**Sustained and widespread gene delivery to the corneal epithelium
via *in situ* transduction of limbal epithelial stem cells using lentiviral
and AAV vectors**

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Short Title

In vivo limbal epithelial stem cell gene delivery

Abstract

Corneal epithelial dystrophies are typically characterised by symptoms such as pain, light sensitivity and corneal opacification leading to impaired vision. The development of gene therapy for such conditions has been hindered by an inability to achieve sustained and extensive gene transfer, as the epithelium is highly replicative and has evolved to exclude foreign material. We undertook a comprehensive study in mice aiming to overcome these impediments. Direct injection of lentiviral vector within the stem cell niche resulted in centripetal streaks of epithelial transgene expression sustained for >1 year, indicating limbal epithelial stem cell transduction *in situ*. Extent of transgene expression varied markedly but at maximum covered 26% of the corneal surface. Following intrastromal injection, adeno-associated viral (AAV) vectors were found to penetrate Bowman's membrane and mediate widespread, but transient (12-16 days) epithelial transgene expression. This was sufficient, when applied within a Cre-Lox system, to result in recombined epithelium covering up to ~80% of the corneal surface. Finally, systemic delivery of AAV2/9 in neonatal mice resulted in extensive corneal transduction, despite the relative avascularity of the tissue. These findings provide the foundations of a gene therapy toolkit for the corneal epithelium which might be applied to correction of inherited epithelial dystrophies.

Introduction

The epithelium is the outermost layer of the cornea and thus forms the most anterior surface of the eye and is a barrier to prevent infiltration by pathogenic material. A healthy epithelium is vital for maintaining the optical properties of the cornea. It provides a smooth, protective outer surface, helping to minimise light scatter, maintain hydration and prevent infection and thus contributing to corneal transparency¹. The corneal epithelium is organised into 5-7 layers, with a basal monolayer of columnar cells adhered tightly to the underlying basement membrane, and several layers of more apical cells that become increasingly differentiated, larger, flatter and more tightly adhered to each other. The epithelium is a highly replicative tissue undergoing constant renewal to counterbalance the continuous desquamation of cells from the corneal surface. Only the most basal monolayer of epithelial cells, the transient amplifying cells (TACs), are replicative, however these cells do not possess unlimited replicative capacity²⁻⁵. They in turn are renewed by limbal epithelial stem cells (LESCs) a population of adult stem cells located at the periphery of the cornea. The TAC lineages of LESCs undergo centripetal migration within the epithelium, driven by several proposed mechanisms, such as population pressure from the periphery due to the increased proliferative capacity of LESCs / early TACs, or an increased rate of cell loss from the central cornea, and chemotaxis^{4,6}. When engineered to express a reporter, LESC lineages can be visualised as streak-like patterns extending from limbal to central cornea⁷⁻¹¹.

Gene therapy is conventionally applied to the correction of inherited genetic defects, and a number of such conditions have been described affecting the epithelium. Individually these are rare, however many of them display an increased prevalence within the Japanese population¹²⁻¹⁴. Gelatinous drop-like corneal dystrophy (GDLD) is inherited in an autosomal recessive pattern, caused by a loss of function in the gene TACSTD2^{15,16} and is both among the most prevalent (1 in 33,000 in Japanese populations) and most severe of the epithelial dystrophies. Its primary phenotype is the accumulation of sub-epithelial amyloid deposits that render the cornea opaque¹⁷⁻¹⁹ and until recently the only treatment commonly applied to manage GDLD was anterior lamellar keratoplasty (ALK). However, this procedure does not resolve the disease permanently as ALK only replaces the central portion of the cornea allowing the recipient's limbal stem cells to remain *in situ*. Healthy graft epithelium is therefore replaced over time by the recipient's diseased epithelial cells via the process of normal corneal renewal. Recurrence of the condition thus often occurs within 1-2 years²⁰. An alternative which has shown some initial promise for the treatment of inherited epithelial dystrophies is the supplementation of ALK surgery with allogeneic LESC transplantation^{21,22}. Over the last two decades, LESC transplantation has become established as a successful treatment for limbal

stem cell deficiency caused, for instance, by alkali burns. Whilst limbal epithelial transplants can be effective in this context²³, their long-term efficacy and mechanism of action is unclear, especially in the case of allogeneic transplantation. It is often assumed or implied that reconstitution of the epithelium results from successful engraftment of the transplanted stem cells, but this may only be partially true. Studies that have genetically characterised restored corneal epithelial tissue following allogeneic LESC grafts have found that in many cases the restored healthy tissue is derived from the recipient rather than the donor^{24–26}. This suggests the engrafted cells may largely function to support or restore the recipient's remaining endogenous limbal stem cells. Thus, this approach may not be optimal for the long-term treatment of inherited epithelial dystrophies. Moreover, many allogeneic LESC grafts eventually fail in the long term (5+ years), possibly due to an incomplete/failed engraftment or insufficient immunosuppression^{27,28}.

To date, the development of corneal epithelial directed gene therapy has been limited. In order to be effective a corneal epithelial gene therapy must overcome the challenge of mediating a stable gene delivery to a dynamic tissue that has evolved to exclude infectious agents and, to date, the most promising results have been achieved with lentiviral vectors. In the adult human corneal rim these have been shown to mediate efficient gene transfer to the corneal epithelium *ex vivo* with epithelial transgene expression being shown at 3 days post transduction²⁹. The most sustained expression demonstrated thus far lasted 6-weeks. This was achieved *in vivo* by topical delivery of a lentiviral vector to the rat cornea following epithelial scarification³⁰, however, the efficiency of transduction appeared limited. The same study also provided the only example of application of AAV to target the corneal epithelium that we are aware of at present. Transgene expression resulting from topical application of an AAV2/2 vector was found to be more limited than that obtained using the lentiviral vector, both in terms of both extent and duration, which was around 2 weeks. In terms of application, a promising siRNA-based gene therapy has been developed for Meesmann epithelial dystrophy. The siRNA was shown to mediate specific and substantial (63%) knockdown of the mutant allele within patient epithelium *ex vivo* and was able to phenotypically correct a transfected epithelial cell line³¹. However, the authors note that the therapeutic siRNA lacks a suitable delivery vehicle for efficacious application *in vivo*.

There remains therefore significant scope for both improvement and application of gene transfer to the corneal epithelium and here we present a comprehensive study examining multiple approaches aiming to mediate an extensive and sustained gene delivery to this tissue via gene delivery to LSCs *in situ*.

Materials and Methods

Animals

The mouse strains used were wild type C57BL/6J and Ai9 (Jackson Laboratory 007909) mice, which contain a loxP-flanked tdTomato reporter gene at the Gt(ROSA)26Sor locus. All mice were maintained under a 12 h light–dark cycle. Unless otherwise stated all animals used were adult (10+ weeks). WT animals were all females, while Ai9 mice were mixed sex. All experiments were approved by the local Institutional Animal Care and Use Committees (UCL, London, UK) and conformed to the guidelines on the care and use of animals adopted by the Society for Neuroscience and the Association for Research in Vision and Ophthalmology (Rockville, MD, USA).

rAAV vector preparation and titre

Recombinant AAV vectors were produced via triple transient transfection. The plasmid construct, AAV serotype-specific packaging plasmid and helper plasmid were mixed in a ratio of 1:1:3 at 20 µg total DNA per ml of DMEM, were mixed with Polyethylenimine (Polysciences Inc.) to a final concentration of 50 mg/mL and incubated for 10 min at room temperature to form transfection complexes. These were added to HEK293T cells at 50 µg DNA per 175cm² and left for 72 h. The cells were collected, concentrated and lysed by freeze–thaw (4x) in TD buffer to release the vector. All AAV serotypes except AAV2/9 were purified by affinity with an AVB Sepharose column (GE Healthcare) and eluted with 50mM Glycine pH2.7 into 1M Tris pH 8.8. AAV2/9 was purified by a combination of size exclusion and anion exchange. Sephacryl S300 and Poros HQ50 respectively (GE Healthcare). Vectors were washed in 1x PBS-MK and concentrated to a volume of 100–150 µl using Vivaspin 4 (10 kDa) concentrators. Vector genome (vg) based titres were determined by quantitative real-time PCR (qPCR) using an ITR binding assay as previously described (Aurnhammer et al, 2012).

Lentiviral vector preparation and titre

HIV1 based lentiviral vectors were also produced by triple transient transfection. The plasmid construct, second-generation packaging plasmid and envelope plasmid were mixed in a ratio of 4:1:3 at 32 µg per ml of Opti-MEM were mixed with Polyethylenimine (Polysciences Inc.) to a final concentration of 50 mg/mL and incubated for 10 min at room temperature to form transfection complexes. These were added to HEK293T cells at 80 µg DNA per 175cm² and left for 72 h. Vector was purified and concentrated from supernatant medium by filtration

(0.45µm) and ultracentrifugation (90,000 rcf) at 48 and 72 h. Virial particle (vp) based titres were determined by infection of HEK293T cells with a serial dilution of concentrated vector followed by enumeration of infectious events.

Intraocular injections

Prior to all surgeries mice were anaesthetised with a single intraperitoneal (IP) injection of a 0.01-ml/g mixture of Domitor (1mg/ml medetomidine hydrochloride), ketamine (100 mg/ml), and water at a ratio of 5:3:42. After all surgeries anaesthesia was reversed and a 1% chloramphenicol ointment (FDC International) applied topically to the cornea.

Intrastromal injections were performed under observation thorough an operating microscope. Vector or control solution was injected with a 34-gauge hypodermic needle mounted on a 5 µL Hamilton syringe (Hamilton). The needle tip was positioned (bevel up) about a half, to quarter of the corneal radius in from limbus and penetrated the cornea in this position. The needle was then pushed into the cornea taking care to remain within the stroma. Once the needle tip was positioned centrally a volume 2 µL of vector or control solution was injected. Upon injection the cornea became opaque in the area of the injected bleb (usually extending over a majority cornea), this was completely resolved 24hrs post injection.

Limbal subepithelial injections were also performed under observation thorough an operating microscope. The eye was mildly prolapsed and secured in position with an elasticated Vinyl drape. Vector or control solutions were injected using a needle pulled from borosilicate glass capillaries (1.0mm O.D. x 0.78mm I.D. – Harvard Apparatus) and an injection holder set (IM-H1 Narishige). The pulled capillaries had a tip $\varnothing < 10\mu\text{m}$ and a long taper morphology. Flow of solution to be injected was continuous, driven by ~1MPa of air pressure. Whilst solution was flowing the multiple injections were performed (~30-50) around the entire limbal region of the cornea. Due to the continuous flow neither the total volume injected or the volume per injection could be accurately measured. Precise vector dose is therefore unknown.

Systemic delivery of AAV2/9 via intraperitoneal injection

These injections were all performed upon P0 mice within 12 hours of birth. Injections were performed under observation thorough an operating microscope and using a 34-gauge hypodermic needle mounted on a 10 µL Hamilton syringe. The animal was physically restrained and the needle guided into the peritoneal cavity through the abdominal wall. Injection volume was 10 µL.

***In vivo* imaging**

Prior to all *in vivo* imaging mice were anaesthetised to minimise any motion blurring. GFP fluorescence was imaged by Scanning Laser Ophthalmoscopy (SLO). The instrument used was a Spectralis HRA2 with a 55° lens (Heidelberg engineering) modified for use with animals with increased 488 excitation laser power. All images were taken using the 25° angle setting. Infrared (790nm) images were taken using a power setting of 75% and 30 images were averaged and normalized. GFP (488nm) images were taken using a power setting of 95% and 30 images were averaged and normalized. A single infrared image was taken to visualise the positioning of the eye. GFP expression was visualised by compositing multiple 488 channel images from multiple focal planes. RFP fluorescence was imaged by Fundoscopy. The instrument used was a slit lamp (SC-16, Keeler) modified for use with animals and with a filter set to detect RFP (excitation: 550±15nm, emission: 580LP). Images were taken using an attached DC500 digital camera and associated software (Leica). Prior to imaging corneal refractive power was neutralized by placing a 5 mm coverslip on the cornea covered with a coupling medium solution (Viscotears, Novartis Pharmaceuticals). All images were taken at 25x magnification and the following exposure times were used: Bright field: 0.2s, RFP: 30s

Histology

For corneal flat mounts eyes were enucleated *post mortem* and the cornea isolated by an orbital cut just posterior to the limbus. Tissue was fixed in 4% paraformaldehyde for 1 h before 4 evenly spaced radial cuts were made with a scalpel. Tissue was then counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with DAKO fluorescent mounting media. For corneal sections, eyes were enucleated *post mortem* the globe was punctured with the tip of a 30G needle and then fixed in 4% paraformaldehyde for 1 h before embedding in optimal cutting temperature medium. Twenty µm cryosections were cut in sagittal orientation, counterstained with 4',6-diamidino-2-phenylindole (DAPI) and mounted with DAKO fluorescent mounting media (DAKO, S3023, Denmark). All images were acquired by confocal microscopy (Leica DM5500Q). CD31 staining was achieved using Rat α Mouse CD31 antibody (BD, MEC13.3) according to protocol outlined in Cao et al, 2011³².

Quantification of transgene expression within the epithelium by confocal imaging of flat mounts

An area measuring 480 x 960 µm (2x4 40x fields of view) covering an area starting in the central cornea and extended toward a randomly determined quadrant was usually used for

213 analysis. Images were taken at a z-resolution of 1 μ m through the whole depth of the epithelium
214 into the apical stroma. The peak of DAPI staining was found to correlates well with the dense
215 basal epithelial cell layer, and an automated process could thus be used to align multiple
216 samples to this reference point in the z-axis. In cases where this failed (usually due to poorly
217 flattened tissue) the sample was aligned manually. The z-planes that constituted the area of
218 interest were selected and a pixel intensity threshold set manually that well separated
219 fluorescent signal from background. Data to be compared all used the same threshold value
220 and all other aspects of the imaging were kept consistent. An automated process then counted
221 all pixels above threshold intensity in the z-slices of interest.

Results

Intra-epithelial injection of integrating lentiviral vector transduces LESC and results in sustained transgene expression within their lineages

The optimal means by which to mediate pan-epithelial transgene delivery is to target LESC which are responsible for the renewal and maintenance of the tissue. We therefore tested the efficacy of injecting an integrating HIV1-based lentiviral vector (LNT-SFFV-eGFP, titre of 1×10^7 vp/mL) directly into the murine LESC niche around the entire circumference of the corneoscleral junction. We used an ultrafine glass needle to make around 30 injections per eye.

In total, 94 eyes were injected and of these 88 were suitable to undergo complete analysis, the remainder were either culled for histology or died prior to completion of the experiment. For the first 10-12 weeks post injection (WPI) eyes were assessed weekly or fortnightly by scanning laser ophthalmoscopy (SLO) for the development of the streak-like patterns of transgene expression indicative of eTAC or LESC transduction. After this period, follow up became monthly and was continued until at least 52 WPI. If, after 12 WPI, an eye did not display any streaks for more than two consecutive time points, follow up was discontinued. **Figure 1A** shows this data as a Kaplan-Meier survival curve. Such a long period of follow up was selected to definitively differentiate between LESC and eTAC transduction. At 52 WPI, 32 % of the eyes had sustained streaks for the whole period, strongly indicating LESC transduction. The extent of resulting epithelial transgene expression varied substantially. The best example is shown in **Figure 1B** in which ~25% of the corneal surface is covered by epithelium expressing GFP. A more typical example is shown in **Figure 1C** in which coverage was ~7%. Due to the lack of a specific pan-epithelial promotor, the ubiquitous SFFV promotor was used. Transgene expression might not therefore be expected to be restricted to the epithelium and indeed stromal expression was also seen. Whilst stromal and epithelial expression could be reliably distinguished on the basis of spatial and temporal patterning, confocal microscopy of corneal flat mounts *post mortem* confirmed the premise that the streaks were indeed epithelial (**Figure 1D**).

Intrastromal injection of AAV (but not lentiviral) vector mediates transient epithelial transduction across Bowman's membrane

Although the transgene expression achieved by injecting a lentiviral vector directly into the limbus is superior to any other transduction achieved to date, the injections proved technically demanding. Thus, the success rate was relatively low (~32% at 52 WPI) and the best

transduction achieved remained limited in terms of spatial extent (~25% of the corneal surface). A potentially more reproducible approach is to target the epithelium from the basal direction through Bowman's membrane via an intrastromal injection. Lentiviral and AAV vectors were both assessed for this ability to transduce the corneal epithelium via this route of administration. Intrastromal injections of either Lenti-SFFV-eGFP, (1×10^6 vp / eye) or AAV2/8[Y733F]-CMV-eGFP (1×10^{11} vg / eye) were carried out in adult mice, n=4 eyes per vector. Animals were culled at 4 DPI and transgene expression assessed on cryosections. In all cases the lentiviral vector-mediated transgene expression was restricted to the stroma (**Figure 2A**), but AAV vector-mediated expression was seen in both the stroma and the epithelium (**Figure 2B**). In contrast to lentiviral vectors, rAAV vectors do not efficiently integrate their genomes into that of the host³³. In the highly replicative epithelium, transgene expression would be expected to decline over time due to loss of transduced cells from the corneal surface and to the dilution of vector episomes upon cell replication. We did indeed observe such an effect; we noted that at 8 DPI AAV mediated epithelial transgene expression appear maximal (**Figure 2C**) and by 18 DPI (**Figure 2D**) had been almost entirely lost.

Having shown that AAV is able to penetrate Bowman's membrane and mediate transient transduction of the epithelium, we examined which AAV serotype was best suited to for this purpose. Six AAV serotypes were assessed: AAV2/5, AAV2/8, AAV2/9, AAV2/8[Y733F], AAV[ShH10] and AAV[Anc80] titre matched to a dose of 1.4×10^{11} vg / eye, n=6 eyes per serotype. Animals were culled at 8DPI and corneas were flat mounted. A region of each cornea was selected for analysis by confocal microscopy, a representative orthogonal projection of such a region is shown in **Figure 2E**. Transgene expression (eGFP signal above threshold) in the epithelium of this region was then quantified (**Figure 2F**). All the AAV serotypes tested mediated corneal epithelial expression. However, AAV8[Y733F] and AAV[ShH10] mediated substantially more transgene expression than the other serotypes tested.

Intrastromal injection of AAV-Cre mediates genomic recombination in LSCs that is propagated to their lineages

Any transduction of LSCs achieved via intrastromal injection of AAV would not be visualised with an eGFP transgene due to the lack of vector integration and the temporal limitations outlined above. The Cre-Lox recombination system, however, allows permanent expression of a marker gene to result from transient expression of Cre recombinase. This system was therefore used to assess the ability of intrastromal AAV to transduce LSCs. Intrastromal injections (1×10^{11} vg / eye) of AAV2/8[Y733F]-CMV-Cre were performed in Ai9 mice whose

cells express the fluorescent tdTomato transgene upon exposure to Cre. Sagittal sections of injected corneas at 21 DPI show extensive RFP expression both in the central cornea (**Figure 3A**) and at the basal corneoscleral junction where the LSCs are located (**Figure 3B**). A small cohort (n=4) was assessed by fundoscopy fortnightly for 17 weeks, a period that was sufficient to be highly indicative of LESC transduction based upon our lentiviral data. All four eyes developed centripetal streaks of recombined cells and sustained them over the entire period of follow up. Even within this small cohort it was clear that both the extent and reliability of LESC transduction improved upon that achieved via intra-epithelial injection of a lentiviral vector. **Figure 3C** shows the greatest spatial extent of recombined epithelium observed (~82% of corneal surface) and **Figure 3D** the least (~22%). The latter figure is equivalent to maximum extent achieved with a lentiviral vector. Across the whole group, the mean (± 1 SD) extent was $53\% \pm 25.0\%$. It was also noted that prior to 10 WPI, epithelial streaks became increasingly more defined over time. This perhaps represents the extinction of lineages derived from late, central TACs leaving solely those derived from LSCs and early, more limbal TACs. Again, due use of a ubiquitous promoter both stromal and epithelial recombination was both expected and observed, and thus histology was used to confirm localisation of tdTomato within the epithelium (**Figure 3E**).

Intraperitoneal injection of AAV2/9 at P0 mediates gene delivery to all layers of the cornea including the epithelium

AAV2/9 has been shown to result in widespread somatic gene delivery if delivered systemically at early postnatal time points³⁴. We therefore assessed its ability to transduce the cornea following systemic injection in neonatal mice. Intraperitoneal injections (7.2×10^{11} vg per animal) of AAV2/9-CMV-Cre were performed in P0 Ai9 mice.

At 28 DPI, extensive tdTomato expression was seen in flat-mounted corneas (**Figure 4A**). To better visualise the distribution of expression with regard to corneal layers, a pixel intensity threshold (127-255) was applied to each z-plane of the confocal image and each plane was then false coloured by depth (**Figure 4B**) with ImageJ's³⁵ ICA look up table. Layer specific Z-stacks were also taken at a higher magnification. The majority of tdTomato expression is localised within the stroma (**Figure 4B & D**), but multiple islands of epithelial expression are also seen which appear to result from clonal expansion (**Figure 4B & E**) and some expression was also seen in the endothelium (**Figure 4E**). Over time these islands of epithelial expression developed into centripetal streaks (**Figure 4F-H**) which persist until at least 21 weeks post injection and many of which originate in the limbus.

Discussion

We have presented a comprehensive study of gene transfer to corneal epithelial cells utilising several different routes of vector administration and both lentiviral and AAV-based vector systems. We achieved substantial improvements over the results achieved to date both in terms of extent and duration of transgene expression. Intra-epithelial injection of lentiviral vector at the LESC niche is the most intuitive and direct of these approaches and thus is likely the most suitable for further development and clinical application. This approach gave rise to epithelial transgene expression that was sustained for more than a year and thus very likely resulted from LESC transduction. Technically, however, these injections were demanding and the extent of transduction achieved, whilst superior to any documented to date, remained somewhat limited. In the human eye, which is larger and possesses visible landmarks³⁶ to indicate the LESC niche, it is likely that the procedure may prove less challenging. However, the human LESC niche is also substantially more complex than the murine, being associated with specialised structures within the limbus, which might complicate the procedure^{37,38}. Therefore, optimisation within larger animal model, such as the pig, with a limbal anatomy more closely resembling that of the human may be required to develop the procedure further.

A gene therapy in which an integrating vector is used to correct a genetic disorder via transduction of an adult stem cell resulting in therapeutic gene expression throughout the entire resulting cell lineage is a well-established approach. Indeed, some of the first successful clinical applications of gene therapy used this methodology, transducing hematopoietic stem cells *ex vivo* for the treatment of severe combined immune deficiency^{39,40}. More recently, the technique has been applied to the epithelial stem cells of the skin in the correction of junctional epidermal bullosa⁴¹. In such cases, however, the stem cells are extracted from the patient and transduced *ex vivo*, followed by re-engraftment. This approach presents the potential advantages of allowing selection and amplification of transduced clones prior to engraftment⁴¹ and avoiding direct exposure of the patient to the gene therapy vector. However, the effectiveness of this strategy is entirely dependent upon the feasibility of achieving functional engraftment. In the case of LESC the clinical data to date casts some doubt upon the efficiency of donor cell engraftment^{24–26}, thus gene transfer to the adult stem cell *in situ* may constitute a superior approach for the correction of epithelial dystrophies.

Our second approach was to apply the technically simpler technique of intrastromal injection to transduce the epithelium across the physiological barrier of Bowman's membrane. Whilst lentiviral vectors were found to be unable to penetrate this barrier, AAV vectors mediated substantial transduction, possibly due to their much smaller size allowing dissemination across the membrane. To date there has been little evidence of AAV having any substantial efficacy

in the corneal epithelium³⁰. Nonetheless, we found that not only was AAV able to mediate substantial epithelial transduction, but that all AAV serotypes tested were able to do so, albeit with some variation in efficacy. This would suggest that this tropism is relatively robust and not dependent upon any single mechanism of viral entry, adding to the likelihood that some serotypes of AAV will maintain this tropism across species. The murine Bowman's membrane is known to be relatively thin in comparison with that of the human^{42–44}, and thus it is possible that AAV dissemination across this more substantial barrier would be more limited than that observed here. However, developing serotypes able to overcome the physical barriers present within a tissue has been a major focus of AAV vector development and engineering. Specifically, within the eye, achieving a substantial transduction of photoreceptors via intravitreal injection of vector has been a longstanding objective^{45,46}. This requires the AAV virion to penetrate the inner limiting membrane and ~200µm of neural tissue, which several engineered serotypes have now been shown capable of doing. Recent experiments in the anterior segment revealed that AAVAnc80 can penetrate Descemet's membrane and mediate transduction of the corneal stroma following an intracameral injection⁴⁷. These data suggest that some AAV serotypes may be able to mediate epithelial transduction even across the more substantial barrier posed by a human Bowman's membrane, although this still needs to be established. The intrastromal injection technique applied here (complete stromal hydration) would likely be undesirable within the clinical context. A more applicable technique would be to make multiple small volume intrastromal injections around the limbal circumference in order to maximise vector delivery to LSCs and minimise the risk of stromal scarring within the visual axis.

The major limitation of rAAV within a replicative tissue is its inability to replicate its genome upon host cell division and indeed in the corneal epithelium we observed this resulting in loss of transgene expression over time. We observed that near complete loss of epithelial transgene expression took around 18 days, in agreement with previous estimates of ~3 weeks for complete epithelial turnover⁹. Loss of transgene expression therefore proceeded too rapidly to determine the ability of AAV vectors to transduce LSCs. We therefore combined intrastromal injection of AAV with a Cre recombinase system and showed that not only was AAV-mediated recombination sustained for long enough to be highly indicative of LESC transduction, but that the extent of recombined lineages also began to approach pan-epithelial coverage. The Cre recombinase system is a laboratory tool unsuitable for clinical application, however, the key attribute of system utilised here is the induction of a permanent genomic change via transient transgene expression. This property is shared by the much more clinically applicable CRISPR gene editing system and moreover sustained expression of Cas9 may not be desirable due to an increased risk of off-target cleavage⁴⁸. These results suggest that

combining CRISPR technology with the ability of AAV to mediate extensive but transient LESC transduction *in situ*, as demonstrated here, might be a promising future avenue for the correction of epithelial dystrophies.

Intrastromal and limbal sub-epithelial injections attempted to deliver vector either directly within the target LESC niche or close to it. Whilst such approaches provide an obvious benefit in terms of cellular targeting and transduction efficiency, they are also invasive and technically demanding procedures. It has previously been demonstrated that AAV2/9 has the ability to mediate widespread transgene delivery seemingly throughout the entire murine soma (including some evidence of corneal transduction) when delivered systemically at an early post-natal timepoint⁴⁹. Recently this facet of AAV2/9 has been exploited in the treatment of young infants with spinal muscular atrophy 1 with some very promising results⁵⁰. We therefore examined the potential corneal application of this route of administration in more detail, specifically with regard to the corneal epithelium and LSCs. Systemic propagation of vector likely occurs via dissemination in blood and within the retina it has been shown that the extent of transduction resulting from systemic AAV2/9 delivery is closely correlated with the extent of retinal angiogenesis at the point of injection⁵¹, a finding mirrored in our study (**Figure S1B**). Since the cornea is largely an avascular tissue, supplied only by the limbal vascular arcades (**Figure S1A**), the degree of transduction we observed is surprising. Perhaps especially in the epithelium, in which scattered recombined TAC clones were seen in both central and limbal locations with no apparent preference to the latter. Recombined clones developed into centripetal streaks over time, many of which originated in the limbus and were sustained until at least 18 weeks post injection highly indicative of LESC transduction. The extent of the epithelial transduction achieved is not as great as by either of the other methods presented here, but remains notable in comparison to previous studies and has the potential to be further improved upon. The intraperitoneal route of systemic administration was chosen for its technical simplicity, but it is possible that an intravenous injection more proximal to the corneal vasculature might result in an improved corneal transduction. With regard to potential clinical application, it is worth considering that the human LESC niche is far more intimately associated with the vascular system than that of the mouse³⁷. Thus, greater efficacy might be achievable in the human context. Whilst not the focus of this study, the stromal transduction we observed is noteworthy, being both far more extensive than that in the epithelium and also displaying a clear correlation between the degree of transduction and the proximity to the limbal blood supply. This stromal transduction might be of particular application to the mucopolysaccharidoses, syndromic conditions for which a systemic approach would be eminently suitable and whose corneal phenotypes have proved resistant to correction via otherwise promising treatment methodologies^{52–54}.

435 To conclude, here we have presented an extensive comparison of vectors and administration
436 routes in order to begin to develop a gene delivery toolkit for the corneal epithelium with
437 potential applications in the correction of corneal epithelial dystrophies by gene therapy.

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Author Disclosure Statement

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Figure Legends

Figure 1 – Intra-epithelial injection of lentiviral vector mediates sustained epithelial transgene expression via LESC transduction

A: Percentage of injected eyes displaying centripetal streak-like patterns of transgene expression over time. (n= 88). **B:** SLO images of the eye in which the most extensive transgene expression was seen at **i)** 7 weeks, **ii)** 18 weeks, **iii)** 30 weeks, **iv)** 42 weeks, **v)** 52 weeks post injection. **C:** SLO images of the eye showing a more typical extent of transgene expression at **i)** 7 weeks, **ii)** 18 weeks, **iii)** 30 weeks, **iv)** 42 weeks, **v)** 73 weeks post injection. White: eGFP, Eye delimited by red circle. **D:** Confocal image of the streak highlighted in Cv at 73 weeks post injection. Z-projection through the basal epithelium. White: eGFP, Red: Phalloidin, Blue: DAPI, Scale bar: 250µm

Figure 2 – Intrastromal injection of AAV mediates transient trans-Bowman's epithelial transduction

A: Representative transverse section of the central cornea 4 days after intrastromal injection of Lenti-SFFV-eGFP. **B:** Representative transverse section of the central cornea 4 days after intrastromal injection of AAV8[Y733F]-CMV-eGFP. Red dotted line demarcates stroma from epithelium, white arrow towards epithelium. **C:** Representative transverse section of the central cornea 8 days after intrastromal injection of AAV8[Y733F]-CMV-eGFP. **D:** Representative transverse section of the central cornea 18 days after intrastromal injection of AAV8[Y733F]-CMV-eGFP. **E:** Orthogonal view of a confocal z-stack used to generate data in (F), XY plane set at stroma/epithelial transition, white arrows in XZ and YZ show direction of pixel quantification. Green: eGFP, Blue: DAPI, Scale bars: 50µm. **F:** Quantification of the epithelial transgene expression mediated by different serotypes of AAV. One-way ANOVA with Bonferroni's correction. Mean ± S.D., * p ≤ 0.05, ** p ≤ 0.01, n ≥ 5

Figure 3 - Intrastromal injection of AAV can mediate genomic modification of LSCs within Ai9 mice

A: Representative transverse section of the central cornea 21 days after intrastromal injection of AAV8[Y733F]-CMV-Cre. **B:** Representative transverse section of the limbal corneoscleral junction 21 days after intrastromal injection of AAV8[Y733F]-CMV-Cre. Red: tdTomato, Blue: DAPI, Scale bar: 100µm. **C:** Fluorescent funduscopy of the eye in which the most extensive recombination was seen at **i)** 4 weeks, **ii)** 8 weeks, **iii)** 11 weeks, **iv)** 13 weeks, **v)** 17 weeks post injection. **D:** Fluorescent funduscopy of the eye in which the least extensive recombination was seen at **i)** 4 weeks, **ii)** 8 weeks, **iii)** 11 weeks, **iv)** 13 weeks, **v)** 17 weeks

post injection. **E:** Confocal image of an entire cornea at 17 weeks post injection. Z-projection through the epithelium. Red: tdTomato, Blue: DAPI, Scale bar: 500µm

Figure 4 - Intraperitoneal injection of AAV2/9 at P0 mediates gene delivery to all layers of the cornea including the epithelium

A: Representative whole corneal flatmount 28 days after intraperitoneal injection of AAV2/9-CMV-Cre. Z-projection through the whole cornea, solid white line demarcates the tissue. Red: tdTomato, Scale bar: 500µm. **B:** Z-projected flatmount presented in (A), false coloured on the basis of depth in the z-axis. Calibration bar shows colours assigned to each z-plane at a resolution of 3µm. White arrows highlight “islands” of epithelial tdTomato expression. Vector mediated recombination in the **C:** endothelium **D:** stroma and **E:** basal epithelium. Z-projection through each layer, Red: tdTomato, Blue: DAPI, Scale bar: 100µm. **F:** Fluorescent funduscopy of a representative eye at i) 9 weeks ii) 12 weeks iii) 15 weeks iv) 18 weeks and v) 21 weeks post injection. **G:** Confocal image of an entire cornea at 20 weeks post injection, z-projection through the cornea. **H:** Higher magnification of area highlighted in (G), single 3µm z-plane through the basal epithelium. Red: tdTomato, Blue: DAPI, Scale bar: 250µm

Figure S1 – Correlation of systemic AAV9-mediated transduction with tissue vasculature

A: The extent of the corneal vasculature at P0; restricted to the limbus, as in the adult. White line delimits the cornea, Red: CD31, Scale bar: 250µm. **B:** Representative transverse section through the retina 28 days after P0 intraperitoneal injection of AAV8[Y733F]-CMV-Cre. Red: tdTomato, Blue: DAPI, Scale bar: 250µm.

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646 **Figure 1**

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Figure 2

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Figure 5

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665 **Supplementary Figure 1**