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Treatment of Inherited Eye Defects by Systemic Hematopoietic Stem Cell Transplantation

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PURPOSE. Cystinosis is caused by a deficiency in the lysosomal cystine transporter, cystinosin (*CTNS* gene), resulting in cystine crystal accumulation in tissues. In eyes, crystals accumulate in the cornea causing photophobia and eventually blindness. Hematopoietic stem progenitor cells (HSPCs) rescue the kidney in a mouse model of cystinosis. We investigated the potential for HSPC transplantation to treat corneal defects in cystinosis.

METHODS. We isolated HSPCs from transgenic DsRed mice and systemically transplanted irradiated *Ctns*^{-/-} mice. A year posttransplantation, we investigated the fate and function of HSPCs by *in vivo* confocal and fluorescence microscopy (IVCM), quantitative RT-PCR (RT-qPCR), mass spectrometry, histology, and by measuring the IOP. To determine the mechanism by which HSPCs may rescue disease cells, we transplanted *Ctns*^{-/-} mice with *Ctns*^{-/-} DsRed HSPCs virally transduced to express functional CTNS-eGFP fusion protein.

RESULTS. We found that a single systemic transplantation of wild-type HSPCs prevented ocular pathology in the *Ctns*^{-/-} mice. Engraftment-derived HSPCs were detected within the cornea, and also in the sclera, ciliary body, retina, choroid, and lens. Transplantation of HSPC led to substantial decreases in corneal cystine crystals, restoration of normal corneal thickness, and lowered IOP in mice with high levels of donor-derived cell engraftment. Finally, we found that HSPC-derived progeny differentiated into macrophages, which displayed tunneling nanotubes capable of transferring cystinosin-bearing lysosomes to diseased cells.

CONCLUSIONS. To our knowledge, this is the first demonstration that HSPCs can rescue hereditary corneal defects, and supports a new potential therapeutic strategy for treating ocular pathologies.

Keywords: cystinosis, hematopoietic stem cell, corneal cystine crystals, tunneling-nanotubes

Corneal disease is a significant cause of blindness worldwide,¹ and corneal transplant for hereditary or acquired diseases is limited by low availability of donor corneas and risks associated with transplantation, such as immune rejection, cataract, and glaucoma.² Stem cell transplantation represents an attractive alternative for focal corneal repair, for example with limbal epithelial stem cells to repair corneal epithelium,³ and with mesenchymal stem cells (MSCs) that have been shown to migrate and promote corneal healing, secrete soluble factors, and suppress inflammation and angiogenesis.⁴ However, repairing hereditary eye deficiencies with stem cells has remained relatively unexplored. We have been investigating the therapeutic potential of hematopoietic stem and progenitor cells in a mouse model of an inheritable lysosomal storage disease, cystinosis.

Cystinosis is an autosomal recessive lysosomal storage disorder in which cystine accumulates within lysosomes due to impairment of the transmembrane lysosomal cystine transporter, cystinosin, encoded by the ubiquitously expressed gene *CTNS*.^{5,6} Cystine accumulation leads to the formation of cystine crystals within tissues, causing cell death and eventually organ degeneration.^{7–10} Kidneys and eyes are

the primary tissues impacted by the disease in mice and humans^{11,12}; *Ctns*^{-/-} mice develop ocular pathology similar to humans.^{11,13,14} The main ocular manifestation is crystal deposition in the cornea, which begins in infancy, increases with age, and gradually leads to photophobia, blepharospasm, keratopathy, and recurrent corneal erosions.¹⁵ In older patients, filamentous keratopathy, band keratopathy, and peripheral corneal neovascularization also are observed.^{15–17} A similar spectrum is seen in the mouse model, with central corneal opacification and loss of the corneal cellular architecture, and eventually phthisis bulbi.^{11,13,14,18} Crystals also accumulate in conjunctiva, lens, sclera, ciliary body, iris, trabecular meshwork, choroid, retinal pigment epithelium, and retina, and can cause retinopathy.^{19,20} The current treatment for cystinosis is the drug cysteamine, but taken orally it does not improve ocular manifestations of the disease, presumably due to the lack of bioavailability in the cornea.²¹ Topical treatment with cysteamine hydrochloride eye drops or a more recent gel formulation is effective in reducing corneal crystal density and alleviating symptoms,^{15–17,22–24} but patients still exhibit significant crystal accumulation and

associated ocular morbidity, suggesting the need for more potent and durable therapeutics.²⁴

Hematopoietic stem and progenitor cells (HSPCs) are multipotent cells capable of self-renewal and are easily mobilized from bone marrow into the circulatory system. These cells enhance reepithelialization of corneal wounds after alkali injuries in mice,²⁵ but beyond this finding limited data exist to indicate the potential of HSPCs for reversing ocular pathology.

We demonstrated previously that systemic transplantation of wild-type HSPCs resulted in a significant decrease of cystine levels in tissues and the long-term preservation of the kidney in *Ctns*^{-/-} mice.²⁶⁻²⁸ The objective of the present study was to test whether systemic HSPC transplantation also could treat eye pathology in *Ctns*^{-/-} mice. We showed that HSPCs differentiate into macrophage progeny into ocular tissues, deliver cystinosin product locally, and improve corneal defects in *Ctns*^{-/-} mice.

METHODS

Mice

The C57BL/6 *Ctns*^{-/-} mice were provided by C. Antignac (Inserm U1163, Paris, France) and bred continuously at University of California, San Diego (UCSD). Transgenic mice constitutively expressing GFP (C57BL/6-Tg[ACTB-EGFP]10sb/J) or DsRed (B6.Cg-Tg[CAG-DsRed⁺MST]1Nagy/J) were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and both were cross-bred with the C57BL/6 *Ctns*^{-/-} mice to produce transgenic GFP *Ctns*^{-/-} and DsRed *Ctns*^{-/-} mice. All protocols were approved by the UCSD Institutional Animal Care and Use Committee (IACUC) and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Bone Marrow Cell (BMC) Isolation, Transduction, and Transplantation

Bone marrow cells were flushed from the long bones of 6- to 80-week-old GFP-transgenic mice or DsRed *Ctns*^{-/-} mice. Sca1⁺ HSCs were isolated as described previously.²⁹ One million GFP cells were directly transplanted in lethally irradiated (8 Gy) DsRed *Ctns*^{-/-} mice by tail vein injection while DsRed *Ctns*^{-/-} cells were first transduced with SIN lentivirus expressing the fused protein CTNS-GFP before transplantation in lethally irradiated (8 Gy) *Ctns*^{-/-} mice as described previously.²⁹ To analyze bone marrow engraftment of the transplanted cells, fresh blood was treated with red blood cell lysis buffer (eBioscience, San Diego, CA, USA) and subsequently analyzed by flow cytometry to the GFP-expressing cells.

Fluorescence Microscopy

Eyes were fixed in 5% formaldehyde for 2 to 3 hours and equilibrated in 20% sucrose overnight. Eyes were frozen in Tissue-Tek Optimal Cutting Temperature buffer at -80°C (Sakura Finetek USA, Inc., Torrance, CA, USA) and 20-µm sections were cut. Sections were stained with 4',6-diamidino-2-phenylindole (DAPI). The Keyence BZ-X700 microscope (Keyence, Osaka, Japan) was used to capture the pictures and the Keyence software was used to perform image stitching and 3D reconstitution.

In Vivo Confocal Microscopy (IVCM) of the Cornea and Cystine Crystal Quantification

Images of corneal nerves were collected using the Heidelberg Retina Tomograph (HRT) 3 with Rostock Cornea Module

(Heidelberg Engineering, Heidelberg, Germany) and a custom built small animal platform as described previously.³⁰ Briefly, general anesthesia was induced using 2.5% isoflurane in oxygen and GenTeal gel (Novartis Pharmaceuticals Corp., East Hanover, NJ, USA) was placed on both eyes for laser light coupling and to prevent the eye from drying. To evaluate the effectiveness of the HSC transplantation, the microscope objective was positioned at the center apex of the cornea, the depth adjusted to 25 µm and one volume stack of 40 images (384 × 384 pixels, 1 µm lateral resolution) collected. To evaluate formation of cystine crystals, the microscope operator identified easily recognizable physical attributes located as near to the apex as possible. The operator then used these attributes as landmarks to collect identical volume scan images in the same area of the cornea each week for 6 weeks. Images were imported into Matlab (MathWorks, Natick, MA, USA) and cystine crystals in each layer picture was quantified using ImageJ software (available in the public domain at <http://imagej.nih.gov/ij/>; National Institutes of Health [NIH], Bethesda, MD, USA). The threshold was adjusted to recognize specifically the cystine crystals and pixels were quantified.

IOP Measurement

Intraocular pressure was measured using the Icare TONOLAB tonometer (Icare, Vanda, Finland), as described by the manufacturer. Briefly, mice were placed on a stand with eyes positioned approximately 1 to 4 mm away from the TONOLAB tonometer, which was securely fixed to a ring stand. Corneas were aligned centrally with the TONOLAB tonometer probe and five measurements were collected and averaged from each mouse eye. Before IOP measurements, eyelashes were trimmed carefully and eyes were lightly moistened with balanced salt solution to avoid IOP measurement obstruction and errors, sometimes caused by corneal drying and tonometer probe-corneal sticking. In addition, to maintain steady readings during this procedure, mice were placed under anesthesia using an isoflurane-oxygen mixture.

Cystine Content Measurement

Explanted eyes were ground in 500 µL of N-ethylmaleimide (Fluka Biochemika, Bushs, Switzerland) at 650 µg/mL using the Precellys 24 homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). Proteins were precipitated using 15% 5-sulfosalicylic acid dihydrate (Fluka Biochemika), resuspended in NaOH 0.1 N and measured using the Pierce BCA protein assay kit (Pierce, Rockford, IL, USA). The cystine-containing supernatants were sent to the UCSD Biochemical Genetics laboratory for measurement by mass spectrometry as described previously.²⁸

Reverse Transcription and Quantitative PCR (RT-qPCR)

The RNA from explanted eyes was isolated using the RNeasy kit (Qiagen, Venlo, The Netherlands) according to manufacturer's instructions. One microgram of RNA was reverse transcribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The *Ctns*-specific qPCR was performed using 2 µL of cDNA, 2X SsoAdvanced Universal Probes Supermix (Bio-Rad Laboratories, Inc.), and primers and probe were obtained from Applied Biosystems (Carlsbad, CA, USA): *Ctns* oligo 1: 5'-TTGTGGTGCAGTCGGTATC-3', *Ctns* oligo 2: 5'-AGCTTGATGTAGGAGAAGCAGAAGA-3', and *Ctns* probe: 5'-CACATGGCTCCAGTTC-3', and 18S primer mix on a CFX96 (Bio-Rad Laboratories, Inc.). The expression level of *Ctns* gene, expressed as fold change, was calculated using the

$\Delta\Delta Ct$ method between the target gene and an endogenous control (18S).

Histology and Central Cornea Thickness (CCT) Measurement

Explant eyes were fixed in formalin and embedded in paraffin. Sections (10 μm) were stained with hematoxylin and eosin. Using a light microscope (Keyence), pictures of the central cornea were acquired ($\times 20$) and CCT as well as the epithelial layer thickness were measured using Keyence software at 3 different parts of the cornea and averaged.

Immunofluorescence Analysis

Eyes were fixed in 5% formaldehyde for 2 to 3 hours and equilibrated in 20% sucrose overnight. Eyes were frozen in Tissue-Tek Optimal Cutting Temperature buffer at -80°C (Sakura Finetek USA) and 20- μm sections were cut. Sections were stained with DAPI and with Rat-anti-F4/80 (14-4801-82, eBioscience), Rat-anti-MHC Class II [NIMR-4] (ab25333, Abcam) and Mouse-anti-CD11c [3.9] (ab11029; Abcam, Cambridge, UK) antibodies. To quantify HSPCs-derived cells (DsRed) and F4/80⁺ cells, three sections per animal were used and cells were counted manually in the entire cornea. To visualize the nanotubes, all pictures were acquired using a $\times 63$ objective on an Olympus FluoView1000 (Olympus, Center Valley, PA, USA) or Leica TCS SP5 II confocal microscope (Leica Microsystems, Buffalo Grove, IL, USA). Images were processed with ImageJ or Volocity software (Perkin Elmer, Waltham, MA, available in the public domain at www.perkinelmer.com) to generate 3D reconstructions from optical slices (Z-stacks).

Statistics

We summarized continuous data as arithmetic means \pm SEM. Group comparisons were made using *t*-test. Associations between blood engraftment and *Ctns* expression or cystine content was investigated with linear regressions. The χ^2 test was used to identify whether IOP above or below 20 mm Hg was dependent on cell engraftment. All analyses were performed using Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered as statistically significant.

RESULTS

HSPCs Migrate to Different Regions of the Eye in *Ctns*^{-/-} Mice

Lethally irradiated *Ctns*^{-/-} mice between 1.5 and 3.5 months of age were transplanted with wild-type DsRed-expressing bone marrow stem cells to study the fate and impact of HSPCs on the corneal defects in cystinosis. Nontreated *Ctns*^{-/-} mice and lethally irradiated *Ctns*^{-/-} irradiated mice transplanted with DsRed *Ctns*^{-/-} HSPCs were used as controls. At 1 year after transplantation, bone marrow-derived cells were present through various parts of the eyes of *Ctns*^{-/-} mice, including the cornea, retina, lens, sclera, and ciliary margin (Fig. 1a), demonstrating that systemically injected HSPCs migrate to and integrate in the diseased eyes.

To determine the level of donor bone marrow stem cell engraftment, DsRed-positive cells were quantified in peripheral blood samples by flow cytometry; engraftment ranged from 16 to 95.4% (Low, $n = 7$, mean = $27.91\% \pm 12.24\%$; High, $n = 7$, mean = $85.46\% \pm 6.09\%$). Mice with high levels of engraftment ($>50\%$; High) exhibited more abundant bone marrow-derived cells within the eyes compared to mice with a low level of

engraftment ($<50\%$; Low; Fig. 1a). This histologic observation was confirmed by RT-qPCR quantitative analysis of *Ctns* expression (derived from the donor cells), demonstrating that *Ctns* expression in the eyes of the treated *Ctns*^{-/-} mice increased with increasing engraftment levels ($F_{1,9} = 9.635$, $P = 0.0146$; Fig. 1b). Since we previously showed that a correlation exists between level of donor-derived blood cell engraftment and long-term preservation of the kidney,²⁸ we assessed the subsequent structural, biochemical, and functional effect of HSPCs on corneas in the *Ctns*^{-/-} mice by differentiating between the High and Low engraftment groups, compared to age-matched wild-type and nongrafted *Ctns*^{-/-} controls.

Prevention of Corneal Cystine Crystal Accumulation in *Ctns*^{-/-} Mice With High HPSC Engraftment

First, tissue cystine content was measured by mass spectrometry in whole enucleated eye. A significant decrease in cystine levels was observed in high ($n = 6$) and low ($n = 3$) treated mice compared to *Ctns*^{-/-} controls ($n = 3$, reductions of 85% and 73%, respectively; Fig. 2a). To evaluate corneal cystine crystals, we performed IVCN in live mice, scanning through the entire cornea, differentiating the epithelium, Bowman's membrane, stroma, Descemet's membrane, and endothelium (Fig. 2b). Crystals in each layer then were quantified using ImageJ software (Fig. 2c). Because there is asymmetry of crystal deposition between the two eyes of the same animal,¹⁸ we analyzed each eye individually. At 1 year of age, all *Ctns*^{-/-} mouse controls (nontreated or transplanted with *Ctns*^{-/-} HSPCs, $n = 4$) exhibited abundant cystine crystals within all layers of the cornea, with the majority being present in the anterior stroma and endothelium layers. The *Ctns*^{-/-} mice with low level of engraftment (Low; $n = 5$) showed a partial reduction of crystal counts, and mice with high engraftment levels (High; $n = 5$) exhibited almost a complete resolution of crystals from the epithelial layer to the mid-stroma (100% and 72% clearance, respectively; Fig. 2c). These data showed, for the first time to our knowledge, that systemic HSPC transplantation has a significant beneficial impact on corneal cystine crystals and also suggested that some minimum level of *Ctns*-expressing bone marrow-derived cells must be present in the cornea to resolve the corneal cystine crystals in the stroma and epithelium.

Cystine crystal levels in the posterior stroma also were significantly reduced in the High engraftment mice, by 28% (Fig. 2c), a less dramatic reduction than with the anterior cornea. No reduction in the posterior stroma was found in Low engraftment animals. These data might be explained by the kinetics of the formation of the corneal cystine crystals that were shown to first appear in the peripheral posterior stroma/corneal endothelium.¹⁸ Thus, crystal formation may occur within the endothelium before HSPC transplantation, and our data suggested that, although HSPCs prevent progression to stromal layers, they do not remove crystals already present in the endothelium at 2 months.

Rescue of Corneal Atrophy in HSPC-Treated *Ctns*^{-/-} Eyes

Corneas, and in some cases the entire eye, degenerate over time in *Ctns*^{-/-} mice.^{11,13,14,18} To evaluate whether HSPCs prevent corneal atrophy in *Ctns*^{-/-} mice, we measured the CCT in hematoxylin-stained eye sections, as described previously.³¹ We found that the cornea of old *Ctns*^{-/-} mice (approximately 13 months of age; $n = 4$) was significantly thinner than in wild-type animals ($n = 4$), an effect most

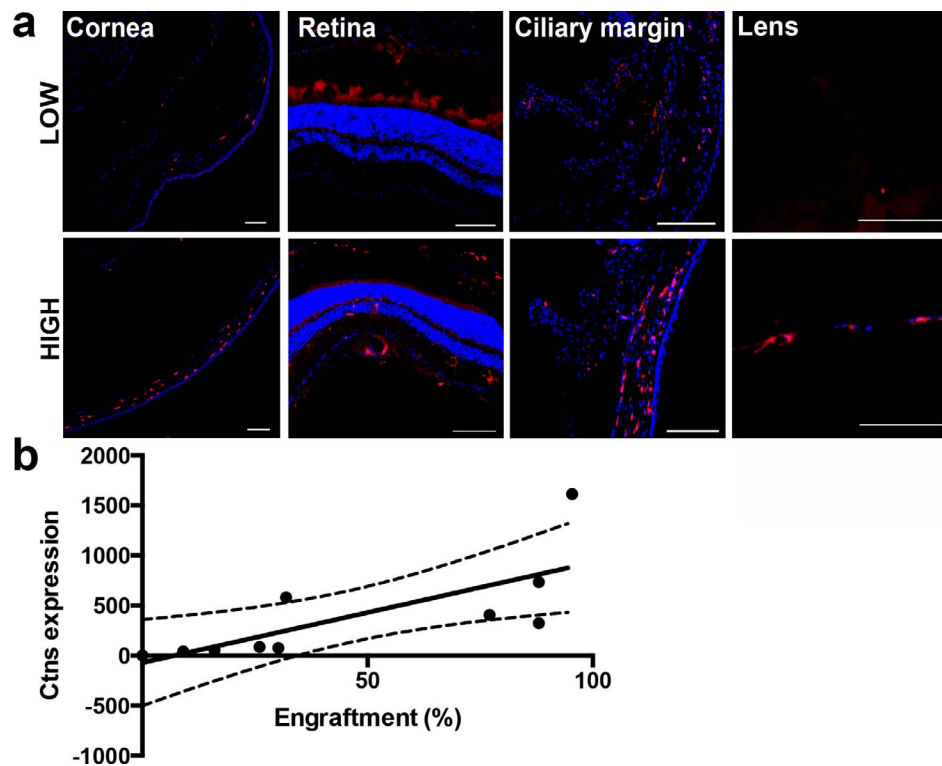


FIGURE 1. Ocular engraftment of transplanted HSPCs depends on donor-derived blood cell engraftment. (a) Systemic DsRed-expressing HSPC transplantation resulted in the presence of bone marrow-derived cells (seen in red) in the whole eye as shown in sagittal sections of the eyes of mice with donor-derived blood cell engraftment <50% (Low) compared to >50% (High). More abundant HSPC-derived cells were seen when donor-derived blood cell engraftment in High than in Low mice in the different eye compartments. Scale bars: 100 μ m. (b) Linear regression analysis of Ctns expression in the whole eye showing that Ctns expression increases with the level of transplanted DsRed-HSPCs. The linear regression interrupted lines correspond to 95% confidence.

apparent in the epithelial layer (Fig. 3a). Interestingly, HSPC transplantation ($n = 6$) prevented the reduction of CCT and allowed maintenance of normal epithelial structure in mice with a high level of engraftment (Fig. 3b). Data from Ctns^{-/-} mice with low levels of engraftment also showed an improvement of corneal structure, but histological sections were only available from two animals (data not shown). Thus HSPC engraftment prevents late corneal atrophy in cystinosis.

Rescue of Elevated IOP in HSPC-Treated Ctns^{-/-} Eyes

Cystinosis leads to IOP fluctuations and elevation in mice and humans.^{13,24} IOP measures in Ctns^{-/-} mice were significantly higher compared to wild-type mice (see Table), reaching greater than 20 mm Hg in 5 of 54 eyes. In contrast, high- and low-HSPC engraftment in HSPC-treated Ctns^{-/-} mice demonstrated decreased IOP compared to Ctns^{-/-} mice, with no observed measures above 20 mm Hg ($P = 0.006$, χ^2 test).

Mechanism of Corneal Rescue by HSPC Transplantation in Ctns^{-/-} Mice

We recently showed that the cellular mechanism of kidney repair in HSPC-transplanted cystinosis mice involved lysosomal cross-correction via tunneling nanotubes (TNTs) after HSPC differentiation into macrophages.²⁹ Here, we asked whether the same mechanism is observed in eyes. Confocal microscopy showed that most HSPC progeny in the cornea differentiated into mature macrophages, as confirmed by the colocalization of all bone marrow-derived DsRed-positive cells with F4/80

(Fig. 4c). Quantification of F4/80-positive cells within corneas of nontransplanted animals showed that Ctns^{-/-} mice exhibit approximately 75% more macrophages in the corneas compared to wild-type mice, presumably due to the underlying tissue injury and degeneration associated with cystinosis (Figs. 4a, 4b). We then compared the level of tissue engraftment and differentiation potential of wild-type HSPCs and Ctns^{-/-} HSPCs by quantifying and characterizing the DsRed-positive cells in the cornea of Ctns^{-/-} mice transplanted with either DsRed wild-type HSPCs and DsRed Ctns^{-/-} HSPCs. We found that the level of engraftment and phenotype of transplanted wild-type and Ctns^{-/-} bone marrow-derived cells within the cornea was equivalent in the two groups (Figs. 4c, 4d). These data suggested that wild-type HSPCs show no advantage in migration, differentiation, or integration in ocular tissues compared to Ctns^{-/-} donor cells. However, more endogenous, DsRed-negative F4/80-positive cells were found in corneas from animals transplanted with Ctns^{-/-} HSPCs compared to wild-type HSPCs-transplanted mice (Fig. 4d). These results suggested that tissue injury in Ctns-deficient mice leads to

TABLE. IOP in Control and HSPC-Transplanted Mice

| | N | Eyes Closed | Average IOP, mm Hg | χ^2 Test, IOP > 20 mm Hg; P Value |
|---------------------|----|-------------|--------------------|--|
| Wild-type | 44 | 1 | 11.51 \pm 3.72 | 0.006 |
| Ctns ^{-/-} | 54 | 7 | 15.17 \pm 7.17 | |
| Low | 16 | 1 | 13.12 \pm 2.96 | |
| High | 56 | 4 | 12.65 \pm 3.30 | |

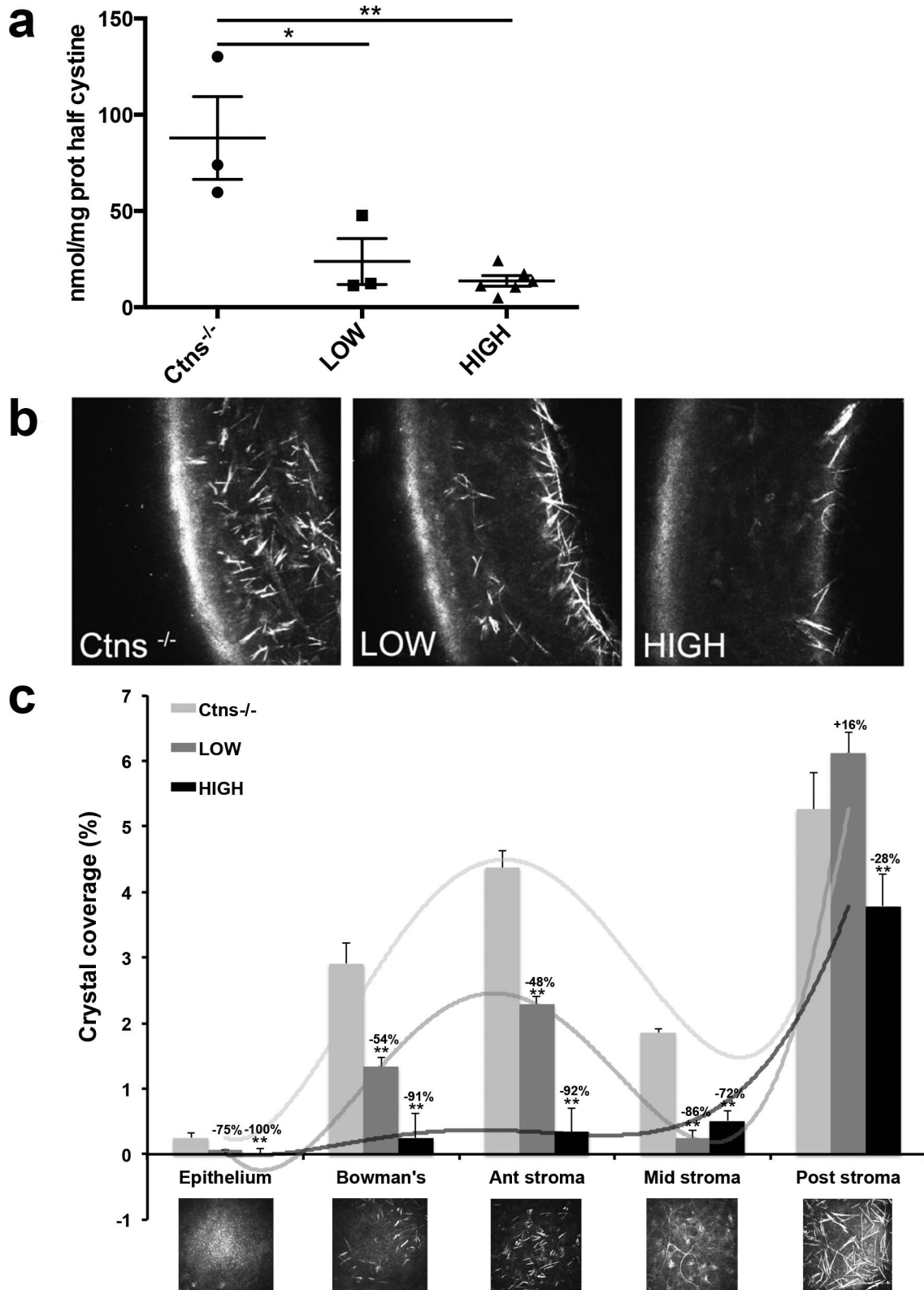


FIGURE 2. Transplantation of HSPC leads to ocular cystine and corneal cystine crystal clearance. (a) Cystine levels measured in the whole eye were significantly reduced in the HSPC-transplanted mice, Low and High, compared to the Ctns^{-/-} controls. Error bars: SEM (*P < 0.05, **P < 0.005). (b) Lateral cornea ICM representations of Ctns^{-/-} controls and Low and High HSPC-transplanted mice. Abundant cystine crystals were observed in the whole cornea in Ctns^{-/-} mice in contrast to Ctns^{-/-} with high level of engraftment where residual crystals can be seen only in the posterior stroma. (c) Surface crystal quantification within each layer of the full ICM cornea scans from both eyes of Ctns^{-/-} controls and transplanted (Low and High) mice. Transplantation of HSPC with high level of engraftment prevents almost completely cystine crystal formation within the superior layers of the cornea (epithelium to middle stroma). The mice with low level of engraftment exhibit significant but more modest crystal reduction. Error bars: SEM (*P < 0.05, **P < 0.005).

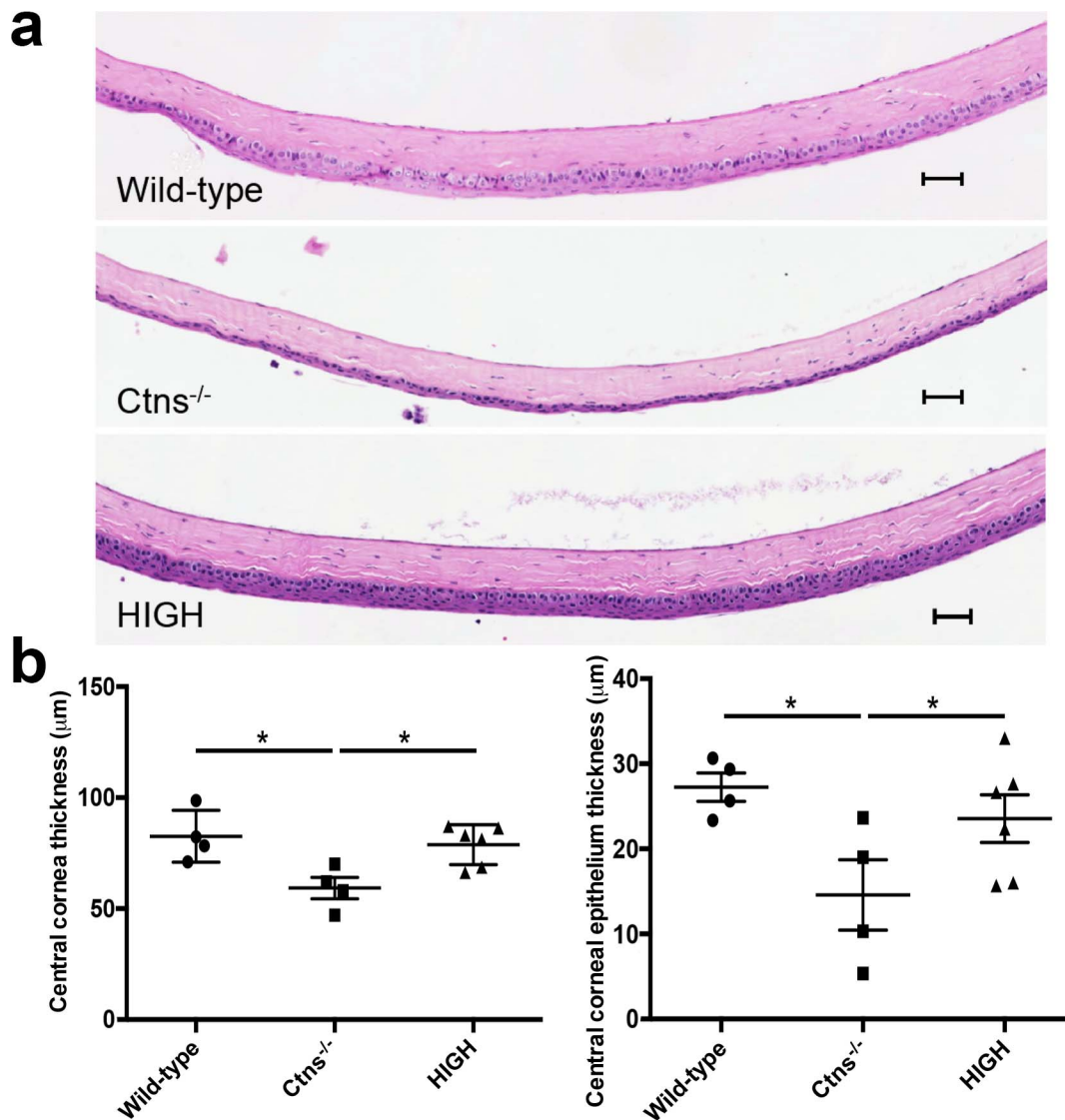


FIGURE 3. Corneal structure is preserved in HSPC-transplanted *Ctns*^{-/-} mice. (a) Representative sagittal hematoxylin and eosin sections of the central cornea of wild-type mice, nontreated *Ctns*^{-/-}, and highly engrafted *Ctns*^{-/-} (High) mice. The *Ctns*^{-/-} mice exhibit markedly reduced CCT compared to wild-type animal, while treated *Ctns*^{-/-} mice presented with CCT comparable wild-type. Scale bars: 200 μm. (b) Thickness measurement of the central cornea and central corneal epithelium of wild-type, *Ctns*^{-/-}, and transplanted (High) *Ctns*^{-/-} mice showing that HSPC transplantation prevents corneal reduction. Error bars: SEM (**P* < 0.05).

chronic inflammation, which is reduced when the macrophages express a functional *Ctns* gene associated with tissue rescue. Further characterization of the macrophages showed that most of the macrophages expressed MHC-II (Fig. 4e). We also observed that few HSPCs (~10%) colocalized with CD11c suggesting that some of the HSPCs also differentiated into dendritic cells (Fig. 4e).

Higher magnification confocal images showed that bone marrow-derived macrophages form TNT structures. We identified TNTs generated from bone marrow-derived cells in the cornea sclera margin, ciliary margin and retina (Fig. 5a). To test if lysosomal cross-correction occurs in the cornea, we transplanted normal *Ctns*^{-/-} mice with DsRed *Ctns*^{-/-} HSPCs stably expressing cystinosin-eGFP fusion protein or with DsRed *Ctns*^{-/-} HSPCs as control. Eyes were analyzed 7 months posttransplantation by confocal microscopy. No GFP-positive lysosomes were identified in *Ctns*^{-/-} mice transplanted with DsRed *Ctns*^{-/-} HSPCs alone (Fig. 5b, upper). In contrast, we observed cystinosin-eGFP-bearing lysosomes within the DsRed-

positive bone marrow-derived cells as expected in mice transplanted with cystinosin-eGFP-HSPCs and also within host corneal cells, directly demonstrating lysosomal transfer from the HSPC-derived cells to the adjacent diseased cells (Fig. 5b, lower). Thus protein transfer from donor to host cells is detected in the corneas of HSPC-treated *Ctns*^{-/-} mice, providing evidence for lysosomal cross correction in the eye.

DISCUSSION

Here we report for the first time that systemic HSPC transplantation can treat corneal defects in the context of a degenerative, hereditary metabolic disorder, cystinosis. This treatment led to a long-term prevention of corneal cystine crystal deposition, preservation of corneal structure, and reduction in IOP in the *Ctns*^{-/-} mice. These results demonstrate that HSPCs have the capacity to produce bone marrow-derived cells that home to and integrate into injured/diseased eyes, to

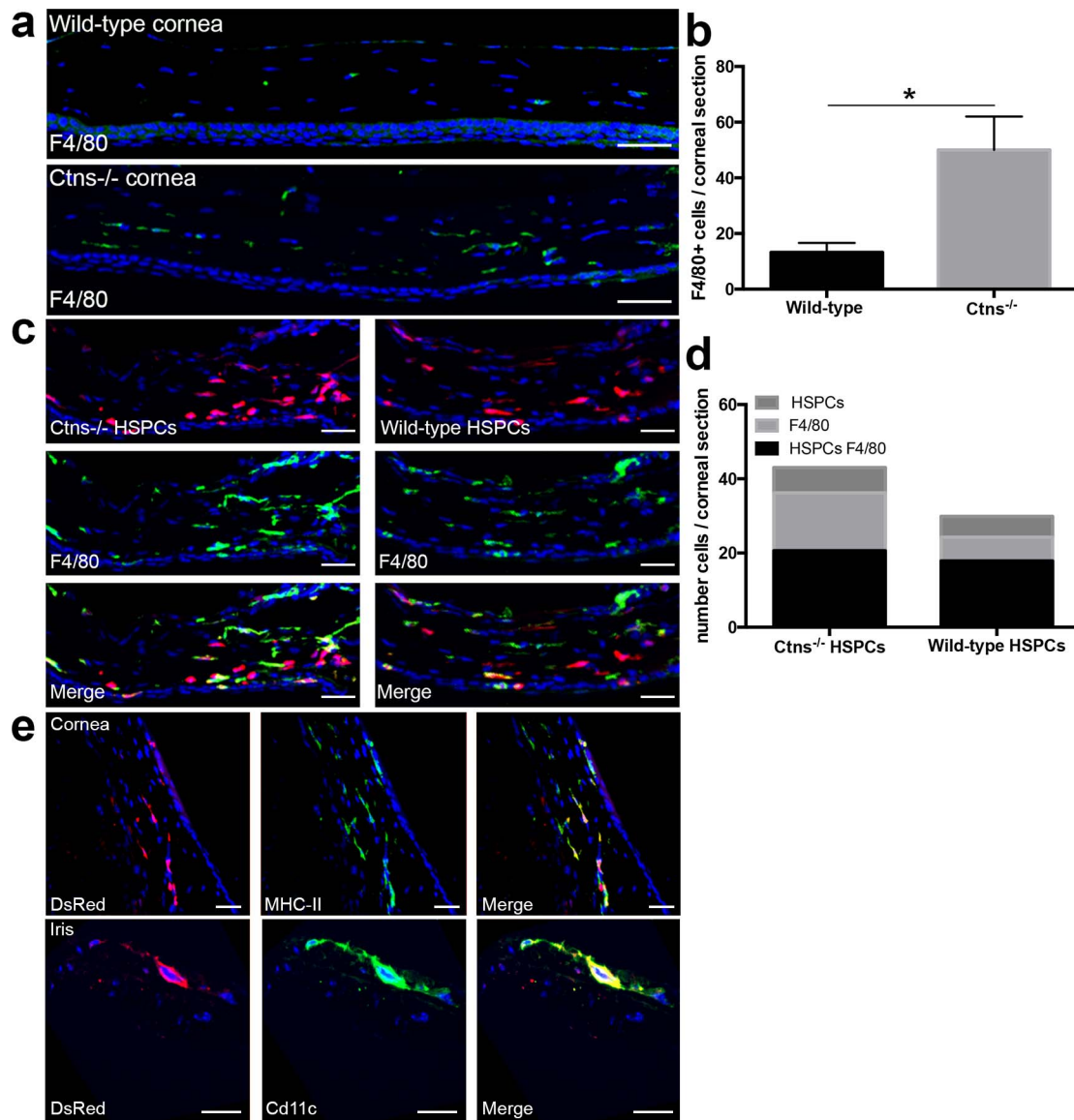


FIGURE 4. Hematopoietic stem and progenitor cells differentiate into phagocytic cells within the cornea. (a) Representative confocal pictures of corneal sections from wild-type and Ctns^{-/-} mice. Nuclei were stained with DAPI are seen in blue and F4/80⁺ cells are seen in green. (b) Quantification of F4/80-positive cells within corneas of wild-type ($n = 4$) and Ctns^{-/-} ($n = 4$) mice. (c) Representative confocal pictures of corneal sections from Ctns^{-/-} mice transplanted with either DsRed Ctns^{-/-} HSPCs or DsRed wild-type HSPCs. Nuclei were stained with DAPI are seen in blue, HSPCs-derived cells are seen in red, and F4/80-positive cells are seen in green. (d) Quantification of DsRed-positive HSPC-derived cells (HSPCs), host F4/80-positive cells (F4/80), and F4/80-positive and DsRed-positive HSPC-derived cells (HSPCs F4/80) within the corneas of Ctns^{-/-} mice transplanted with Ctns^{-/-} DsRed HSPCs ($n = 5$) or wild-type DsRed HSPCs ($n = 7$) mice. (e) All the DsRed-expressing bone marrow-derived colocalize with the antigen presenting cells (APC) marker MHC-II class II (seen in green, upper). Few of them expressed CD11c (seen in green, lower) indicating that some of them differentiated into dendritic cells. Error bars: SEM (* $P < 0.05$). Scale bars: (a, c) 50 μ m and (e) 30 μ m.

actively participate in the preservation of eye function and structure over prolonged periods of time. These results are particularly important considering that they demonstrate this correction in the avascular cornea tissue after systemic transplantation. We present in this study 1-year posttransplantation data, but there is no indication that results would not extend to the entire life span of the study animals, as previously shown for the kidney.²⁸ Therefore, transplantation of normal, CTNS-expressing HSPCs in patients affected with cystinosis may represent a long-term treatment that can preserve disease-affected tissues, including the kidneys and the eyes. Based on these data, ocular exams will be included as important efficacy endpoints in an upcoming hematopoietic stem cell-based human clinical trial for cystinosis. We do not know whether

transplanting HSPCs into aged Ctns^{-/-} mice with advanced ocular deficits will rescue established tissue damage, an important future study when considering the potential for therapeutic impact when treating adults with cystinosis.

We found that long-term preservation of ocular function and structure in the Ctns^{-/-} mice were dependent on achieving a relatively high level of donor-derived blood cell engraftment of Ctns-expressing cells (>50%), similar to our prior studies of kidney function.³² Improvement in some measures was noted in treated mice with a low level of engraftment (<50%) compared to Ctns^{-/-} controls, but for corneal crystal accumulation, an outcome comparable to wild-type was achieved only following high levels of engraftment. Thus, it seems that a critical load of properly engrafted HSPCs is required to achieve a meaningful

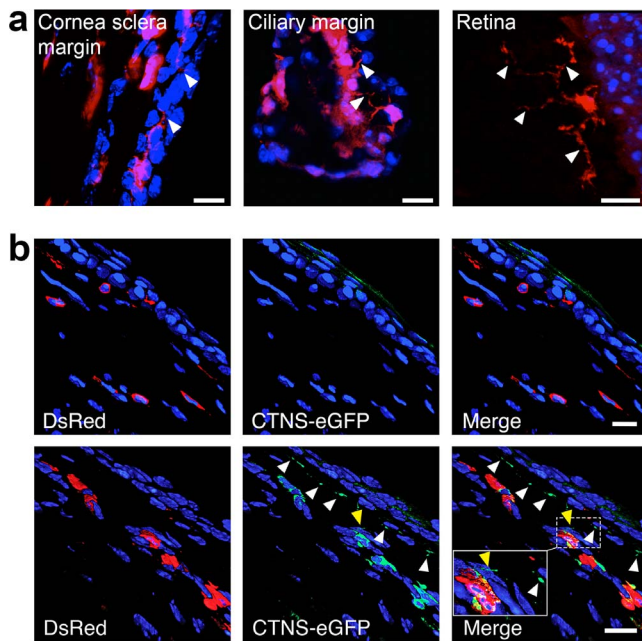


FIGURE 5. HSPCs-derived macrophages transfer cystinosin-bearing lysosomes via TNTs to the adjacent disease cells. **(a)** Tunneling nanotubes generated from the DsRed bone marrow-derived cells were observed in the cornea (sclera margin and ciliary margin) and retina (arrows). **(b)** Representative confocal pictures of the cornea of *Ctns*^{-/-} mice transplanted with *Ctns*^{-/-} DsRed HSPCs as control (upper) or with *Ctns*^{-/-} DsRed HSPCs stably expressing the fusion protein CTNS-eGFP (lower). Green vesicles are observed within the DsRed-bone marrow-derived cells in the mice treated with CTNS-eGFP-HSPCs but also in the no color host cells (white arrowheads) showing that lysosomes containing CTNS-eGFP could be transferred. Inset shows a TNT containing cystinosin-bearing vesicles (yellow arrowhead). Scale bars: (a) 10 μ m, (b) 15 μ m.

integration of disease-correcting bone marrow-derived cells within the eyes. Thus, maximizing engraftment will have to be considered for the clinical application of this strategy. As a major complication associated with allogeneic HSPC transplantation is graft-versus-host disease, which also can lead to severe ocular damage,³³ a clinical trial currently being developed by our group for cystinosis will use autologous transplantation of ex vivo gene-modified HSPCs using a lentiviral vector containing *CTNS* cDNA.²⁶

The major pathological ocular symptom described in cystinosis patients is the presence of corneal cystine crystals that result in photophobia, keratopathy, and recurrent corneal erosions.¹⁵ In vivo confocal analysis revealed abundant cystine crystals first in the posterior stromal-endothelium layers and later progressing anteriorly, similar to previous descriptions.^{13,14} Interestingly, we observed that the crystal accumulation in the middle stroma of *Ctns*^{-/-} mice was lower than the anterior and posterior stroma. This differed from one study of 8 patients where the highest amount of cystine crystals was found in the anterior stroma and less in the mid- and posterior stroma,²⁴ although this could be explained by breakdown of crystals and/or scarring.¹⁸ Studying the time course of corneal engraftment of the HSPC-derived cells will be important to determine differences in tissue response between the anterior and posterior stroma.

Our data revealed significantly lower amounts of crystals within the anterior region of the cornea (epithelium and upper stroma) in high HSPC engrafted *Ctns*^{-/-} mice. A previous study performed on *Ctns*^{-/-} mice treated with topical cysteamine

applied 4 times daily for 4 weeks beginning at 5 months showed that the drug only delays the formation of cystine crystals; the cysteamine-treated group still showed a 15% increase in the crystal volume index.¹⁸ Similar results were observed in patients using a newer gel formulation of topical cysteamine.²⁴ Our data suggested that HSPC transplantation could be a more efficient alternative, almost totally preventing crystal formation in the cystinotic eyes. However, the prevention of cystine crystal accumulation was observed to a lesser extent in the posterior than in the anterior cornea. This may be explained by prior findings that engrafted HSPC-derived cells localize initially in the anterior stroma and later migrate to the posterior stromal layers.³⁴ Thus, we hypothesize that by the time the HSPCs reach the posterior stroma, crystal formation and accumulation has occurred and cannot be reversed. Note that HSPC-derived cells also were present in the sclera, ciliary margin, iris, retina, choroid, and to lesser degree the lens, all previously identified sites of crystal accumulation.¹⁴ Cystine clearance in these regions also may have increased in HSPC-engrafted animals, as suggested by the significant decrease of global ocular cystine content in the HSPC treated mice (Fig. 2a).

Central corneal thickness was thinner in the *Ctns*^{-/-} mice, and restoration of physiological corneal thickness was found in the treated mice with high level of engraftment. Thin corneas may lead to an underestimation of IOP,^{35,36} but increased IOP in these nontreated *Ctns*^{-/-} mice may have been caused by corneal sclerosis or scarring.³⁷ In contrast, physiological IOPs were observed in the HSPC-engrafted *Ctns*^{-/-} mice. It also should be noted that pupillary block may elevate IOP in cystinosis patients and in the mouse model.^{13,38} This is caused by a deposition of cystine crystals in the iris, which can lead to a seal between the iris and lens, bulging the iris forward, and closing the flow of aqueous fluid out the trabecular meshwork.^{13,39} Thus, in addition to corneal-related IOP lowering, it also is possible that lower IOP we observed in HSPC engrafted *Ctns*^{-/-} mice was due to lower crystal accumulation in the iris, where HSPC-derived cells were found (Fig. 4e). We also found HSPC-derived cells in the ciliary margin (Fig. 5a) and at locations in or around the trabecular meshwork, but it is not clear whether these cells have a role in lowering IOP since most documented cystinosis cases conclude that IOP increases as a result of crystal accumulation in the iris and not decreased trabecular meshwork outflow.³⁹ These data emphasized the powerful impact of HSPC transplantation on the long-term structural and functional preservation of the eye.

We showed that most of the HSPC-derived bone marrow-derived cells that migrated to the eye were F4/80⁺ MHC class II⁺ macrophages, with a few dendritic cells. Interestingly, we observed that these macrophages generated TNTs as observed previously within the kidney.²⁹ These membrane extensions are heterogeneous cell-connecting tubules that have been involved in pathogen spreading and intercellular communication.^{40,41} In vivo existence of TNT was reported initially in MHC II-positive cells present in mouse corneal stroma.⁴² The function of these nanotubes has been attributed to the transfer of small molecules, such as MHC and/or MHC-antigen complexes, to help the propagation of immune responses.⁴²⁻⁴⁴ kely to provide lysosomal cross-correction by delivering cystinosin-bearing lysosomes to the adjacent diseased corneal cells, thereby providing functional cystine clearance mechanisms for *Ctns*^{-/-} cells, similar to the TNT-mediated crossing of the tubular membrane that halts kidney degeneration.²⁹ Therefore, TNTs generated by MHC class II⁺ bone marrow-derived cells, which penetrate corneal layers, are likely leading to the our presented findings of structural and functional preservation in corneas. Cystinosin-containing microvesicle

transfer from the bone marrow-derived macrophages to the host cells is an alternative route for cystinosis cross-correction. Other groups have reported that microvesicles/exosomes shed by CTNS-expressing cells could lead to significant cystine decrease in cystinotic cells *in vitro*.^{45,46} This mechanism also has been described in co-culture assays of mucopolysaccharidosis VII fibroblasts with umbilical MSCs and as the mechanism responsible for the rescue of the corneal defects in mice after direct corneal transplantation of the MSCs.⁴⁷ However, we previously demonstrated using coculture assays of wild-type MSCs or macrophages with Ctns-deficient fibroblasts that, while microvesicle transfer could lead to a substantial decrease of cystine in the fibroblasts (approximately 19% clearance), TNT-mediated lysosomal transfer was a more efficient route (approximately 75% clearance).²⁹

Taken together, these results suggested that, rather than replacing defective corneal tissues with transplanted or stem cell-derived tissues, the transfer mode of action between gene-corrected, HSPC-derived donor cells and diseased cells and tissues may be an efficient alternative to correct genetic deficiencies and stabilize ocular pathology. Future directions should include determining if there is a therapeutic window for disease correction, if HSPCs delivered directly within the eyes have the same therapeutic potential as systemic delivery, and if similar therapeutic efficacy can be detected in human patients.

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