Successful Clinical Implementation of Corneal Epithelial Stem Cell Therapy for Treatment of Unilateral Limbal Stem Cell Deficiency

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ABSTRACT

The corneal epithelium is maintained by a population of stem cells known as limbal stem cells [LSCs] due to their location in the basal layer of the outer border of the cornea known as the limbus. Treatment of limbal stem cell deficiency [LSCD] has been achieved with transplantation of ex vivo expanded LSCs taken from a small biopsy of limbus. This is a relatively new technique and as such, specific national or international guidance has yet to be established. Due to the lack of such specific guidance, our group has sought to minimise any risk to the patient by adopting certain modifications to the research methodologies in use at present. These include the replacement of all non-human animal products from the culture system and the production of all reagents and cultures under Good Manufacturing Practice [GMP] conditions. In addition, for the first time, a strictly defined uniform group of patients with total unilateral LSCD and no other significant ocular conditions has been used to allow the success or failure of treating LSCD to be attributable directly to the proposed stem cell therapy.

A prospectively designed study with strict inclusion and exclusion criteria was used to enrol patients from our database of patients with unilateral LSCD. Eight eyes of 8 consecutive patients with unilateral total LSCD treated with ex vivo expanded on human amniotic membrane (HAM) autologous LSC transplant with a mean follow up of 19 (RANGE) months were included in the study. Postoperatively, satisfactory ocular surface reconstruction with a stable corneal epithelium was obtained in all eyes [100%]. At last examination, best corrected visual acuity improved in 5 eyes and remained unchanged in 3 eyes. Vision impairment and pain scores improved in all patients [p<0.05]. This study demonstrates that transplantation of autologous limbal epithelial stem cells cultured on HAM without the use of non-human animal cells or products is a safe and effective method of reconstructing the corneal surface and restoring useful vision in patients with unilateral total LSCD.

INTRODUCTION

The cornea is the clear dome-shaped window at the front of the eye and its clarity and regular surface is vital for the transmission and focussing of light onto the retina allowing accurate visual perception. Corneal disease represents the second most common cause of world blindness after cataract [1]. The surface of the cornea is made up of an epithelium which is continuous with that of the surrounding conjunctiva. The transition between the corneal and conjunctival epithelia is formed by the limbal epithelium. There is now a substantial body of evidence, both scientific and clinical, pointing to the basal layer of the limbus epithelium as the location for putative corneal epithelial stem cells,
CESCs, also known as limbal stem cells, LSCs [2,3] although recent finding suggest a diffuse distribution of LSCs on the ocular surface in certain mammals [4]. The limbal epithelium acts as a reservoir for the replacement of corneal epithelial cells that are normally continually lost from the corneal surface into the tear film. In addition, the limbal epithelium is thought to exert a “barrier” function in preventing the migration of conjunctival epithelium and its blood vessels onto the surface of the cornea. Upon significant injury to the limbal epithelium and the LSCs contained therein, the corneal epithelium cannot renew itself and conjunctival epithelium can encroach onto the corneal surface, a process called “conjunctivalisation”. This results in persistent epithelial defects and neovascularisation of the cornea; chronic inflammation; scarring and loss of vision. This is known clinically as limbal stem cell deficiency [LSCD]. Corneal vascularisation and opacity have been estimated to cause blindness in 8 million people [10% of total blindness] worldwide each year [1] and various forms of LSCD contribute to this total. A large number of ocular surface diseases, both acquired and congenital, share features of partial or complete LSCD [2]. This means that the diagnosis of LSCD must be considered in all cases of significant corneal epithelial disease and vascularisation as only the replacement of the LSC population will be effective in treating these conditions.

The management of LSCD depends on whether the patient has partial or total LSCD. In partial LSCD, there is still limited presence of functioning LSCs and when the visual axis is covered with normal corneal epithelium and the patient is relatively asymptomatic with good vision, conservative [medical] management is indicated [3]. However, in partial LSCD, where there is central corneal involvement with decreased vision, significant irritation and persistent epithelial defect, surgical management including mechanical debridement of conjunctival epithelium from the corneal surface and/or amniotic membrane transplantation may be indicated [5]. However, in total LSCD, where there is no evidence of functioning LSCs, the only treatment is surgical and involves a stem cell therapy that allows the replacement of the damaged or absent LSC population.

In the past, central corneal transplants were used with limited long-term success to treat extensive LSCD [6-8]. The main reason for failure is the inability to maintain a healthy corneal epithelium, which relies solely upon a healthy recipient limbus. In total LSCD, recipient limbal epithelium has to be restored in the first instance, so that a healthy corneal epithelium can be maintained. This can only be achieved by the transplantation of healthy limbal epithelium, using either large whole tissue limbal epithelial grafts [9-13] or, more recently, ex vivo expanded limbal epithelial graft from small biopsies of limbal epithelium which can be taken from patients’ other healthy eye [in the case of unilateral LSCD: autograft] or healthy eye of living related or cadaveric donors [in the case of bilateral LSCD: allograft [14]. The requirement of much smaller amounts of tissue for the ex vivo expansion process means that patients with unilateral LSCD has two main advantages. Firstly, the small amounts of tissue required for ex vivo expansion are much less likely to damage the LSC population of the healthy donor eye than previously used autologous whole tissue grafts that required large quantities of limbal tissue for direct transplantation. Secondly, the small biopsies needed can be taken from the fellow eye of the patient with unilateral LSCD, or even if the disease is bilateral to an extent, providing there are remaining areas of healthy limbus in one of the eyes. This means the tissue is autologous and eliminates the requirement of systemic immune suppression compared to previously used whole tissue allografts. More recently, ex vivo expansion of oral mucosa epithelium has been used successfully to transplant onto the ocular surface of rabbits and subsequently in humans with total bilateral LSCD which will potentially provide treatment for autologous stem cell therapies for patients with total bilateral LSCD [15-18].

The ex vivo expansion of limbal epithelium prior to transplantation is a relatively new technique and as such, specific national or
international guidance has yet to be established although regulatory bodies encourage strategies that minimise any risk to the patient [19]. Due to the lack of such specific guidance, our group has sought to minimise any risk to the patient by adopting certain modifications to the research methodologies in use at present. These include firstly, the replacement of all non-human animal products from the culture system and secondly, the production of all reagents and cultures under Good Manufacturing Practice [GMP] conditions. These modifications have been used to produce ex vivo expanded limbal epithelium to then treat a uniform group of patients whom all have total unilateral LSCD and no other significant ocular conditions. This meant that all treatments were autologous, requiring no immunosuppression, thus any success or failure of the stem cell therapy could be attributed directly to our intervention and not due to the effects of concomitant immunosuppression or ocular co-morbidity.

Up to now, the initial growth of limbal epithelial cells in culture required the concomitant use of non-human animal cells and products including a mouse 3T3 fibroblast feeder layer for co-culture and foetal calf serum (FCS) in the growth medium and this poses two problems: Firstly, since such a transplant would be a potential xenograft, the patient may require immunosuppression to prevent rejection of the tissue. Secondly, and more importantly, the use of non-human animal products in tissue destined for human transplantation has the potential to produce inter-species pathogen transfer [20,21]. This latter risk would be augmented further on a background of immunosuppression. The successful culture of LSCs has been established on extracellular matrix components including collagen IV coated shields [22], laminin and fibronectin [23], human limbal fibroblasts [24] and human amniotic membrane (HAM) [25-30]. In addition, defined serum free media has been successfully used for expansion of LSCs, although the limbal cell proliferation was reduced in comparison to serum containing media [31]. Instead, human autologous serum (HAS) has been successfully used to replace the need for FCS in epithelial growth medium for the culture of a variety of epithelial cell types including skin [32], oral mucosa [32] and cornea [34].

In this study we report for the first time successful ex vivo expansion of limbal epithelial cells using a completely non-human animal product free system that combines usage of HAM as a matrix and HAS as replacement for FCS in culture medium. Such a system of ex vivo expansion was fully validated and refined over a three year period in the laboratory prior to implementation in our first patients. The stem cell therapy was fully studied and approved by all the relevant ethical and regulatory bodies in the UK prior to human translation. The ex vivo expanded autologous limbal epithelium was then transplanted into 8 patients with unilateral total LSCD. Successful treatment of LSCD was proven for each patient in this study both from reversal of the clinical signs and also by the conversion of impression cytology specimens of the central cornea from a conjunctival phenotype preoperatively to an exclusive corneal phenotype postoperatively.

### MATERIALS AND METHODS

#### Ethics and Regulatory Statements

The ex vivo expansion of autologous human limbal epithelium for the treatment of LSCD was approved by the Human Tissue Authority [HTA], Local Research Ethics Committee [LREC] and Newcastle upon Tyne Hospitals NHS Foundation Trust’s Research and Development Department and New Procedures Committee. For more detail, see Supplementary Information Annex 1.

#### Patient demographics

8 patients [that fulfilled all the inclusion and exclusion criteria; Supplementary Information Annex 1] were included in this study. Detailed patient demographics are shown in Supplementary Information Table 1. Seven of the patients were male and one female. The mean age was 43 [range 16 - 73]. Mean follow up was 19 months [range 12 - 30]. All patients had total unilateral LSCD confirmed by both clinical examination and impression cytology with cytokeratin profiling. Consent was obtained from patient 1 to provide clinical
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Study protocol
The key steps used for *ex vivo* expansion of limbal stem cells and subsequent transplantation are outlined in Supplementary Information Figure 1. In summary, following diagnosis of LSCD, the technique involved taking a small limbal biopsy from the contralateral healthy eye of a patient with unilateral LSCD and expanding the LSC population *ex vivo* and then transplanting this enlarged population into the surgically prepared diseased fellow eye.

Impression cytology
All patients underwent objective assessment of corneal epithelium in both eyes by impression cytology. This was performed to confirm the diagnosis of total LSCD in the affected eye and to confirm *absence* of any features of LSCD in the fellow healthy eye. For more information on this technique please refer to Supplementary Information Figure 2 and Supplementary Information Annex 1.

Manufacture of human autologous serum [HAS]
Following a negative infectious disease screen for hepatitis B and C, HIV, HTLV and syphilis, the patient was eligible to proceed to have blood taken for HAS preparation/production. Although no universally accepted system of HAS production exists, the technique we used is based on the optimised protocol [36]. HAS produced with this protocol has been used and validated in our preceding laboratory studies and shown to support limbal epithelium expansion while maintaining LSC morphology, colony forming efficiency [CFE] and expression of putative LSC markers [data not shown]. All HAS production was carried out within the GMP Stem Cell Laboratory using the protocol outlined in Supplementary Information Figure 3.

Plating of human amniotic membrane
HAM for clinical transplantation was obtained from the NHSBT Tissue Services (London & Liverpool, UK). For plating of explants on HAM, please refer to Supplementary Information Annex 1.

*Ex vivo expansion protocol of LSCs on HAM*
Limbal biopsy was performed as shown in Supplementary Information Annex 2 and Supplementary Information Figures 3 and 4. In brief, the epithelial medium from the previously plated HAM was removed from the culture well. The limbal biopsy was placed on the centre of the plated HAM and gently pressed downward to promote the attachment and 1.5 ml of epithelial medium was gently added to the culture well very slowly. The culture was examined and subsequently fed with 2ml of epithelial medium on alternate days up to 12-14 days [Supplementary Information Figure 5]. The area of explant outgrowth was marked on the underside of the culture well at the time of each feed to allow subsequent measurement of growth rate [Supplementary Information Figure 6]. On the 7-10th day of culture, 1 ml of the culture medium was sent for microbiological analysis. Early explant outgrowth was observed between days 2 and 3; full coverage of the extent of the HAM was usually reached between 12 and 14 days. For control purposes, *ex vivo* expansion of LSCs using 3T3 feeder cells and foetal calf serum as described in [37] was carried out simultaneously in the initial experiments [data not shown].

Transplantation protocol
Transplantation of the *ex vivo* expanded limbal tissue on to HAM took place between 12 and 14 days following the initial limbal biopsy. Full informed consent was obtained from all patients prior to surgical intervention. For full details of protocol refer to Supplementary Information Annex 3 and Supplementary Information Figure 7.

Follow up protocol
Post-operatively, patients were treated with a combination of preservative-free 0.5% chloramphenicol antibiotic eye drops [Minims, Chauvin, UK], 1% prednisolone acetate steroid eye drops [Moorfields Eye Hospital, London] and autologous serum [produced from the patient's own blood by NHSBT, UK]. The
Successful laboratory ex vivo expansion of limbal epithelial cells using a combination of HAM and HAS

Human limbal epithelial cells were successfully cultured on HAM as both explant [Figure 1A and B] and single cell suspension cultures [Figures 1C and D] in fetal calf serum [FCS] containing media. The outgrowth areas were measured at weekly intervals and this indicated that both the explant [Figure 1E; p<0.0005; n=3] and suspension cultures showed successful increase in growth [data not shown]. Comparison between the two culture types indicated that the limbal explant culture epithelial outgrowth covered the amniotic membrane much earlier than the suspension cultures [Figure 1F; p<0.005; n=3]. We investigated whether the ex vivo single cell expansion of limbal epithelial cultures could be achieved on 3T3 feeders using medium supplemented with HAS as a replacement for FCS. Morphological observations showed that the HAS cultures were identical to co-cultures using FCS [Figure 2A]. Cell counts performed on both the FCS and HAS containing primary cultures showed that those with HAS had significantly higher cell counts [Figure 2B; p<0.05; n=3]. However, there was no statistically significant difference [n=3] between the colony forming efficiency (CFE) of the limbal epithelial cells from cultures established using either FCS or HAS [Figure 2C] or p63 expression [Figure 2D].

For clinical applications, we combined the explant culture method on HAM with medium supplemented with HAS. Morphological observations, flow cytometry analysis for expression of p63 and CFE assays indicated that there were no statistically significant differences between the two culture methods [data not shown]. A comparison of the outgrowth areas from explant on HAM using FCS and HAS [Figure 2E] showed that the HAS containing cultures had significantly larger outgrowths [p<0.005; n=3]. This was
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corroborated by higher cell counts in the HAS
ex vivo expanded cultures [data not shown].

Prior to carrying out cultures of limbal explants for patients with total unilateral LSCD, a series of cultures using cadaveric human limbal explants were first established in the GMP Stem Cell Laboratory. The three trial cultures of 1.5 mm by 1.5 mm cadaveric human limbal explants on HAM using human serum containing epithelial medium grew significantly better under GMP conditions than in the standard tissue culture laboratory [Supplementary Information Figure 8; p<0.00005; n=3].

Successful transplantation of ex vivo expanded limbal epithelial cells into patient with LSCD: first case study
The first transplantation of autologous ex vivo expanded limbal epithelial cells under GMP conditions was performed on a 45 year old Caucasian male who had developed all the clinical features of total LSCD (following a severe chemical injury to the eye) including: [i] significant epithelial defect; [ii] significant peripheral and central corneal vascularisation; [iii] marked corneal opacity [Grade 4-no iris details visible] and [iv] total loss of Palisades of Vogt [Figures 3A and B; for a full clinical history see Supplementary Information Annex 4]. The right eye examination was entirely normal [Figure 3C]. The patient’s subjective pain score was 5/10 and the visual impairment score was 7/10 with regards to the left eye [using the scale provided in Supplementary Information Figure 9] and 0/10 in either case for the normal right eye. Impression cytology of both central corneas confirmed left total LSCD and normal right corneal epithelium [Figures 3D and E].

Right limbal biopsy was obtained from the patient as previously described in Supplementary Information Annex 2. A sheet of ex vivo expanded epithelium was established at 14 days after initial biopsy. On day 14, the patient underwent total superficial keratectomy to remove all the abnormal corneal epithelium from his left LSCD eye. Histological and immunohistochemistry analysis confirmed that the removed tissue was highly vascular, irregularly arranged with multiple goblet cells and with strong expression of CK19 and lack of CK3 expression confirming complete absence of a corneal epithelium phenotype [Figure 4]. The ex vivo expanded limbal epithelium sheet on HAM was secured in place (stromal side down) with four 10-0 nylon sutures. Also, the explant was positioned in the limbus at 12 o’clock to allow the highest concentration of LSCs to be placed in the natural protective niche environment [38]. This was followed by placement of a second HAM on top of the first HAM containing the LSC explant (stromal side down). At the time of surgery, excess cultured limbal epithelium on HAM was excised and divided into two halves: one for histology and immunohistochemical analysis and the second for ultrastructural analysis of the cultured epithelium by transmission electron microscopy [TEM]. This analysis confirmed that a normal limbal epithelium is formed on the HAM [Figure 5A]. This epithelium is 2-3 cells layers thick although thicker at the edges of the culture specimen. The basal layer of cells adjacent to the HAM shows characteristics indicating an actively expanding LSC phenotype including: [i] small cuboidal cells with undifferentiated appearance; [ii] high expression of the putative LSC markers p63, ABCG2 & vimentin; [iii] low expression of the CK3 differentiation marker and [iv] high expression of the cell proliferation marker Ki67. TEM confirmed the light microscopy findings demonstrating the formation of a primitive epithelium with a prominent basal layer of cuboidal cells with a high nucleus: cytoplasm ratio [Figure 5B]. The basal cells are attached to their basement membrane via hemidesmosomes and to each other via desmosomes. The superficial squamous cells which are away from the HAM showed early differentiation demonstrating primitive microplicae formation in keeping with a corneal epithelial phenotype [Figure 5B].

Following the stem cell transplantation procedure, the patient was treated topically with eye drops as outlined in Supplementary Information Annex 3. No systemic immunosuppression was needed since all transplanted tissue was autologous in nature.
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Within 1 week of surgery, the patient’s left eye was significantly less red and more comfortable [Supplementary Information Figure 10]. Initially, there was mild peripheral vascularisation of the superficial HAM but this HAM melted within the first four weeks to reveal a healthy avascular layer of transplanted ex vivo expanded tissue which formed a stable epithelium with no epithelial defect. This was associated with an absence of ocular surface inflammation and a comfortable eye over the next 12 months with no side effects. We then elected to proceed to central full thickness corneal graft to replace the previously scarred stroma. Such a graft would be expected to succeed since it would now be reepithelialised by healthy host limbal epithelium. The patient therefore underwent a left HLA-matched penetrating keratoplasty 1 year after the stem cell graft [data not shown]. The removal of the central corneal button gave us a unique opportunity to perform detailed analysis of the corneal epithelium which must have been generated from the previously transplanted autologous LSCs as our clinical data had shown a complete absence of normal corneal epithelium on the ocular surface for over 10 years. The histology and immunohistochemical analysis showed that a multilayered stratified non keratinising epithelium was formed on a basement membrane [Supplementary Information Figure 11]. PAS staining revealed the complete absence of glycogen containing goblet cells found in the previously conjunctivalised corneal surface. The p63 staining demonstrated the presence of proliferating LSC-like cells in the basal epithelium which were not present in the most superficial layers. In addition, all the cells of the corneal epithelium now expressed the corneal specific marker, CK3. TEM revealed that the ultrastructure of the generated epithelium shows all the features of a normal central corneal epithelium [Supplementary Information Figure 12]. It shows in detail a uniform stratified epithelium. The basal layer of cells are columnar and rest on a basement membrane and are attached to it by means of hemidesmosomes. The epithelial cells are attached to one another by desmosomes. The most superficial layer of squamous cells is differentiated to show multiple microplicae.

The long term follow up data of all these outcome measures is shown in Figures 6A and 6B. In summary, we can conclude for the first case study that transplantation of ex vivo expanded autologous limbal epithelium using an explant technique on intact HAM cultured with an non-human animal cell free, GMP compliant system, leads to successful and long term [at least 2 years] reversal of LSCD. This is shown by the successful objective outcome measures including normal impression cytology [CK19 negative] and absence of epithelial defect following transplantation. In addition there is improvement of Snellen’s visual acuity, reversal of corneal vascularisation and decreased corneal opacity. ADD significant decrease in pain and visual impairment scores.

Summary of clinical results from transplantation of ex vivo expanded limbal epithelial cells in eight patients with unilateral total LSCD

Eight patients, including the above first case, [that fulfilled all the inclusion and exclusion criteria; Supplementary Information Annex 1] were included in this study [for patient demographics refer to Supplementary Information Table 1]. All patients underwent limbal biopsy of their healthy fellow eye using our standard biopsy protocol. Excellent outgrowths were obtained for each patient with initial outgrowths noted by day 2-3. The growth of the explant culture was marked at each feed day and the results of the growth are shown in Supplementary Information Figure 13. The patients have been followed up for an average of 19 months following LSC treatment [Range 12 -30 months]. The primary subjective measure was reduction in ocular discomfort. Improvement in patient’s vision was a secondary subjective outcome measure as the majority of patients had significant corneal stromal scarring, secondary to the original disease and recurrent inflammation caused by LSCD. The subjective outcomes are shown in Figures 7A and 7B. These results clearly show that in all cases, there has been a significant improvement in both subjective outcomes. The
pain scores reduced from 7.25 [SD=1.28] pre-treatment to 0.75 [SD=0.89] at the end of the follow up period which is highly significant [p<0.0001, Student t test]. The visual impairment scores were also reduced from 7.63 [SD=1.30] pre-treatment to 3.00 [SD=2.45] which was also highly significant [p=0.00033, Student t test].

A summary of primary and secondary objective outcome measures is also shown in Figure 7C. From this it is clear that 100% of patients had successful reversal of their LSCD as shown by change of their central corneal epithelium impression cytology from a conjunctival phenotype pre-operatively to a corneal phenotype post-operatively as well as successful re- epithelialisation following transplantation of ex vivo expanded LSC with no significant epithelial defects in any patient at the end of the study period. There was also a significant improvement in secondary objective measures such as Snellen’s visual acuity [Figure 7D], reversal of central corneal vascularisation and reduction of corneal capacity.

By the end of the follow up period, satisfactory ocular surface reconstruction was obtained in all eyes [100%], as confirmed by clinical examination and impression cytology. However, three of the eight eyes developed localised conjunctival invasion of the cornea (less than 3 clock hours) within the first 6-12 months. All three patients underwent sector epitheliectomy where the tongue of conjunctival in-growth was scraped off the corneal periphery combined with HAM transplant as a biological bandage (stroma side down) allowing surrounding LSCs to reform the limbal barrier. This was successful in reversing the localised conjunctivalisation without the need for additional LSC transplant.

**DISCUSSION**

The first use of ex vivo expanded LSCs for the treatment of LSCD in human subjects was described by Pellegrini and co-workers who used a culture system of LSCs grown on mouse J2-3T3 fibroblasts with FCS supplemented media [14]. Since then, several studies have been reported on the transplantation of ex vivo cultured LSCs for the treatment of LSCD [14, 25-28, 39-51]. It is difficult to draw conclusions from existing studies because of the lack of standardisation in terms of patient selection [such as total and partial LSCD used in the same study], cause of LSCD (acquired and congenital), unilateral and bilateral cases, source of initial tissue [allo- and auto-graft transplants in the same study], methods of ex vivo expansion [explant or single cell; HAM or 3T3 fibroblast co culture or both], the surgical management [method of superficial keratectomy, the use of a second HAM as a bandage, contact lens protection or both] and post operative management [use of HAS or not]. These factors represent a major deficiency in this field of LSC therapy and also a major obstacle to interpret the results. We have aimed to improve this by describing, a novel, fully validated non-human animal cell free system under GMP conditions with well defined inclusion and exclusion criteria and specific subjective and objective outcome measures.

Our system of ex vivo expansion uses a very specific and well defined technique of intact HAM stretched onto glass coverslips together with an explant technique. The culture medium used was modified epithelial growth medium where the FCS was replaced by HAS. This combination has been used successfully over the last few years in our laboratory studies where we have shown that LSCs can grow from the limbal explant onto the HAM [38]. The GMP culture process used shows categorically that a system of ex vivo expansion of very small amounts of limbal tissue can be used to produce large sheets of cells which can successfully regenerate the corneal epithelium in patients with total LSCD. Analysis of the expanded sheets at the time of surgery shows that they contain an epithelium with a basal layer of cells which have a LSC like morphologic appearance and express a signature of putative LSC markers. These findings are in agreement with our previous detailed laboratory analysis of ex vivo expanded cultures [52, 38, 2] using explant cultures on intact HAM.
To ensure the subsequent success of transplanted cells, the host environment must be made conducive to cell survival. Thus, we took a great deal of effort to remove all the abnormal conjunctival epithelium on the diseased eye and minimise bleeding. We also placed an additional HAM on top of the cell culture which we feel not only would protect the transplanted cells from physical trauma but may produce additional LSC niche cues that prolong the life span and maintenance of clonogenicity of epithelial progenitor cells [29] while inhibiting inflammation [52] and vascularisation [52] and promoting corneal epithelialisation [53]. At the time of surgery, the abnormal conjunctival epithelium was removed and subsequently analysed both histologically and with immunohistochemistry to provide tissue diagnostic proof that no normal corneal epithelium existed in our patients pre-operatively.

Post-operatively, in addition to topical steroid and antibiotics, we also treated all patients with up to a one year course of autologous serum which has been shown to have significant epitheliotropic effects [35] and be a useful adjunct to ocular surface reconstruction [55]. Close post-operative follow up of the patient is vital, particularly in the first 12 months. Rapid and stable epithelialisation took place in all our patients so that there were no epithelial defects within a few days post transplantation. If there was a localised area of active peripheral vascularisation representing an area of incoming conjunctival epithelium through a defect in the limbal barrier [as was the case in 3 patients], we used the technique of sequential sector conjunctival epitheliectomy described by Dua et al [56] to excise the small incoming tongue of conjunctival epithelium and allow the transplanted LSCs to restore the limbal barrier [which proved successful in each case].

Successful surgical treatment of LSCD was proven for each patient in this study both from reversal of the clinical signs, in particular the re-surfacing of the corneal surface with a stable epithelium and also by the conversion of impression cytology specimens of the central cornea epithelium from a conjunctival phenotype pre-operatively to a corneal phenotype post-operatively. There was a marked improvement in subjective symptoms in all patients, many of whom had suffered years of persistent ocular pain and significant visual reduction. Interestingly, those patients who had a fairly recent diagnosis of total LSCD had marked improvement in objective visual acuity since the main issue of conjunctivalisation had been reversed. However, for those with a several year history of LCSD, the visual improvement was not as marked since persistent epithelial breakdown and inflammation combined with recurrent infection led to marked corneal stromal scarring. Although these patients had a stable epithelium after LSC transplantation, they would require a corneal transplant to restore vision.

In conclusion, we have succeeded in using an non-human animal product free GMP compliant autologous LSC ex vivo expansion technique to successfully reverse LSCD within a controlled population and showed 100% in predefined subjective and objective outcome measures. However, there still are few unanswered questions. Firstly, the initial patients transplanted using these techniques have only completed just over 2 years post operative follow up in this study and we are still unsure about very long term outcomes which are pending, although results so far are very encouraging. Although a healthy epithelium can be produced using this technique in an eye with no observable pre-existing LSCs, there are as yet no direct ways of accurately identifying LSCs in vitro or in vivo due to the absence of a specific marker. Although HAM appears to be a very effective means of culturing LSCs, it is however a biological substrate which is impossible to standardise in terms of its structure (e.g., thickness), physiological properties and handling and there is scope for finding reliable and consistent alternatives for a culture substrate. A previous study has used a fibrin support for transplantation of ex vivo cultured LSCs in combination with FCS and 3T3, although their success rate was slightly lower than ours (success was achieved in 14 out of 18 patients). It remains to be investigated whether a combination of the fibrin support with our
culture conditions will provide a fully standardised system with equal efficiency in treatment of patients with LSCD [57].

The role of the niche in maintaining the optimal environment for the protection and maintenance of LSCs is becoming better understood. In addition, in our patient group who have total LSCD, the putative LSC niche, the palisades of Vogt, have been severely damaged and yet the LSC therapy is successful in reconstituting a normal corneal epithelium. It may be that the new niche may be distributed throughout the corneal epithelium as suggested with other mammals [4]. We can only speculate on this point until we have developed techniques to directly identify LSCs in vivo. We envisage a time when better understanding of the LSC niche will allow us to use more specific substrates and culture media to increase the efficiency and longevity of future LSCD treatments.

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See www.StemCells.com for supporting information available online.
Figure 1. Human limbal explant and cell suspension cultures on human amniotic membrane. [A] Macroscopic picture of culture showing two inner red rings indicating previous day’s growths and the present outgrowth as the outer ring. [B] Phase contrast micrograph of the culture showing the explant in the upper right corner and the epithelial outgrowth on the amniotic membrane. Scale-bar = 200 µm. [C] Macroscopic picture of culture showing multiple limbal epithelial colonies highlighted by the black asterisks. [D] Phase contrast micrograph of the culture showing two limbal epithelial colonies as highlighted by the white arrows. The 200 µm scale-bar is shown. [E] Epithelial outgrowth areas from limbal explants on amniotic membrane with increasing time in culture. The number of days is shown on the x-axis and the epithelial outgrowth areas from the human limbal explants are shown on the y-axis. [F] The number of days for the cultured epithelial cells to cover the amniotic membrane in both limbal explant and cell suspension cultures. The type of culture [explant or cell suspension] on amniotic membrane is shown on the x-axis and the number of days to cover the entire amniotic membrane is shown on the y-axis [* indicates p<0.005; n=3].
Figure 1

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Figure 2. Comparison of HAS to FCS. [A] Phase contrast micrograph of human limbal epithelial cells co-cultured with 3T3 fibroblasts using human serum containing medium. Early epithelial colonies resembling holoclone like colonies can be seen highlighted by the white arrows. The 200 µm scale-bar is shown. [B] Total viable cell counts of human limbal epithelial cells co-cultured with 3T3 fibroblasts using foetal calf serum or human serum in the growth medium. The different culture conditions [foetal calf serum or human autologous serum] are shown on the x-axis and the total cell count on the y-axis [* indicates p<0.05; n=3]. [C] Colony forming efficiencies of human limbal epithelial cells co-cultured with 3T3 fibroblasts using foetal calf serum or human serum in the growth medium. The different culture conditions [foetal calf serum or human serum] are shown on the x-axis and the colony forming efficiency on the y-axis. [D] Colony forming efficiencies of human limbal epithelial cells co-cultured with 3T3 fibroblasts using foetal calf serum or human serum in the growth medium. The different culture conditions [foetal calf serum or human serum] are shown on the x-axis and the colony forming efficiency on the y-axis. [E] Explant outgrowths from limbal explants on human amniotic membrane using foetal calf serum and human serum. The outgrowth areas at weekly intervals were measured. The day of outgrowth is shown on the x-axis and the outgrowth area is shown on the y-axis.
Figure 2

A

B

C

D

E

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Figure 3. Pre-operative assessment of Patient 1. [A] Pre-operative clinical photograph of left eye with total LSCD showing complete corneal vascularisation and significant corneal opacification. [B] Fluorescein staining of the same eye observed with cobalt blue filter reveals an irregular epithelial surface with diffuse epithelial late staining. [C] The fellow right eye is unaffected with a normal corneal examination. [D] Normal corneal impression cytology with minimal cellularity with firm impression and no CK19 positive cells. [E] Impression cytology showing extensive cellularity and CK19 positive staining diagnostic of LSCD.
Successful stem cell therapy for unilateral LSCD

Figure 3

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Figure 4. Superficial keratectomy specimen at time of LSC transplantation surgery. [A] Clinical photograph of the left eye of patient 1 showing the removal of abnormal epithelium from the surface of left eye with total LSCD. [B] Low power histology of the excised central conjunctivalised corneal epithelium stained with H&E showing a very abnormal multilayered epithelium which contains multiple blood vessels [arrows] which are absent in normal cornea – Scale bar=500µm. [C] Higher power H & E stained excised epithelium showing in more detail a very thickened epithelium with irregular surface and loose sloughing epithelium with multiple goblet cells [arrows.] [D] PAS stained excised epithelium clearly demonstrating the glycogen containing goblet cells [arrows]. [E] Immunohistochemistry of the excised epithelium demonstrates the lack of the corneal specific marker cytokeratin K3 and [F] the heavy expression of the conjunctival marker, CK19. Scale bars for C-F = 100µm.
Successful stem cell therapy for unilateral LSCD

Figure 4

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**Figure 5.** Analysis of ex vivo expanded epithelium of patient 1. [A] Summary panel showing the light microscopic and immunohistochemistry appearances for ex vivo expanded epithelium of patient 1. H & E staining reveals an epithelium with a basal layer of tightly packed cuboidal cells which express high levels of p63, ABCG2, Vimentin & Ki67. Conversely, the basal layer is devoid of high CK3 expression. [B] TEM of cultured epithelium from patient 1. [a] The ex vivo cultured epithelium [EE] sits on the HAM. The basal cells are much smaller and cuboidal [red outline] with high N/C ratios [blue outline] compared with the large columnar cells of adult corneal epithelium. Areas of the expanded epithelium [shown by the dotted rectangles] were viewed at higher magnifications to reveal [b] the presence of microplicae [MP] on the superficial epithelial cells [c] the presence of desmosomes connecting the basal cells together and [d] hemidesmosomes connecting the basal cells to the basement membrane.
Figure 5A

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Figure 5B

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Figure 6. Summary of outcomes of *ex vivo* expanded limbal epithelium transplantation for Patient 1. [A] Our treatment protocol produces reversal of LSCD by all measures including impression cytology, reversal of epithelial defects, corneal vascularisation, corneal opacity and Snellen vision. Corresponding subjective scores show a corresponding reduction in pain and visual impairment scores. [B] Microphotographs showing the patients affected eye before and after the limbal and corneal transplant.
Successful stem cell therapy for unilateral LSCD

B

<table>
<thead>
<tr>
<th>Pre operative</th>
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<tr>
<td>Following limited transplant</td>
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<tr>
<td>Following corneal transplant</td>
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Kolli et al. 2009
**Figure 7.** Graphs to show subjective and objective outcomes of *ex vivo* expanded LSC transplantation. [A] Pain scores shown above and visual impairment scores shown below. [B] Improvement in subjective outcomes as result of LSC transplantation. [C] Objective outcomes from pre-operative values [Preop] to post-operative values at the end of the follow up period [Postop].* For patient 1 who underwent a penetrating corneal transplant 1 year after LSC transplant, the pre corneal graft values of visual acuity and corneal opacity were used to prevent bias from the second procedure. [D] Changes in LogMAR Visual Acuity [this is a linear scale allowing statistical comparison of vision where lower values correspond to better visual acuity] following *ex vivo* expanded limbal epithelium for the treatment of total LSCD.
Successful stem cell therapy for unilateral LSCD

Figure 7B

Subjective pain score

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Subjective vision score

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<th>Preop. CK19 negative</th>
<th>Postop. CK19 positive</th>
<th>Postop. CK19 negative</th>
<th>Impression Cytology</th>
<th>Significant Epithelial Defect</th>
<th>Snellen Decimal &amp; (Snellen Visual Acuity)</th>
<th>Corneal/Vascularisation (Central Area)</th>
<th>Corneal Opacity</th>
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<td>0.17*</td>
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<td>++++</td>
<td>+++ (5 after PN)</td>
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<td>0.62</td>
<td>0.02</td>
<td>Present</td>
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<td>++++</td>
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<td>Absent</td>
<td>0.62</td>
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<td>Absent</td>
<td>+++</td>
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</tr>
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**Figure 7C**

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**Figure 7D**

**Objective Visual Acuity Change Following Stem Cell Treatment**

![Graph showing visual acuity change](Kolli et al. 2009)