Ectopic Epithelial Implants following Surface Ablation of the Cornea

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Purpose. To determine the direct contribution of the epithelium to the generation of complications using a phototherapeutic keratectomy model.

Methods. A mouse model with a genetically labeled epithelium was used to determine whether any epithelium-derived cells persist in the stroma up to 1 month after surgery. Also, gross histology and macrophotography of excimer-ablated rabbit corneas were analyzed for evidence epithelial ingrowths into the stroma.

Results. Epithelium-derived cells were present in the wounded stroma 1 month after surgery. Micrographs taken during the first 4 days during healing evidenced epithelial invasion of the stroma in one and sometimes more locations in the same cornea. Gross histology also revealed that the epithelial invasions can result in complete delamination of stromal tissue and subsequent inclusion of the stromal material in the epithelium. The epithelial inclusions ultimately created a highly irregular corneal surface.

Conclusions. Ectopic epithelia are a known complication of LASIK and LASIK-like procedures. The data presented here indicate that ectopic epithelia are also a complication of surface ablation techniques. The knowledge that these complications are present following surface ablations provides a new understanding of the biological response to surface ablation techniques and suggests new avenues of study to improve clinical outcomes of those for whom LASIK-based techniques are not an option. (*Invest Ophthalmol Vis Sci.* 2012;53:7760–7765) DOI:10.1167/iovs.12-10768

For some patients requiring or desiring refractive surgery, surface ablation (SA) techniques remain the only option. Efforts to improve clinical outcomes of SA surgeries have been progressing on at least two fronts: (1) improvements in the laser systems, and (2) improvements in understanding the biological response to corneal wounding. The accuracy and reproducibility of the initial reshaping of the stroma is good; however, understanding and predicting how the cornea responds and subsequently changes both the initial ablation profile and corneal opacity are not as well understood. The situation can be summarized as "the physics works," but the biological generation of complications and deviations from the correction are still in need of improvement. While much is known about the cellular and molecular biology of corneal wound healing,[1–6] improvements beyond this basal knowledge are constantly sought in order to explain the observed variance in optical and refractive outcomes following SA-based surgeries.

One well-documented biological complication from another class of laser surgery is ectopic epithelial invasion of the LASIK flap following LASIK or LASIK-like procedures.[7–9] The creation of the flap gives rise to both to epithelial activation and it provides an avenue through which the migrating epithelium can invade the stroma. The direct consequences of these ectopic epithelia are increased opacity and surface irregularity at the ectopic site[7] and a direct contribution to total light reflection by the ectopic cells themselves.[9] Ectopic epithelia have been experimentally implanted into LASIK flaps in order to reveal more detailed molecular and immunohistological data.[10] The introduction of epithelial cells into the LASIK flap increases keratocyte proliferation and myofibroblast differentiation, which indicates that the overall effect would be expected to generate more light reflecting haze.[11]

To date, no reports have been found indicating that surface ablation techniques suffer from ectopic epithelial implantation. Using a mouse model with a genetically labeled epithelium and gross histology of early time points during healing in a rabbit model, we have demonstrated that phototherapeutic keratectomy (PTK) surface–ablated mice and rabbits do in fact present with ectopic epithelial deposits and, in some instances, stromal inclusions in the epithelium. The work presented herein provides the first evidence for the occurrence of stromal invasion by the epithelium, the persistence of ectopic epithelial cells in the stroma, and the generation of surface irregularities resulting from the epithelial invasion of the stroma.

Materials and Methods

All of the animals used herein were treated in a manner consistent with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Tracing Experiment

Reporter Mice. In order to test whether the epithelium contributes cells to the final scar, a genetically labeled reporter mouse model was used. The reporter mice employ the Cre-recombinase system[12] to deinhibit a β-galactosidase reporter enzyme.[13] For this cell tracing experiment, the Cre-recombinase transgene was under control of the PAX6 promoter. This promoter is active in various tissues in the eye, but in the cornea, it is solely active in the corneal epithelium.[14] In the corneas of the reporter mice, cells derived from the epithelium will

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Table 1. Primers for the Mice with Genetically Labeled Corneal Epithelium

Name	Sequence
R26R-F	5′-TTT CCA CAG CTC GCG GTT GAG GAC-3′
R26R-R	5′-CTA AAG CGC ATG CTC CAG ACT GCC-3′
Cre-F	5′-GCC GTA AAT CAA TCG ATG AGT-3′
Cre-R	5′-TGA CGG TGG GAG AAT GTT AAT-3′

possess β-galactosidase activity, while cells derived from the stroma will not.

The pups were genotyped and marked by ear punch prior to weaning (postnatal day 21). Tail biopsies were collected with sharp, bead-sterilized, surgical scissors. Silver nitrate was applied to the cut tail for hemostasis. Genomic DNA was isolated from the tail biopsies using a PCR kit (Sigma REDExtract-N-Amp Kit; Sigma-Aldrich, St. Louis, MO). The primers (Integrated DNA Technologies, Inc., San Diego, CA) listed in Table 1 were used to detect the presence of both necessary transgenes. The PCR reactions were always run with a negative control comprised solely of 20 uL of master mix (Qiagen, Valencia, CA). The PCR amplified samples were resolved on a 1.5% agarose gel with 100 base pair ladder (Qiagen). The β-galactosidase transgene has a 220 base pair amplicon, while the PAX6-Cre has a 270 base pair amplicon. Pups with at least one of each of the two necessary transgenes were selected for experimentation.

Excimer Laser Wounding. Mice were generally anesthetized with 3.5% isoflurane/oxygen and the eye to be wounded was locally anesthetized via a drop of tetracaine (Bausch & Lomb, Tampa, FL). The whiskers were cut with scissors and the eyelid and lashes were gently pushed out of the way with a cotton swab. The mouse was then oriented beneath the excimer laser (Nidek EC5000; Nidek, Inc., Freemont, CA). A 1.0-mm diameter by 24-μm deep transepithelial PTK excimer wound was created. The mouse was periodically observed in the following weeks to determine whether and to what extent the wound scarred.

Cell Tracing Tissue Harvesting, Processing, and Sectioning. At the terminal time point, the mice were anesthetized as before and euthanized by cervical dislocation. Each globe was immediately enucleated via blunt dissection with fine-tipped forceps and placed in fresh 10% neutral buffered formalin on ice. The globes were punctured with a 25-gauge needle after 30 minutes to improve fixative penetration. The globes were intentionally under-fixed for 1 hour at 4°C to preserve the enzymatic activity of the β-galactosidase reporter. The globes were then placed in 30% sucrose in PBS overnight at 4°C to cryoprotect the tissue. In order to improve cryosectioning, each globe was grossly prepared by cutting open the posterior retina, removing the lens, and injecting embedding medium into the globe with a blunt-tipped needle and syringe. The tissue was then submerged in embedding medium and rapidly frozen. The frozen blocks were stored at −20°C until cryosectioning. Ten micron sections were cut and mounted on poly-L-lysine–coated slides. The slides were air dried and then stored at −20°C until staining.

Table 2. Incidence of Sloughing and Ectopic Epithelia in Rabbit Corneas

Time Point (post-Sx)	Sloughing (eyes with/total eyes)	Ectopic Epithelium (eyes with/total eyes)
1 Day	6/6*	3/4
2 Day	3/4	3/4
3 Day	n/a†	2/2
4 Day	n/a†	2/4

* One rabbit was observed at this time point, but not euthanized.
† Macrophotographs were not taken at these time points.

β-Galactosidase Detection. The sections were rehydrated and washed 3 times in PBS. The slides were then incubated in 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) staining solution (2 mM $MgCl_2$; Sigma-Aldrich); 5 mM $K_4(CN)_6$-$3H_2O$ (Sigma-Aldrich); 5 mM $K_3(CN)_6$ (Sigma-Aldrich); 1 mg/mL X-Gal (Promega, Madison, WI), in PBS pH 7.4 at 37°C in a humidified chamber overnight. The slides were then washed 3 times with PBS, counterstained with nuclear fast red (Vector Labs, Burlingame, CA). The sections were dehydrated and permanently mounted with mounting medium (Permount; Fisher Scientific, Waltham, MA).

Gross Observation of Early Time Points of Healing

Excimer Laser Wounding. Each rabbit was anesthetized using inhaled isoflurane and each eye was topically anesthetized using one

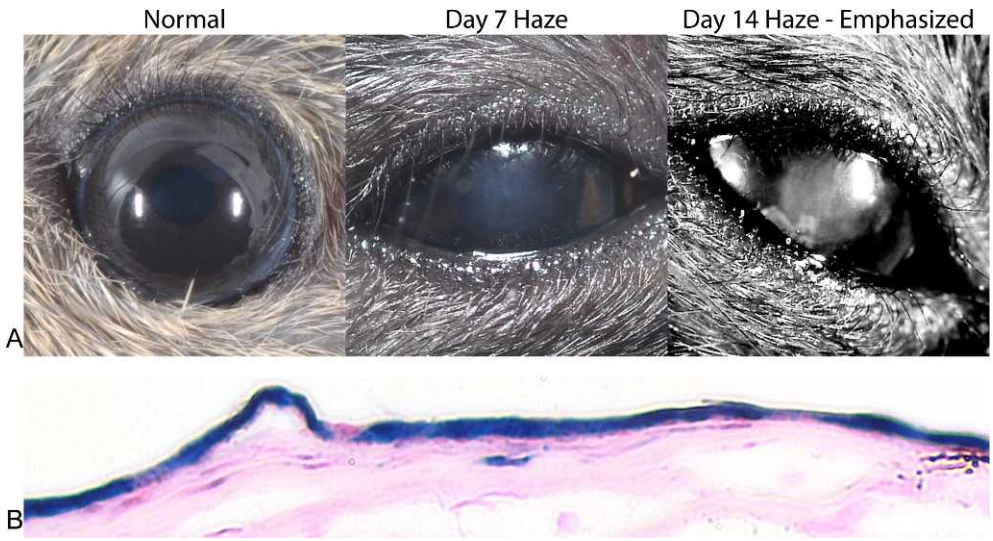

Figure 1. Genetically marked mouse epithelial cells—ectopic epithelial cells persist. (**A**) A series of macrophotographs prior to and after haze begins to form. (**B**) At 32 days postwounding, a single strong positive blue mass comprised of two cells in the anterior center of the stroma are surrounded by cells without β-galactosidase activity indicating that there is an "island" of epithelium-derived cells surrounded by cells that are not epithelial derived. These data suggest that the direct contribution to light reflection by the ectopic epithelia is low, and that the surrounding myofibroblasts are still the predominant source of light reflection.

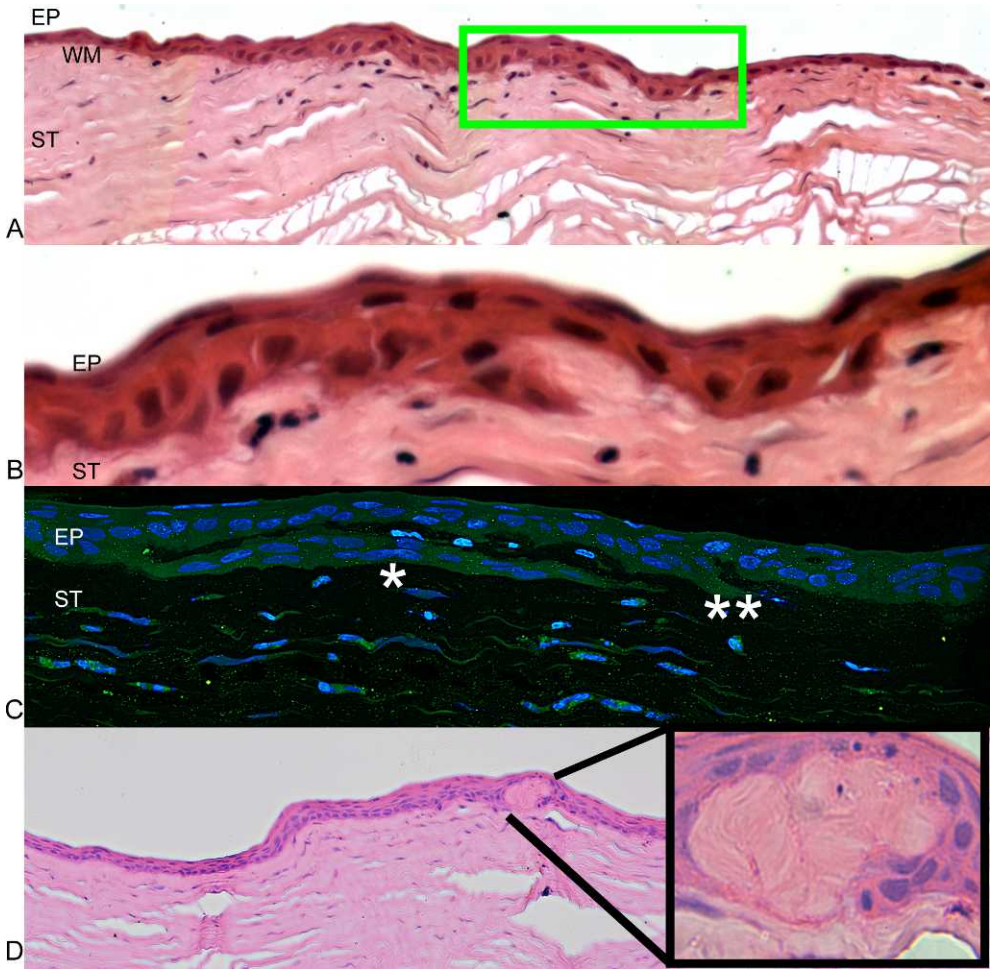

Figure 2. Evidence for epithelial invasion of rabbit stroma during wound closure. Epithelial invasion of the stroma during reepithelialization. (**A**) An H&E mosaic of a day 1 postwounding cornea. (**B**) A closer look of the highlighted region where three epithelial cells are entering a stromal "flap." (**C**) An autofluorescence and DAPI image of a day 2 postwounding cornea where the initial invasion has progressed significantly (*) and another invasion has begun (**). (**D**) An H&E low power mosaic of a day 4 postwounding cornea, the invading epithelial front has effective delaminated several lamellae and generated an included collagenous mass (*detail in inset*).

drop of tetracaine (Bausch & Lomb). The eye was exposed and the central thickness of the cornea was measured by ultrasonic pachymetry ($n = 5$ central measurements per eye). Each eye then received a central 6.0-mm diameter, 125-μm deep, transepithelial excimer laser PTK wound with no transition zone (Nidek EC5000; Nidek, Inc.) resulting in an approximate +6.0-diopter (D) correction depending on the actual initial epithelial thickness. All rabbits received a daily dose of oral meloxicam (0.2 mg/kg; Boehringer Ingelheim, St. Joseph, MO) until wound closure or euthanization. The terminal time points observed and the number of eyes in this study are available in Table 2.

Gross Histology. The rabbits were euthanized and their corneas harvested. An 8-mm region surrounding the wound was harvested via punch biopsy. The 8-mm punched cornea was fixed, paraffin embedded and 4- to 5-μm sections were cut and mounted. Paraffin sections were deparaffinized with xylenes and rehydrated though a graded ethanol series. The sections were stained with hematoxylin and eosin (H&E), or with DAPI containing medium (Vector Labs). The H&E slides were imaged using standard illumination while the DAPI stained sections were imaged such that the green channel was intentionally overexposed to display autofluorescence differences between the epithelium and stroma.

Immunohistology. Paraffin sections were deparaffinized as above. The sections were blocked with 10% normal horse serum (NHS). The primary α-smooth muscle actin (α-SMA) antibody (A5228; Sigma-Aldrich) was diluted (1:500) in 1% NHS (Vector Labs) in PBS + Tween 20 0.05% (PBST) and was incubated for 1 hour at room temperature on the one section while the negative control remained in blocking solution. The slides were washed three times for 5 minutes with wash buffer. The sections were then incubated with a fluoresceinated secondary horse anti-mouse IgG (Vector Labs) for 30 minutes at room temperature. Following the final washes, the slides were mounted with DAPI containing medium (Vector Labs) and the slide cover was fixed in place with nail hardener.

Results

Cell Tracing

A visible opacification was present by day 7 (Fig. 1A). Even though the scar appeared to be mature by day 7, the eyes were not harvested until day 32 to ensure that a mature scar had formed. Only one region in the wound possessed a strong positive signal; and upon closer inspection, the β-galactosidase positive region appeared to be two cells in close juxtaposition (Fig. 1B). The majority of the cells in the wound surrounding the two positive cells are β-galactosidase negative, nuclear fast red positive cells indicating that they were not epithelial-derived cells.

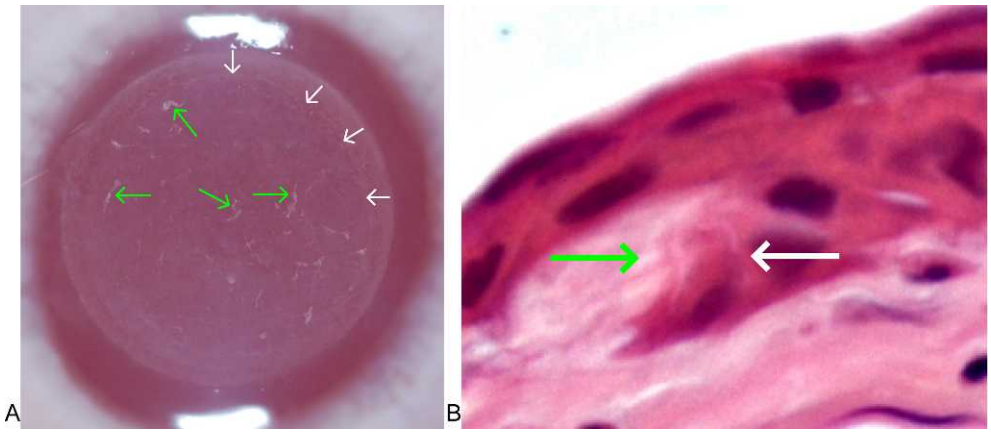

Figure 3. Mechanism of stromal invasion in rabbit corneas. A potential mechanism for the observed epithelial invasion of the stroma. (**A**) A cornea one day after PTK surgery. The *white arrows* indicate the migrating epithelial front while the *green arrows* indicate peeling residual stromal lamellae. (**B**) If the migrating epithelial front aligns with the peeling stromal lamellae, then the epithelial cells can enter and become embedded in the stroma or generate delaminated stromal inclusions in the epithelium.

Gross Observation of Early Time Points of Healing

Gross Histology. Within 24 hours following wounding, there is evidence of a significant number of neutrophils and epithelial invasion of stromal lamellae (Figs. 2A, 2B). One day later, there is evidence of continued migration of epithelial cells between stromal lamellae, and evidence of new regions of stromal invasion (Fig. 2C). Sections from a total of 14 eyes indicated that 10 eyes had at least one ectopic epithelium or epithelial front invading a stromal lamellae (Table 2). On day 4 postwounding, one eye had evidence of the epithelium effectively "bulldozing" several stromal lamellae and creating a stromal inclusion within the central epithelium (Fig. 2D). Only sections from two separate eyes from two separate rabbits had evidence of these inclusions, both were at day 4 postwounding.

Corneal Images. In the days following surgery, the corneas were dried and photographed immediately prior to euthanization. The migrating epithelial front is visible (white arrows, Fig. 3A), as are some attached proteinaceous structures that resemble peeling skin (green arrows, Fig. 3A). These peels are presumably residual stromal flaps that can serve as avenues for stromal invasion if approached at the correct angle by the migrating epithelium (Fig. 3B). The cornea immediately after surgery is finely textured, but grossly smooth (Fig. 4). However, in the days which follow, the proteinaceous masses appeared in most corneas observed (Table 2), suggesting that these structures are generated secondary to the excimer ablation. The severity of the sloughing varied considerably in the amount of apparent mass peeling off of the surface.

Discussion

It is known that ectopic epithelia can give rise to vision degrading optical aberrations following LASIK surgery. With the data presented here, ectopic epithelia can now be considered a potential complication of surface ablation techniques as well. When the migrating epithelium enters a stromal layer, at least two complications have now been witnessed: either a scar promoting ectopic epithelial implant, or a refractive aberration inducing stromal inclusion in the epithelium.

The generation of the stromal flaps may be caused by incomplete layer removal by the laser and expansion via tear film proteinases, or they may be generated entirely by proteinases. Further work is necessary to identify the source of generation and expansion of these defects. Matrix metalloproteinase-1 (MMP-1), a collagenase, has been demonstrated to be necessary for hepatocyte growth factor–mediated

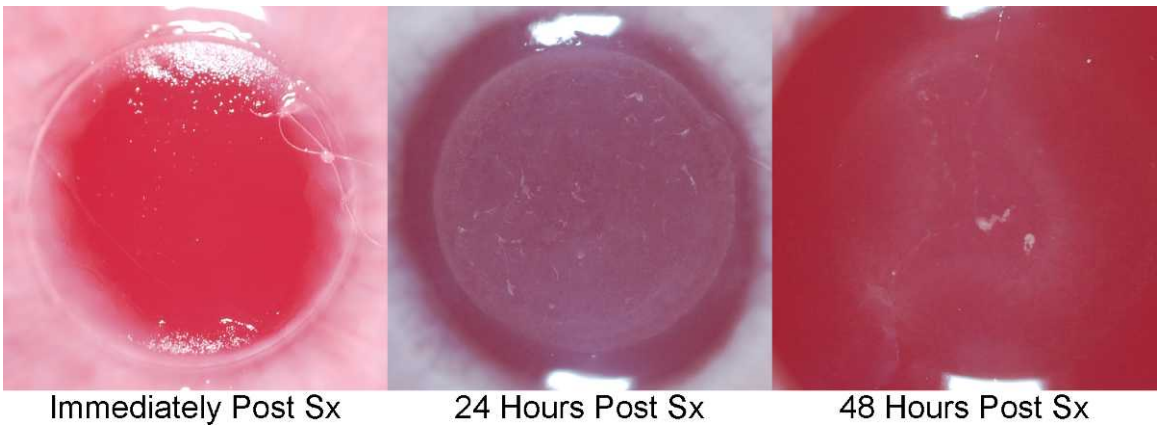

Figure 4. Macrophotographs of rabbit corneas after PTK. The stromal surface is finely textured immediately after surgery, but grossly smooth without any apparent stromal flaps. However, 24 hours later, there are grossly apparent flaps of stromal material. The presence of the flaps only after 24 hours and beyond suggests that the process is secondary to the laser surgery.

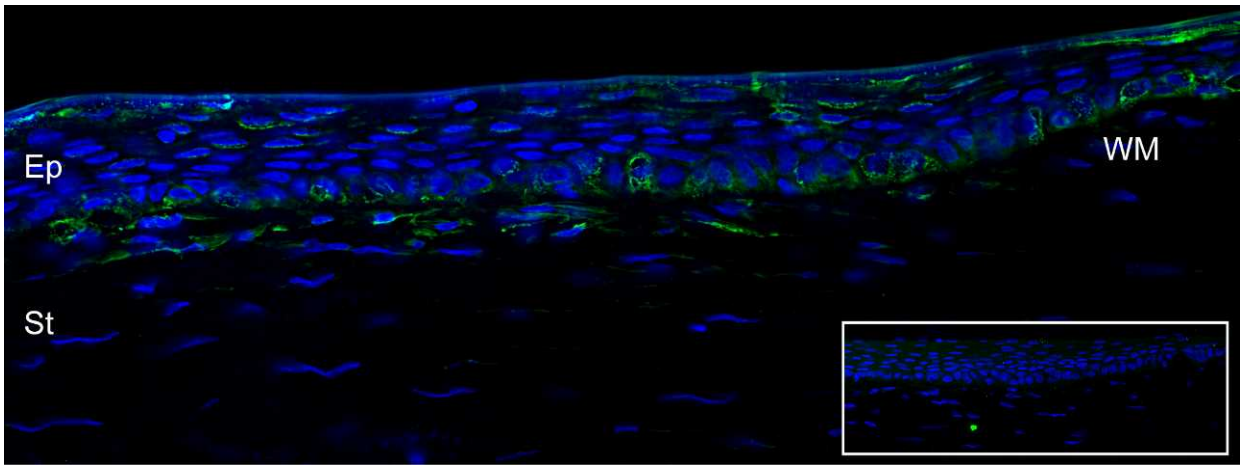

FIGURE 5. A micrograph from a day 5 postwounding rabbit cornea α-SMA (*green*) and DAPI (*blue*). α-SMA is present in both the epithelium and stroma though the cytoplasmic distribution differs between the cells in that the epithelial α-SMA is more diffuse/globular than the fibrous organization in the stromal myofibroblasts. Ep, epithelium; St, stroma; WM, wound margin.

epithelial migration. It could be that this necessary enzyme facilitates the invasion into the stroma once the epithelium is caught by one of the observed flaps. The necessity of the enzyme suggests that the proteinases might not be a good therapeutic target, and that at least one set of trials with collagenase inhibitors have not produced significant beneficial results to date.[15]

Epithelial invasion of the stroma might also explain the link between increased haze with a less smooth stromal surface[4]; as is seen experimentally following irregular PTK.[5,6] Ablating the wound surface in this manner would most certainly generate many severed stromal lamellae into which the epithelium could easily enter and be stimulated to generate more scar-inducing cytokines. If this is the case, then irregular PTK might not be a good model for clinically relevant defects following laser surgery in that it emphasizes this particular source of complications. The evidence presented here also suggests that control of stromal smoothness via control of the initial ablation might not be sufficient to ensure a smooth stromal surface throughout wound healing. While laser technologies have improved with time, the fact remains that the cornea is an irregular material comprised of interwoven fibrils of collagen[16] and that even a well-controlled cut will not leave complete stromal lamellae intact. While it is well known that immediate postsurgical roughness will drastically degrade the refractive surgery outcomes, the macrophotographic evidence presented herein indicates that postsurgical, host-derived roughening can occur secondary to the initially well-controlled laser ablation.

Ectopic epithelial cells can contribute to the overall light scatter in LASIK,[9] though the overall direct contribution appears to be small. The direct contribution of the ectopic epithelial cells seen in this study has yet to be investigated. The evidence from the cell tracing experiment reported here would suggest it is low, while the initial gross histology data from the rabbit corneas suggest it might be higher. It has been reported that in photorefractive keratectomy (PRK)–ablated corneas treated with mitomycin-C (MMC), that there was a residual haze not remedied by MMC, indicating a source of haze other than myofibroblasts.[17] Haze generation caused directly by epithelial implants would not be addressed by MMC, though it remains to be demonstrated whether the haze in these cases is cellular or matrix derived. In seeking to quantify the incidence of ectopic epithelia using historical SA samples, looking for implanted epithelium using α-SMA and seeking regions within the fibrotic scar that were α-SMA negative would not suffice. Epithelia produce α-SMA in response to proscarring growth factors, though it is distributed in a more globular pattern that the fibrous pattern seen in myofibroblasts.[18] This is seen during wound healing in the cornea as well (Fig. 5) where the basal epithelium has a diffuse staining for α-SMA versus the more organized appearance in the stromal myofibroblasts. Previous work with experimentally implanted epithelia indicate that keratin 3 (K3) can differentiate the ectopic epithelium from the surrounding stroma for at least 1 month after implantation.[10] Future inclusion of micrographs stained for K3 could begin to elucidate the incidence of ectopic epithelia following surface ablations in corneas derived from experimental models and from donor cornea tissues from those who had an SA surgery prior to death.

The appearance of substantial stromal material in the epithelium now provides another mechanism for the generation of higher order refractive aberrations. Neither the magnitude of effect nor the frequency of incidence with significant numbers of samples was addressed in this study. Of particular interest was that the stromal mass appeared to be

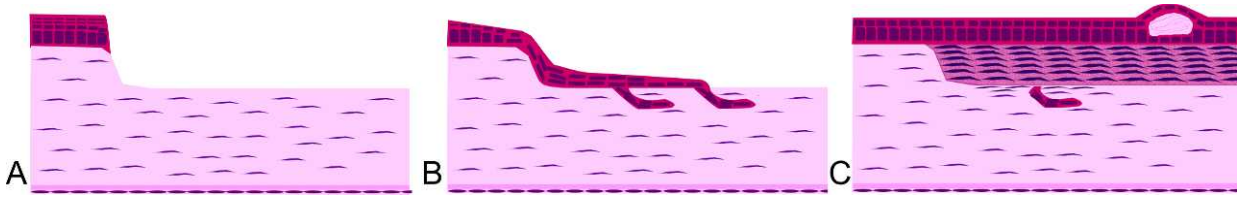

FIGURE 6. Consequences of ectopic epithelium. (**A**) The initial wound. (**B**) The migrating epithelial front occasionally invades the stroma (days 1–3). (**C**) The epithelial invasion can either remain as an implant, which is surmounted by stroma-derived myofibroblasts and newly synthesized matrix, or it can delaminate the stromal lamellae entirely and create an aberrant inclusion in the epithelium.

acellular. If this is the case, then clinical examination via in vivo confocal microscopy of sites on the cornea with such surface aberrations might reveal acellular deposits in the epithelium, which may be easily addressed via an epithelial scrape to remove the mass.

The experiments performed herein were conducted by a nonclinician researcher using a clinic-grade laser in good repair under the guidance of a clinical ophthalmologist and in accord with the manufacturer's instructions. While it does remain a possibility that there may have been postsurgical roughness, it was not grossly observable until the wounds were observed 24 hours later. This very strongly indicates that the primary source of the observed flaps was secondary to wounding, though it remains a possibility that the biological response amplified unobservable roughness, which could have been created during lasing. Additionally, these experiments were conducted in model animals, which are different from humans. However, these model animals share the same general stromal structure of interwoven stromal lamellae that overlap one another in a rotated alternating pattern with some lamellar interweaving. This basic general tissue structure would still be expected to result in incompletely dissected or ablated stromal lamellae in humans, and these lamellae would face similar proteolytic stresses during wound healing. That said, additional experiments are being planned to seek out the incidence of these complications using donor corneas in a manner similar to that which was done by Dawson et al. with corneas that had a history of LASIK.[9] However, unlike the LASIK corneas, we would likely need to employ immunofluorescent identification given that a highly reflective necrotizing ectopic epithelial cells present in a PRK or PTK cornea would be surrounded by similarly highly reflective myofibroblasts, making identification via ex vivo confocal microscopy much more difficult.

The discovery of this new source of complications that can cause light scatter and refractive abnormalities gives rise to a new model to explain the sources of complications following surface ablation techniques. Following the ablation (Fig. 6A), the epithelium migrates to cover the defect and occasionally, it can enter the stroma at one or more of the residual stromal flaps (Fig. 6B). The invasion can be resolved in at least two distinct ways (Fig. 6C): (1) the epithelium stops at some point and becomes an ectopically implanted "island" or "peninsula" of cells (Fig. 1B); or (2) it can continue to migrate and completely delaminate the stromal lamellae generating a contour inducing inclusion in the epithelium (Fig. 2D). Within this model, the myofibroblasts are still believed to contribute the preponderance of light scatter, but the ectopic epithelia are expected to give rise to additional myofibroblasts in the region of implantation based on the results of surgically implanted epithelia in LASIK flaps.[10] Knowledge of these complicating events may enable further refinements and improvements in clinical outcomes following surface ablation techniques—though additional work is necessary to better determine the incidence of these complications and the magnitude of their effects.

Acknowledgments

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