Translational

Refractive Lenticule Re-Implantation after Myopic ReLEx: A Feasibility Study of Stromal Restoration after Refractive Surgery in a Rabbit Model

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Purpose. To investigate the potential of refractive lenticule (RL) storage and re-implantation in vivo as a method for reversing RL extraction (ReLEx) and restoring corneal stromal volume.

Methods. ReLEx [−6.00 diopter (D) correction] was performed on six New Zealand White rabbits in one eye. Each extracted RL was tagged and orientated before storage at −80°C for 28 days. Each RL was then re-implanted autologously in the correct orientation after flap relifting. All animals were monitored for 28 days before being euthanized for immunohistochemical analysis. Unoperated fellow eyes were used as controls. All animals had regular pre- and postoperative slit lamp photography, in vivo confocal microscopy, anterior segment optical coherence tomography (AS-OCT), keratometry, and topography.

Results. No intra-operative complications occurred and RL re-implantation was performed without complication. A mild intrastromal haziness was noted on day 3 after re-implantation (corneal haze grade: 2.20 ± 0.45), but corneas were clear on day 28 (0.20 ± 0.27). RL re-implantation restored central corneal thickness, and keratometric and topographic indices to near pre-operative values. Wound healing processes, marked by fibronectin and tenascin, and a few inflammatory cells were present along the re-implanted lenticular interfaces. No myofibroblasts formation, and Ki67- and TUNEL-positive cells were observed in the corneal stroma on postoperative day 28.

Conclusions. RL storage and re-implantation is a feasible technique for restoring stromal volume after myopic ReLEx, and may provide a method for restoring tissue in ectatic corneas, or provide an opportunity for further refractive surgery and presbyopic treatment. (*Invest Ophthalmol Vis Sci.* 2012;53:4975–4985) DOI:10.1167/iovs.12-10170

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Excimer laser corneal refractive surgery has improved the visual function and quality of life of millions of patients.[1] LASIK, the commonest corneal refractive surgery, due to its excellent visual results together with rapid and painless postoperative visual recovery, is an intrastromal refractive technique that first requires the creation of a stromal flap allowing intrastromal corneal excimer laser ablation. The excimer laser, a 193 nm argon fluoride laser, achieves emmetropia by sculpting the human cornea with submicron precision through a process of photo-ablation that breaks molecular bonds and photo decomposes corneal tissue into atomic constituents.[2] This process causes a permanent loss of corneal stromal tissue, and results in reduced postoperative corneal thickness. Over the last decade, the femtosecond laser (FSL) has become the instrument of choice for safe and precise flap creation during LASIK.[3]

The FSL is an ultrafast pulse, near infrared 1064 nm laser, which dissects tissue through a process of precise photodisruption and plasma cavitation formation.[4] This contrasts with the excimer laser, which photo-ablates, or vaporizes, tissue into the desired shape. Until recently, the predominant utility of the FSL has been in LASIK flap creation; however, introduction of refractive lenticule extraction (ReLEx), an all-in-one FSL refractive technique performed with the Visumax FSL system (Carl Zeiss Meditec, Jena, Germany), marks a new approach utilizing the precision and accuracy of FSL technology, allowing the entire refractive procedure of flap creation and stromal tissue removal to be performed with one single laser.[5]

The proprietary technology of the Visumax FSL laser system currently limits refractive corrections up to 10 diopter (D) of myopia and 5 D of myopic astigmatism by precise dissection of an intrastromal refractive lenticule (RL) that is then manually removed by the surgeon. A curved, low suction applanation cone that causes minimal corneal distortion on contact with the cornea aids the precision of the laser.[6,7] Visumax FSL-based ReLEx surgery broadly encompasses a number of techniques of lenticule extraction that are performed either with a flap akin to a LASIK flap [femtosecond lenticule extraction (FLEx)], or without a flap through a small incision (<4 mm) [small incision lenticule extraction (SMILE) or a variant of FLEx-pseudo SMILE, in which, although the full flap laser incisions are made, the flap is not fully lifted and the lenticule is instead extracted through a partial incision that is larger than in SMILE]. In our own experience and in reported series of ReLEx (FLEx and SMILE), the Visumax FSL has demonstrated excellent safety and refractive outcomes similar to those with LASIK.[8–11]

Having performed ReLEx in a number of patients at our center, we hypothesized that it might be possible to cryopreserve the extracted lenticule for a period of time and then re-implant it back into the host cornea as a method of autologous stromal volume restoration. This could potentially

be of benefit in patients with evidence of corneal ectasia, where stromal volume could be restored in areas of thinning. The lenticule and host cornea may then be cross-linked following lenticule replacement to further arrest the ectatic process in these patients. Alternatively, previous myopic treatments might be reversed and patients could be given monovision at the onset of presbyopia. Such a lenticule might also potentially be used as an allogeneic biological stromal refractive inlay in presbyopic emmetropes, also as a treatment for presbyopia. Previously, we have demonstrated that the stromal keratocytes within extracted lenticules remain viable and can be stimulated to proliferate under appropriate cell culture conditions, even after a prolonged period of cryopreservation at –80°C.[12] In this present study, we describe the findings of a feasibility study of autologous cryopreserved lenticule implantation following myopic correction in a rabbit model of ReLEx (FLEx).

Methods

Animals

Six 12- to 15-week-old New Zealand White rabbits (3–4 kg in weight) were obtained from the National University of Singapore and housed under standard laboratory conditions. The rabbits underwent –6.00 D ReLEx (FLEx) correction in one eye that was selected at random. Contralateral eyes were used as unoperated controls. Extracted lenticules were stored at –80°C for 28 days, after which autologous re-implantation of the stromal lenticule was performed. Animals were anesthetized with xylazine hydrochloride (5 mg/kg intramuscularly; Troy Laboratories, Smithfield, Australia) and ketamine hydrochloride (50 mg/kg intramuscularly; Parnell Laboratories, Alexandria, Australia) during both ReLEx (FLEx) and the re-implantation procedure. The rabbits were euthanized under anesthesia 28 days after the re-implantation procedure by overdose intracardiac injection of sodium pentobarbital. All animals were treated according to the guidelines of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The study protocol was approved by the Institutional Animal Care and Use Committee of SingHealth (Singapore).

Refractive Lenticule Extraction (FLEx) Procedure

ReLEx (FLEx) was performed using the Visumax FSL as previously described, in which the FSL performed a hinged LASIK-type flap incision that was lifted up, and the underlying refractive stromal lenticule peeled away.[13] Briefly, the laser was visually centered on the pupil. A small sized interface cone was used in all cases. In order, the main refractive and nonrefractive FSL incisions were performed in the following automated sequence: the posterior surface of the lenticule (spiral in pattern), the anterior surface of the lenticule (spiral out pattern), followed by the flap side cut. The FSL parameters were: 120-μm flap thickness, 7.5-mm flap diameter, 175 nJ power for lenticule and 160 nJ for the flap, with side cut angles at 90 degrees were used in this experiment. The spot distance and tracking spacing were set at 3 μm/3 μm for lenticule, 2 μm/2 μm for lenticule border, 3 μm/3 μm for flap, and 2 μm/2 μm for flap side cut. Following completion of the laser sequence, a spatula (Seibel spatula; Rhein Medical, Inc., Petersburg, FL) was inserted under the flap near the hinge and the flap was lifted, the stromal lenticule was gently undermined with the spatula, and was then grasped with forceps and extracted. The flap was then repositioned and flap striae smoothed out. A bandage contact lens (Bausch & Lomb, Rochester, NY) was placed over the flap and the eyelid was closed with a temporary tarsorrhaphy for 3 days (6/0 silk suture).

Storage and Re-Implantation of Stromal Lenticule

Stromal lenticules were carefully transferred on to rigid gas permeable (RGP) contact lenses (Bausch & Lomb) with careful attention to maintaining anatomical lenticular orientation. A marking at the 12 o'clock position was made on the RGP lens to indicate the corresponding anatomical position of the lenticule on the cornea before extraction. The contact lens was placed in a lens case and the well was filled with a stock freezing solution containing 10% fetal bovine serum (FBS; Sigma, St. Louis, MO) and 20% dimethyl sulfoxide (DMSO; Sigma). Freezing of the RGP and contact lens case containing the stromal lenticule was carried out at a controlled cooling rate within a cryocontainer (Mr. Frosty; Thermo Fisher Scientific, Roskilde, Denmark) in a –80°C freezer overnight, and transferred into liquid nitrogen the following day for long term storage.

The re-implantation of the lenticule was performed 28 days after the initial ReLEx (FLEx) procedure. The lenticule/RGP was allowed to warm to room temperature and then washed with balanced salt solution (BSS-Plus; Alcon, Forth Worth, TX). Rabbits were anesthetized and a Seibel spatula was inserted under the ReLEx (FLEx) flap near the hinge. Flap adhesions were released by sweeping under the flap and the flap was then lifted. The lenticule was transferred directly onto the exposed stromal bed by sliding it from the RGP contact lens. The 12 o'clock orientation of the lenticule on the stromal bed was carefully observed during re-implantation. The flap was then replaced and a bandage contact lens (Bausch & Lomb) was placed over the cornea and the eyelid was closed with a temporary tarsorrhaphy for 3 days. Gentamicin sulphate (40 mg/ml; Shin Poong Pharmaceutical, Seoul, South Korea) and Dexamethasone sodium phosphate (4 mg/ml; Hospira, Lake Forest, IL) of 1 mL each were injected subconjunctivally following the re-implantation procedure. Prednisolone acetate (1%; Allergan, Irvine, CA) and Tobramycin (0.3%; Alcon) drops were administered four times a day for 1 week.

Corneal Imaging: Slit Lamp Photography, AS-OCT, and Corneal Topography

Slit lamp photographs, corneal topography, and anterior segment optical coherence tomography (AS-OCT) scans were taken before ReLEx (FLEx), 28 days after ReLEx (FLEx), and at 3, 14, and 28 days after lenticule re-implantation. Slit lamp photographs were taken with a Zoom Slit Lamp NS-2D (Righton, Tokyo, Japan). Post re-implantation corneal clarity was assessed by adopting a grading method reported by Fantes et al.[14] Corneal cross-sectional visualization was performed by using the Visante AS-OCT (Carl Zeiss Meditec) and corneal topography was captured by using a handheld videokeratographer (Oculus, Lynnwood, WA).

In Vivo Confocal Microscopy

In vivo confocal microscopy was performed before ReLEx (FLEx), 28 days after ReLEx (FLEx), and at 3, 14, and 28 days after lenticule re-implantation, using the Heidelberg retinal tomography HRT3 with Rostock corneal module (Heidelberg Engineering GmbH, Heidelberg, Germany). A carbomer gel (Vidisic; Mann Pharma, Berlin, Germany) was used as the immersion fluid. All corneas were examined centrally with at least three *z*-axis scans from epithelium to endothelium. In vivo confocal micrographs were analyzed with the Heidelberg Eye Explorer version 1.5.1 software (Heidelberg Engineering GmbH). As previously described, semiquantitative analysis of the reflectivity level of the flap interface was performed by measuring the mean gray value of the reflective particles using the ImageJ software (software available at http://rsbweb.nih.gov/ij/index.html).[13]

Tissue Fixation and Sectioning

After euthanization, the rabbit corneas were excised from the globe and embedded in an optimum cutting temperature (OCT) cryocompound (Leica Microsystems, Nussloch, Germany). Frozen tissue blocks were stored at –80°C until sectioning. Serial saggital corneal 10-μm sections were cut using a cryostat (Microm HM550; Microm, Walldorf,

Germany). Sections were placed on polylysine-coated glass slides and air dried for 15 minutes.

Immunofluorescent Staining

Sections were fixed with 4% paraformaldehyde (Sigma) for 15 minutes, washed with 1X PBS, blocked with 4% bovine serum albumin (Sigma) in 1X PBS, 0.15% Triton X-100 (Sigma) for 1 hour, and incubated with either mouse monoclonal antibody against cellular fibronectin (catalog no. MAB1940; Millipore, Billerica, MA) diluted 1:400, tenascin-C (ab88280; Abcam, Cambridge, UK) diluted 1:200, CD18 (NB100-65,303; Novus Biologicals, Littleton, CO) diluted 1:100, α-smooth muscle actin (α-SMA; N1584; Dako Cytomation, Glostrup, Denmark) diluted 1:50, Thy-1/CD90 (sc-53116; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:50 in the blocking solution, or with prediluted mouse monoclonal antibody against Ki67 (08-0156; Invitrogen, Carlsbad, CA) at 4°C overnight. A probe (Alexa Fluor 488 phalloidin; Invitrogen) was used to detect filamentous actin. After washing with 1X PBS, the sections were incubated with goat anti-mouse Alexa Fluor 488 conjugated secondary antibody (Invitrogen) at room temperature for 1 hour. Slides were then mounted with UltraCruz Mounting Medium containing 4′,6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotechnology). For negative controls, nonimmune serum was used in place of the specific primary antibody. Sections were observed and imaged with a fluorescence microscope (Zeiss Axioplan 2; Zeiss, Oberkochen, Germany).

TUNEL Assay

To detect apoptotic cells, a fluorescence-based TUNEL assay (In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as mean ± SD where appropriate. The *P* value was determined using the two-tailed Student's *t*-test with the Microsoft Excel 2007 software (Microsoft, Redmond, WA). Data were considered to be statistically significant when *P* was less than 0.05.

Results

Slit Lamp Photography

Slit lamp photographs showed that corneal clarity progressively improved from day 3 to 28 following lenticule re-implantation with minimal stromal inflammation, infiltration, or diffuse lamellar keratitis (DLK) (Fig. 1A, upper panel). Retro illumination photography revealed irregularities or microstriae within the lenticule on postoperative day 3. These were predominantly seen at the periphery of the lenticule (Fig. 1A, bottom panel). These irregularities had disappeared by day 14 after re-implantations, and by day 28, the re-implanted corneas were comparable to unoperated control corneas in terms of clarity (Fig. 1A, bottom panel). Corneal clarity gradually improved from post re-implantation day 3 (2.20 ± 0.45) to day 28 (0.20 ± 0.27) (Fig. 1D). There was a statistical significant difference in corneal clarity between day 3 and pre-operative corneas ($P < 0.001$), and between day 14 and pre-operative corneas ($P < 0.05$). The corneal clarity on day 28 was comparable to before the ReLEx (FLEx) surgery.

Stromal Volume Restoration

On AS-OCT, corneas appeared edematous compared with control eyes on day 3 after re-implantation, but returned to normal at subsequent time points (Fig. 1B). The anterior and posterior borders of the lenticule were easily discernible on postoperative day 3. AS-OCT corneal pachymetry before ReLEx (FLEx), 28 days after ReLEx (FLEx), and 28 days after re-implantation was measured at 351.67 ± 11.90 μm, 274.73 ± 11.64 μm, and 361.00 ± 18.13 μm, respectively (Fig. 1C).

In Vivo Confocal Microscopy

The anterior border (top panel), lamellae (middle panel), and posterior border (bottom panel) of the re-implanted corneal lenticule on postoperative days 3, 14, and 28 were clearly identifiable on confocal microscopy (Fig. 2). The anterior and posterior border of the lenticule showed increased light reflectance and was acellular on day 3 after re-implantation. Interspersed small particles with variable size and reflectivity were observed at the lenticule's anterior and posterior border, which were likely to be after surgical debris. On day 14, the reflective layer at both interfaces was less prominent and keratocytes were visible, particularly at the posterior interface of the lenticule. Increased numbers of keratocytes had appeared at the anterior border of the lenticule on day 28. The keratocytes within the center of the lenticule remained quiescent and did not seem to change in terms of their morphology and activity from postoperative day 3 to 28. Quantification of the relative reflectivity level of the lenticule's anterior and posterior borders are shown in Figures 2A and 2B respectively, and were seen to decrease over the duration of the study. The intensity of the reflective layer at the lenticule's anterior decreased from 117.09 ± 20.67 on post re-implantation day 3 to 83.73 ± 14.15 on day 28, and reduced from 105.15 ± 12.87 on day 3 to 90.09 ± 14.10 on day 28 at the lenticule's posterior. Significant difference ($P < 0.05$) was observed between day 3 and control, and between day 14 and control at both interfaces.

Corneal Topography and Keratometry

Corneal topographic maps (Fig. 3) showed obvious flattening consistent with the –6.00 D treatment 28 days after the initial ReLEx (FLEx) procedure. Twenty eight days after lenticule re-implantation, corneas were steepened centrally in all cases, and topographic maps appeared similar to corneas before ReLEx (FLEx). Mean keratometry values for operated eyes were as follows: 48.0 ± 2.3 D before ReLEx (FLEx), 42.0 ± 2.0 D 28 days after ReLEx (FLEx), and 45.6 ± 1.8 D 28 days after lenticule re-implantation. Based on these values, the mean keratometry after re-implantation was 2.4 ± 0.7 D less than the unoperated keratometry before ReLEx (FLEx).

When the keratometry of the control nonoperated fellow corneas was measured, we found a baseline mean keratometry of 48.6 ± 1.9 D. The mean keratometry at the final time point in control eyes (28 days after lenticule re-implantation in the fellow eye) was 46.7 ± 1.3 D. These measurements demonstrate an overall flattening and commensurate reduction in the mean keratometry of –1.9 ± 1.0 D in control eyes over the 56 day time course of this study. This is a previously documented natural aging phenomenon that occurs as the rabbit matures.[15–17] When this natural reduction in keratometry was applied to the operated eyes, the final keratometry following re-implantation was –0.6 ± 0.8 D from the pre-operative correction.

Immunohistochemistry

On day 28 after re-implantation, fibronectin was expressed along the anterior and posterior border of the lenticule (Fig. 4, top panel). Leukocyte integrin β2 (CD18), an inflammatory marker and mediator of polymorphonuclear leukocyte (PMN) migration within the corneal stroma,[18] was seen expressed

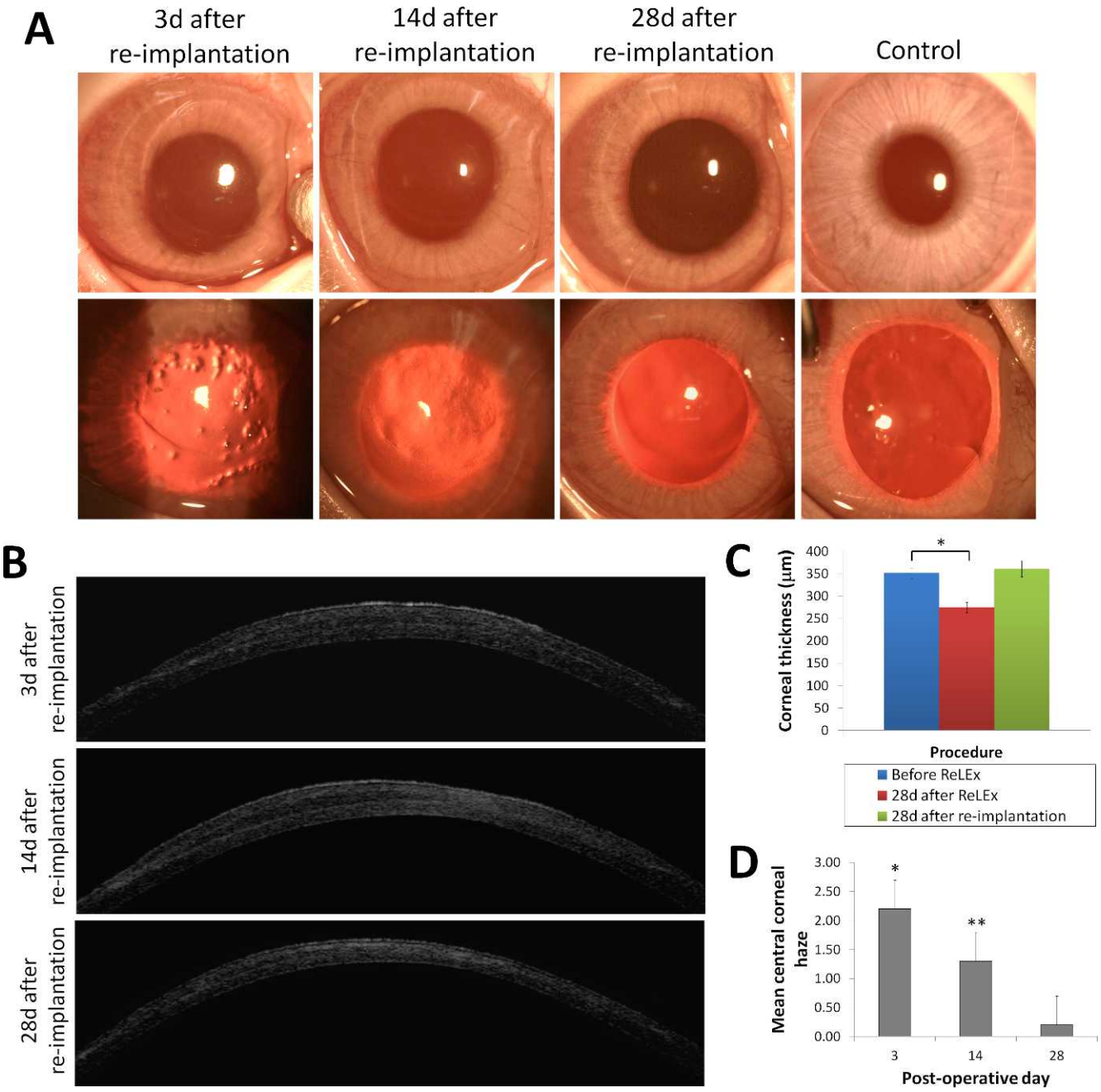

Figure 1. Slit lamp microscopy, AS-OCT, and pachymetry of the postoperative corneas. (**A**) The top panel shows slit lamp photographs of the nonoperated cornea (control) and cornea on day 3, 14, and 28 after lenticule re-implantation. The bottom panel shows retro illumination photographs of the control and postoperative corneas. (**B**) Temporal AS-OCT images of postoperative corneas shows resolving tissue edema over time. (**C**) Bar graph showing the corneal thickness before ReLEx procedure, 28 days after ReLEx, and 28 days after lenticule re-implantation. (**D**) Quantification of central corneal haze on day 3, 14, and 28 after lenticule re-implantation. Corneal clarity or haze is graded on a scale of 0–4 (from 0 being completely clear to 4 being completely obscured). Statistical significance was obtained by comparing postoperative to pre-operative corneal clarity. Error bars represent SD. $^{*}P < 0.001$, and $^{**}P < 0.05$.

only by a few cells and predominantly found at the interfaces of the lenticule (Fig. 4, middle panel). Tenascin-C could be detected within the lenticule, and was mainly expressed along lenticule's anterior interface (Fig. 4, bottom panel).

No proliferating Ki67-positive cells were observed within the lenticule and corneal stroma (Fig. 5, top panel), and only a few apoptotic TUNEL-positive cells were found within the lenticule of the re-implanted cornea (Fig. 5, middle panel). No apoptotic epithelial cells were present in the cornea after re-implantation (Fig. 5, middle panel). Cell migration, indicated by the relatively strong staining of phalloidin indicating the intracellular assembly of filamentous actin, could be seen within the re-implanted lenticule (Fig. 5, bottom panel). This was more abundant in the posterior portion of the lenticule.

There were no myofibroblasts detected in the re-implanted cornea, which was indicated by the absence of α-SMA (Fig. 6,

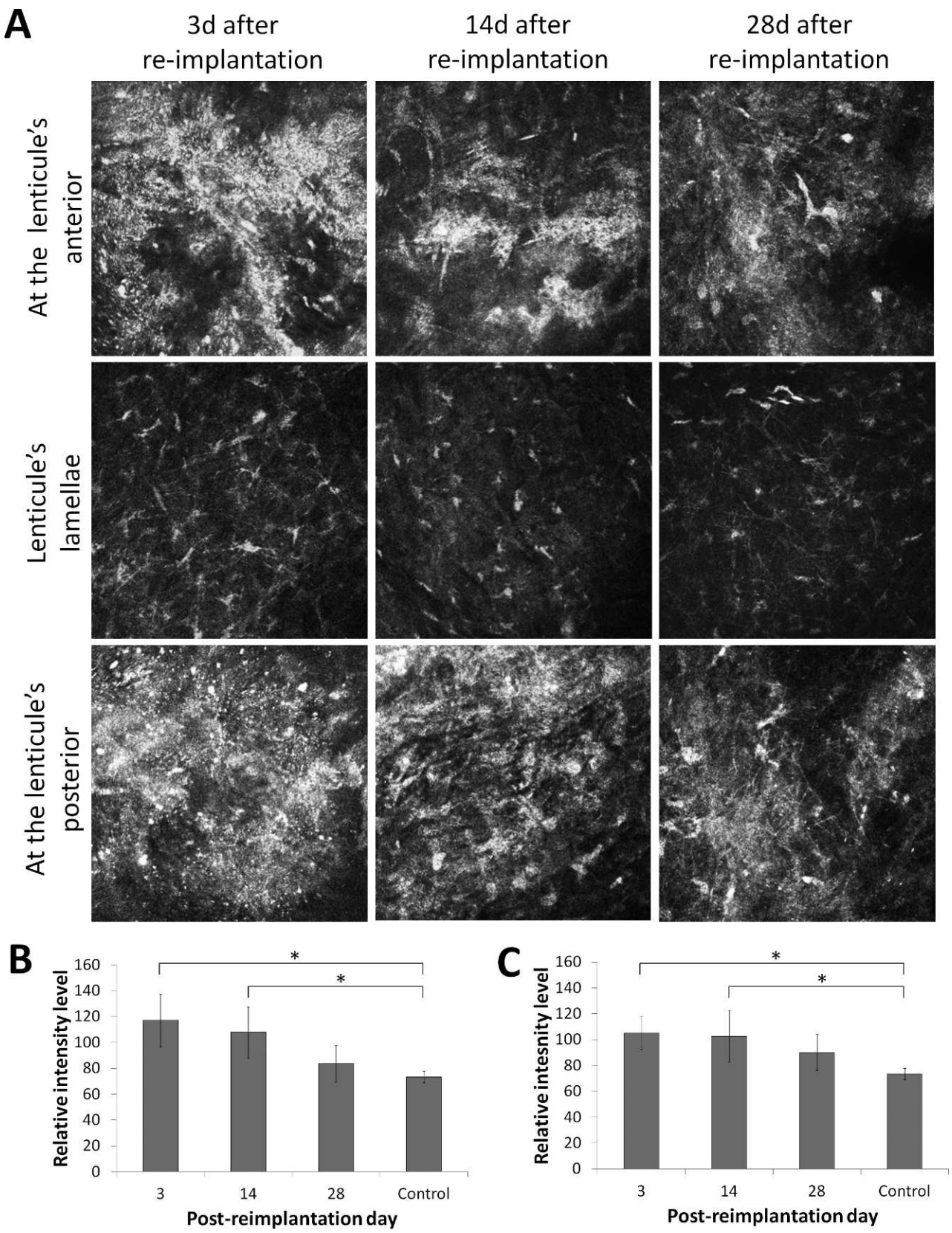

Figure 2. In vivo confocal micrographs of the corneas on day 3, 14, and 28 after lenticule re-implantation. (**A**) The top panel shows the anterior border of the lenticule within the re-implanted cornea. The middle panel shows the presence of quiescent keratocytes within the lenticule's lamellae. The bottom panel shows the posterior interface of the lenticule. Repopulation of anterior and posterior borders of the lenticule occurs by day 28. (**B**) Bar graph showing the mean reflectivity level of the lenticule's anterior interface on day 3, 14, and 28 after lenticule re-implantation. (**C**) Bar graph showing the mean reflectivity level of the lenticule's posterior plane on day 3, 14, and 28 after lenticule re-implantation. Error bars represent SD. $^*P < 0.05$.

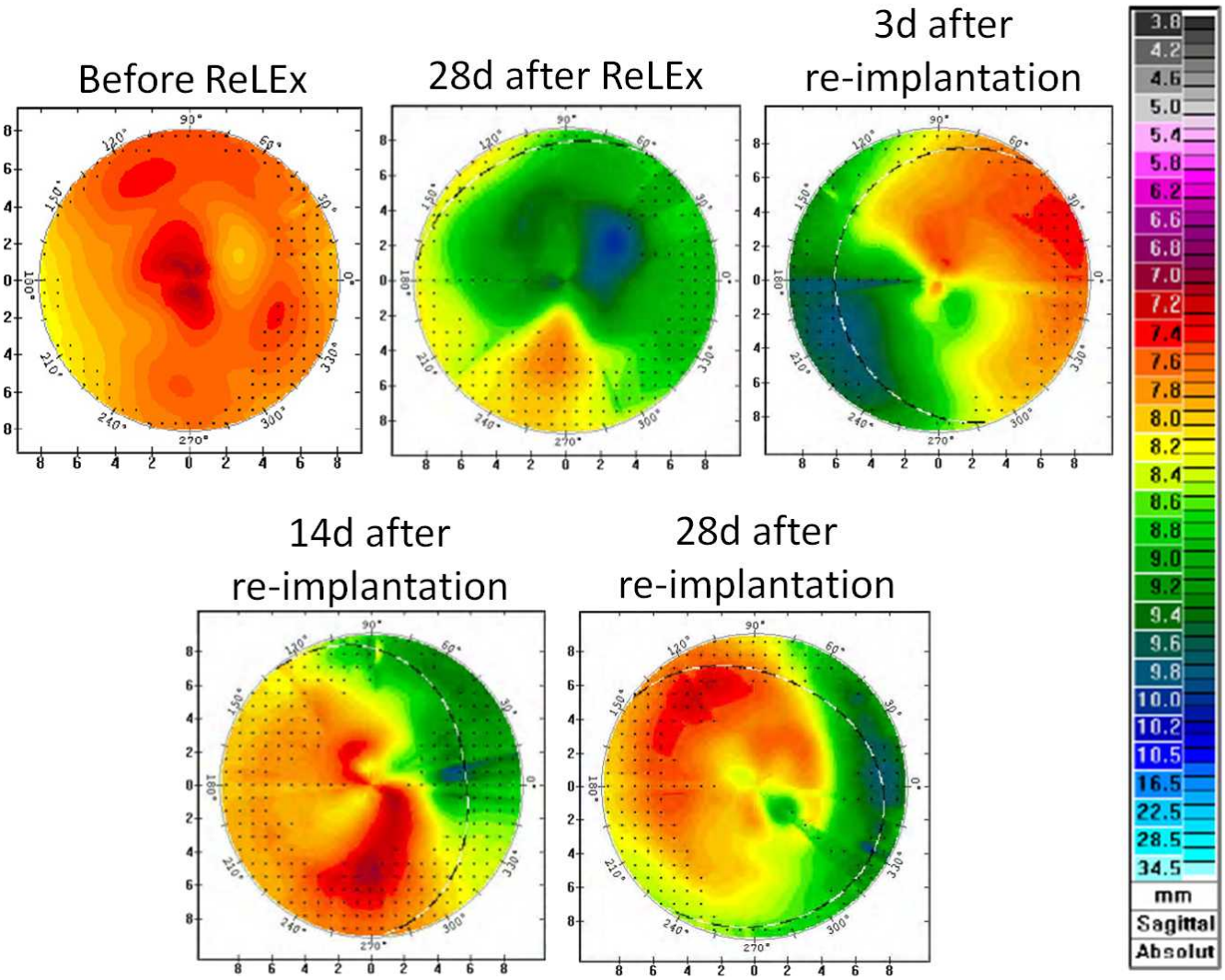

Figure 3. Corneal topography before ReLEx procedure, 28 days after ReLEx, and 3, 14, and 28 days after lenticule re-implantation. There is initial flattening of the cornea consistent with the –6.00 D ReLEx correction initially, however, by day 28 post lenticule re-implantation topographic indices are similar to the unoperated state.

top panel). Thy-1–positive fibroblasts were also only scarcely present in the operated cornea, and were typically found within the lenticule (Fig. 6, bottom panel).

Discussion

This study, for the first time, demonstrates the potential of stromal volume restoration following FSL refractive surgery using autologous stromal tissue stored at the time of initial surgery. We have previously demonstrated, using human corneal tissue, that cells within these extracted lenticules remain viable, and are capable of proliferation under culture conditions following storage at –80°C for a prolonged period of time.[12] This would allow the patient to augment the volume of their own stroma with tissue containing autologous cells, thereby circumventing the risk of immune rejection of the transplanted tissue. The technique is applicable at present only to ReLEx (FLEx, SMILE, and pseudo-SMILE), which utilize the Visumax FSL to correct ammetropia by intrastromal lenticular extraction.

As described previously, the Visumax FSL performs corneal refractive surgery by precisely dissecting an intrastromal RL that is manually extracted by the surgeon. This contrasts with the excimer laser that achieves the same result through photoablation of stromal tissue. The significant benefit of an FSL-only refractive technique compared with the excimer laser is that the FSL delivers significantly lower levels of energy to adjacent stromal tissue, and is, there by, likely to account for the weaker wound healing response demonstrated by our group after –6.00 D and greater myopic corrections with FLEx compared with LASIK in rabbits.[13]

In this study, we utilized DMSO, a ubiquitous and nontoxic cryoprotectant, often used in the cryostorage of embryonic and haematopoietic stem cells, to prevent intralenticular cell damage during freezing in liquid nitrogen.[19] We used FBS in the cryoprotectant solution, and alternatives may need to be explored if this technique were to be adopted for human use to avoid the use of xeno-derived products and risk of zoonosis. The technique of lenticular storage described here, which utilized a simple rigid gas permeable lens with an orientation mark to indicate the 12 o'clock position of the lenticule on the eye, would also need to be optimized for clinical use especially in patients with toric lenticules, with the development of dedicated cryopreservation lenticular cases, which maintain the anatomical curvature of the lenticules and the appropriate

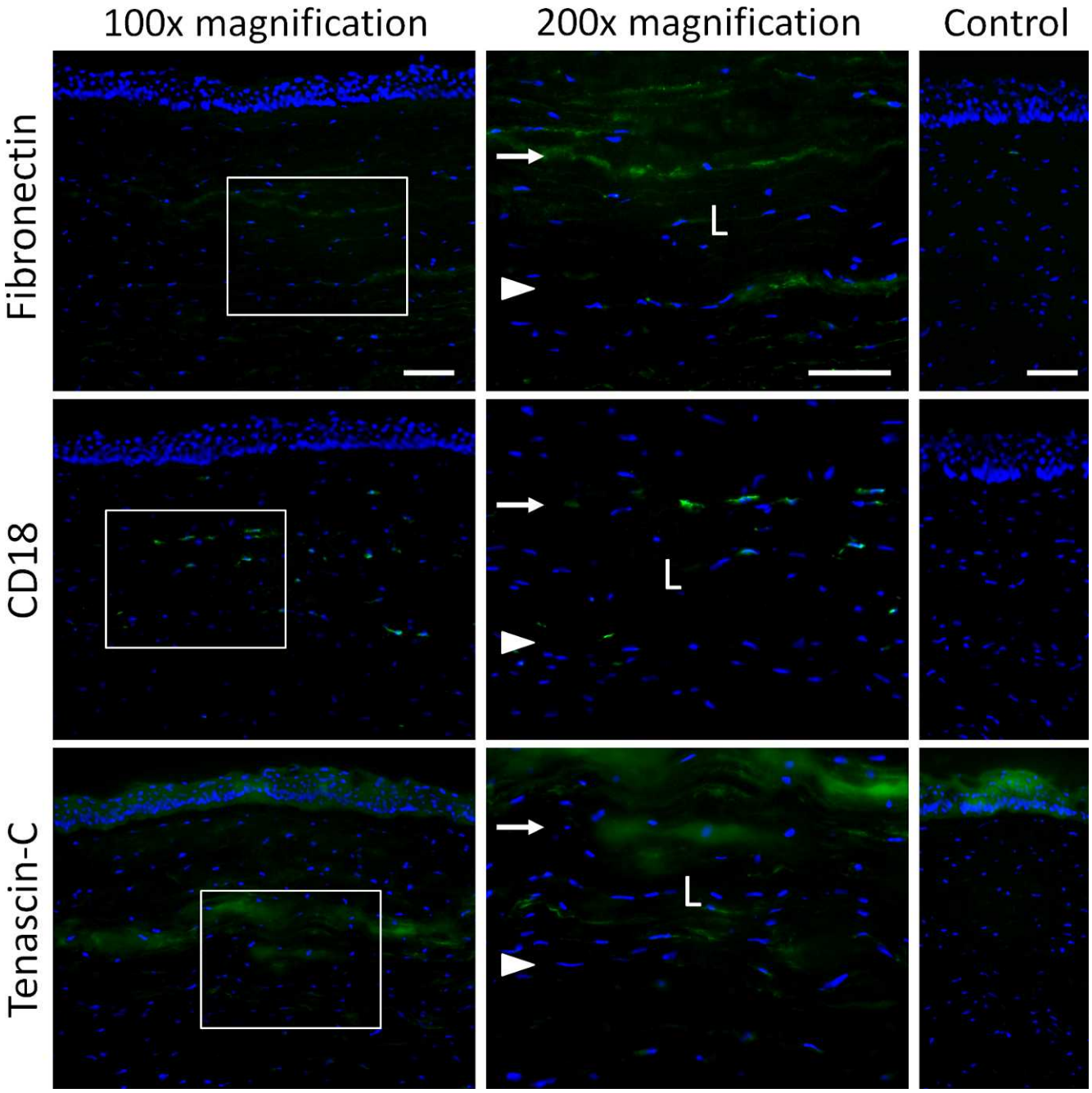

Figure 4. Expression of fibronectin, CD18, and tenascin-C in the corneas on day 28 after lenticule re-implantation. Images in the middle column are the magnified images within the white boxes found in the left column. Staining of the nonoperated corneas (control) is shown in the right column. Arrowheads show the lenticule's posterior interface and arrows show the lenticule's anterior interface. L indicates the re-implanted lenticule within the corneal stroma. Sections were counterstained with DAPI, which stained the nuclei (*blue*). Scale bars: 50 μm.

axial orientation of the lenticules. Lenticular orientation is more critical if a spherocylindrical ReLEx treatment is applied, where correct repositioning of the lenticule would be required if the pre-operative refraction were to be restored. However the true importance of precise lenticular re-implantation remains to be seen, as one potential application of this technique could be the opportunity for further laser refractive surgery to provide a presbyopic patient with monovision, in which case, orientation may not be as critical as induced astigmatism may be corrected at the time of further surgery.

Despite initial tissue edema and microstriae within the first few days of lenticule implantation, corneas were clear and comparable to unoperated fellow eyes by day 28. Corneal thickness (351.67 ± 11.90 μm) before ReLEx (FLex) was restored in all eyes after the lenticule was replaced. The very slight increase in thickness at the end of the study period could be due to mild residual edema (361.00 ± 18.13 μm), but was not statistically significant and may normalize over a longer observation period. Another explanation of the slight increase in corneal thickness would be the mild epithelial hyperplasia that usually takes place after central flattening of the cornea either by FSL refractive surgery or resection.[20] Clarity of the re-implanted cornea returned to control levels by 28 days after lenticule replacement and was matched by a commensurate

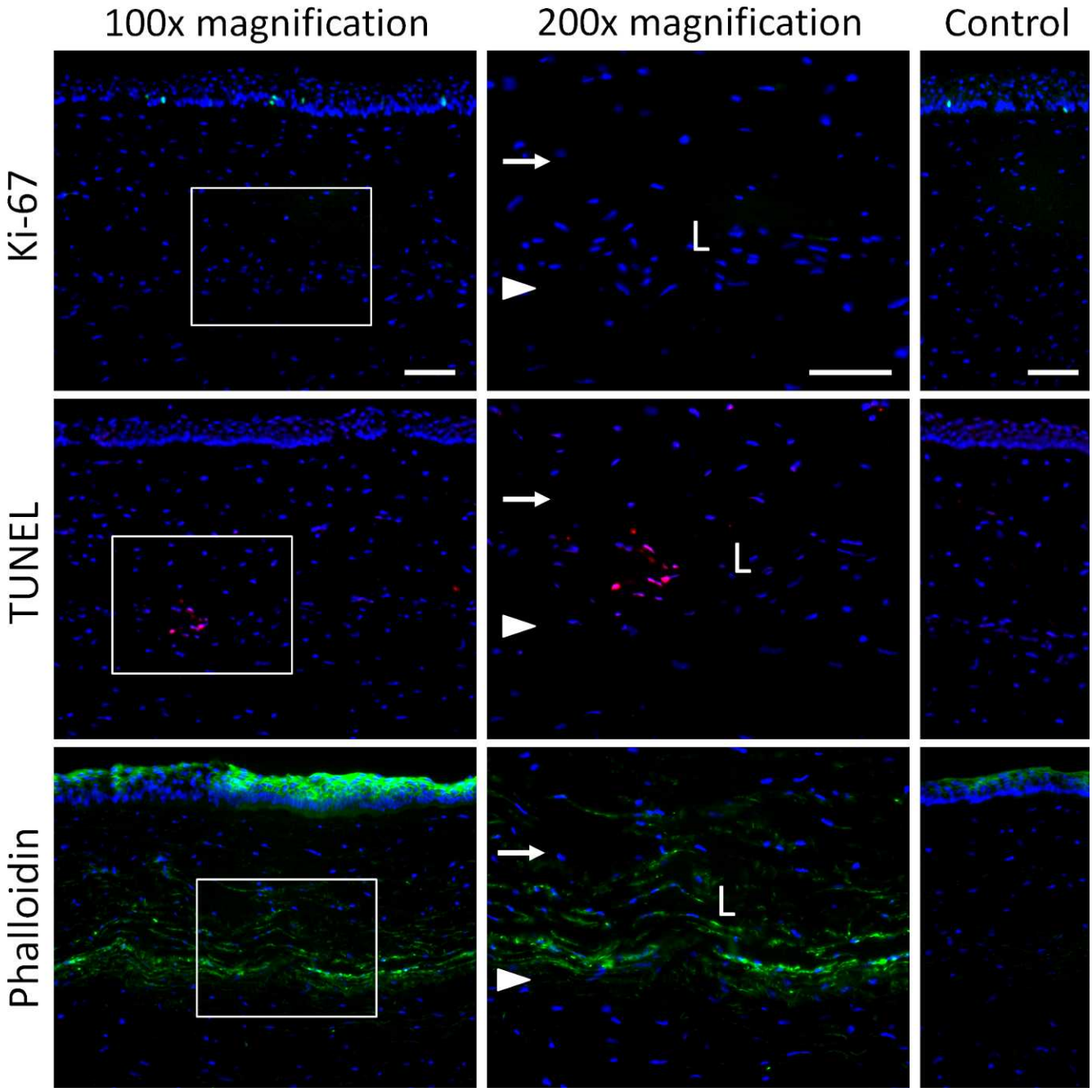

Figure 5. Fluorescent staining of Ki67, TUNEL, and phalloidin in the corneas on day 28 after lenticule re-implantation. Images in the middle column are the magnified images within the white boxes found in the left column. Staining of the nonoperated corneas (control) is shown in the right column. Arrowheads show the lenticule's posterior interface and arrows show the lenticule's anterior interface. L indicates the re-implanted lenticule within the corneal stroma. Sections were counterstained with DAPI, which stained the nuclei (*blue*). Scale bars: 50 μm.

reduction in interface reflectivity based on confocal microscopy measurements. Myofibroblasts and fibroblasts, both cell types implicated in scarring and haze formation in the cornea, were largely absent on immunohistochemical staining. Previous well-characterized models of corneal wound healing in rabbits after excimer laser refractive surgeries have demonstrated abundant expression of these cell types by 28 days postoperatively.[21,22] Temporal confocal cellular analysis demonstrated resident keratocytes within the lenticule body and repopulation of the anterior and posterior lenticular borders by day 28 after implantation. Repopulation of the lenticular borders appears to occur through migration of adjacent keratocytes, rather than by keratocyte proliferation as indicated by a relative absence of Ki67 staining cells (a marker of cell proliferation), together with positive staining for cellular actin (phalloidin staining), a contractile cytoskeletal element within the cell body. The lack of a proliferative keratocyte response is probably partly as a result of the fact that the lenticule itself contains a viable resident population of cells.

We have previously demonstrated a comparatively minor inflammatory response at 24 hours after a –6.00 D myopic ReLEx (FLEx) correction compared with a similar excimer laser LASIK treatment. This is probably as a result of the significant difference in stromal energy delivery between the FSL and

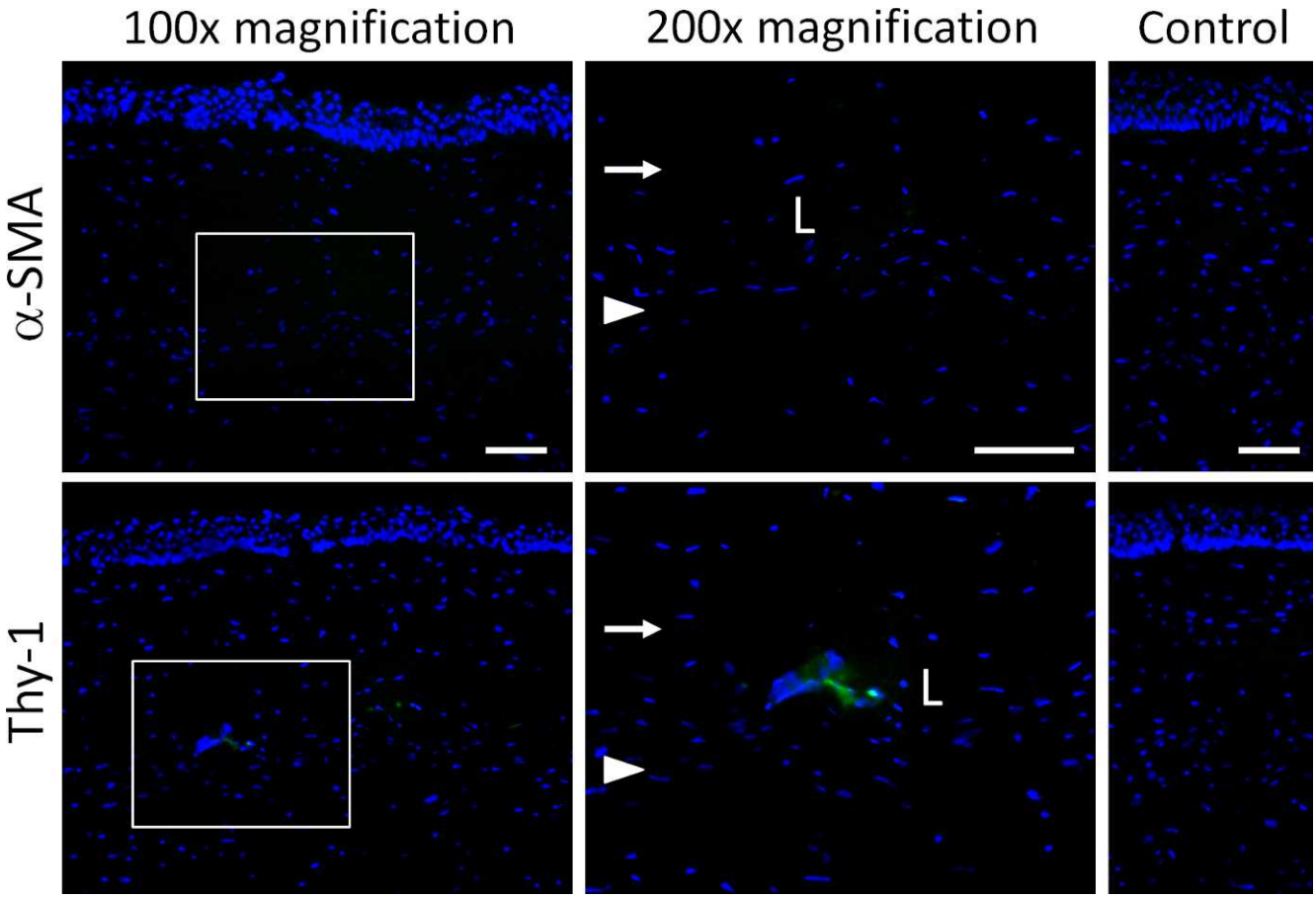

Figure 6. Expression of α-SMA and Thy-1 in the corneas on day 28 after lenticule re-implantation. Images in the middle column are the magnified images within the white boxes found in the left column. Staining of the nonoperated corneas (control) is shown in the right column. Arrowheads show the lenticule's posterior interface and arrows show the lenticule's anterior interface. L indicates the re-implanted lenticule within the corneal stroma. Sections were counterstained with DAPI, which stained the nuclei (*blue*). Scale bars: 50 μm.

excimer lasers.[13] Perhaps due to this reason, ReLEx does not appear to incite a significant corneal wound healing response and we observed sparse inflammatory cell presence, even following lenticule re-implantation (CD18). This weak healing stimulus, probably also explains why there is very little extracellular matrix deposition following lenticule re-implantation (i.e., fibronectin and tenascin). This relative lack of inflammation and wound healing responses will clearly be advantageous with regards to maintenance of corneal clarity and refractive accuracy in refractive stromal reimplantation procedures.

Current methods for stromal volume restoration, particularly in cases of corneal ectasia after refractive surgery, are mainly limited to various techniques of anterior lamellar keratoplasty (ALK) involving the removal of part or all of the host stroma and replacement with donor stromal tissue.[23] These techniques are technically and surgically demanding, time consuming, and are usually performed by corneal transplant surgeons, necessarily limiting their applicability to specific indications. In addition, use of donor corneal tissue involves some risk of graft rejection.[24] Epikeratophakia, a technique in vogue in the 1990s, was another method for stromal volume restoration used in the treatment of keratoconus. The epikeratophakia technique involved the removal of host corneal epithelium and fixation by suture of a cryolathed donor corneal lenticule on to the anterior stroma, over which the host epithelium would heal.[25,26] The failure to widely adopt this technique resulted from a number of postoperative complications including interface scarring between the lenticule and host cornea, and poorly predictable astigmatic outcomes. In contrast to these methods, the technique of refractive stromal lenticule re-implantation described here clearly has significant advantages in terms of lamellar accuracy and refractive correction. Significantly, it respects many of the benefits of corneal wound healing by allowing the lenticule to be placed under a stromal flap without undue epithelial injury, in a manner similar to a flap lift for enhancement performed after conventional LASIK treatment. Risk of epithelial ingrowth, which we did not observe in any study cases, is likely to be no greater than with conventional LASIK.[27] If keratectasia occurs in the patient who underwent a ReLEx procedure, the use of the patient's own autologous tissue also circumvents the risk of tissue rejection and the need for prolonged topical immunosuppression postoperatively. In patients who develop post LASIK keratectasia, stored lenticules that are not personally reserved by the original ReLEx patients could be donated to these patients (with appropriate informed consent from the donor, and also appropriate serological testing) to restore corneal volume under the LASIK flap. Being allogeneic, there would be a small risk of stromal rejection, similar to immunological risk after an ALK procedure. Unlike ALK or deep ALK (DALK), however, the intrastromal insertion technique is surgically easier and relatively more straightforward to perform, and could be done by any surgeon experienced with LASIK or ReLEx surgery.

Temporal topographic indices during this study demonstrated post implantation mean keratometry values that were within −0.6 ± 0.8 D of values before ReLEx (FLEx). While this is promising and shows that pre-operative corneal topography can be largely restored after lenticule implantation, there are a number of potential sources of inaccuracy in our data. Significantly, although handheld topography scans were captured on the corneal center, actual ReLEx (FLEx) treatments were difficult to center accurately and consistently on the pupil center of the anaesthetized rabbit due to a tendency for the rabbit eye to roll eccentrically when applanated with the treatment cone of the Visumax FSL. Even though attempts were made to fixate the globe with forceps while applying suction, it was not fully possible to control eye position, resulting in slightly eccentric treatments. This is unlikely to pose a problem in a conscious patient focused on the fixation light during actual ReLEx.

Lenticule storage and re-implantation is a novel technique for stromal volume restoration that may be utilized in a number of potential clinical situations. In cases of post refractive surgery corneal ectasia, the technique offers the possibility to restore corneal stromal volume with autologous tissue, theoretically any time after surgery, and may also be combined with collagen cross-linking for added structural re-enforcement.[28] A more ubiquitous clinical scenario is that of a previously myopic patient who has undergone refractive surgery to near emmetropia, and then finds, with time, that near visual tasks become more difficult as accommodation is lost through presbyopia. Current excimer-based refractive surgeries result in permanent stromal tissue loss, thereby, limiting the possibility of further corneal surgery; however, lenticule replacement could restore the patients previous refractive state, and would offer the possibility of re-implantation of an autologous lenticule reshaped to a +1.5 or +2.0 D power, in the nondominant eye, thus, enabling monovision. Finally, several synthetic corneal refractive inlays have recently demonstrated some promise in the treatment of presbyopia, but issues with regards to polymer biocompatibility, stromal haze, and nutritional issues with corneal melting are still a potential concern. An alternative approach here could be the use of these stored lenticules as an autologous biological intrastromal inlay in the same manner as these synthetic inlays, thus, obviating biocompatibility and nutritional issues.[29]

In conclusion, this study demonstrates proof of principle of reversibility of an FSL corneal refractive procedure. Up until now, excimer-based refractive surgeries, such as LASIK, have offered excellent visual results, albeit with permanent loss of stromal tissue and without any possibility of reversibility. The potential option of stromal lenticule storage after ReLEx offers patients the unique opportunity to bank their tissue in case of future need, or to donate their tissues to others in need.

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