Visualizing the Fate of Transplanted K14-Confetti Corneal Epithelia in a Mouse Model of Limbal Stem Cell Deficiency

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PURPOSE. Therapies for limbal stem cell deficiency (LSCD) include stem cell (SC) grafts that regenerate the damaged ocular surface. However, the fate of transplanted cells is ill-defined. We addressed this limitation using primary corneal epithelial cells from K14-Confetti mice.

METHODS. Cultures of primary corneo-limbal epithelia were generated from K14-Confetti (n = 6) and wild-type (WT) (n = 3) mice. Cell phenotype and function was ascertained by immunofluorescence, flow cytometry, quantitative PCR and colony formation. K14-Confetti cells were nurtured on fibrin and transferred onto WT mice with experimentally induced LSCD (n = 16) to determine the site of implantation, longevity, and phenotype.

RESULTS. Transgenic and WT cells derived from explanted corneal tissue displayed no phenotypic or functional differences. K14-Confetti corneo-limbal epithelia that engrafted in recipient LSCD WT mice formed 107 ± 36 fluorescent clones at 2 weeks postprocedure, which decreased to 70.0 ± 5.5 by 6 weeks (P = 0.15). Furthermore, cells commonly implanted in the periphery (P < 0.05) and some generated clones that migrated centripetally. However, a normal corneal epithelial phenotype was not restored. We speculate this is due to insufficient SCs being seeded within grafts, and shows evidence of both cell loss from the implants and transdifferentiation into K8-conjunctival and K10-cutaneous epithelia after transplantation.

CONCLUSIONS. This study successfully tracked the fate of transplanted corneo-limbal epithelia in a mouse model of LSCD by intravital microscopy. Our data shed new light into how donor cells behave, the positions they take, how long they survive, and potential mechanisms of loss from the ocular surface. This information is important for improving future animal models, to render them clinically relevant.

Keywords: cornea, Confetti, cell culture, limbus, stem cells, transplantation
animals that either had their transgenes induced in vivo, and were therefore fluorescent at the time of cultivation, or were administered 4-hydroxy-tamoxifen (4OH-TAM) in vitro. No morphological, phenotypic, or functional differences were observed when compared with cells cultivated from wild-type C57BL/6 (WT)-derived mice. Transgenic multicolored cells were cultivated on fibrin gels and grafted into WT recipient mice with experimentally induced LSCD to establish a visual fate-map of their ability to implant and develop long-lived clones. This investigation provides vital clues as to the potential mechanism of donor cell loss and possible SC-graft failure in patients with LSCD who are receiving fibrin-based SC therapies.

**METHODS**

**Confetti Mice**

All procedures on mice were performed in accordance with the University of New South Wales Animal Care and Ethics Committee guidelines (ACEC no. 14/89B), the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Confetti mice, tamoxifen (TAM) induction and genotyping have been described previously.1 WT mice were generated from our colony and were either Cre or Confetti negative; this minimized the risk of graft rejection, eliminated the need for immunosuppression, and rendered recombination impossible.

**Explant Culture and Expansion of Murine Primary Corneal Epithelial Cells**

Four-week-old Confetti or WT mice were euthanized, their eyes enucleated, sterilized in 2% iodine (Sigma-Aldrich Corp., St. Louis, MO, USA), and cultured as previously described.17 Please see Supplementary Methods for full details of culture conditions.

**Confetti Transgene Induction In Vitro**

4OH-TAM (Sigma-Aldrich Corp.) was prepared by dissolving in 100% ethanol at 37°C. In brief, cells were exposed to increasing concentrations of 4OH-TAM (0.1–20 μM) in defined keratinocyte serum-free media (dKSFM) (Thermo Fisher, Waltham, MA, USA) for 30 minutes to 8 hours, washed in PBS, and fresh dKSFM was added. Conditioned media was assayed 24 hours after treatment for lactate dehydrogenase (LDH) using an LDH detection kit (Abcam, Cambridge, UK) and cell viability assessed by propidium iodide (Sigma-Aldrich Corp.) staining and flow cytometry. Cultures were visualized on a Nikon Eclipse TiE (Coherent-Scientific, Santa Clara, CA, USA) and images processed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).

**Immunofluorescence**

Immunofluorescence was performed on PFA-fixed cultured corneal epithelial cells or PFA-fixed and frozen mouse eyes as previously described,18 with appropriate primary and secondary antibodies (Supplementary Table S1). Samples were viewed on an LSM780 (Carl Zeiss, Oberkochen, Germany) confocal microscope and images were analyzed with ImageJ software.

**Flow Cytometry**

Flow cytometry was performed as previously described with minor modifications.18 Please see Supplementary Methods for full details.

**Quantitative PCR**

RNA from cultured primary mouse corneal epithelial cells was extracted as previously described18 using the RNAgent Total RNA Extraction Kit (Promega, Madison, WI, USA) and reverse transcribed into cDNA using the SuperScript III RT system (Thermo Fisher) with oligo dT primers (Supplementary Table S2).

** Colony-Forming Assay**

Colony-forming assays were performed as previously described.16 Please see Supplementary Methods for full details.

**Cultivation of Mouse Primary Corneal Epithelial Cells on Carrier Scaffolds**

Fibrin gels (Tisseel; Baxter Healthcare, Deerfield, IL, USA) were prepared as previously described20 in glass chamber slides (BD Biosciences, San Jose, CA, USA). Confetti cells (1.2 × 10⁵ per gel) were seeded and allowed to expand for 7 days.

**Epithelial Debridement and Grafting**

Anaesthetized 6- to 10-week-old WT mice received limbal-limbal epithelial debridement to the right eye using an Algerbrush II rotating burr (Alger Equipment Co., Inc., Lago Vista, TX, USA) as previously described,21 followed by a graft of either fibrin gel alone, or in conjunction with Confetti cells between passages 5 and 10. Mice were monitored by intravitral microscopy for 6 weeks. For full details please see Supplementary Methods.

**Statistical Analysis**

A 2-way Welch’s t-test with unequal variance or a 2-way ANOVA with Tukey’s multiple comparison test was performed, otherwise statistical tests are given in figure legends. Prism v7.02 (GraphPad, La Jolla, CA, USA) was used for all analyses.

**RESULTS**

**Expansion and Transgene Induction of Confetti Corneal Epithelium In Vitro**

Initially, we used corneas from Confetti mice in which the transgenic reporters were not expressed, finding cells emerged from tissue explants within 6 to 10 days (Fig. 1A). Cultures were composed of small, densely packed cells, among which was a scattering of large vacuolated cells, indicative of differentiation (Figs. 1A–C). Cells were subcultivated for 25 generations, during which time they retained their proliferative activity and heterogeneity (Figs. 1B, 1C). Cultures were examined at passage (P9) and P13 by flow cytometry, finding that 14.5% ± 1.5% were K14⁺ (Figs. 1D, 1E), a result corroborated qualitatively by immunofluorescence (Fig. 1F). To our knowledge, this is the first successful long-term culture of corneal epithelial cells from Confetti transgenic mice.

Cultures were administered 4OH-TAM to induce the transgenes in vitro. Cells treated with 1 or 5 μM of 4OH-TAM for 1 hour demonstrated no significant morphological changes (Figs. 1H, 1I). Likewise, there was no significant change in toxicity, as indicated by LDH release (Fig. 1L) and cell viability (Fig. 1M) relative to untreated cells. However, cells exposed to 10 μM or 20 μM 4OH-TAM for 1 hour became larger and rounder (Figs. 1J, 1K, respectively), and released significantly higher levels of LDH (P < 0.01 and P < 0.001, respectively) (Fig. 1L). Moreover, 20 μM 4OH-TAM for 1 hour significantly
FIGURE 1. Expansion and in vitro transgene induction of K14CreER<sup>12</sup>-Confetti-derived primary corneal epithelial cells. Corneo-limbal tissue explants were placed in culture and epithelial cells emanated from this biomaterial within 6 to 10 days (A). The same cells were serially cultivated and imaged by phase-contrast microscopy at P5 (B) and P25 (C). The hatched white line in (A) demarcates the edge of the explant. Some cells were collected at the indicated passage and assessed for K14 content by flow cytometry (D, E). Histograms represent K14 (red) and a relevant isotype Ig control (blue) at P8 (D) and P13 (E). Other cells were assessed for K14 protein by immunofluorescence (F, red) and nuclei counterstained with 4',6-diamidino-2-phenylindole (DAPI) (G, blue). Cells incubated with an isotype control Ig had negligible reactivity (G). Primary corneo-limbal donor cell fate after transplantation.
epithelium from Confetti mice were exposed to varying concentrations of 4OH-TAM (1–20 μM) at specific times, ranging from 30 minutes to 8 hours, and toxicity assessed by cell morphology (H–K), secreted LDH (L), and cell viability by propidium iodide staining with flow cytometry (M). Control cells were exposed to an appropriate concentration of vehicle (ethanol) (H inset, L, M). Bars in (L, M) represent mean ± SD (n = 3 replicates/group) and statistical analysis performed using a Welch’s t-test with unequal variance. **P < 0.01, ***P < 0.001. Five days after cells were exposed to 1 μM 4OH-TAM for 1 hour, they were examined under wide-field fluorescence microscopy and displayed high levels of Confetti reporter protein expression including CFP (N), YFP (O), and RFP (P). A merged image (Q) shows that most cells are genetically labeled. Scale bars: (A–G) 50 μm, (F G) 20 μm, (K) 50 μm, (Q) 50 μm.

Phenotypic and Functional Analysis of Confetti Corneal Epithelial Cells

Next, the phenotype and function of fluorescent Confetti cells were compared with cells derived from WT mice. WT mice were generated from our colony and did not contain either the Cre or Confetti transgenes, and were therefore not fluorescent. Immunofluorescence, flow cytometry, and quantitative PCR demonstrated no significant differences in protein or gene expression in WT, in vitro, and in vivo TAM-induced cells (Figs. 3A–D, Table). Likewise, colony-forming efficiency (a measure of SC activity) was similar among WT, in vitro, and in vivo TAM-induced cells (0.13% ± 0.04%, 0.14% ± 0.03% and 0.16% ± 0.04%, respectively) (Fig. 3E). These results demonstrate that irrespective of the TAM treatment, cultivated cells were predominantly of a basal limbal phenotype and further confirm that the Confetti transgene does not have an impact on cell function.

Induction of LSCD in WT Mice

The AlkGrbII IIa transgenic mouse was used to remove the corneal epithelium beyond the limbus (Fig. 4A), including any resident LESC, to generate LSCD-like disease in WT mice.21–23 Within 24 hours, a significant leukocyte infiltrate was noted throughout the corneal stroma, which remained denuded (Fig. 4B). At 48 hours after wounding, reepithelialization from the peripheral cornea was obvious, together with a reduction in the inflammatory infiltrate (Fig. 4C). By 4 weeks, reepithelialization was complete, inflammation had subsided, and PAS+ goblet cells were present throughout the corneal stroma (Fig. 4D). Immunofluorescence performed on tissue harvested 4 weeks after wounding demonstrated the absence of K12, indicating that cells covering the stroma were not of a corneal lineage (Fig. 4E). The conjunctival-specific K8/18 and K13 were intensely expressed within the epithelium that now covered the cornea (Figs. 4F, 4G), associated with goblet-like cells (Fig. 4F) and squamous epithelium (Fig. 4G), respectively. CD31+ vascular endothelial cells were observed throughout the corneal stroma (Fig. 4H), indicating the presence of a persistent angiogenic response. The infiltration of conjunctival epithelium, goblet-like cells, and blood vessels are hallmark features of LSCD.6

Transplantation of Fluorescent Confetti Cells in a Mouse Model of LSCD

Before grafting, it was important to show the ability of cultured cells to transfer from their carrier-scaffold to another surface. Fluorescent Confetti cells adhered to and expanded on pre-fabricated fibrin gels, after which they transferred to culture plastic substratum where they further proliferated (Supplementary Fig. S2).

Following epithelial debridement of the right eye, mice received grafts comprising either fibrin alone (Fig. 5A, rows 1 and 2) or fibrin with Confetti cells (Fig. 5A, rows 3 and 4). Confetti cell grafts generated 107.5 ± 56 fluorescent clones at 2 weeks after injury, which diminished to 70 ± 5.5 at 6 weeks; however, this was not statistically significant (P = 0.15) (Fig. 5B). At 2 weeks post-transplantation, the peripheral cornea (zones 4 and 5) contained significantly more fluorescent colonies than the center (zone 1) (P = 0.007 and P = 0.03, respectively) (Fig. 5D); however, cells were evenly distributed across the cornea by 6 weeks after grafting (Fig. 5E). Corneas of mice that received fibrin only demonstrated the absence of K12, together with the presence of K8/18+ goblet-like cells and CD31+ stroma blood vessels, indicating conjunctivalization had occurred (Fig. 5E, rows 1 and 2). Similarly, mice that received Confetti donor cells demonstrated no K12 expression, and the epithelium was populated with K8/18+ goblet-like cells, together with stromal blood vessels at both 2 and 6 weeks after grafting (Fig. 5F, rows 3 and 4). Some K8/18+ goblet-like cells expressed Confetti fluorescent proteins, indicating they arose from donor cells (Fig. 5F, rows 3 and 4, Supplementary Fig. S3). Interestingly, the eyelids of mice that received Confetti cells were also fluorescent, and this persevered for 6 weeks (Fig. 5A, row 4). Fluorescent eyelids displayed K10 (Figs. 5G, 4H) concurrent with RFP expression (Fig. 5G), indicating donor cells dislodged from the ocular surface following grafting, embedded in the eyelids, and underwent trans-differentiation, as K10 was not displayed in cultures used to prepare grafts (Fig. 5I). However, compared with the eyelids of Confetti mice that received TAM in vivo, both K14+ cell density and the percentage of fluorescent epithelial cells were both significantly reduced (P < 0.0001) when viewed with intravitreal or confocal microscopy, respectively (Supplementary Fig. S4).

Discussion

Herein, we described the first report detailing the cultivation of corneal epithelial cells from Confetti mice and their subsequent transplantation in a mouse model of LSCD. In this system, Confetti reporter proteins label K14+ cells and their progeny with a kaleidoscope of color, facilitating real-time observation and fate-mapping of donor cells after grafting. Initially, we used mice in which the transgenic reporter was not activated, and to our knowledge performed the first in vitro transgene induction of Confetti-derived primary epithelial cells after administering 4OH-TAM. Cultures were also generated from animals that had the transgenes induced in vivo; under these conditions, no phenotypic or functional differences were noted compared with WT-derived cells. Next, experimental LSCD was induced in WT mice, in which Confetti cells were exposed to varying concentrations of 4OH-TAM (1–20 μM) at specific times, ranging from 30 minutes to 8 hours, and toxicity assessed by cell morphology (H–K), secreted LDH (L), and cell viability by propidium iodide staining with flow cytometry (M).
In vitro expansion and fluorescent reporter protein expression in Confetti corneo-limbal epithelia. Corneal explants derived from Confetti mice that had been administered TAM 7 days before euthanasia were placed in culture plates and submerged in KSFM. Cells emerged at day 6 (A, C) and continued expanding even after explants were removed at day 12 (B, D). Cultures were imaged using phase-contrast (A, B) and wide-field fluorescence (C, D) microscopy. Hatched white lines (A–D) represent the edge of the tissue explant and extent of cell expansion. Solid box (D and inset) shows a magnified view of cells emerging from the explant. Cells displayed the correct combinations of fluorescent proteins, as anticipated from the possible recombination events (E–J, representative of three independent cultures). Scale bars: (A–D) 100 μm, (D) (inset) 50 μm, (E–J) 20 μm.

**Figure 2.**
FIGURE 3. Phenotypic and functional analysis of Confetti-derived primary corneo-limbal epithelial cells. Investigations were carried out on PFA-fixed epithelial cells derived from corneo-limbal tissue explants from WT C57BL/6 (A, row 1; B–E, green bars), and homozygous Confetti mice whose cells were exposed to 4OH-TAM in vitro (A, row 2; B–E, blue bars) or from in vivo TAM-injected mice (A, row 3, B–E, red bars). The expression of K14, ΔNp63, K12, and K8/18 and K13 proteins and mRNA was examined by immunofluorescence (A, B), flow cytometry (C), and quantitative PCR (D). Immunofluorescence was also performed on PFA-fixed limbal (A, row 4) and corneal (A, row 5) tissue sections. Hatched
respectively (Figs. 3A–C, Table), both markers of basal limbal keratins33,34 also were profiled and likely due to differences in assay sensitivity. Conjunctival 30% to 50% and 50% to 80% of cells were K14 origin. Immunofluorescence and flow cytometry indicated that determine whether cultures were indeed corneo-limbal in vitro, which generated persistent fluorescent clones (Figs. 1H–T). TAM (4OH-TAM) was used to induce the Confetti transgenes in mice (Fig. 3, Table). As K14 is localized to both basal limbal1,2,28 and conjunctival epithelium,29 it was necessary to compare their phenotype with those generated from WT mice (Fig. 3, Table). As K14 is localized to both basal limbal1,2,28 and conjunctival epithelium,29 it was necessary to determine whether cultures were indeed corneo-limbal in origin. Immunoﬂuorescence and flow cytometry indicated that 30% to 50% and 50% to 80% of cells were K14 and ΔNp63δ, respectively (Figs. 3A–C, Table), both markers of basal limbal progenitors26,31 whereas <10% of cells were immunoreactive for the corneal differentiation marker K12 (Figs. 3A–C, Table). Notably, our initial cultures displayed 2-fold lower K14 expression, potentially because they were analyzed at later passages (Figs. 1D, 1E), indicating a progressive loss of K14 over subsequent generations, similar to that found for K15 and P-cadherin expression.32 Differences in ΔNp63 expression were also noted when cells were analyzed with immunofluorescence and flow cytometry (Figs. 3B, 3C), although this is likely due to differences in assay sensitivity. Conjunctival keratins33,34 also were proﬁled and <10% of cells were found to be K13δ, whereas 30% to 50% were K8/18β (Figs. 3A–C, Table). Although these results suggest conjunctival cells may have contaminated our cultures, we believe this is unlikely, as every precaution was taken to ensure complete removal of the conjunctiva from enucleated eyes by carefully resecting this tissue from intact corneas. A potential explanation is that in vitro cultivation modiﬁed gene and protein expression proﬁles.32,33 Curiously, both K13δ and K8/18β were found in suprabasal35 and basal36 human limbal epithelia, respectively, and K8/18 coexpressed with K14, K12, and Muc5AC in compound niches within the peripheral murine cornea.37 Furthermore, it is hypothesized that K8/18 is a marker of limbal progenitor cells capable of differentiating into conjunctival or corneal epithelia.37 Our phenotypic assays suggested we cultivated basal limbal epithelial cells with limited primitive characteristics.18,39 Others have generated similar undifferentiated cultures, speculating this is due to calcium in the media, which at high concentrations induces differentiation.17,24,35–38 ΔK5FM contains low calcium (<0.1 mM), which likely aids in maintaining a precursor phenotype. In this regard, cultures from transgenic and WT mice harbored comparable SC activity (Fig. 3E), a result congruent with previous investigations.18,39 Overall, these studies demonstrate that neither the presence nor induction of the transgene affected cell phenotype or function, indicating cultures derived from Confetti and WT mice are equivalent. The former system is advantageous for fate-mapping grafted cells due to the stable incorporation of SC-speciﬁc fluorescent reporters,26 which allows mice to be monitored in real time by intravitreal microscopy.

The pathological characteristics that developed in our LSCD model included a loss of normal corneal epithelia, encroachment of conjunctival epithelium that harbored PAS-positive goblet cells, inflammation, and neovascularization (Fig. 4). These features are similar to those described in other mouse models that use mechanical13,22 and chemical injury40–42 and are akin to the clinical signs displayed by patients with LSCD.6 Although there are ample reports of LSCD generated in mice, few studies have examined whether corneal epithelial restoration actually ensued following SC-based transplantation. After grafting our Confetti cells into WT recipient mice, intravitreal and confocal microscopy revealed a scattering of fluorescent clones, more so in the periphery, some of which developed into migratory streaks (Fig. 5A, rows 3 and 4; Supplementary Fig. S5) similar to those present in healthy corneas.1–3 Over time, their numbers diminished, possibly due to the heterogeneous population of cells that were implanted, most of which were likely to be TACs with limited proliferative potential.43 Notably, grafts containing a higher proportion of SCs are more successful,12 and therefore future investigations will focus on enhancing the quality of our starting population by cell-sorting for Confetti ﬂuorescence in conjunction with selective cell-surface antigens.44–46 Additionally, we used cells between passages 5 and 10 for transplant experiments, which may have led to lower numbers of SCs being present. However, our primary cells could be subcultivated up to 25 times, indicating that SCs are still present even at later generations.

Similar to previous reports, the corneal epithelium (as assessed by K12 expression) was not completely restored in mice that received fibrin alone or fibrin in conjunction with Confetti cells (Fig. 5). A recent report detailed the transplantation of transgenic hair follicle-derived SCs in LSCD mice, demonstrating patchy K12 expression.21 Furthermore, the presence of host-derived K12 cells indicated that sectors of limbus must have remained intact, despite this being a model of LSCD.21 Others used cultured murine ABCB5+ LESCs carried on fibrin scaffolds to demonstrate K12 expression in approximately 30% of the regenerated epithelium, with no indication as to the phenotype of the K12 cells.13 In the above-mentioned studies (including ours), donor cells were implanted immediately after injury; exposing them to a hostile, inflammatory, and angiogenic microenvironment. It is possible that grafted cells may have fared better if the ocular surface...
was allowed to stabilize for several weeks before transfer. Furthermore, it is likely that the corneal injury inflicted in our mouse model damages the limbal niche, meaning grafted cells are precluded from taking up residence in their typical microenvironment, and are therefore restricted from receiving their normal survival and differentiation signals. Finally, during transplantation, donor cells are distributed across the ocular surface; hence, even if the limbus is not damaged, they are unlikely to trek backward (centrifugally) into the limbus due to the stronger centripetal forces generated by the cornea.

We also identified K8/18+ goblet-like cells across grafted corneas, a subset of which were Confetti+ (Fig. 5E; Supplementary Fig. S3), indicating they originated from donor cells. The obvious question is how did they evolve from transplanted corneo-limbal epithelia? Goblet cell differentiation has been observed in mice that received a 2.5-mm mechanical wound to the cornea,45 and in wounds to the oral mucosa51 epithelia can transdifferentiate into a corneal phenotype, implying a level of plasticity exists in ectodermally derived tissue; however, the molecular mechanism governing this process is not well defined.46

The other surprising observation was the presence of Confetti donor cells in the eyelid epidermis of recipient WT mice. These cells persisted for 6 weeks and expressed the K12+ corneal epithelial cell clusters were detected in the superficial epidermis near the eyelid margin, but did not proliferate into large clones, as was apparent on the corneal surface (Supplementary Fig. S4). To explain this observation, recipient mice underwent total body irradiation.47 Curiously, self-sustaining K12+ corneal epithelial cell clusters can be maintained in the eyelid without reimplantation of limbal cells.48 The other surprising observation was the presence of Confetti donor cells in the eyelid epidermis of recipient WT mice. These cells persisted for 6 weeks and expressed the K12+ corneal epithelial cell clusters were detected in the superficial epidermis near the eyelid margin, but did not proliferate into large clones, as was apparent on the corneal surface (Supplementary Fig. S4). To explain this observation, recipient mice underwent total body irradiation.47 Curiously, self-sustaining K12+ corneal epithelial cell clusters can be maintained in the eyelid without reimplantation of limbal cells.48

All results represent the mean % of positive cells ± SD. IF immunofluorescence; Flow, flow cytometry; NS, not significant when tested using a 2-way ANOVA with unequal variance.

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**TABLE.** Quantification of Immunofluorescence and Flow Cytometry on WT and Confetti Primary Corneal Epithelial Cells

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**FIGURE 4.** Induction of LSCD. WT C57BL/6 mice were subjected to total corneo-limbal epithelial debridement using an Algerbrush II rotating burr, which extended into the conjunctiva. Eyes were enucleated, formalin-fixed, and paraffin-embedded before PAS staining with hematoxylin counterstaining (A–D). Images were acquired from the peripheral-to-central cornea at 0 (A), 24 (B), and 48 (C) hours and 4 weeks postinjury (D). Sections were counterstained with DAPI (blue). Hatched white line in (E–H) represents the epithelial/stroma boundary. Scale bars: (A–D) 100 μm, (E–G) 50 μm, (H) 25 μm.

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FIGURE 5. Grafting Confetti corneo-limbal epithelial cells onto severely wounded WT C57BL/6 mice. WT mice were subjected to total corneo-limbal epithelial debridement, then immediately received a graft consisting of either fibrin alone (A, rows 1 and 2) or fibrin together with fluorescent corneo-limbal epithelial cells (A, rows 3 and 4) and animals were monitored at 2 (A, rows 1 and 3) and 6 (A, rows 2 and 4) weeks by intravital microscopy. *Hatched white circle (A, row 1) represents the IOL that autofluoresces. The total number of fluorescent clones was counted across the cornea at both 2 and 6 weeks (B). *Histogram bars (B) represent the mean number of fluorescent clones ± SD (n = 4 corneas/time point) and statistical analysis was performed using a Welch’s t-test with unequal variance. To establish whether clones preferentially implanted in specific regions, corneas were digitally segregated into five equidistant concentric circular zones (C) and fluorescent colonies were counted at 2 (D) and 6 (E) weeks after grafting. Zone 5 (red) encompasses the limbus, whereas zone 1 (green) represents the central cornea (C). *Colored histogram bars (D, E) represent the mean clonal density per region ± SD (n = 4 corneas/time point) and correspond to the equivalent circular region in (C). Statistical analysis was performed using a 1-way ANOVA with unequal variance. (D) *P < 0.05, **P < 0.01. Immunofluorescence was performed for K12 (green), K8 (green), and CD31 (red) (F) on tissue sections obtained from the corneas of mice receiving fibrin only (F, rows 1 and 2) or fibrin together with Confetti cells (F, rows 3 and 4). Confetti reporter proteins (RFP or YFP) also are displayed (F, rows 3 and 4). *Hatched white lines (F) represent the corneal epithelial (epi)/stromal boundary. As eyelids in WT mice that received Confetti cell grafts fluoresced (A, rows 3 and 4), they were dissected and examined by immunofluorescence for Confetti reporter expression (RFP, red) in conjunction with the epidermal-specific antigen K10 (yellow) (G). K10 expression (yellow) also was examined in normal mouse epidermal tissue (H) and in cultured mouse primary corneal epithelial cells (I). *Hatched white lines (G, H) demarcate the cutaneous epidermal (epi)/dermal junction. All tissue sections or cells were counterstained with DAPI (blue). EL, eyelids. Scale bars: (A) 400 μm, (F) 40 μm, (G, H) 40 μm, (I) 10 μm.
scanning by intravital fluorescence microscopy, and their liver, lung, and spleen were enzymatically dissociated for analysis by flow cytometry; however, no Confetti cells were detected (data not shown). Two plausible explanations can be offered for the integration and survival of these cells on the recipients’ eyelids. First, sutures were anchored in a large fold of eyelid skin to ensure complete closure and minimize the chance of self-removal. However, this forced segments of epidermis to come into contact with the graft. Alternatively, donor cells may have transferred via external means, for example by itching or grooming, a behavior that is amplified upon injury. This highlights the need for additional graft protection to prevent cell loss, potentially via a therapeutic contact lens or high highlights the need for additional graft protection to prevent cell loss, potentially via a therapeutic contact lens or

P keratinization has been observed in Tgf signaling may represent a downstream or independent pathway capable of initiating K10 expression. It is possible that in previous studies using mouse models or indeed in patients with LSCD who received similar fibrin grafts, transplanted cells may have been lost or displaced in a comparable manner; however, without the capacity to visualize this depletion, determining their fate is virtually impossible. Once donor Confetti/K10+ cells implant in the epidermis, a credible mechanism of cutaneous keratin switching may involve suppression of key transcription factors, including Dkk2, Notch1, and Pax6. Alternatively, corneal keratinization has been observed in Tgfr2 conditional knockout mice without loss of Pax6 expression, indicating that TGFβ may represent a downstream or independent pathway capable of initiating K10 expression.

First and foremost, our experimental paradigm was to establish and provide proof-of-concept of a long-term primary culture that stably expressed an array of reporter proteins, an animal model of LSCD that recapitulated the clinicopathological features of humans, a graft strategy that could be applied to mice, and the ability to monitor donor cells in real time. In all areas, our investigations were successful, despite recognizing several limitations. Two plausible mechanisms of cell loss and subsequent graft failure also were identified, which would not have been possible without the ability to visualize transplanted cells. Taken together, we have generated important new information that improves our understanding of donor cell fate during transplantation and their ability to regenerate corneal epithelial tissue, and in the future may inform the development of more successful SC therapies for patients with LSCD.

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**References**
