

EXTENDED REPORT

Corneal stromal changes following reconstruction by ex vivo expanded limbal epithelial cells in rabbits with total limbal stem cell deficiency

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Aim: To study corneal stromal changes and the presence of myofibroblasts after transplantation of ex vivo expanded limbal epithelium.

Methods: A state of limbal deficiency was induced in 16 rabbits. After transplantation with autologous ex vivo expanded limbal epithelium on amniotic membrane (AM), their clinical outcomes were classified as success, partial success or failure according to surface smoothness, stromal clarity, and vascularisation. Clinical outcomes were correlated with phenotypic outcomes of corneal, conjunctival, or mixed epithelium, defined by expression of K3 keratin or MUC5AC. Immunostaining was performed with antibodies against collagen IV, fibronectin, and α -smooth muscle actin (α -SMA) to assess stromal wound remodelling.

Results: Rabbits were sacrificed after a mean follow up of 10 (SD 3.3) months. Collagen IV, expressed in the basement membrane of all three groups, was found in the stroma of the partial success, but not in that of the success or the failure. Fibronectin was absent in the success and the failure, but expressed in the stroma of the partial success. α -SMA was expressed in superficial stroma of the partial success, but suppressed in areas with AM remnants.

Conclusion: Restoration of a clear and transparent cornea is associated with a normal corneal epithelium and complete wound remodelling. In contrast, wound healing remains active and incomplete in conjunctivalised corneas, which remain opaque with myofibroblasts.

One major advance made in treating patients suffering from total limbal stem cell deficiency is to transplant epithelial cells that have been expanded from a small limbal biopsy cultured on amniotic membrane.^{1–5} We have recently demonstrated the efficacy of this new surgical procedure in a rabbit model of unilateral total limbal stem cell deficiency.⁶ After more than 1 year follow up, we noted that the control group receiving amniotic membrane transplantation (AMT) alone showed 100% (n=10) clinical failure with vascularised and scarred corneas. In contrast, AMT with ex vivo expanded limbal epithelial cells showed 26% (11/42) clinical success and 19% (8/42) partial success (both (p<0.0001), resulting in total and partial recovery of a smooth, avascular, and clear cornea, respectively.⁶ Furthermore, clinical success is correlated with the recovery of a normal corneal epithelial phenotype (p<0.05), and clinical failure is correlated with the maintenance of a conjunctival epithelial phenotype (p<0.005) (Ti *et al*, submitted, 2003).

During the above investigation, we were intrigued by the finding that the amniotic membrane with limbal epithelial cells was initially opaque but became transparent after healing. For this reason, we investigated changes of extracellular matrix components during wound healing. Specifically, we analysed the expression of collagen IV (Coll-IV), a basement membrane component, which is expressed by injured corneal stromal fibroblasts.⁷ We also studied fibronectin (Fn), a provisional matrix component, which is expressed by stromal fibroblasts during early and active wound healing.⁸ Furthermore, we determined whether fibroblasts expressed α -smooth muscle actin (α -SMA), a marker of myofibroblasts,⁹ responsible for corneal wound contraction.¹⁰ The significance of our findings is further discussed.

MATERIALS AND METHODS

Special materials

Monoclonal antibodies against Fn (Sakajama, Japan), Coll-IV (Southern Biotechnicals, AL, USA), and α -SMA (Dako, Carpinteria, CA, USA) were obtained. DAPI mounting media Vectashield was from Vector Laboratories (Burlingame, CA, USA).

Rabbit model of total limbal deficiency

The study included a total of 16 rabbits, which were a part of a study protocol that was approved by the animal research committee of the University of Miami. Two additional normal uninjured rabbits were added as normal controls. Left eyes of the 16 rabbits were rendered total limbal stem cell deficient as previously described^{11,12} by 1-n-heptanol and mechanical debridement of the cornea epithelium and surgical lamellar removal of a circular rim of limbus. The state of limbal deficiency was confirmed in all these rabbits using impression cytology¹³ and based on a published diagnostic criterion.¹⁴

Preparation of rabbit AM, limbal biopsy, and AM culturing

Rabbit AM was harvested from 28 day pregnant rabbits under sterile conditions, prepared and preserved as previously described. After thawing and rinsing in HBSS, the AM was fastened onto inserts.¹⁵ A limbal biopsy was obtained and cultured in the AM containing inserts as previously described.⁶

Surgical transplantation

Following complete pannus removal in 16 rabbits, 14 left eyes received AM transplantation with expanded epithelial cells⁶

in a manner as previously described.⁶ Two left eyes underwent AM alone without expanded epithelium as a control.

Clinical and phenotypic outcomes

According to the clinical outcome, each eye was classified as “success” if a smooth, avascular and clear cornea was restored; as “partial success” if more than two quadrants of the cornea was smooth, clear and avascular; and as “failure” if more than three quadrants of the cornea remained irregular, vascularised, and scarred. All corneas were then subjected to immunostaining using antibodies to cornea specific keratin K3¹⁶ and to conjunctival goblet cell specific MUC5AC.¹⁷ The resultant epithelium following reconstruction was defined as “corneal” if positive to K3 but negative to MUC5AC, as “conjunctival” if positive to MUC5AC but negative to K3, or as “mixed” if it was partly positive to K3 and partly to MUC5AC.

Immunostaining

Sections of corneoscleral buttons were fixed in cold acetone for 10 minutes at -20°C . After incubation in BSA to decrease non-specific staining, sections were incubated overnight with the following primary antibodies in an appropriate dilution: mouse anti-Fn (1:60), mouse anti- α -SM (1:100), and goat anti-Coll-IV (1:50). The binding with the primary antibody was detected by a rhodamine conjugated secondary antibody. Finally, sections were mounted in anti-fading solution containing DAPI for nuclear counterstaining.

RESULTS

Follow up and clinical outcome

All 16 experimental rabbits were followed up for 10 (SD 3.3) months (range 7–13 months). The clinical outcome of the two eyes receiving AM transplantation alone was graded as “failure” as reported.⁶ The remaining 14 eyes receiving ex vivo expansion were graded as “success” ($n = 6$, follow up of 11.8 (0.4) months), “partial success” ($n = 4$, follow up of 12 (1.1) months), and “failure” ($n = 4$, follow up of 6.1 (0.9) months). Two normal uninjured rabbit eyes were also included as a control.

Phenotypic outcome and stromal changes

Normal control group

In the normal uninjured cornea, the epithelium was stratified and rested on a Bowman’s membrane, and the stroma was compacted with keratocytes without vascularisation (Fig 1A). The full thickness of the corneal epithelium expressed K3 keratin (Fig 1B) as reported.¹⁶ Immunostaining to Coll-IV was positive in the basement membrane of the corneal and conjunctival epithelia as a thin line, which under higher magnification was thinner in the peripheral and central cornea (Fig 1C) but thicker and wavy in the conjunctiva (Fig 1D). No staining was observed in the corneal stroma. Coll-IV was also present in the basement membrane of blood vessels of the subconjunctival stroma and the Descemet’s membrane (not shown). No immunoreactivity against Fn was seen in the corneal basement membrane or stroma (Fig 1E), while a weak reactivity to Fn was noted in blood vessels of the conjunctiva (Fig 1F, indicated by a white arrow), consistent with the notion that serum Fn was present. Positive α -SMA staining was only found in the smooth muscle of blood vessels of the conjunctiva (similar to other groups shown below, Fig 2G).

Success group

In this group, all six corneas showed a smooth and clear reconstructed surface without vascularisation (Fig 2A for an example). These corneas were associated with restoration of a corneal epithelial phenotype, which was positive to K3

keratin (Fig 2B, arrow marks the limbus), but negative to MUC5AC, which was a mucin expressed by conjunctival goblet cells (Fig 2C, arrow marks the limbus). The corneal stroma had an organised lamellar architecture and lack of inflammatory cell infiltration and vascularisation. In some cases, an eosinophilic layer of matrix, corresponding to the area where AM was applied, was noted immediately subjacent to the epithelium (not shown). Immunostaining to Coll-IV resembled what was found in the normal control. It stained a thin subepithelial basement membrane in the central and peripheral corneal areas and Descemet’s membrane, but not the stroma (Fig 2D). In addition, it stained a thicker basement membrane in the conjunctival area, and the basement membrane of blood vessels in the peripheral cornea, limbal, and conjunctival stroma (Fig 2E, marked by white arrowheads). Immunostaining to Fn was negative (Fig 2F). Immunostaining to α -SMA was positive in the smooth muscle of blood vessels of the limbal and conjunctival stroma (Fig 2G), but not in the regressing blood vessels of the peripheral cornea (Fig 2G, indicated by bracket).

Partial success group

Corneas of this group showed a smooth clear and avascular surface in one area and a hazy vascularised surface in another area (for examples see Fig 3A and F). In some corneas with partial success, the overlying epithelium was of a corneal phenotype in one side (Fig 3B, white arrowhead shows the beginning of K3 keratin expression), and of a conjunctival epithelium with positive expression of MUC5AC in the other side (not shown). The stroma under the corneal epithelium had a normal density of keratocytes and did not have inflammatory cells or vascularisation (not shown). In contrast, the stromal area under a conjunctival epithelium had increased intrastromal cell infiltration and was vascularised. Two rabbits in this group showed AM remnant in the corneal stroma. One of them, AM remnant was in the centre of a granuloma in the superficial stroma (Fig 3C, demarcated by asterisks). Immunostaining to Coll-IV showed a thin basement membrane in the area with a corneal epithelium, but a thick and wavy basement membrane in the area with a conjunctival epithelium (Fig 3D and G). Importantly, immunostaining to Coll-IV was clearly detected in the superficial corneal stroma of the conjunctivalised area but not in the area where the corneal epithelium was restored (Fig 3D and G, marked by arrows). Coll-IV was also found the amniotic basement membrane of AM remnant (Fig 3D, asterisk). Immunostaining to Fn was strongly positive in the anterior stroma regardless of the presence of AM (Fig 3H). Immunostaining to α -SMA was negative when there was AM remnant (Fig 3E), but was uniformly positive in the superficial stroma when there was no AM (Fig 3I).

Failure group

All four corneas with failure following ex vivo expansion showed diffuse vascularisation and stromal haze (for an example see Fig 4A). They were covered by a conjunctival epithelium containing goblet cells with MUC5AC positive goblet cells (Fig 4B), but negative to K3 keratin (not shown). The underlying stroma showed loss of lamellar structure and was infiltrated with blood vessels. Histological sections showed that AM could not be identified in all corneas (not shown). Strong immunostaining to Coll-IV was found in the basement membrane (Fig 4C), and in blood vessels that infiltrated in the corneal stroma (Fig 4C and inset showing high magnification). Immunostaining to Fn was negative in the corneal stroma of all failure corneas (Fig 4D). Immunostaining to α -SMA was not seen in the corneal stroma nor in infiltrating blood vessels (Fig 4E).

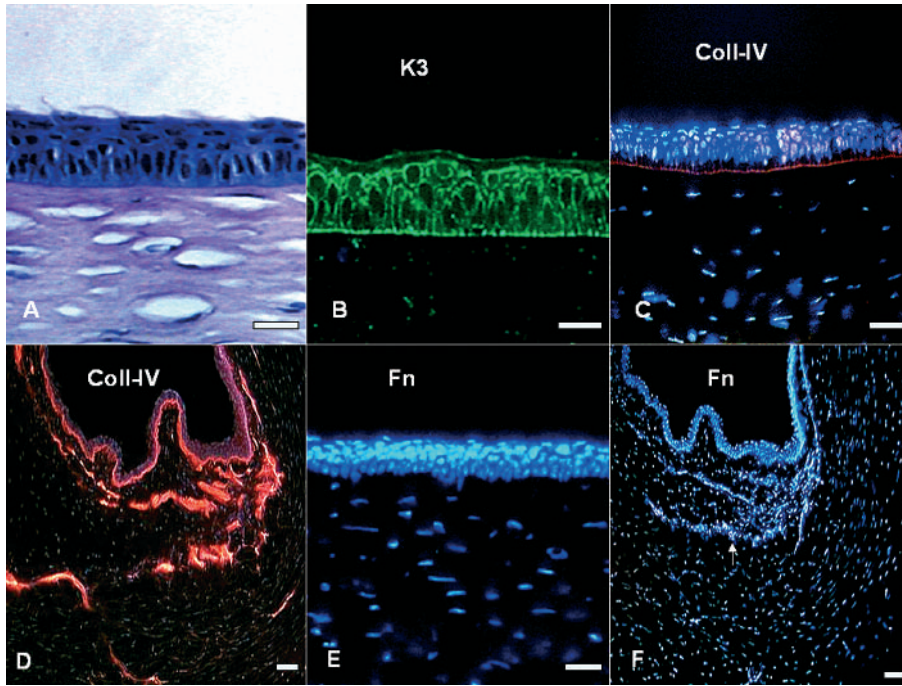


Figure 1 Normal control cornea. Haematoxylin staining (A). Keratin 3 (K3) is expressed in the full thickness of the corneal epithelium (B). Collagen IV staining in the basement membrane of corneal epithelium was weak and thin (C) while thicker and wavy in the conjunctival region and the underlying stromal blood vessels (D). Fibronectin (Fn) staining was negative in the entire cornea (E) and conjunctiva (F) except for the lumen of blood vessels (arrow). Bar 50 μ m.

Failure following AMT alone

The two corneas that failed after AM transplantation alone also showed diffuse vascularisation and stromal haze (for an example see Fig 5A). They were covered by a conjunctival epithelium containing goblet cells with MUC5AC positive goblet cells (not shown). The underlying stroma showed loss of lamellar structure and was infiltrated with blood vessels. Histological sections showed that AM remnants could be found in one cornea (Fig 5A, B, and D, demarcated by brackets). Strong immunostaining to Coll-IV was found in the subepithelial basement membrane, the subepithelial region corresponding to AM remnant, and superficial stroma subjacent to or near AM (Fig 5C and D, marked by arrows). Immunostaining to Fn was negative in AM remnant, but was

positive in the epithelial basement membrane, superficial stroma, and subjacent blood vessels of the area near AM remnant (Fig 5E, marked by asterisk). Immunostaining to α -SMA was negative in the entire stroma including infiltrating blood vessels (Fig 5F).

DISCUSSION

The ex vivo expansion of limbal stem cells is a new technique that may help restore corneal transparency in patients with total limbal SC deficiency. This report together with our earlier one⁶ clearly illustrates its potential efficacy in restoring the normal corneal phenotype and promoting stromal wound healing and remodelling, leading to transparency in a rabbit model with total limbal SC deficiency.

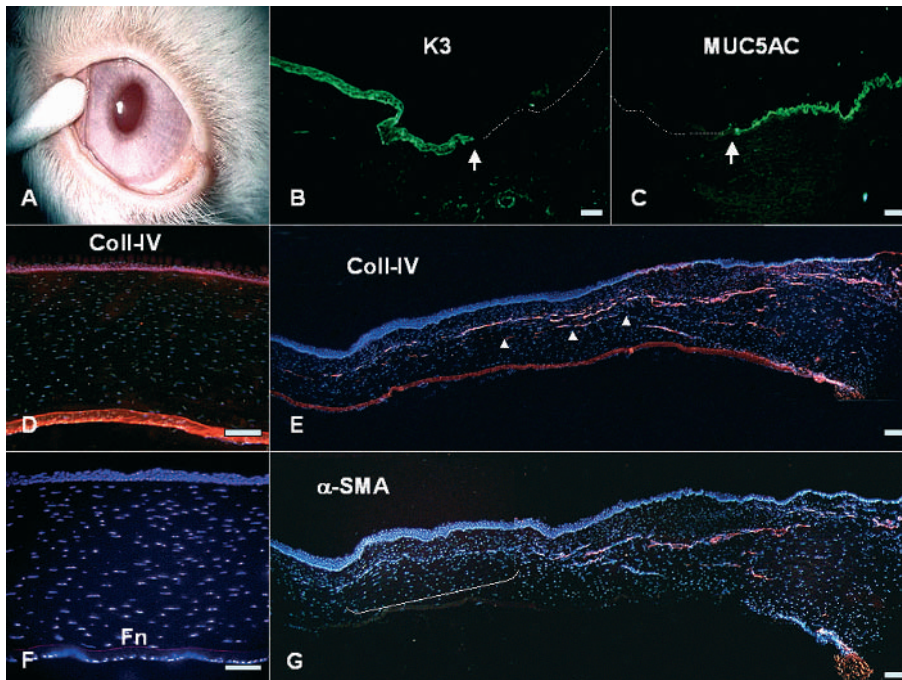


Figure 2 Cornea with success. External appearance shows that the reconstructed surface is smooth and clear without vascularisation (A). Keratin 3 (K3) is expressed in the reconstructed corneal epithelium (B, arrow marks the limbus). MUC5AC positive goblet cells are limited to the conjunctival region (C, arrow marks the limbus). Coll-IV is expressed in the corneal basement membrane and Descemet's membrane but not in the stroma (D). Coll-IV is also expressed in the stroma of peripheral cornea and limbal region (E). Immunoreactivity against fibronectin (Fn) was negative (F). α -SMA was expressed in the smooth muscle of blood vessels of the limbal and conjunctival stroma (G). Bar 50 μ m.

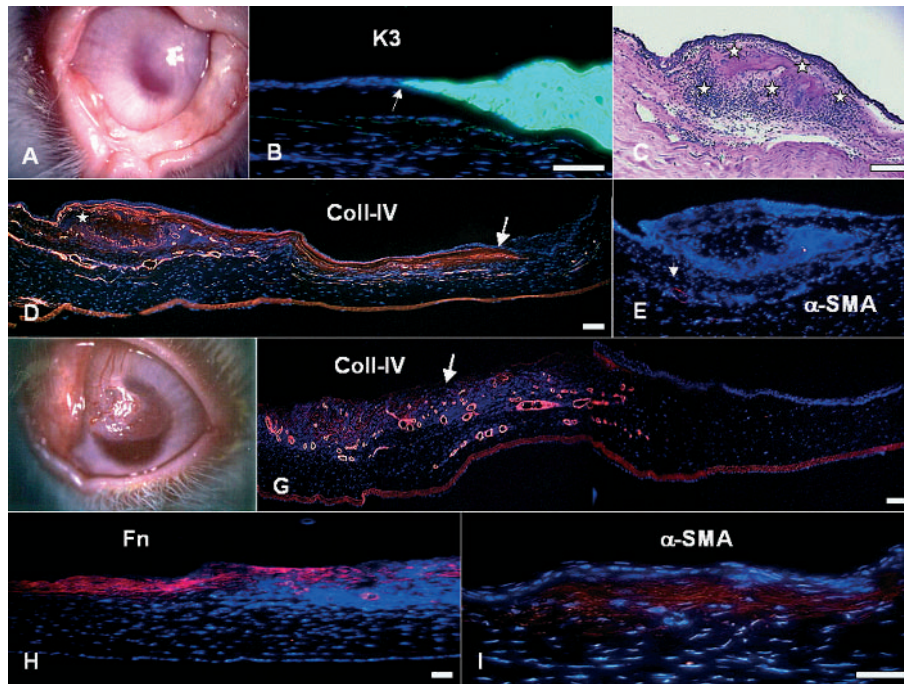


Figure 3 Corneas with partial success. Slit lamp appearance shows that part of these corneas is smooth and clear and the other is vascularised and opaque (A, F). Keratin 3 (K3) is expressed only in the smooth and clear area (arrow marks the border between corneal and conjunctival phenotypes) (B). Haematoxylin and eosin staining shows AM remnant (marked by asterisks) (C). Coll-IV is expressed in the corneal basement membrane, AM remnants and the superficial corneal stroma (D). α -SMA was only expressed in the smooth muscles of blood vessels in the limbal stroma (arrowhead marks a blood vessel) (E). Coll-IV is expressed in superficial stroma and intrastromal blood vessels (G). Fibronectin (Fn) was highly expressed in the stroma and blood vessels of granuloma (H). α -SMA expression suggests the presence of myofibroblasts in the granuloma (I). Bar 50 μ m.

After a long term follow up of 11.8 (0.4) months, we have confirmed that a normal stromal architecture with the lack of Coll-IV and Fn deposition, identical to that of normal corneas, was restored in those corneas with successful restoration of a corneal epithelial phenotype. Similarly, a normal stromal architecture was also restored in the area with a corneal epithelium but lost in the area with conjunctival epithelium of those corneas with partial success. In the latter area, the superficial stroma was deposited with Coll-IV and Fn. Deposition of Coll-IV was also found in rabbit corneal stroma injured by alkali burns or laceration, was associated with activated stromal fibroblasts, and was thought to be the reason for forming opaque granulation tissue.⁷ In the early stage of wound healing, fibronectin

exuded from the plasma or tears and secreted by activated fibroblasts is deposited in the corneal stroma as a provisional matrix. Fn is completely reabsorbed when the wound healing process is completed.¹⁸ We thus surmise that wound remodelling remained incomplete in corneas with partial success. To our surprise, we did not observe deposition of Coll-IV or Fn in the corneal stroma of the failure group no matter whether the failure resulted from ex vivo expansion or not. Therefore, we conclude that continuous wound remodelling is not associated with the presence of a conjunctival epithelium.

That was the reason why we also looked into the presence of myofibroblasts, which characteristically express contractile α -SMA in cytoskeletons and integrin α 5 β 1 on cytoplasmic

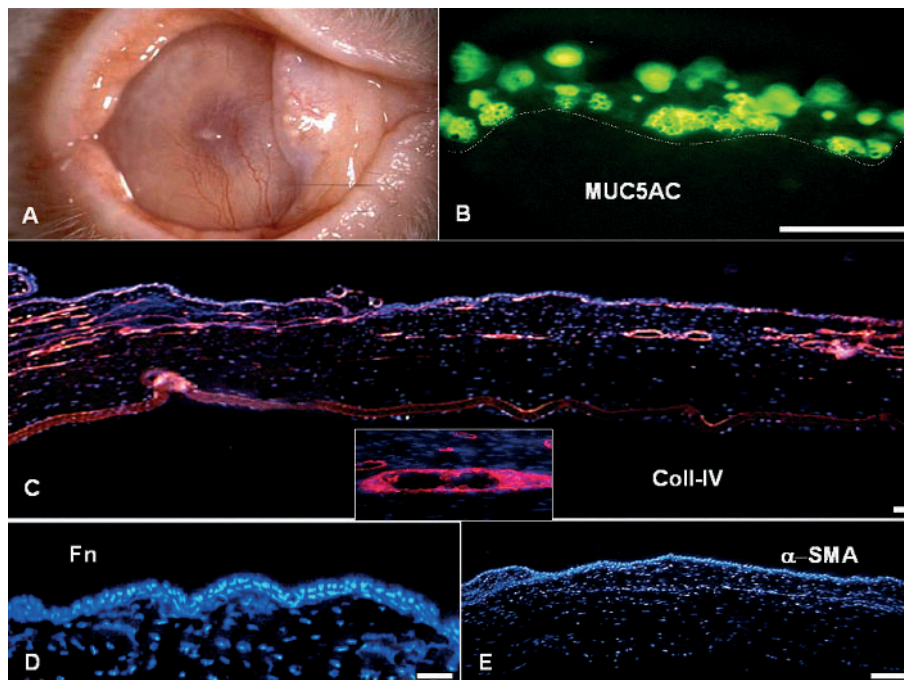


Figure 4 Cornea with failure following ex vivo expansion. Slit lamp appearance shows corneal haze, vascularisation, and inflammation (A). MUC5AC staining of the goblet cells confirms conjunctival invasion of the corneal surface (B). Coll-IV is expressed in the corneal basement membrane and intrastromal blood vessels. Inset shows the higher magnification of blood vessel wall (C). Fibronectin (Fn) and α -SMA were expressed only in stromal blood vessels (D and E, respectively). Bar 50 μ m.

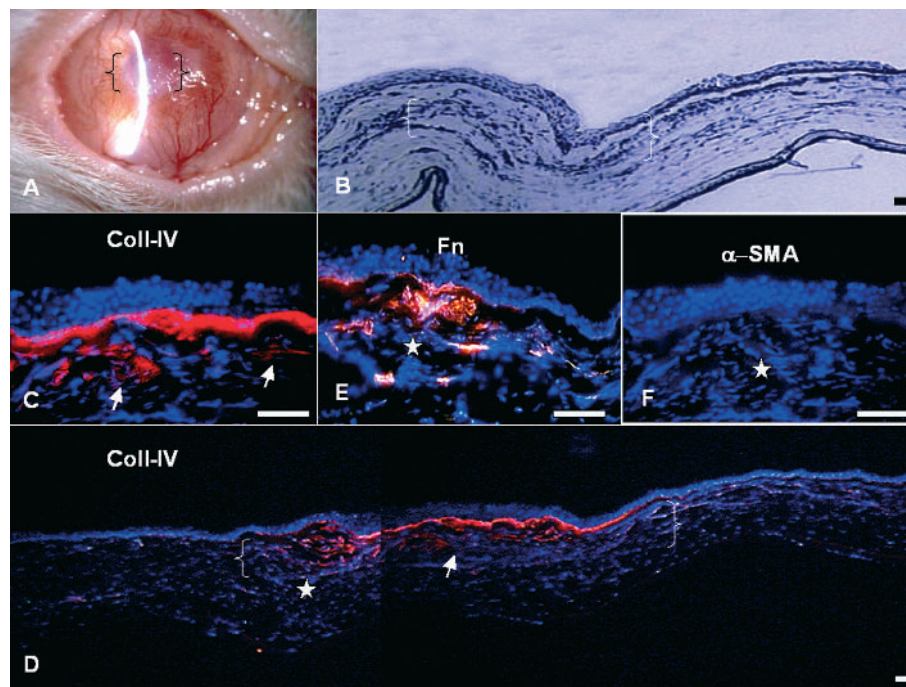


Figure 5 Cornea with failure following AM transplantation alone. Slit lamp appearance shows corneal haze and vascularisation. AM remnants are seen in the stroma (enclosed by brackets) (A). Haematoxylin stain shows the presence of AM remnants in the cornea (enclosed by brackets) (B). Coll-IV is highly expressed in the corneal basement membrane and stroma (marked by arrows) (C). Coll-IV is also found in AM remnants (enclosed by brackets) (D). (Fibronectin (Fn) is also found in the corneal stroma with AM remnants (E). α -SMA is not expressed in this area (F). Bar 50 μ m.

membranes, and deposit EDA domain containing cellular fibronectin in the pericellular matrix, and collagens and proteoglycans in the stromal matrix (for reviews see Jester *et al*¹⁰). In this report, we noted that expression of α -SMA was absent in the stromal fibroblasts in corneas with successful reconstruction, similar to that of normal corneas. In contrast, α -SMA positive myofibroblasts were found in the same area deposited with Coll-IV and Fn in corneas with partial success. This result supports the notion that complete remodelling is associated with the absence of myofibroblasts, and that incomplete remodelling leads to the persistence of myofibroblasts. Again, we did not detect any myofibroblasts in failure corneas, further confirming that corneal stromal opacity in failure corneas was not the result of myofibroblasts. Besides the reason that a conjunctival epithelium covering the cornea is not optically as sound as the corneal epithelium, the opacity in failure corneas might also be caused by intense infiltration of blood vessels. These blood vessels contained Coll-IV (in the basement membrane) but not α -SMA (which is also found in smooth muscle cells), indicating that they were capillaries (Figs 3–5). The other contributing factor to stromal opacity might be infiltration of chronic inflammatory cells.

It is intriguing to note that expression of α -SMA in corneas with partial success was aborted in the area with AM remnant (Fig 3). This finding suggests that formation of myofibroblasts is prohibited by AM, a finding consistent with our previously reports showing that myofibroblasts are inhibited by AM in vivo and in vitro¹⁹ and that TGF β (tumour necrosis factor β) signalling and myofibroblast differentiation are downregulated by AM stroma.^{20–21} Taken together, we believe investigations into the mechanism whereby corneal stromal transparency can be achieved by restoration of a corneal epithelial phenotype and complete remodelling of AM will unveil additional applications in the future.

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Proprietary interest: SCGT and his family are more than 5% shareholders of TissueTech, Inc, which owns US Patent Nos 6,152,142 and 6,326,019 on the method of preparation and clinical uses of human amniotic membrane, distributed by Bio-Tissue, Inc.

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