounded hTECs were incubated overnight with the phosphokinase array membrane, washed to remove unbound proteins and then incubated with a mixture of biotinylated detection antibodies. Streptavidin-HRP and chemiluminescent detection reagents were applied and the signal produced at each captured spot quantified using ImageJ (from Wayne Rasband, National Institute of Health (NIH), USA).

2.7. Histology and immunofluorescence analyses

Biopsy specimens from the hTECs treated with DMSO (negative control), C646 alone, GDC-0068 alone, or the combination of C646 + SC79, were fixed with BOUIN solution (Produits Chimiques ACP; St-Leonard, QC, Canada) and embedded in paraffin. Microtome sections (5 µm thick) were stained with Masson trichrome for histologic analysis. For immunofluorescence analyses, biopsy specimens from hTECs treated with C646 + SC79 or DMSO (control) were embedded in optimal cutting temperature compound (SomaGen, Edmonton, AB, Canada), frozen in liquid nitrogen, and stored at –80 °C until use. Cryosections (8 µm thick) were fixed with acetone 100% (10 min at –20 °C) and then incubated for 45 min with either a mouse primary antibody directed against alpha-smooth muscle actin (α-SMA; 1:400; DAKO, Burlington, ON, Canada), collagen type I (1:500; Sigma, Oakville, ON, Canada) or fibronectin (1:200; Sigma, Oakville, ON, Canada), or with a rabbit primary antibody directed against collagen type IV (1:200; Abcam, Toronto, ON, Canada). All antibodies were diluted in PBS containing 1% bovine serum albumin. Samples were washed with PBS before addition of peroxidase-conjugated AffiniPure Goat secondary antibody against either mouse or rabbit IgG (1:800, Thermo Fisher Scientific Inc., Rockford, IL, USA) and further incubated for 30 min. 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.). Negligible background was observed for controls (primary antibodies omitted).

2.8. Electron microscopy

hTECs were fixed in 2.5% glutaraldehyde (Canemco, Lakefield, Québec, Canada) and processed for transmission electron microscopy (TEM), as described [35]. Briefly, the glutaraldehyde-fixed hTEC was washed in cacodylate buffer, postfixed in 1% osmium tetroxide, stained with 0.5% uranyl acetate, dehydrated in a graded series of ethanol solutions, and embedded in Poly/Bed 812. Thin sections were processed and stained with uranyl acetate

and lead citrate. Tissues were visualized using a JEOL JEM-1230 (Tokyo, Japan) transmission electron microscope at 80 kV.

2.9. Quantitative PCR (qPCR)

Quantitative PCRs were conducted using total RNAs prepared for microarray analyses exactly as recently reported [15]. The DNA sequence of all the primers used is listed in *Supplementary Table 1*.

2.10. Statistical analyses

Student's *t*-test was employed to determine statistical significance for comparison of the groups in qPCR analyses. The values are represented as mean \pm standard error of the mean (SEM) and $P < 0.05$ was considered significant.

3. Results

3.1. Damage to the tissue-engineered human cornea alters the pattern of HCECs expressed genes

Wound healing is a process involving activities such as cell migration and proliferation. Migration of corneal epithelial cells requires expression of both extracellular matrix (ECM) proteins and matrix metalloproteinases (MMPs) that ensure appropriate remodeling of the ECM during the wound healing process [36–38]. We recently evaluated the feasibility of using hTECs comprising a stroma and a stratified epithelium produced using human corneal epithelial cells (HCECs) isolated from donors of different ages, as a model for studying the molecular mechanisms of corneal wound healing in an environment close to the *in vivo* condition [15]. Punch biopsy-wounded hTECs entirely heal in 6–7 days and display a newly produced epithelium that stratifies similarly to the normal corneal structure near the wound margin and in the middle of the neo-epithelium (*Fig. 1A* and B) demonstrating that hTECs appropriately respond to injury and that it clearly represents an appropriate model to study the mechanisms of corneal wound healing.

Binding of ECM components with their corresponding integrins triggers the activation of intracellular signaling pathways such as the JAK/STAT [23], mitogen-activated protein kinases (MAPKs) or phosphatidylinositol-3OH kinase (PI3K)/Akt [39] pathways (*Fig. 1C*). To investigate whether expression of the genes coding for the major mediators of these signal transduction pathways is altered during corneal wound healing, we wounded hTECs and harvested the epithelial cells from two different areas on the wounded corneas after 4-more-days of culture at the air–liquid interface: i) the central area of 8 mm in diameter (wounded) that contains the neoepithelium, and ii) the external ring, an area that surrounds the unwounded internal ring from diameter 14 to 19 mm (*Fig. 1A*). As negative controls, epithelial cells were also isolated from the same two areas of unwounded hTECs. Total RNA was then isolated from each cell sample and used for gene profiling analyses on microarrays. Each experimental condition (wounded and unwounded) were conducted in triplicate, using HCECs isolated from donors of different ages (44, 52 and 71 years old). Comparison between HCECs from the central area of wounded corneas paired with its corresponding region in the unwounded cornea identified a total of 1120 genes whose expression is deregulated by more than 4-fold (condition A in *Supplementary Fig. 1*). The total number of deregulated genes dropped to 894 when data from the external area are compared between the wounded and unwounded hTECs (condition B in *Supplementary Fig. 1*). Of all these genes, 356 were commonly deregulated in both the central and external wounded hTECs (condition A/B in *Supplementary Fig. 1*).

We next searched the microarray data files to sort out genes encoding the mediators from the ERK5, JAK/STAT, MAPK, JNK and PI3K/Akt pathways whose expression is the most deregulated in HCECs from the central and external areas of the wounded corneas relative to their unwounded substitutes. No significant alterations were observed in the expression of mediators from the ERK5 and JAK/STAT pathways, respectively (*Fig. 2A*). However, expression of a few mediators from the MAPK (such as FAK (PTK2B), FIN, MRAS), JNK (FAK, RAC3) and PI3K (FAK, AKT3) pathways were observed (*Fig. 2A*). Interestingly, some mediators, such as FAK and AKT, had their expression considerably increased whereas others, such as RAC3 and CREB seek their expression reduced in the wounded (central) area of hTECs. The increase in the expression of AKT3 and PTK2B, as well as the reduction in CREB expression observed by microarray in the central region of wounded hTECs was also validated by qPCR (*Fig. 2B*).

3.2. Corneal wound healing differently alters the phosphorylation status of CREB and AKT

To confirm the contribution of the PI3K/AKT and CREB signaling pathways to corneal wound healing in our hTEC model, phosphorylation levels of protein mediators from different pathways were checked using the phospho-kinase proteome profiler array (*Fig. 3A*). An increase in the phosphorylation status of Akt and P53 family members was observed in the central wound, together with a decrease in the phosphorylation of CREB, ERK1/2, STAT5, Src, Lyn, Fyn, HSP27 and Hck (*Fig. 3B*).

As the phospho-kinase array can only detect the phosphorylated proteins and does not take into consideration their corresponding unphosphorylated state, we next examined the levels of total Akt (t-Akt) and CREB (t-CREB) protein, together with the level of their phosphorylated counterpart (p-Akt and p-CREB) in the proteins extracted from both the central and external regions of our wounded hTECs by Western blot analysis. Confirming the results obtained previously, we observed a significant increase in the p-Akt/t-Akt ratio in the central compared to the external area of our wounded hTECs (4.45 vs 1.61, respectively; *Fig. 3C*). Furthermore, and consistent with the phospho-kinase arrays, the p-CREB/t-CREB ratio was considerably lower in the central wound (0.40 in the central wounded area vs 1.66 in the external area). As CREB is a direct target of Erk kinases [40], we also determined the phosphorylation level of these mediators in our wounded hTECs. Consistent with the data from the phospho-kinase arrays (*Fig. 3B*), and as for CREB, the level of phosphorylated Erk1/2 (p-Erk1/2) was strongly reduced in the central area relative to the external area of our wounded hTECs (0.60 vs 11.09, respectively; *Fig. 3C*). Therefore, activation of the PI3K/Akt pathway is coordinated with the suppression of the CREB pathway during wound healing of hTECs.

3.3. Wound closure time is reduced by the CREB inhibitor C646 and the AKT agonist SC79

As pharmacological inhibitors and agonists of the PI3K/Akt and CREB are commercially available, we next evaluated the impact of altering the activation of these signal transduction pathways on the dynamic of wound closure using our wounded hTECs as a model. Approximately 20% of the wounded surfaces were remaining 4-days post-wounding on the hTECs exposed solely to the vehicle (DMSO) (which have been used as controls in *Fig. 4A*). Indeed, DMSO-exposed hTECs required between 6 and 7 days to reach complete closure of the wounds. Wounded hTECs supplemented with 36 nM GDC-0068, an inhibitor of Akt, had a dramatically reduced wound closure capacity (more than 50% of the wounded areas were remaining at 4 days post-wounding) when compared

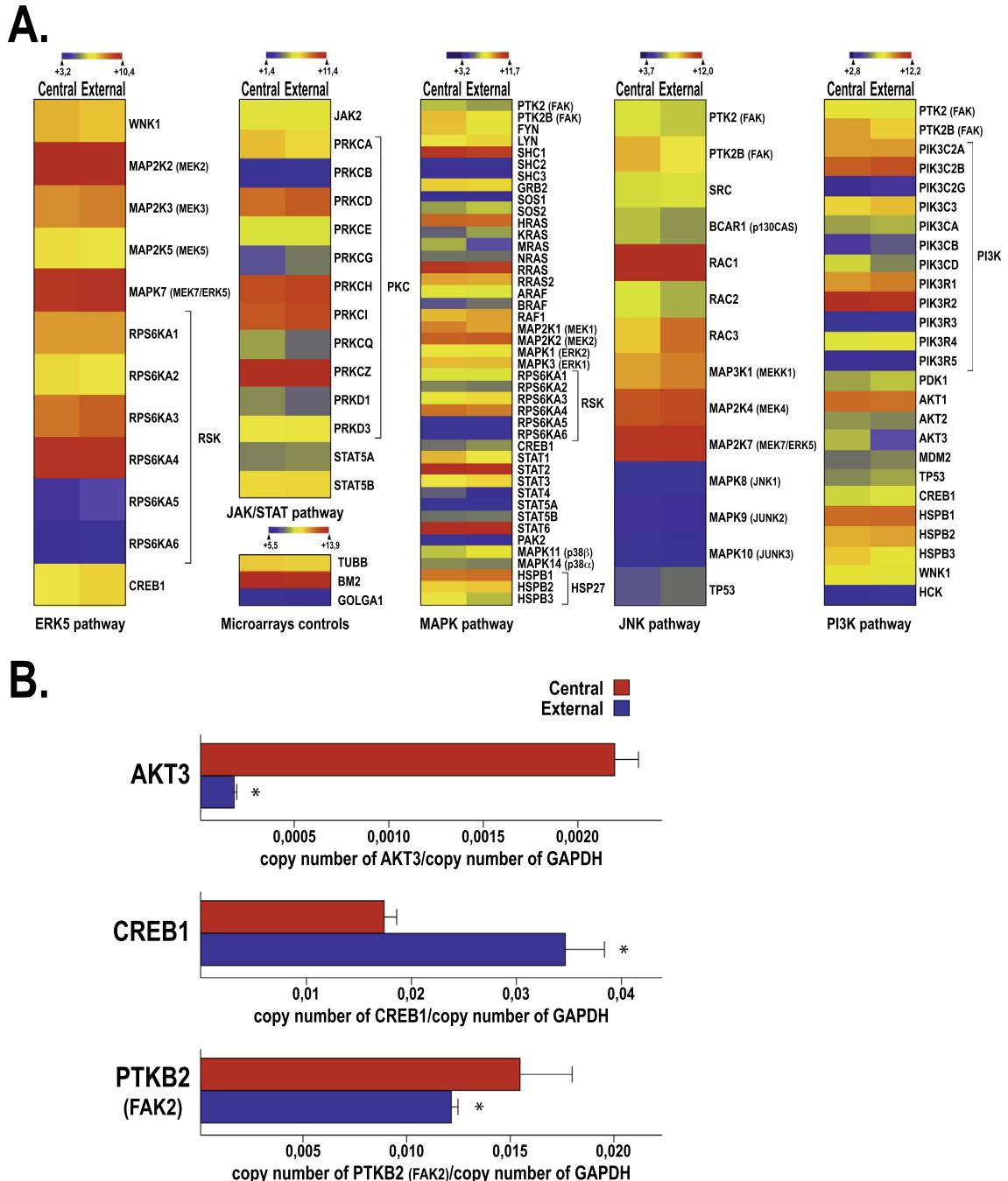


Fig. 2. Analysis of signal transduction mediator's gene expression in response to corneal damage. A: Heatmap representation of the expression profiles of the genes coding for the major protein mediators from the ERK5, JAK/STAT, MAPK, JNK and PI3K signal transduction pathways between central (wounded) and external (unwounded) areas of hTECs four days after wounding. 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. Microarray data for the housekeeping genes TUBB (tubulin β -chain), β -microglobulin (B2M) and golgin subfamily A member 1 (GOLGA1) that are expressed respectively to moderate, very high and low levels in all types of cells are also shown. **B:** qPCR analysis conducted for the genes encoding the mediators AKT3, CREB1 and FAK that have been identified as being deregulated between the central and external areas of wounded hTECs. Data were normalized to GAPDH and their statistical relevance analyzed by one-way ANOVA (Dunnett's post-test; $P < 0,05$).

to wounded hTECs exposed solely to the vehicle (DMSO) (Fig. 4A). On the other hand, wound closure was significantly accelerated (only 8% of the wounded areas were remaining at 4 days post-wounding) when wounded hTECs were maintained in the presence of 800 nM C646, an inhibitor of p300/CREB binding protein (CBP) [41]. We then treated our wounded hTECs simultaneously with the C646 inhibitor (800 nM) and the Akt agonist SC79 (22 μ M) and again monitored the dynamic of wound closure. As in the

previous experiment, near 22% of the wounded surfaces were remaining at 4 days post-wounding on the hTECs exposed solely to DMSO (Fig. 4B). On the other hand, co-incubation of wounded hTECs with both C646 and SC79 resulted in the complete closure of the wounds at 4 days post-wounding. We therefore conclude that inhibition of the CREB pathway together with activation of the PI3K/Akt pathway significantly reduces wound closing time in our wounded hTEC substitutes.

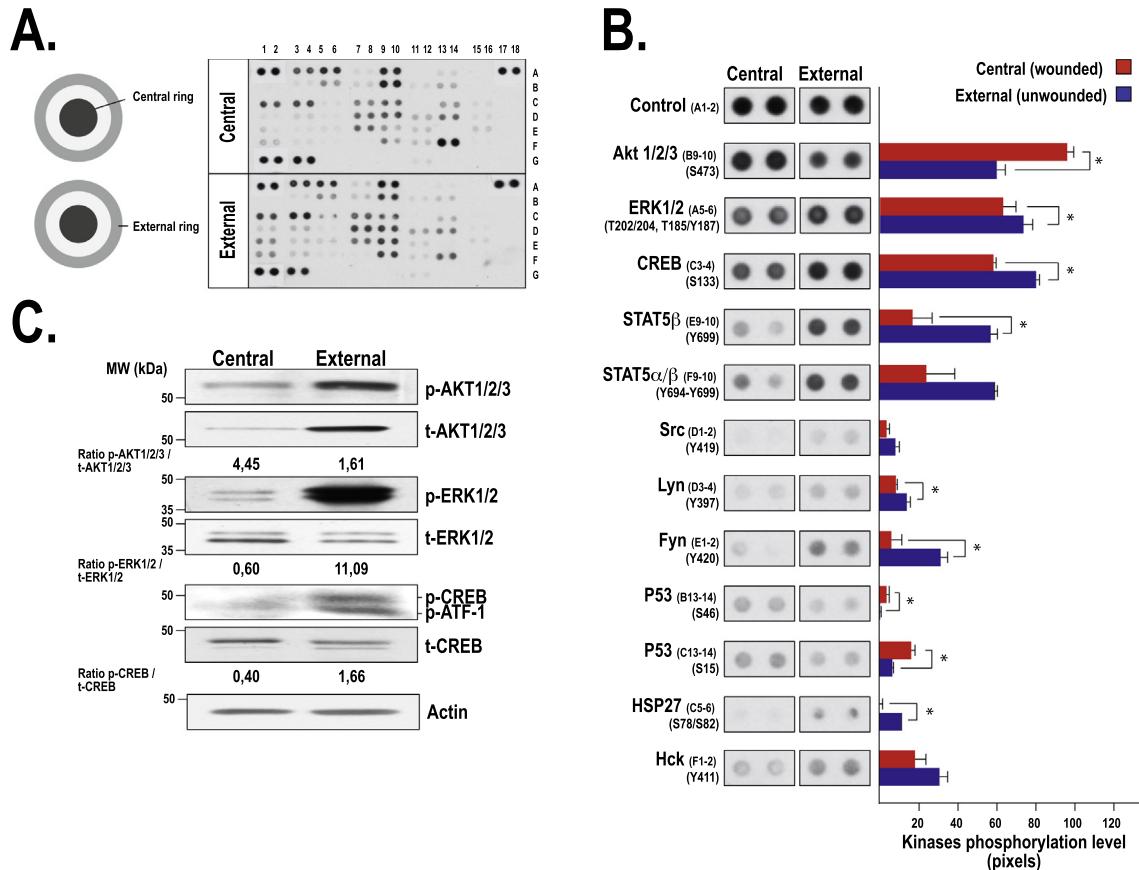


Fig. 3. Impact of corneal wound healing on the phosphorylation of signal transduction mediators. **A:** Cell lysates prepared from both the central (wounded) and external (unwounded) areas of wounded hTECs were used as sources of proteins for conducting the phosphokinase assays as indicated in *Materials and Methods*. The result obtained for each blotted membrane is shown. **B:** The duplicate spots corresponding to the protein mediators whose phosphorylation is the most altered by wound healing (Akt 1/2/3, ERK1/2, CREB, STAT5 β , Src, Lyn, Fyn, P53, HSP27 and Hck), are shown for both the central (left) and external (right) areas of the wounded hTECs are shown. The intensity of each spot as well as that of the positive control was quantified using *Image J* software and expressed as a ratio to the positive control. * $P < 0.05$ vs. each control. **C:** Cell lysates from the central and external areas of wounded hTECs were analyzed by immunoblotting to confirm the phosphokinase array results for the mediators AKT1/2/3, ERK1/2, CREB and STAT5. Actin was used as the loading control.

3.4. Histologic and ultrastructural analysis of C646/SC79-treated wounded hTECs

After wounding, hTECs are placed over two supplementary fibroblast sheets (F3 and F4 on Fig. 1A) to allow reepithelialization over a natural matrix. Four days after wounding, epithelial cells migrated to regenerate a neoepithelium. Histologic analysis of our wounded, DMSO-treated hTECs revealed the presence of a stratified epithelium with a structural organization very similar to the normal cornea (i.e., a basal layer covered by five or six layers of differentiating cells; Fig. 4C). Suprabasal epithelial cells at the tip of the migrating epithelial tongue (MET) migrated over the basal layer to make contact with the tissue-engineered stroma. The MET was however clearly shorter when wounded hTECs were added the Akt inhibitor GDC-0068. On the other hand, hTECs treated with C646 had a much longer MET that increased further when both C646 and SC79 were added together. Electron microscopic examination of the wounded hTECs revealed the presence of an organized epithelial-stromal junction (ESJ) in the unwounded side of both the DMSO-exposed (Fig. 5A and A') and C646/SC79-treated hTECs (Fig. 5C and C'), with the presence of many hemidesmosomes (black arrowheads) that attach basal corneal epithelial cells to the underlying fibroblast sheets along the continuous BM (Fig. 5A and A'). In the DMSO-treated hTECs, a discontinuous, disorganized BM was observed in the MET with the presence of a few hemidesmosomes (Fig. 5B and B'). In addition, the stromal compartment underneath the epithelium was made-up of numer-

ous collagen fibers (Supplementary Fig. 2). Therefore, these results indicate that the formation of an ESJ resumed after reepithelialization of wounded hTECs.

We then examined the expression of the ECM components CI, CIV and FN that become typically deregulated during corneal wound healing by indirect immunofluorescence analyses on wounded hTECs exposed or not to the C646/SC79 combination. As can be seen on Fig. 6, the hTECs stroma as well as that of the native cornea, expresses CI. As previously reported [42], staining for CIV was present throughout the entire stroma in the MET region of control wounded hTECs (DMSO) as in the fibroblast sheets (F3 + F4) before their addition under the reconstructed cornea. CIV became essentially expressed along the basal membrane (BM) upon incubation with C646 + SC79, a localization similar to that seen in the native cornea. FN staining was observed along the BM and more diffusely into the stroma of hTECs and the native cornea. On the other hand, none of our hTECs nor the native cornea express α -SMA, which has been used in order to ensure that stromal keratocytes have not differentiated into α -SMA $^{+}$ myofibroblasts (staining was also conducted on a section from a native human saphenous vein as a positive control).

3.5. C646 alters the phosphorylation but not the DNA binding capacity of CREB in wounded hTECs

As there are very little details regarding the impact of CREB phosphorylation on its DNA binding properties, we examined

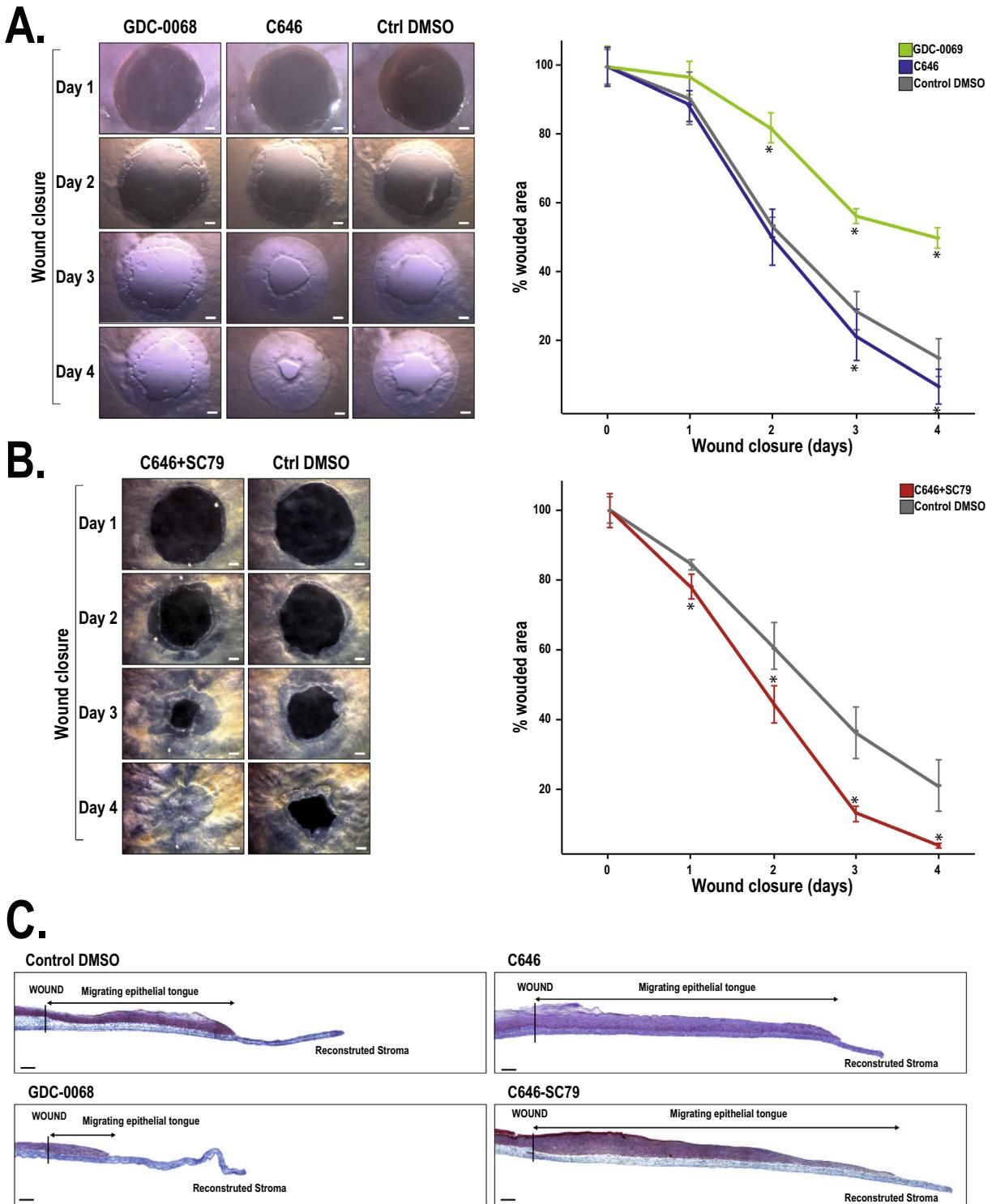


Fig. 4. Impact of GDC-0068, C646 and SC79 on wound closure of human TECs. **A:** Human TECs (one out of four representative hTECs is shown for each condition) were wounded and maintained in culture medium supplemented with GDC-0068 or C646 (left). Wound surfaces remaining for each condition were determined at each day and plotted on graph (right). Control wounded hTECs were exposed solely to the vehicle (DMSO). Scale bars: 1 mm. **B:** Wounded hTECs were incubated with both C646 and SC79 (left; one out of six representative hTECs is shown for each condition). Wound surfaces remaining were determined at each day and plotted on graph (right). Control wounded hTECs were exposed solely to the vehicle (DMSO). Scale bars: 1 mm. **C:** Composite images showing a complete histological view of wounded TECs grown in the presence of GDC0068, C646 or both the combination of C646 and SC79 at 4 days following corneal damage (sections were stained with Masson trichrome; cells are pink and collagen is bluish). The wound margin created by the biopsy punch is indicated. Scale bar: 100 μ m.

whether inhibition of CREB with C646 would also alter its capacity to recognize its high affinity target site in DNA. HCECs were therefore grown as monolayers and scratch-wounded before they were added either C646 or SC79, or the combination of both. As a control, scratch-wounded HCECs were also exposed solely to the vehicle

(DMSO). Phosphorylated CREB was easily detected in unwounded HCECs grown without any added compounds (NC) or in the wounded HCECs exposed solely to the vehicle (DMSO) (pCREB, Fig. 7A). Interestingly, and in agreement with the data from the kinase array conducted on the wounded and unwounded

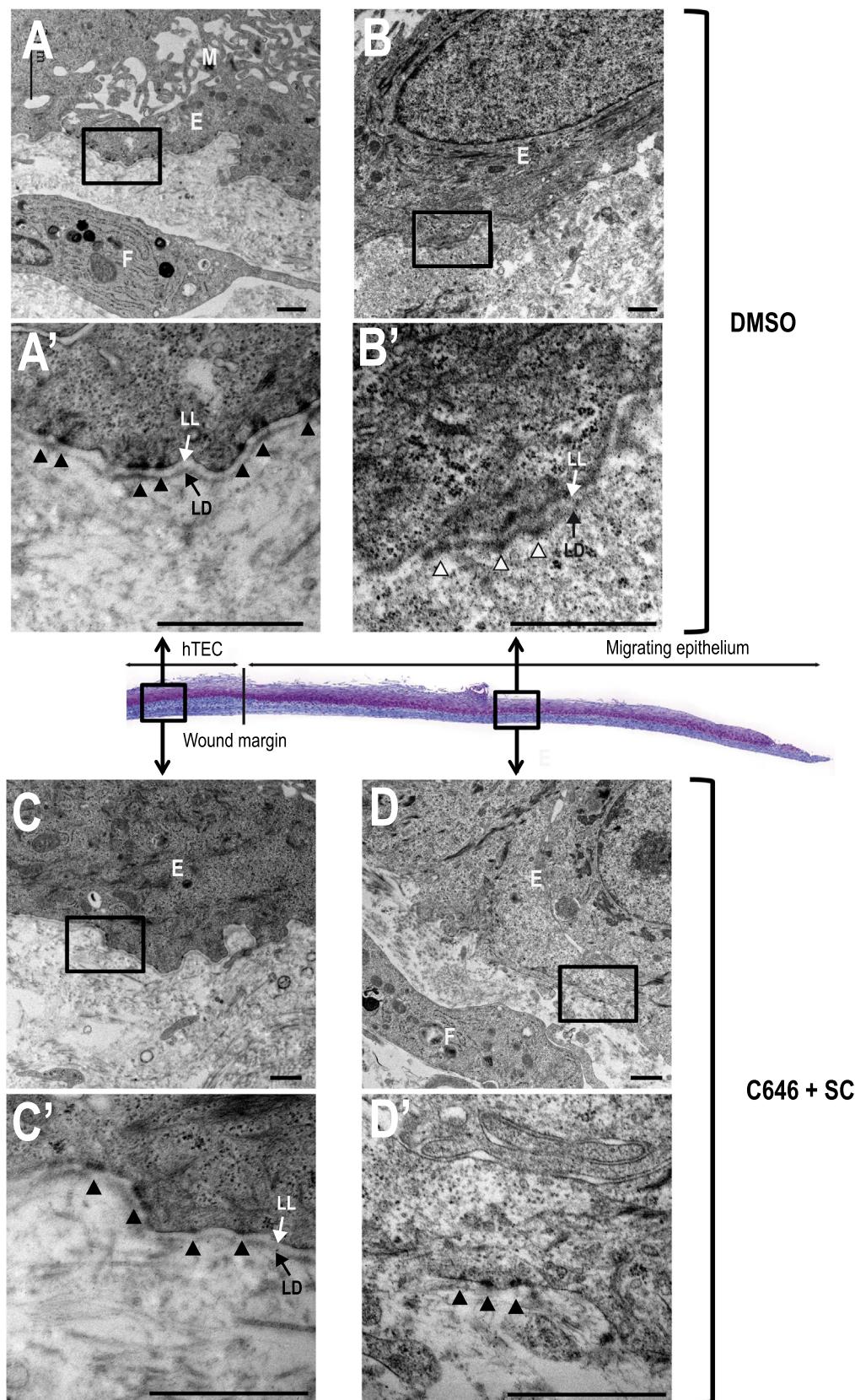


Fig. 5. Transmission electron microscopic analysis of C646/SC79-treated wounded hTECs. Electron microscopic characterization was made on the neoeplithelium of wounded hTECs treated with DMSO (negative control; panels A–B) or with the combination of C646 and SC79 (panels C–D). Higher magnification (A', B', C' and D') shows the structure of hemidesmosomes (arrowheads) present at the epidermal-stromal junction. BM, basement membrane; E, epithelial cells; M, microvilli; C, collagen fiber; Scale bars: 1 μm.

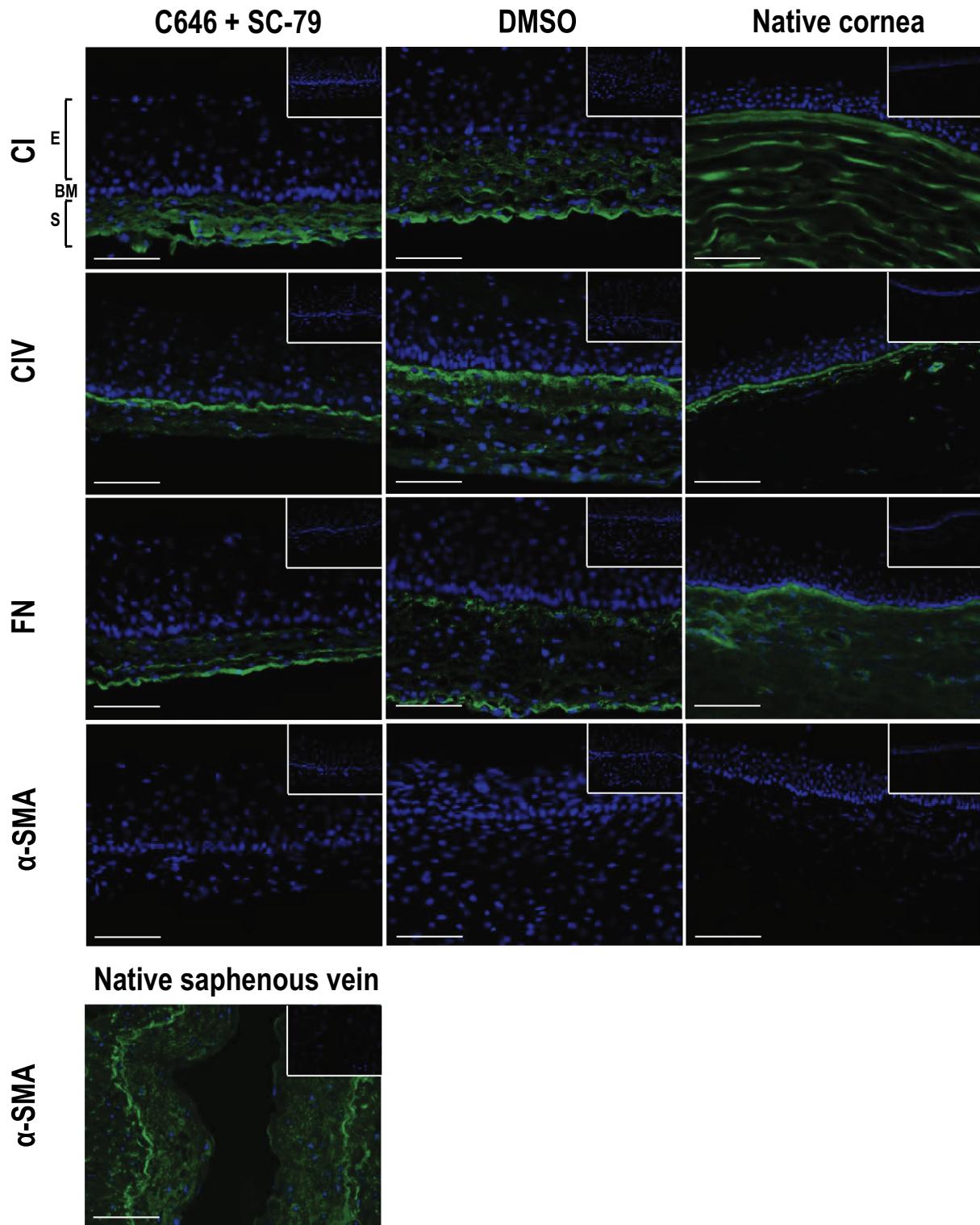


Fig. 6. Immunofluorescence analysis of the ECM from C646/SC79-treated wounded hTECs. Expression of α -smooth muscle actine (α -SMA), collagen I (Col I), collagen IV (CIV) and fibronectin (FN) was evaluated by indirect immunofluorescence (IF) analyses conducted on hTECs 4 days after wounding (DMSO and C646+SC79) or on human native corneas (used as controls; a section from a human blood vessel (saphenous vein) was also used as a positive control for α -SMA). A positive staining appears in green on the photographs. The corneal epithelium (E), basement membrane (BM) and stroma (S) are shown. Nuclei were counterstained with Hoechst 33258 reagent (blue). Each IF negative control is shown in the upper right corner of each panel. Scale bar : 100 μ m.

hTECs, we also observed a weak reduction in the amount of pCREB when the HCEC monolayer is scratch-wounded. As expected, the presence of the Akt agonist SC79 did not alter the phosphorylation status of CREB in wounded HCECs. On the other hand, no phosphorylated CREB could be detected when scratch-wounded HCECs

were grown in the presence of C646, either alone or in combination with SC79 (Fig. 7A). The absence of phosphorylated CREB when HCECs are grown with C646 did not result from a corresponding reduction in the expression of total CREB proteins as the total CREB antibody could detect amounts of CREB proteins in C646 treated

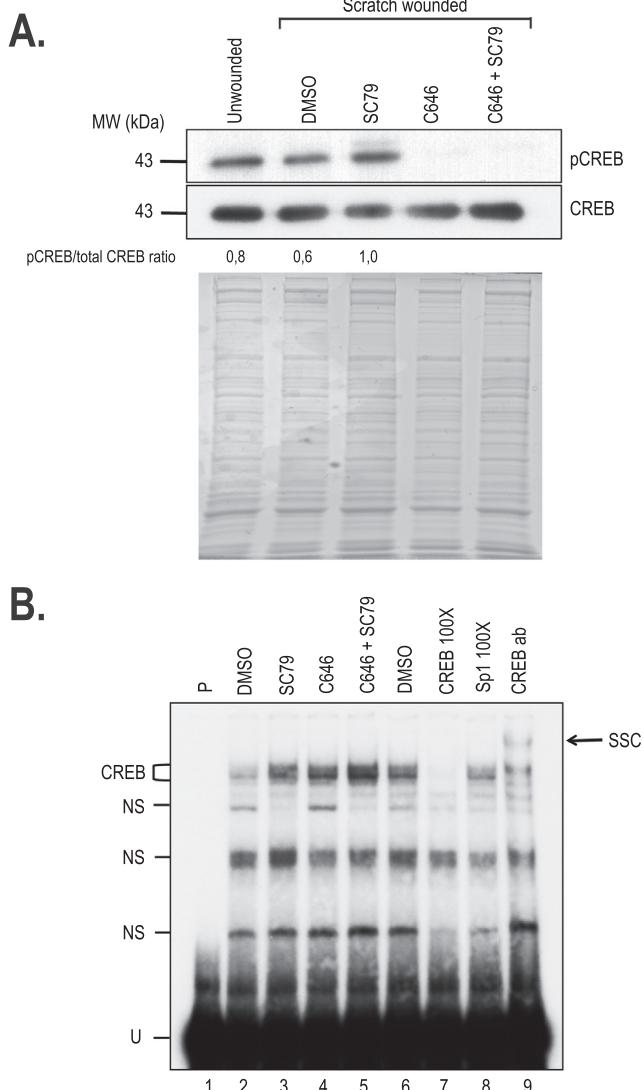


Fig. 7. Analysis of CREB expression and DNA binding in nuclear extracts prepared from monolayers of HCECs. A. Western blot analysis of both phospho-(p-CREB) and total-CREB (CREB) proteins in total cell extracts prepared from scratch-wounded monolayers of HCECs exposed to the vehicle (DMSO) or to SC79, C646 or both. Proteins from unwounded HCECs were also used as a control. The ratio of p-CREB/CREB is presented. Coomassie blue staining of each protein extract is also presented (below the Western; used as a protein load control). **B.** Nuclear proteins from scratch-wounded HCECs cultured either with the vehicle (DMSO), SC79, C646 or both SC79 and C646 were incubated with the CREB labeled probe and formation of DNA-protein complexes analyzed by EMSA. When indicated, nuclear proteins from wounded HCECs grown with DMSO were also incubated alone (DMSO) or in the presence of a 100-fold molar excess of unlabeled competitor oligonucleotides bearing the high affinity target site for either CREB or Sp1, or with a polyclonal antibody directed against CREB (last track), prior to addition of the CREB labeled probe. The position of the DNA-protein complexes corresponding to CREB bound to the labeled probe is indicated as well as that of a supershifted complex (SSC) yielded by the addition of the CREB antibody. U: unbound fraction of the labeled probe.

cells very similar to those of the DMSO control, wounded HCECs. However, neither C646 nor SC79 resulted in a decreased binding of CREB to its high affinity DNA target site in EMSA (compare the CREB-DNA complex from nuclear extracts prepared from wounded HCECs grown with DMSO (Fig. 7B, lanes 2 and 6) with those from wounded HCECs grown with C646 and/or SC79 (Fig. 7B, lanes 3, 4 and 5)). Specificity for the formation of the CREB DNA-protein complex was confirmed by the ability of an unlabeled double-stranded

oligonucleotide bearing the CREB target site (lane 7), but not one bearing the DNA binding site bound by the positive transcription factor Sp1 (lane 8), to compete for the formation of the CREB complex in EMSA (lane 6). The slow migrating complex observed in EMSA was further demonstrated to result from the binding of CREB to the DNA labeled probe by supershift in EMSA as the addition of an antibody directed against CREB caused a reduction in the formation of the CREB complex but also yielded a new, supershifted complex (SSC; Fig. 7B) that results from the formation of a DNA-CREB-antibody ternary complex. Therefore, decreased in the phosphorylation of CREB does not alter its DNA binding properties *in vitro*.

4. Discussion

Wound healing of the human cornea is a complex process that requires cell migration and proliferation. Corneal wound healing is characterized by major changes in the composition of the ECM such as a massive transitory secretion of fibronectin combined with a reduction in the secretion of collagens and laminins [43–45]. This ECM remodeling is required to allow fast migration of corneal epithelial cells in order to rapidly cover the damaged area. In the present study, we used the hTEC as a model to study the signal transduction pathways, and their participating protein mediators, that are involved in corneal wound closure. We demonstrated that expression of a few protein mediators from both the PI3K/Akt and MAPK pathways was altered during wound healing of damaged hTECs. Increased phosphorylation of Akt combined to a reduced phosphorylation of the CREB mediators were among the most striking alterations observed. By supplementing the culture medium of wounded hTECs with both the CREB inhibitor C646 and the Akt agonist SC79, we could considerably accelerate wound closure in our model.

A few human models of corneal wound healing have been described [12,46,47]. *Ex vivo* organ culture models [46,47] take advantage of the native tissue but the limited availability and inter-individual variability restrict the number of analyses that can be performed simultaneously. In contrast, a large number of conditions and amount of treated cells is possible with the scratch assay. However, epithelial cells are in contact with the growth medium that contains numerous supplements and growth factors since HCECs are cultured submerged in the culture medium. The hTEC has the advantage of a 3D structure that renders possible the culture at the air–liquid interface. Under this condition, the medium containing the growth factors is not in direct contact with epithelial cells since it is only present under the reconstructed stroma. Consequently, the epithelium is nourished indirectly, a condition that better mimic the *in vivo* condition seen with the native cornea.

Our gene profiling analyses revealed alterations in the expression of a few genes coding for mediators from both the MAPK and PI3K/Akt pathways, including genes, such as PTK2 and members of the Akt family, whose expression is increased in the central wounds, whereas others, such as CREB, are decreased in wounded hTECs. PTK2 encodes for the kinase FAK (focal adhesion kinase) that is activated through a yet poorly understood mechanism possibly involving FAK clustering, autophosphorylation of FAK at Y397 [48] and a mechanical linkage of integrins to the actin cytoskeleton [49]. This increased expression of PTK2 is also consistent with the coordinated increase in the expression of the $\beta 1$ -associated integrins $\alpha 1$, $\alpha 5$, $\alpha 6$ and $\alpha 11$ that we also observed in our wounded hTECs (Supplementary Fig. 3). Under normal circumstances, activation of FAK leads to the downstream activation of the ERK kinases [50], a required step in the activation of a number of transcription factors such as c-Jun [51], AP-1, NFkB, Nrf2 [52] and Sp1 [53]. However, our results demonstrate clearly that phosphorylation of ERK

kinases is considerably reduced (more than 18-fold reduction) in the central wound relative to its level in the external area of wounded hTECs. We previously reported that expression of the $\alpha 5$, $\alpha 6$ and $\alpha 9$ integrin subunits were negatively regulated by the transcription factor NFI [54–56]. Interestingly, Zheng et al. reported that expression of the NFI isoform NFIA was induced in an ERK-dependent manner in mouse neurons [57]. This raises the interesting possibility that the increased expression observed for both the $\alpha 5$ and $\alpha 6$ integrin genes in our wounded hTECs may result, at least in part, from the coordinated reduction in NFI activity in response to a reduction in ERK activation.

The kinase arrays conducted in this study demonstrated that both the activation of Akt and the suppression of the CREB activity are required in order to ensure proper wound closure of punch-damaged hTECs. Phosphorylated Akt as being involved in wound healing is no surprise. Akt, also referred to as protein kinase B, is a serine threonine kinase that plays many critical roles in biological processes such as cell survival, proliferation, migration, angiogenesis and metabolism. It is activated downstream of phosphatidylinositol 3-kinase (PI3K) [58] by a variety of growth factors and cytokines. The upregulated levels of Akt that have been reported in exaggerated wound-healing responses [59], together with decreased Akt signaling in chronic, non-healing wounds [60] all point to the central role played by Akt in wound healing. Indeed, Xiao et al. recently demonstrated that treatment of dorsal skin wounded C57BL/6 mice with ozone oil facilitated the wound healing by increasing fibroblast migration via PI3K/Akt/mTOR signaling pathway *in vivo* [61]. Similar observations were also reported for wound healing of the rat skin, the impaired wound healing observed for the skin of diabetic rats being reported to result from a dysfunction in the Akt/mTOR pathway [62]. Most interestingly, high glucose was reported to suppress Akt phosphorylation in a ROS sensitive manner, a process that also delayed corneal epithelial wound healing in the porcine cornea [63]. On the other hand, much less is known about the impact CREB phosphorylation has on wound healing. We initially thought that the negative action of phosphorylated CREB on HCECs proliferation in wounded hTECs could rely on its ability to increase the expression of the antiproliferative molecule p21 as target sites for CREB have been identified in the promoter region of the p21 gene [64]. However, close comparison of the microarray data between the external region and central wound revealed no significant variation in the expression of the p21 gene (Supplementary Table 2). Somehow consistent with our results, CREB phosphorylated at its serine 133 residue has been recently found to act as a negative regulator of PDGF-BB-stimulated vascular smooth muscle cells (VSMCs) proliferation [65]. Similarly, a migration-inhibitory function for CREB was also reported in glioma cells, a process that apparently depends on a CREB-miR-9 negative feedback minicircuitry [66]. Interestingly, CREB was found to simultaneously play a pro-proliferative and anti-migratory role in such cells, a process described by these authors as the “go or grow” process. Suppression of CREB by miR-150, that also demonstrates the ability to also suppress expression of EP300, was reported to increase migration and invasiveness of HCT116 cells, a mechanism that could also be reproduced by knocking-down CREB expression by CRISPR/Cas9 [67]. However, and to our knowledge, we are the first to demonstrate that both these mechanisms (suppression and activation of CREB and Akt activities, respectively) occur simultaneously while wound healing of hTECs is proceeding.

Blocking the action of CREB with the C646 inhibitor together with increasing Akt activation with the SC79 agonist is particularly interesting as it significantly reduced wound closure time from 7 days (which is required for complete closure of control hTECs exposed to DMSO) to only 4 days (when hTECs are exposed to both C646 and SC79) in our hTEC model. Analysis of the ECM composi-

tion underneath the wounded hTEC neo-epithelium exposed to C646 + SC79 revealed that as for the native cornea, they also express CI, CIV and FN. Staining for CIV was present throughout the entire stroma in the wounded hTEC (DMSO) but interestingly became essentially expressed along the basal membrane (BM) upon incubation with C646 + SC79 suggesting a more mature tissue structure resembling that observed with the native, unwounded cornea. Type IV collagen is the BM basic structural component that forms the backbone to which other ECM components attach [68]. This is particularly interesting in that culturing embryonic stem cells on CIV has been shown to induce their differentiation into corneal epithelial progenitor cells, a process dependent on the expression of the transcription factor Pax6 [69,70].

Corneal wounds account for 37% of all visual disabilities and 23% of medical consultations for ocular problems in North America [71]. Each day, about 2000 U.S. workers have a job-related eye injury requiring medical treatment. Severe recurrent and persistent corneal wounds secondary to ocular diseases such as trauma, autoimmune diseases, and chemical alkali burns, can progress to corneal perforation with a risk of eye loss [72]. Moreover, one million laser vision corrective procedures are performed each year using refractive surgery (primarily photorefractive keratectomy (PRK) and laser *in situ* keratomileusis (LASIK)) in USA [73]. According to the US Food and Drug Administration (FDA), epithelial defects reach 0.5% in patients treated with LASIK but a more realistic non-FDA estimate reported defects ranging between 5 and 22.6% depending on the study [74]. The cellular and molecular regulatory phenomena associated with postoperative wound healing are likely to be involved in the adverse effects observed after these surgeries [75] but their underlying mechanisms yet remain to be elucidated. Our demonstration that wound closure could be considerably accelerated by simultaneously suppressing CREB activation with C646 whereas that of Akt is increased with SC79 is particularly interesting as it may provide a new therapeutic tool in the treatment of many of these wound-healing related corneal pathologies. Meanwhile, as both mRNA expression of the gene encoding Fyn, a protein kinase that plays an important role upon activation of the integrin-dependent MAPK signalization pathway, and Fyn phosphorylation are considerably reduced in wounded hTECs (Figs. 2A and 3C), further characterization of this mediator might prove an interesting avenue in the understanding of the molecular mechanisms that contribute to corneal wound healing.

5. Conclusions

In this study, we demonstrated that hTECs is an attracting substitute in order to study expression of the genes encoding the many mediators of the signal transduction pathways that play pivotal functions in the response of corneal epithelial cells toward wound healing. Most of all, the Akt and CREB genes might prove to be therapeutically interesting targets and may also find applications in wound healing of other tissues, such as the skin.

Acknowledgments

The authors would like to thank Mr. Gaëtan Le-Bel, Mrs. Amélie Lavoie and Mrs. Manel Benhassine for their technical assistance, and the Institut de Biologie Intégrative et des Systèmes (IBIS; Université Laval) for their help with electron microscopy.

Funding

This work was supported by the Canadian Institutes for Health Research (CIHR) Grant MOP-12087 and FDN-143213 (L.G.), the Fondation des Pompiers du Québec pour les Grands Brûlés

(FPQGB), the Fonds de Recherche du Québec-Santé (FRQS), and the Réseau de thérapie cellulaire et tissulaire (ThéCell) du FRQS. The Banque d'yeux Nationale is partly supported by the Réseau de Recherche en Santé de la Vision from the 'Fonds de Recherche du Québec-Santé' (FRQS). C.C. was supported by a studentship from the FRQS. L.G. is the recipient of a Tier 1 Canadian Research Chair on Stem Cells and Tissue Engineering and a Research Chair on Tissue-Engineered Organs and Translational Medicine of the Fondation de l'Université Laval.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.actbio.2018.04.021>.

References

- [1] Y.B. Lin, M.F. Gardiner, Fingernail-induced corneal abrasions: case series from an ophthalmology emergency department, *Cornea* 33 (7) (2014) 691–695.
- [2] S.Y. Lee, Y.H. Kim, D. Johnson, B.J. Mondino, B.A. Weissman, Contact lens complications in an urgent-care population: the University of California, Los Angeles, contact lens study, *Eye Contact Lens* 38 (1) (2012) 49–52.
- [3] D.J. Bunker, R.J. George, A. Kleinschmidt, R.J. Kumar, P. Maitz, Alkali-related ocular burns: a case series and review, *J. Burn Care Res.* 35 (3) (2014) 261–268.
- [4] N. Gupta, M. Kalaivani, R. Tandon, Comparison of prognostic value of Roper Hall and Dua classification systems in acute ocular burns, *Br. J. Ophthalmol.* 95 (2) (2011) 194–198.
- [5] M.S.G.K. Vemuganti, S. Das, Update on pathologic diagnosis of corneal infections and inflammations, *Middle East Afr. J. Ophthalmol.* 18 (4) (2011) 277–284.
- [6] J.W. Hong, J.J. Liu, J.S. Lee, R.R. Mohan, R.R. Mohan, D.J. Woods, Y.G. He, S.E. Wilson, Proinflammatory chemokine induction in keratocytes and inflammatory cell infiltration into the cornea, *Invest. Ophthalmol. Vis. Sci.* 42 (12) (2001) 2795–2803.
- [7] B.S. Kang, M.K. Kim, W.R. Wee, J.Y. Oh, Infectious keratitis in Limbal stem cell deficiency: Stevens-Johnson syndrome versus chemical burn, *Cornea* 35 (1) (2016) 51–55.
- [8] J.T. Daniels, J.K. Dart, S.J. Tuft, P.T. Khaw, Corneal stem cells in review, *Wound Repair Regen.* 9 (6) (2001) 483–494.
- [9] H.S. Dua, A. Azuara-Blanco, Autologous limbal transplantation in patients with unilateral corneal stem cell deficiency, *Br. J. Ophthalmol.* 84 (3) (2000) 273–278.
- [10] L. Germain, F.A. Auger, E. Grandbois, R. Guignard, M. Giasson, H. Boisjoly, S.L. Guerin, Reconstructed human cornea produced *in vitro* by tissue engineering, *Pathobiology* 67 (3) (1999) 140–147.
- [11] S. Proulx, J. d'Arc Uwamaliya, P. Carrier, A. Deschambeault, C. Audet, C.J. Giasson, S.L. Guerin, F.A. Auger, L. Germain, Reconstruction of a human cornea by the self-assembly approach of tissue engineering using the three native cell types, *Mol. Vis.* 16 (2010) 2192–2201.
- [12] P. Carrier, A. Deschambeault, M. Talbot, C.J. Giasson, F.A. Auger, S.L. Guerin, L. Germain, Characterization of wound reepithelialization using a new human tissue-engineered corneal wound healing model, *Invest. Ophthalmol. Vis. Sci.* 49 (4) (2008) 1376–1385.
- [13] N. L'Heureux, S. Paquet, R. Labbe, L. Germain, F.A. Auger, A completely biological tissue-engineered human blood vessel, *FASEB J.* 12 (1) (1998) 47–56.
- [14] K. Zaniolo, P. Carrier, S.L. Guerin, F.A. Auger, L. Germain, A tissue-engineered corneal wound healing model for the characterization of reepithelialization, *Methods Mol. Biol.* 1037 (2013) 59–78.
- [15] C. Couture, K. Zaniolo, P. Carrier, J. Lake, J. Patenaude, L. Germain, S.L. Guerin, The tissue-engineered human cornea as a model to study expression of matrix metalloproteinases during corneal wound healing, *Biomaterials* 78 (2016) 86–101.
- [16] C.Y. Liu, W.W. Kao, Corneal epithelial wound healing, *Prog. Mol. Biol. Transl. Sci.* 134 (2015) 61–71.
- [17] C. Streuli, Extracellular matrix remodelling and cellular differentiation, *Curr. Opin. Cell Biol.* 11 (5) (1999) 634–640.
- [18] M.E. Fini, M.T. Girard, M. Matsubara, Collagenolytic/gelatinolytic enzymes in corneal wound healing, *Acta Ophthalmol. Suppl.* 202 (1992) 26–33.
- [19] A.D. Theocaris, S.S. Skandalis, C. Gialeli, N.K. Karamanos, Extracellular matrix structure, *Adv. Drug Deliv. Rev.* 97 (2016) 4–27.
- [20] J. Lake, K. Zaniolo, M. Gaudreault, P. Carrier, A. Deschambeault, R. Bazin, L. Germain, C. Sallesse, S.L. Guerin, Expression of the alpha5 integrin gene in corneal epithelial cells cultured on tissue-engineered human extracellular matrices, *Biomaterials* 34 (27) (2013) 6367–6376.
- [21] L. Koivisto, J. Heino, L. Hakkinen, H. Larjava, Integrins in wound healing, *Adv. Wound Care (New Rochelle)* 3 (12) (2014) 762–783.
- [22] S. Carracedo, N. Lu, S.N. Popova, R. Jonsson, B. Eckes, D. Gullberg, The fibroblast integrin alpha11beta1 is induced in a mechanosensitive manner involving activin A and regulates myofibroblast differentiation, *J. Biol. Chem.* 285 (14) (2010) 10434–10445.
- [23] M.F. Brizzi, P. Defilippi, A. Rosso, M. Venturino, G. Garbarino, A. Miyajima, L. Silengo, G. Tarone, L. Pegoraro, Integrin-mediated adhesion of endothelial cells induces JAK2 and STAT5A activation: role in the control of c-fos gene expression, *Mol. Biol. Cell* 10 (10) (1999) 3463–3471.
- [24] K.L. Yee, V.M. Weaver, D.A. Hammer, Integrin-mediated signalling through the MAP-kinase pathway, *IET Syst. Biol.* 2 (1) (2008) 8–15.
- [25] K.S. Zeller, O. Ideval-Hagren, A. Stefansson, T. Velling, S.P. Jackson, J. Downward, A. Tengholm, S. Johansson, PI3-kinase p110alpha mediates beta1 integrin-induced Akt activation and membrane protrusion during cell attachment and initial spreading, *Cell Signal* 22 (12) (2010) 1838–1848.
- [26] Y.C. Fong, S.C. Liu, C.Y. Huang, T.M. Li, S.F. Hsu, S.T. Kao, F.J. Tsai, W.C. Chen, C.Y. Chen, C.H. Tang, Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway, *Lung Cancer* 64 (3) (2009) 263–270.
- [27] M. Gaudreault, P. Carrier, K. Larouche, S. Leclerc, M. Giasson, L. Germain, S.L. Guerin, Influence of sp1/sp3 expression on corneal epithelial cells proliferation and differentiation properties in reconstructed tissues, *Invest. Ophthalmol. Visual Sci.* 44 (4) (2003) 1447–1457.
- [28] C. Glasson, L. Germain, P. Carrier, S.L. Guérin, C. Sallesse, Auger tissue engineering of cornea, in: G. Bowlin, G.E. Wnek (Eds.), *Encyclopedia of Biomaterials and Biomedical Engineering*, Marcel Dekker, New York, 2004, pp. 1534–1544.
- [29] P. Carrier, A. Deschambeault, C. Audet, M. Talbot, R. Gauvin, C.J. Giasson, F.A. Auger, S.L. Guerin, L. Germain, Impact of cell source on human cornea reconstructed by tissue engineering, *Invest. Ophthalmol. Visual Sci.* 50 (6) (2009) 2645–2652.
- [30] R.J. Roy, P. Gosselin, S.L. Guerin, A short protocol for micro-purification of nuclear proteins from whole animal tissue, *Biotechniques* 11 (6) (1991) 770–777.
- [31] M. Gaudreault, M.E. Gingras, M. Lessard, S. Leclerc, S.L. Guerin, Electrophoretic mobility shift assays for the analysis of DNA-protein interactions, *Methods Mol. Biol.* 543 (2009) 15–35.
- [32] K. Larouche, S. Leclerc, C. Sallesse, S.L. Guerin, Expression of the alpha 5 integrin subunit gene promoter is positively regulated by the extracellular matrix component fibronectin through the transcription factor Sp1 in corneal epithelial cells *in vitro*, *J. Biol. Chem.* 275 (50) (2000) 39182–39192.
- [33] M. Gaudreault, F. Vigneault, S. Leclerc, S.L. Guerin, Laminin reduces expression of the human alpha6 integrin subunit gene by altering the level of the transcription factors Sp1 and Sp3, *Invest. Ophthalmol. Visual Sci.* 48 (8) (2007) 3490–3505.
- [34] M.E. Gingras, K. Larouche, N. Larouche, S. Leclerc, C. Sallesse, S.L. Guerin, Regulation of the integrin subunit alpha5 gene promoter by the transcription factors Sp1/Sp3 is influenced by the cell density in rabbit corneal epithelial cells, *Invest. Ophthalmol. Visual Sci.* 44 (9) (2003) 3742–3755.
- [35] S. Proulx, C. Audet, J. Uwamaliya, A. Deschambeault, P. Carrier, C.J. Giasson, I. Brunette, L. Germain, Tissue engineering of feline corneal endothelium using a devitalized human cornea as carrier, *Tissue Eng. Part A* 15 (7) (2009) 1709–1718.
- [36] M.M. Santoro, G. Gaudino, Cellular and molecular facets of keratinocyte reepithelialization during wound healing, *Exp. Cell Res.* 304 (1) (2005) 274–286.
- [37] K. Philipp, F. Riedel, M. Sauerbier, K. Hormann, G. Germann, Targeting TGF-beta in human keratinocytes and its potential role in wound healing, *Int. J. Mol. Med.* 14 (4) (2004) 589–593.
- [38] L. Lu, P.S. Reinach, W.W. Kao, Corneal epithelial wound healing, *Exp. Biol. Med. (Maywood)* 226 (7) (2001) 653–664.
- [39] K.R. Legate, S.A. Wickstrom, R. Fassler, Genetic and cell biological analysis of integrin outside-in signaling, *Genes Dev.* 23 (4) (2009) 397–418.
- [40] A. Sun, D. Zhuang, H. Zhu, M. Lai, W. Chen, H. Liu, F. Zhang, W. Zhou, Decrease of phosphorylated CREB and ERK in nucleus accumbens is associated with the incubation of heroin seeking induced by cues after withdrawal, *Neurosci. Lett.* 591 (2015) 166–170.
- [41] D. Zhao, S. Fukuyama, Y. Sakai-Tagawa, E. Takashita, J.E. Shoemaker, Y. Kawaoka, Novel p300/CREB-Binding Protein-Specific Inhibitor of Histone Acetyltransferase Attenuates Influenza A Virus Infection, *Antimicrob. Agents Chemother.* 60 (3) (2015) 1902–1906.
- [42] A.F. Laplante, L. Germain, F.A. Auger, V. Moulin, Mechanisms of wound reepithelialization: hints from a tissue-engineered reconstructed skin to long-standing questions, *FASEB J.* 15 (13) (2001) 2377–2389.
- [43] J. Murakami, T. Nishida, T. Otori, Coordinated appearance of beta 1 integrins and fibronectin during corneal wound healing, *J. Lab. Clin. Med.* 120 (1) (1992) 86–93.
- [44] S.J. Kang, E.K. Kim, H.B. Kim, Expression and distribution of extracellular matrices during corneal wound healing after keratomileusis in rabbits, *Ophthalmologica* 213 (1) (1999) 20–24.
- [45] J.D. Zieske, S.C. Higashijima, S.J. Spurr-Michaud, I.K. Gipson, Biosynthetic responses of the rabbit cornea to a keratectomy wound, *Invest. Ophthalmol. Visual Sci.* 28 (10) (1987) 1668–1677.
- [46] D.M. Foreman, S. Pancholi, J. Jarvis-Evans, D. McLeod, M.E. Boulton, A simple organ culture model for assessing the effects of growth factors on corneal re-epithelialization, *Exp. Eye Res.* 62 (5) (1996) 555–564.
- [47] R.S. Chuck, A. Behrens, S. Welllik, L.L. Liaw, A.M. Dolorico, P. Sweet, L.C. Chao, K. E. Osann, P.J. McDonnell, M.W. Berns, Re-epithelialization in cornea organ culture after chemical burns and excimer laser treatment, *Arch. Ophthalmol.* 119 (11) (2001) 1637–1642.
- [48] M. Toutant, A. Costa, J.M. Studler, G. Kadare, M. Carnaud, J.A. Girault, Alternative splicing controls the mechanisms of FAK autophosphorylation, *Mol. Cell Biol.* 22 (22) (2002) 7731–7743.

- [49] Q. Shi, D. Boettiger, A novel mode for integrin-mediated signaling: tethering is required for phosphorylation of FAK Y397, *Mol. Biol. Cell* 14 (10) (2003) 4306–4315.
- [50] J.P. Irigoyen, Y. Nagamine, Cytoskeletal reorganization leads to induction of the urokinase-type plasminogen activator gene by activating FAK and Src and subsequently the Ras/Erk signaling pathway, *Biochem. Biophys. Res. Commun.* 262 (3) (1999) 666–670.
- [51] J.P. Irigoyen, D. Besser, Y. Nagamine, Cytoskeleton reorganization induces the urokinase-type plasminogen activator gene via the Ras/extracellular signal-regulated kinase (ERK) signaling pathway, *J. Biol. Chem.* 272 (3) (1997) 1904–1909.
- [52] A.B. Granado-Serrano, M.A. Martin, G. Haegeman, L. Goya, L. Bravo, S. Ramos, Epicatechin induces NF-kappaB, activator protein-1 (AP-1) and nuclear transcription factor erythroid 2p45-related factor-2 (Nrf2) via phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) and extracellular regulated kinase (ERK) signalling in HepG2 cells, *Br. J. Nutr.* 103 (2) (2010) 168–179.
- [53] J.L. Merchant, M. Du, A. Todisco, Sp1 phosphorylation by Erk 2 stimulates DNA binding, *Biochem. Biophys. Res. Commun.* 254 (2) (1999) 454–461.
- [54] C. Duval, K. Zaniolo, S. Leclerc, C. Salesse, S.L. Guerin, Characterization of the human alpha9 integrin subunit gene: promoter analysis and transcriptional regulation in ocular cells, *Exp. Eye Res.* 135 (2015) 146–163.
- [55] M. Gaudreault, F. Vigneault, M.E. Gingras, S. Leclerc, P. Carrier, L. Germain, S.L. Guerin, Transcriptional regulation of the human alpha6 integrin gene by the transcription factor NFI during corneal wound healing, *Invest. Ophthalmol. Visual Sci.* 49 (9) (2008) 3758–3767.
- [56] M.E. Gingras, B. Masson-Gadair, K. Zaniolo, S. Leclerc, R. Drouin, L. Germain, S. L. Guerin, Differential binding of the transcription factors Sp1, AP-1, and NFI to the promoter of the human alpha5 integrin gene dictates its transcriptional activity, *Invest. Ophthalmol. Visual Sci.* 50 (1) (2009) 57–67.
- [57] S. Zheng, S.M. Eacker, S.J. Hong, R.M. Gronostajski, T.M. Dawson, V.L. Dawson, NMDA-induced neuronal survival is mediated through nuclear factor I-A in mice, *J. Clin. Invest.* 120 (7) (2010) 2446–2456.
- [58] S. Agarwal, C. Achari, D. Praveen, K.R. Roy, G.V. Reddy, P. Reddanna, Inhibition of 12-LOX and COX-2 reduces the proliferation of human epidermoid carcinoma cells (A431) by modulating the ERK and PI3K-Akt signalling pathways, *Exp. Dermatol.* 18 (11) (2009) 939–946.
- [59] S. Aarabi, K.A. Bhatt, Y. Shi, J. Paterno, E.I. Chang, S.A. Loh, J.W. Holmes, M.T. Longaker, H. Yee, G.C. Gurtner, Mechanical load initiates hypertrophic scar formation through decreased cellular apoptosis, *FASEB J.* 21 (12) (2007) 3250–3261.
- [60] I. Goren, E. Muller, D. Schiefelbein, P. Gutwein, O. Seitz, J. Pfeilschifter, S. Frank, Akt1 controls insulin-driven VEGF biosynthesis from keratinocytes: implications for normal and diabetes-impaired skin repair in mice, *J. Invest. Dermatol.* 129 (3) (2009) 752–764.
- [61] W. Xiao, H. Tang, M. Wu, Y. Liao, K. Li, L. Li, X. Xu, Ozone oil promotes wound healing by increasing the migration of fibroblasts via PI3K/Akt/mTOR signaling pathway, *Biosci. Rep.* (2017).
- [62] H. Huang, W. Cui, W. Qiu, M. Zhu, R. Zhao, D. Zeng, C. Dong, X. Wang, W. Guo, W. Xing, X. Li, L. Li, Y. Tan, X. Wu, L. Chen, X. Fu, D. Luo, X. Xu, Impaired wound healing results from the dysfunction of the Akt/mTOR pathway in diabetic rats, *J. Dermatol. Sci.* 79 (3) (2015) 241–251.
- [63] K.P. Xu, Y. Li, A.V. Ljubimov, F.S. Yu, High glucose suppresses epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt signaling pathway and attenuates corneal epithelial wound healing, *Diabetes* 58 (5) (2009) 1077–1085.
- [64] L. Sun, R. Zhao, Y. Bai, R. Cao, F. Wang, S. Yang, Z. Qin, G. Du, CREB is activated in smooth muscle cells isolated from atherosclerotic plaques and reduces smooth muscle cell proliferation via p21-dependent mechanism, *Int. J. Cardiol.* 174 (3) (2014) 764–767.
- [65] L. Sun, R. Zhao, L. Zhang, W. Zhang, G. He, S. Yang, J. Song, G. Du, Prevention of vascular smooth muscle cell proliferation and injury-induced neointimal hyperplasia by CREB-mediated p21 induction: an insight from a plant polyphenol, *Biochem. Pharmacol.* 103 (2016) 40–52.
- [66] X. Tan, S. Wang, B. Yang, L. Zhu, B. Yin, T. Chao, J. Zhao, J. Yuan, B. Qiang, X. Peng, The CREB-miR-9 negative feedback minicircuitry coordinates the migration and proliferation of glioma cells, *PLoS One* 7 (11) (2012) e49570.
- [67] Y.H. Guo, L.Q. Wang, B. Li, H. Xu, J.H. Yang, L.S. Zheng, P. Yu, A.D. Zhou, Y. Zhang, S.J. Xie, Z.R. Liang, C.M. Zhang, H. Zhou, L.H. Qu, Wnt/beta-catenin pathway transactivates microRNA-150 that promotes EMT of colorectal cancer cells by suppressing CREB signaling, *Oncotarget* 7 (27) (2016) 42513–42526.
- [68] J.P. Cleutjens, M.G. Havenith, M. Kasper, M. Vallenga, F.T. Bosman, Absence of type IV collagen in the centre of the corneal epithelial basement membrane, *Histochem. J.* 22 (12) (1990) 688–694.
- [69] M. Kayama, M.S. Kurokawa, H. Ueno, N. Suzuki, Recent advances in corneal regeneration and possible application of embryonic stem cell-derived corneal epithelial cells, *Clin. Ophthalmol.* 1 (4) (2007) 373–382.
- [70] Y. Kumagai, M.S. Kurokawa, H. Ueno, M. Kayama, K. Tsubota, N. Nakatsuji, Y. Kondo, S. Ueno, N. Suzuki, Induction of corneal epithelium-like cells from cynomolgus monkey embryonic stem cells and their experimental transplantation to damaged cornea, *Cornea* 29 (4) (2010) 432–438.
- [71] L. Germain, P. Carrier, F.A. Auger, C. Salesse, S.L. Guerin, Can we produce a human corneal equivalent by tissue engineering?, *Prog Retin Eye Res.* 19 (5) (2000) 497–527.
- [72] S. Das, B. Seitz, Recurrent corneal erosion syndrome, *Surv. Ophthalmol.* 53 (1) (2008) 3–15.
- [73] A. Kim, R.S. Chuck, Wavefront-guided customized corneal ablation, *Curr. Opin. Ophthalmol.* 19 (4) (2008) 314–320.
- [74] D.T. Azar, J.H. Chang, K.Y. Han, Wound healing after keratorefractive surgery: review of biological and optical considerations, *Cornea* 31 (Suppl. 1) (2012) S9–S19.
- [75] M.H. Vesaluoma, W.M. Petroll, J.J. Perez-Santonja, T.U. Valle, J.L. Alio, T.M. Tervo, Laser in situ keratomileusis flap margin: wound healing and complications imaged by *in vivo* confocal microscopy, *Am. J. Ophthalmol.* 130 (5) (2000) 564–573.