CD147 Required for Corneal Endothelial Lactate Transport

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Submitted: March 18, 2014

Accepted: June 10, 2014

Citation: Li S, Nguyen TT, Bonanno JA. CD147 required for corneal endothelial lactate transport. *Invest Ophthalmol Vis Sci*. 2014;55:4673–4681. DOI:10.1167/iovs.14-14386

PURPOSE. CD147/basigin is a chaperone for lactate:H⁺ cotransporters (monocarboxylate transporters) MCT1 and MCT4. We tested the hypothesis that MCT1 and -4 in corneal endothelium contribute to lactate efflux from stroma to anterior chamber and that silencing CD147 expression would cause corneal edema.

METHODS. CD147 was silenced via small interfering ribonucleic acid (siRNA) transfection of rabbit corneas ex vivo and anterior chamber lenti-small hairpin RNA (shRNA) pseudovirus in vivo. CD147 and MCT expression was examined by Western blot, RT-PCR, and immunofluorescence. Functional effects were examined by measuring lactate-induced cell acidification, corneal lactate efflux, [lactate], central cornea thickness (CCT), and Azopt (a carbonic anhydrase inhibitor) sensitivity.

RESULTS. In ex vivo corneas, 100 nM CD147 siRNA reduced CD147, MCT1, and MCT4 expression by 85%, 79%, and 73%, respectively, while MCT2 expression was unaffected. CD147 siRNA decreased lactate efflux from 3.9 ± 0.81 to 1.5 ± 0.37 nmol/min, increased corneal [lactate] from 19.28 ± 7.15 to 56.73 ± 8.97 nmol/mg, acidified endothelial cells ($\text{pH}_i = 6.83 \pm 0.07$ vs. 7.19 ± 0.09 in control), and slowed basolateral lactate-induced acidification from 0.0034 ± 0.0005 to 0.0012 ± 0.0005 pH/s, whereas apical acidification was unchanged. In vivo, CD147 shRNA increased CCT by 28.1 ± 0.9 μm at 28 days; Azopt increased CCT to 24.4 ± 3.12 vs. 12.0 ± 0.48 μm in control, and corneal [lactate] was 47.63 ± 6.29 nmol/mg in shCD147 corneas and 17.82 ± 4.93 nmol/mg in paired controls.

CONCLUSIONS. CD147 is required for the expression of MCT1 and MCT4 in the corneal endothelium. Silencing CD147 slows lactate efflux, resulting in stromal lactate accumulation and corneal edema, consistent with lactate efflux as a significant component of the corneal endothelial pump.

Keywords: CD147, lactate transport, MCT1, MCT4, corneal endothelial pump

The cornea's energy needs are supplied predominantly from glucose.¹ Glucose enters the cornea from the aqueous humor across the corneal endothelium and diffuses through the stroma to the corneal epithelium. Corneal epithelial cells are very glycolytic, converting 85% of glucose to lactate.^{2–4} Therefore, for every 100 glucose molecules entering the cornea, 170 lactate molecules are produced. The accumulation of lactate within the cornea can create an osmotic load that will increase stromal hydration and corneal thickness.⁵ To maintain corneal hydration and transparency, the large quantity of lactate must be simultaneously exported out. Since the surface corneal epithelial cells are impermeable to lactate, lactate efflux must take place across the endothelium. Efflux is driven by the 2-fold [lactate] gradient from cornea to anterior chamber.^{5,6}

Facilitated transport of lactate has been extensively studied in rabbit corneal endothelium.^{7,8} Lactate along with a H⁺ is cotransported through the plasma membrane of many cell types via stereospecific, pH-dependent monocarboxylate transporters (MCT1–4).^{9–18} Recently, we have identified MCT1, -2, and -4 in human,¹⁹ bovine,²⁰ and rabbit²¹ corneal endothelium. Interference with primary or secondary active transporters that regulate intracellular pH (pH_i), reduced buffering by removal of HCO_3^- , or inhibition of carbonic anhydrases slowed lactate fluxes, led to corneal lactate accumulation, and increased corneal thickness, indicating

that lactate efflux is a significant component of the endothelial pump.^{20,21}

CD147, an evolutionary conserved member of the immunoglobulin superfamily, has gained considerable attention for its multiple functions involved in reproduction, neural function, inflammation, and tumor invasion. It is also described in the literature as EMMPRIN, basigin (Bsg), TCSE, M6, OK, 5A11, Gp42, CE9, and neurothelin.^{22–31} CD147 is a highly glycosylated protein with a single-transmembrane domain that serves as a chaperone for proper folding and trafficking of MCT1 and MCT4 to the plasma membrane.^{32–34} CD147 silencing can reduce the malignant potential of pancreatic tumors via the inhibition of MCT-dependent lactate transport.³⁵ Dysfunction of the retina in Bsg^(-/-) mice is ascribed to the failure of plasma membrane integration of MCTs.³⁶ The expression of MCT1 and -4 in corneal endothelium suggests that CD147 is also expressed and will be important for MCT expression and membrane trafficking. In the current study, we examined the effect of CD147 knockdown on the expression of endothelial MCTs and corneal lactate efflux ex vivo as well as corneal [lactate] and corneal thickness in vivo. We found that reducing expression of MCT1 and MCT4 reduces corneal endothelial lactate flux and results in an increase in corneal thickness. These results suggest that maintaining corneal lactate efflux can be considered as an approach to treat corneal edema in dystrophic corneal endothelium.

MATERIALS AND METHODS

Culturing Corneas Ex Vivo and Small Interfering Ribonucleic Acid (siRNA) Transfection

Three CD147 siRNA duplexes were synthesized by Ambion (Life Technologies, Carlsbad, CA, USA) and designated as s444928: 5'-GGAAGGAAGUGGUGAAGGAtt-3', s444929: 5'-GAAUUAAGGCUGUGAAGAAAtt-3', and s444930: 5'-UCAUCUU CAUCUACGAGAAtt-3'. A scrambled nontargeting siRNA was purchased from Invitrogen (Life Technologies, Grand Island, NY, USA). The transfection reagent HiPerFect was purchased from Qiagen (Pleasanton, CA, USA).

New Zealand White rabbit eyes were obtained from Pel-Freez Biologicals (Rogers, AR, USA) and shipped overnight on ice. Corneas were excised with a scleral skirt, and the corneal cup was placed endothelial surface facing up on a ring adaptor that allows the tissue to retain normal curvature within standard six-well culture plates. The transfection mixture containing 10 pmol siRNA and 5 μ L HiPerFect in 100 μ L Opti-MEM (Life Technologies) was applied to the endothelial surface of the cornea and placed in a 37°C, 5% CO₂ incubator for 6 hours. The transfection mixture was then removed, and the cornea was immersed in 2 mL Dulbecco's modified Eagle's medium with 10% bovine calf serum (BCS) and 1% antibiotic/antimycotic. The medium was changed twice per day. Three days post transfection, lactate efflux and corneal [lactate] were measured. Alternately, the endothelium was peeled for extraction of protein and mRNA, for immunofluorescence staining, and for intracellular pH (pH_i) measurements.

Corneal Lactate Efflux

Corneal cups were washed once with PBS and moved (with the culture adaptor) into a Plexiglass incubator warmed to 37°C and gassed with 5% CO₂. One hundred microliters of a bicarbonate-rich Ringer's solution (BR) was pipetted into the endothelial concavity. The BR consisted of (mM) 150 Na⁺, 4 K⁺, 0.6 Mg²⁺, 1.4 Ca²⁺, 118 Cl⁻, 1 H(PO₄)²⁻, 10 HEPES, 28.5 gluconate, 5 glucose, and 28.5 NaHCO₃⁻. A 10- μ L sample was taken, saved for lactate assay, and replenished with 10 μ L fresh BR every 5 minutes for 30 minutes. After washing with PBS once, central cornea thickness (CCT) was measured using an E.T-1 contact lens thickness gauge with 1- μ m resolution (Rehder Development Co., Castro Valley, CA, USA). The cornea was then trephined to a 10-mm central button and immediately placed in a mortar, submerged in liquid nitrogen to stop further metabolism, and pulverized using a pestle. The powder was then collected in an Eppendorf tube filled with 0.5 mL PBS, vortexed vigorously for 30 seconds, and centrifuged at 1800g for 15 minutes. The supernatant was collected for lactate assay, and the pellet was retained for assay standardization. The pellet was dried in a vacuum centrifuge for 2 hours at 30°C and then weighed. Lactate concentration was determined using a lactate assay kit from BioVision Research Products (Milpitas, CA, USA) and represented as nmol lactate/mg dry tissue.

Real-Time RT-PCR

Total RNA was extracted from rabbit corneal endothelium peeled with Descemet's membrane using TRIzol reagent (Invitrogen) followed by RNeasy column (Qiagen) purification. Complementary DNA was generated using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA) at 10 ng RNA/ μ L reverse transcription. Real-time PCR was performed using SYBR Green PCR Master Mix (Agilent Technologies, Eugene, OR, USA). The CD147-specific primers were 5'-TTAAGGCTGTGAAGAAGTCGGAGC-3' and 5'-GCTTCTCGTAGATGAAGATGACGG-3'. β -actin (ACTB) primers

were 5'-TGACCGACTACCTCATGAAGATCC-3' and 5'-CGCACTTCATGATCGAGTTGAAGG-3'. All assays used similar amplification efficiency, and a 2^{- $\Delta\Delta$ Ct} experimental design was used for relative quantification and normalized to ACTB.

Western Blotting

Western blots were produced as described previously.^{20,21} Primary antibodies to CD147 and MCT1, -2, and -4 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti- β -actin, anti-mouse IgG, and anti-rabbit IgG were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Freshly peeled endothelium was disrupted with RIPA lysis buffer solution (50 mM Tris base, 150 mM NaCl, 0.5% deoxycholic acid-sodium salt, 2% SDS, 1% nonyl phenoxypolyethoxyethanol, and protease inhibitor cocktail, pH 7.5). Protein (10 μ g) was separated by SDS-PAGE and transferred to membranes, and relative protein expression level was assessed by densitometric quantitative analysis and normalized to β -actin expression.

Immunofluorescence

As described in previous publications,^{20,21} freshly peeled corneal endothelium was placed with apical surface (anterior chamber facing) up and basolateral side (stromal facing) down on a glass slide, and fixed with 2% paraformaldehyde solution containing 75 mM lysine, 10 mM sodium periodate, and 45 mM sodium phosphate, pH 7.4. Endothelial cells were permeabilized using 0.01% saponin-0.1% Triton X-100 for 10 minutes. The same primary antibodies used for Western blotting were applied, diluted 1:200 with goat serum. Secondary antibodies were Alexa 488-labeled anti-rabbit IgG and Alexa 595-labeled anti-mouse IgG, 1:1000. The tissue was mounted with a glass coverslip using Prolong antifade media (Life Technologies).

Intracellular pH_i

After siRNA transfection, the cornea was trephined to a 10-mm central button and placed in a bicarbonate-free Ringer's produced by equimolar substitution of NaHCO₃ with Na-gluconate. The BF solution was equilibrated with air and adjusted to pH 7.5 and osmolality 295 to 300 mOsm. The endothelial surface was loaded with the pH-sensitive fluorescent dye BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxy-fluorescein) by incubating the tissue in 1 mL BF solution containing 5 μ M BCECF-AM (acetoxymethylester; Life Technologies) at room temperature for 30 minutes. The tissue was then rinsed and incubated in 2 mL BF solution for 30 minutes. The Descemet's-endothelium was peeled from the button using sharp forceps and placed onto a 13-mm-diameter, 45- μ m-thick, 0.2- μ m-pore diameter rigid anodisc filter with apical side up and then mounted in a double-sided perfusion chamber.^{37,38} The chamber was placed on a water-jacketed (37°C) brass collar held on the stage of an inverted microscope and viewed with a long working distance (2 mm) water-immersion objective (40 $\times$; Nikon, Inc., Melville, NY, USA). Apical and basolateral compartments were connected by tubing to hanging syringes that contained BF solution in a 37°C warming box. The flow of the perfusate (approximately 0.5 mL/min) was achieved by gravity. Two independent eight-way valves were used to select the desired perfusate for the apical and basolateral chambers, respectively. Once steady-state pH_i was obtained, the endothelium was pulsed with 30 mM lactate for 25 seconds on the apical or basolateral surfaces or both. Fluorescence was excited alternately at 495 $\pm$ 10 and 440 $\pm$ 10 nm. The fluorescence emission (520–550 nm) ratio (F₄₉₅/F₄₄₀) was obtained at 1 Hz and calibrated against pH_i by the high-K⁺-nigericin technique, as previously described.²⁰ The

FIGURE 1. CD147 was silenced by siRNA transfection. (A), (C), and (E) show representative Western images. (B), (D), and (F) are quantitative analyses of the images. CD147 expression was normalized to β -actin (ACTB). (A) and (B) show effects of three CD147 siRNAs (100 nM) and a scrambled sequence siRNA. In (C) and (D), the optimal dosage of the siRNA s444930 used in a 3-day transfection was tested. In (E) and (F), the effect of s444930 (100 nM) over 0 to 7 days of transfection was tested. (G) and (H) show results of real-time RT-PCR. Asterisk indicates statistical significance; see Results.

initial rate of pH_i change (dpH_i/dt) was determined from a linear fit to the initial 20 seconds following addition of lactate.

CD147 Small Hairpin RNA (shRNA) Knockdown In Vivo

New Zealand White rabbits (age: 8–10 weeks; weight: 2.0–2.5 kg) were purchased from Harlan Laboratories (Indianapolis, IN, USA). All animal procedures were performed in accordance with the Indiana University Animal Care and Use Committee and the ARVO Statement for the Use Animals in Ophthalmic and Vision Research.

Based on the s444930 siRNA sequence, an shRNA targeting rabbit CD147 (GGATCCGTCATCTTCATCTACAAGCACTTCCTGTCAGTTCTCGTAGATGAAGATGATTTTGAATTC) and an shRNA targeting luciferase (GGATCCGGTGCGTTGCTAGTACCAACCCCATCTCTTCCTGTGAGAAATAGGGTTGGTACTAGCAACGCACTTTTGAATTC) were cloned into SI500 plasmid. These two constructs were packaged into lentivector and supplied as pseudoviral particles by SBI (System Biosciences, Inc., Mountain View, CA, USA). The transduction reagent, Transdux, was purchased from SBI.

Before performing anterior chamber injection, rabbits were sedated by intramuscular injection of ketamine-HCl (30 mg/kg) and xylazine-HCl (5 mg/kg). Topical anesthesia and pupil dilation were achieved with two drops of 0.5% proparacaine-

HCl (Akorn, Lake Forest, IL, USA) and one drop of 1% cyclopentolate-HCl (Ocusoft, Rosenberg, TX, USA). A pseudoviral suspension (20 μ L) was prepared with sterile saline solution containing 5×10^6 infectious units (IFU) lenti-shCD147 or lenti-shLuc mixed with Transdux reagent (SBI) and loaded into a Hamilton syringe with a 30-gauge needle (Hamilton Company, Reno, NV, USA). Under a surgical microscope (Nikon, Inc.), the needle was inserted perpendicular to the cornea near the limbus, advanced partway through the stroma, and then turned toward the center of the anterior chamber. With the needle bevel facing the endothelium, the lenti-shRNA suspension was injected over 1 minute. The needle was kept in place for another minute and then slowly removed to prevent leakage. Antibiotic ointment (Fera Pharmaceuticals LLC, Locust Valley, NY, USA) was applied to the injection site. Since pilot studies had shown that unilateral lentiviral injection does not produce a contralateral effect, lenti-shCD147 was injected in the left eye (OS); lenti-shLuc was injected in the right eye (OD), serving as a paired control.

Corneal Thickness Measurement and Azopt Test

Rabbit CCT in vivo was measured using an iVue (Optovue, Inc., Fremont, CA, USA) optical coherence tomography (OCT) imager before viral injection and every other day thereafter over 4 weeks. The OCT measurements can be gathered without sedation by gently restraining the rabbits by wrapping in a towel, while an assistant takes the images. On day 16, an Azopt stress test was performed. Two drops of 1% brinzolamide (Alcon, Fort Worth, TX, USA) were instilled on the ocular surface. The CCT was then measured 1, 3, 5, and 7 hours later. Four weeks after viral injection, the rabbits were killed and corneas collected. Endothelium was quickly dissected for protein isolation, and the remaining cornea was analyzed for lactate content as described above.

Data and Statistical Analysis

Data are presented as mean $\pm$ SD. One-way ANOVAs followed by post hoc tests, independent *t*-tests, or paired *t*-tests were used as stated in Results, and values were considered significant at $P \leq 0.05$.

RESULTS

CD147 siRNA

Figures 1A and 1B show that CD147 protein was significantly reduced by 100 nM s444930 siRNA (82.3%, $P = 0.0014$, ANOVA), while s444928 (7.8%, $P = 0.568$) and s444929 (29.6%, $P = 0.004$) had less effect. Dose analysis indicated that 100 nM siRNA produced the maximum effect (79.3%, $P = 0.0006$) (Figs. 1C, 1D). A time course analysis of CD147 expression over a period of 7 days revealed that HiPerFect-mediated siRNA transfection produced the greatest silencing of CD147 at 72 hours ($P = 0.0003$, Figs. 1E, 1F). Figures 1G and 1H show that the effect of s444930 on CD147 protein expression is consistent with the 77.7% decrease ($P = 0.0003$) in CD147 mRNA, while scrambled sequence siRNA had no effect (1.4% decrease, $P = 0.966$).

The Effect of CD147 Silencing on the Expression of MCT1 and MCT4

Three MCT isoforms (MCT1, MCT2, and MCT4) were detected in rabbit corneal endothelium.²⁰ CD147 is tightly linked to the expression, membrane targeting, and function of MCT1 and MCT4.³² In the present study, we confirmed this association in

FIGURE 2. The effect of silencing CD147 on the expression of MCT1, -2, and -4. (A) and (B) show Western images and densitometry analysis. Rabbit corneas were transfected by using 100 nM CD147 siRNA (s444930) or scrambled siRNA (scRNA) for 3 days. Target genes were normalized to ACTB. Asterisk indicates statistical significance; see Results. (C) Immunofluorescence of CD147 and MCT1, -2, and -4 (magnification: $\times 60$).

ex vivo rabbit corneal endothelium. Western blot and immunofluorescence analyses were used to examine MCT expression in CD147 siRNA (s444930)-treated and control corneas. As shown in Figure 2A, three MCTs were detected at the predicted band size of 50 kDa. Densitometry analysis of these bands showed that CD147, MCT1, and MCT4 protein level, normalized to β -actin, in CD147 siRNA-treated corneas was reduced by 82.3% ($P = 0.0010$), 73.3% ($P = 0.0013$), and

67.2% ($P = 0.006$, ANOVA followed by post hoc testing, $n = 12$ per condition), respectively, while MCT2 was unaffected relative to control corneas treated with scrambled sequence siRNA (scRNA, Figs. 2A, 2B). The protein level in control corneas was similar to that detected in vehicle (no siRNA)-treated corneas (Fig. 2A, lane 1), indicating that scRNA and the transfection reagent had no effect on the expression of CD147 and MCTs.

FIGURE 3. Effects of CD147 silencing on lactate-induced acidification and corneal lactate efflux. Rabbit corneas were transfected with 100 nM siRNA (scRNA or s444930) for 3 days. Dissected Descemet's-endothelium was perfused with BF Ringer's solution and pulsed with 30 mM sodium lactate for 25 seconds. AP, apical surface. BL, basolateral surface. AP+BL, both. (A) Intracellular pH trace in scRNA-transfected corneas recorded for 100 seconds. (B) Intracellular pH trace in s444930-transfected corneas recorded for 100 seconds. (C) Average steady-state pHi (baseline); (D) pHi in response to lactate; (E) dpHi/dt during the first 20 seconds of lactate application. Data shown in the figure are representative of nine independent experiments. Asterisk indicates statistical significance; see Results.

Figure 2C shows immunofluorescence staining of CD147 and MCTs following transfection with vehicle, scRNA, or s444930. Monocarboxylate transporters were clearly visible in control corneas (left column). A similar staining pattern was seen in the corneas transfected with scRNA (middle column). However, staining intensities of CD147, MCT1, and MCT4 were significantly decreased in CD147 siRNA-treated corneas (right column). In contrast, MCT2 staining was not affected, consistent with Western blot analysis. These results indicate that CD147 is required for MCT1 and MCT4 expression.

Effect of CD147 Silencing on Endothelial Cell pH

To examine the physiological effect of CD147 silencing on the corneal endothelium, lactate-induced acidification (LIA) was measured in dissected Descemet's-endothelium from ex vivo rabbit corneas that were transfected with CD147 or scrambled siRNA. As shown in Figures 3A, 3B, and 3C, steady-state (baseline) pHi was lower in s444930- relative to scRNA-treated

corneas (6.83 ± 0.086 vs. 7.19 ± 0.067 , $P = 0.021$, independent *t*-test). When the apical surface was pulsed with lactate, ΔpHi (0.098 ± 0.008 vs. 0.111 ± 0.008) and $\Delta pHi/dt$ (0.0039 ± 0.0005 vs. $0.0043 \pm 0.0005/s$) were not significantly different between siRNA-treated corneas and controls, respectively ($n = 11$, Figs. 3D, 3E). However, when the basolateral surface was perfused with lactate, ΔpHi was 0.037 ± 0.004 pH units in s444930-treated corneas and 0.078 ± 0.006 in scRNA corneas, respectively ($n = 12$, $P = 0.0004$, Fig. 3D). The mean rate of pHi change declined 2.8-fold from 0.0034 ± 0.0005 pHi/s in control to 0.0012 ± 0.0002 $\Delta pHi/s$ in CD147 siRNA-treated corneas ($P = 0.00034$, Fig. 3E). When corneal endothelium was pulsed with lactate on both the apical and basolateral surfaces simultaneously, the average ΔpHi and mean dpHi/dt were similar to values with an apical pulse alone. These results demonstrate that decreased MCT1 and MCT4 expression via CD147 knockdown slowed basolateral lactate

FIGURE 4. Effects of CD147 silencing on corneal thickness, lactate efflux, and stromal [lactate] ex vivo. **(A)** Measurements of central corneal thickness (CCT) at 3 days following siRNA transfection in culture. **(B)** Central cornea thickness change relative to control corneas (no siRNA transfection). **(C)** Corneal lactate efflux (nmol/min) in scrRNA- and s444930-transfected corneas, respectively. **(D)** Corneal [lactate] expressed as nmol/mg dry corneal tissue. Asterisk indicates statistical significance; see Results.

transport. The findings provide direct and specific evidence for the role of CD147 in corneal endothelial lactate transport.

Effects of CD147 Silencing on Corneal Thickness and Lactate Accumulation

If lactate efflux is a significant component of the endothelial pump, then silencing CD147 and the reduced MCT1 and MCT4 expression should cause reduced lactate flux from stroma to anterior chamber, increased stroma [lactate], and an increase in corneal thickness. Figure 4A shows that the average CCT following transfection and 3 days in organ culture was $592 \pm 26.58 \mu\text{m}$ in corneas with no siRNA transfection, $617 \pm 30.299 \mu\text{m}$ in scrRNA corneas, and $784 \pm 39.48 \mu\text{m}$ in s444930 corneas. Figure 4B shows that the increase in CCT was $25.5 \pm 39.2 \mu\text{m}$ with scrRNA ($P = 0.153$) and $192.3 \pm 54.1 \mu\text{m}$ ($P = 0.00047$, ANOVA with post hoc testing) with s444930 relative to no siRNA.

To provide direct and specific evidence for the role of CD147 in the corneal endothelium, we measured lactate efflux

in ex vivo rabbit corneas that were transfected with scrambled or CD147 siRNA. Bicarbonate-rich Ringer's solution (0.1 mL) was added into each corneal cup and lactate was sampled over 30 minutes (Fig. 4C). The average lactate efflux was 3.92 ± 0.81 and 1.56 ± 0.37 nmol/min in control ($n = 11$) and CD147 siRNA-treated corneas ($n = 12$, $P = 0.003$, independent t -test), respectively. Total corneal [lactate] was then determined, and as expected, Figure 4D shows that more lactate was retained in the siRNA-treated corneas. The average concentration of lactate was 19.28 ± 7.15 and 56.73 ± 8.97 nmol/mg dry tissue in scrRNA and siCD147 corneas, respectively ($P = 0.0016$).

Effects of Lenti-CD147 shRNA In Vivo

The feline immunodeficiency virus-based lentiviral vector carrying shCD147 or shLuc can efficiently transfect corneal endothelial cells from the anterior chamber of New Zealand White rabbit eyes.³⁹ A preliminary dose trial had indicated that 5×10^6 IFU lenti-shRNA particles was most effective in silencing

FIGURE 5. Effects of CD147 silencing in vivo. 5×10^6 IFU lenti-shRNA was delivered into the anterior chamber of New Zealand White rabbits. Lenti-shLuc (targeting luciferase) was applied to right eyes (OD), serving as control, and lenti-shCD147 to left eyes (OS). (A) Average central corneal thickness (CCT) over 4 weeks after lenti-shRNA injection. (B) Azopt stress test (two drops of 1% brinzolamide) at day 14 produced 3.3% to 6.5% increases in CCT in OD and OS, respectively. (C) Representative corneal OCT images. (D) The expression of CD147 and MCTs by Western blotting. (E) Quantitative assay of the knockdown of CD147 and MCT1, -2, and -4. β -actin (ACTB) served as loading control. The expression level was expressed as shCD147/scRNA (percentage). (F) The measurement of corneal [lactate] expressed as nmol/mg dry cornea tissue. Asterisk indicates statistical significance; see Results.

CD147 expression. An initial anterior chamber inflammation occurred within 24 hours of injection and resolved in 48 to 72 hours. As shown in Figure 5B, corneal swelling was observed in the eyes receiving CD147 shRNA at around 6 days and reached a steady state at 16 days, which was $28.1 \pm 0.9 \mu\text{m}$ ($P = 0.001$, paired *t*-test) thicker than for paired control eyes. Figure 5C shows representative OCT pictures at 21 days.

In rabbits, the topical carbonic anhydrase inhibitor Azopt (brinzolamide) will cause corneal swelling that is enhanced if the endothelial cell bicarbonate transport is compromised.³⁹ The Azopt stress test was performed at 14 days post injection. Two drops of 1% brinzolamide were applied topically. As shown in Figure 5B, peak changes in corneal thickness occurred at 3 hours. Swelling was $24.4 \pm 3.12 \mu\text{m}$ in shCD147-treated corneas and $12.0 \pm 0.48 \mu\text{m}$ in control, or $6.5\% \pm 0.007$ and $3.3\% \pm 0.007$ swelling, respectively ($P = 0.004$). Figure 5C (bottom row) shows representative OCT pictures. This finding indicates that reduced expression of CD147 and MCT1 and -4 has compromised corneal endothelial function.

To confirm that corneal swelling resulted from reduced CD147 expression, rabbits were killed at 4 weeks post injection and endothelial cell protein was examined by Western blotting. As shown in Figures 5D and 5E, CD147

was reduced by $67\% \pm 0.03$, MCT1 by $60\% \pm 0.06$, and MCT4 by $55\% \pm 0.11$ in shCD147 eyes (OS) in comparison to shLuc eyes (OD). Monocarboxylate transporter 2 was not changed. Consequently, corneal lactate concentration was remarkably different, with 47.63 ± 6.29 nmol/mg dry cornea tissue in OS and 17.82 ± 4.93 nmol/mg in OD ($P = 0.0012$, paired *t*-test) (Fig. 5F).

DISCUSSION

In this study, we examined the role of CD147/basigin in the transport of lactate across the rabbit corneal endothelium. Rabbits have been used as the primary species for studying corneal endothelial function, and comparisons with human cornea have shown good concordance.^{40,41} One difference that has emerged is the apparent greater sensitivity of rabbit cornea to topical carbonic anhydrase inhibitors.²⁰ Rabbit endothelium also has a higher proliferative capacity relative to human, but this is not germane to the current study.

Using cultured ex vivo rabbit corneas, we found evidence supporting the notion that CD147 is required to sustain the expression and function of monocarboxylate transporters (MCT1 and MCT4). Previous studies suggest that MCT1 and

MCT4 are assembled with a CD147 subunit forming a basolateral membrane complex.³² Although the protein abundance of MCT1 and MCT4 can be regulated at the genetic level, proper folding and trafficking require CD147 in targeting MCTs to the plasma membrane.^{42–44} We found that CD147 siRNA directly reduced expression of not only CD147, but MCT1 and -4 as well. This was also shown in retina.^{36,42}

Interestingly, CD147 is a broadly expressed protein with particularly high levels in eye tissues, such as retina pigment epithelium (RPE) and corneal endothelium (CE). In RPE, MCT1/CD147 is polarized to the apical membrane and MCT3/CD147 to the basolateral membrane.⁴² It was reported that targeting of MCT1 and -4 to retinal plasma membrane requires association with 5A11/basigin (CD147). The expression of MCT1, -3, and -4 was severely reduced in Bsg^{-/-} mice,³⁶ which was thought to compromise energy metabolism in the retina, leading to abnormal photoreceptor cell function and degeneration.

Reduced availability of MCT1 and -4 lowered resting corneal endothelial pH_i, reduced and slowed LIA, reduced corneal lactate efflux, and increased corneal thickness. The reduction in resting pH_i can be explained by the reduced ability to move lactic acid (lactate coupled to H⁺) from the cytosol to the extracellular bath. In the kinetic analysis of lactate-dependent cellular acidosis, we found that both lactate-induced ΔpH_i and the rate of pH_i change at the basolateral surface were decreased (Fig. 3) when the membrane expression of MCT1 and MCT4 was reduced. These results are in accordance with the basolateral membrane location of MCT1 and MCT4 in rabbit CE.²⁰ Apical LIA was unaffected. Previous immunofluorescence data indicated that MCT2 is an apical membrane protein.²⁰ However, the current immunofluorescence data (Fig. 2) suggest that it may be present on both basolateral and apical membranes. Only MCT1 through MCT4 have been determined to be lactate:H⁺ cotransporters¹⁶ and MCT3 is not expressed in CE,²¹ suggesting that apical flux is carried by MCT2. Further analysis of MCT2 membrane localization is needed.

Ex vivo experiments showed that at the whole-cornea level, reduced endothelial MCT1 and -4 expression slowed endogenous lactate efflux from the cornea with concomitant increase in retained corneal [lactate]. As expected, this caused an increase in corneal hydration (as measured by increased thickness), which is consistent with previous studies showing that increased stromal [lactate] causes corneal edema due to the increased osmotic pressure within the cornea.^{5,6}

Similar to the pseudo-lentivirus approach used to knock down the sodium bicarbonate transporter (NBCe1),³⁹ we knocked down the expression of CD147 in the New Zealand White rabbits. Within a week, corneal thickness was significantly elevated relative to that in the pseudovirus-luciferase shRNA-injected eyes. This CCT difference was most apparent at 2 weeks, after which it appeared to level off, at least over the 4-week period of observation. The increase in CCT is consistent with the reduced MCT1 and -4 expression and increased corneal [lactate] found in the freshly dissected corneas at 4 weeks post injection.

CD147 knockdown increased the sensitivity to the topical carbonic anhydrase inhibitor Azopt (Figs. 4B, 4C). Unlike what occurs in humans, topical Azopt causes a small amount of corneal swelling in the rabbit that is enhanced in corneas with reduced NBCe1 activity.³⁹ Carbonic anhydrase activity, which catalyzes the hydration of CO₂ and dehydration of bicarbonate, speeds acid buffering that in turn facilitates lactate:H⁺ membrane fluxes. Azopt alone leads to corneal lactate accumulation in the rabbit.²⁰ Reduced NBCe1 activity or, as shown here, reduced MCT-dependent lactate:H⁺ cotransport activity interacts synergistically with the reduction in carbonic anhydrase activity to produce enhanced corneal swelling. Interestingly, although normal human corneas are not sensitive

to topical carbonic anhydrase inhibitors, several clinical reports^{45–49} have shown that in patients with corneal guttata or reduced endothelial cell counts, carbonic anhydrase inhibitors can cause edema and irreversible swelling. One possibility is that human endothelial carbonic anhydrase capacity is much greater than in other species.

The cornea is a significant source of lactate.^{1,3} Because the surface squamous cells are impermeable to lactate, it must diffuse posteriorly across the endothelium and be washed out via aqueous humor flow. This sets up a standing gradient in which stromal [lactate] is ~13 mM and anterior chamber [lactate] ~7 mM.⁵ Therefore, any process that facilitates lactate efflux from the cornea will help maintain stromal hydration. Conversely, any interference with lactate efflux will cause corneal swelling, which was confirmed by our experimental results. Previously, we demonstrated that inhibition of primary active transport, reducing cellular buffering capacity by knocking down Na⁺:2HCO₃⁻ cotransport (NBCe1), pharmacological inhibition of bicarbonate transport, or reducing carbonic anhydrase activity each produced increases in stromal [lactate] and increased corneal thickness.^{21,39} Taken together, these results support the notion that lactate efflux is a significant component of the endothelial pump. A model for this activity was presented previously.^{7,20}

Acknowledgments

The authors thank Michelle Brown, John Sowinski, and Diego G. Ogando for their technical assistance.

Supported by National Institutes of Health Grants RO1EY08834 (JAB), KO8EY019537 (TN), and P30EY019008.

Disclosure: S. Li, None; T. Nguyen, None; J.A. Bonanno, None

References

- Riley MV. Glucose and oxygen utilization by the rabbit cornea. *Exp Eye Res.* 1969;8:193–200.
- Kuhlman RE, Resnik RA. The oxidation of C14-labeled glucose and lactate by the rabbit cornea. *Arch Biochem Biophys.* 1959;85:29–36.
- Riley MV. Aerobic glycolysis in the ox cornea. *Exp Eye Res.* 1969;8:201–204.
- Thoft RA, Friend J. Corneal epithelial glucose utilization. *Arch Ophthalmol.* 1972;88:58–62.
- Klyce SD. Stromal lactate accumulation can account for corneal oedema osmotically following epithelial hypoxia in the rabbit. *J Physiol.* 1981;321:49–64.
- Morley N, McCulloch C. Corneal lactate and pyridine nucleotides (PNS) with contact lenses. *Arch Ophthalmol.* 1961;66:379–382.
- Bonanno JA. Molecular mechanisms underlying the corneal endothelial pump. *Exp Eye Res.* 2012;95:2–7.
- Giasson C, Bonanno JA. Facilitated transport of lactate by rabbit corneal endothelium. *Exp Eye Res.* 1994;59:73–81.
- Bergersen L, Johannsson E, Veruki ML, et al. Cellular and subcellular expression of monocarboxylate transporters in the pigment epithelium and retina of the rat. *Neuroscience.* 1999; 90:319–331.
- Bonanno JA. Lactate-proton cotransport in rabbit corneal epithelium. *Curr Eye Res.* 1990;9:707–712.
- Broer S, Broer A, Schneider HP, Stegen C, Halestrap AP, Deitmer JW. Characterization of the high-affinity monocarboxylate transporter MCT2 in *Xenopus laevis* oocytes. *Biochem J.* 1999;341(pt 3):529–535.
- Broer S, Schneider HP, Broer A, Rahman B, Hamprecht B, Deitmer JW. Characterization of the monocarboxylate trans-

- porter 1 expressed in *Xenopus laevis* oocytes by changes in cytosolic pH. *Biochem J.* 1998;333(pt 1):167-174.
13. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. *Biochem J.* 2000;350(pt 1):219-227.
 14. Gerhart DZ, Leino RL, Drewes LR. Distribution of monocarboxylate transporters MCT1 and MCT2 in rat retina. *Neuroscience.* 1999;92:367-375.
 15. Grollman EF, Philp NJ, McPhie P, Ward RD, Sauer B. Determination of transport kinetics of chick MCT3 monocarboxylate transporter from retinal pigment epithelium by expression in genetically modified yeast. *Biochemistry.* 2000;39:9351-9357.
 16. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem J.* 1999;343(pt 2):281-299.
 17. Halestrap AP, Wilson MC. The monocarboxylate transporter family—role and regulation. *IUBMB Life.* 2012;64:109-119.
 18. Philp NJ, Yoon H, Grollman EF. Monocarboxylate transporter MCT1 is located in the apical membrane and MCT3 in the basal membrane of rat RPE. *Am J Physiol.* 1998;274:R1824-R1828.
 19. Schmedt T, Chen Y, Nguyen TT, Li S, Bonanno JA, Jurkunas UV. Telomerase immortalization of human corneal endothelial cells yields functional hexagonal monolayers. *PLoS One.* 2012;7:e51427.
 20. Nguyen TT, Bonanno JA. Lactate-H(+) transport is a significant component of the in vivo corneal endothelial pump. *Invest Ophthalmol Vis Sci.* 2012;53:2020-2029.
 21. Nguyen TT, Bonanno JA. Bicarbonate, NBCe1, NHE, and carbonic anhydrase activity enhance lactate-H+ transport in bovine corneal endothelium. *Invest Ophthalmol Vis Sci.* 2011;52:8086-8093.
 22. Altruda F, Cervella P, Gaeta ML, et al. Cloning of cDNA for a novel mouse membrane glycoprotein (gp42): shared identity to histocompatibility antigens, immunoglobulins and neural-cell adhesion molecules. *Gene.* 1989;85:445-451.
 23. Biswas C, Zhang Y, DeCastro R, et al. The human tumor cell-derived collagenase stimulatory factor (renamed EMMPRIN) is a member of the immunoglobulin superfamily. *Cancer Res.* 1995;55:434-439.
 24. Fadool JM, Linser PJ. Differential glycosylation of the 5A11/HT7 antigen by neural retina and epithelial tissues in the chicken. *J Neurochem.* 1993;60:1354-1364.
 25. Igakura T, Kadomatsu K, Kaname T, et al. A null mutation in basigin, an immunoglobulin superfamily member, indicates its important roles in peri-implantation development and spermatogenesis. *Dev Biol.* 1998;194:152-165.
 26. Kennedy KM, Dewhirst MW. Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation. *Future Oncol.* 2010;6:127-148.
 27. Muramatsu T, Miyauchi T. Basigin (CD147): a multifunctional transmembrane protein involved in reproduction, neural function, inflammation and tumor invasion. *Histol Histopathol.* 2003;18:981-987.
 28. Nehme CL, Fayos BE, Bartles JR. Distribution of the integral plasma membrane glycoprotein CE9 (MRC OX-47) among rat tissues and its induction by diverse stimuli of metabolic activation. *Biochem J.* 1995;310(pt 2):693-698.
 29. Schlosshauer B, Bauch H, Frank R. Neurothelin: amino acid sequence, cell surface dynamics and actin colocalization. *Eur J Cell Biol.* 1995;68:159-166.
 30. Yoshida S, Shibata M, Yamamoto S, et al. Homo-oligomer formation by basigin, an immunoglobulin superfamily member, via its N-terminal immunoglobulin domain. *Eur J Biochem.* 2000;267:4372-4380.
 31. Le Floch R, Chiche J, Marchiq I, et al. CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. *Proc Natl Acad Sci U S A.* 2011;108:16663-16668.
 32. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. *EMBO J.* 2000;19:3896-3904.
 33. Poole RC, Halestrap AP. Interaction of the erythrocyte lactate transporter (monocarboxylate transporter 1) with an integral 70-kDa membrane glycoprotein of the immunoglobulin superfamily. *J Biol Chem.* 1997;272:14624-14628.
 34. Wilson MC, Meredith D, Fox JE, Manoharan C, Davies AJ, Halestrap AP. Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70). *J Biol Chem.* 2005;280:27213-27221.
 35. Schneiderhan W, Scheler M, Holzmann KH, et al. CD147 silencing inhibits lactate transport and reduces malignant potential of pancreatic cancer cells in vivo and in vitro models. *Gut.* 2009;58:1391-1398.
 36. Philp NJ, Ochriator JD, Rudoy C, Muramatsu T, Linser PJ. Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. *Invest Ophthalmol Vis Sci.* 2003;44:1305-1311.
 37. Bonanno JA, Giasson C. Intracellular pH regulation in fresh and cultured bovine corneal endothelium. II. Na+/HCO3- cotransport and Cl-/HCO3- exchange. *Invest Ophthalmol Vis Sci.* 1992;33:3068-3079.
 38. Bonanno JA, Yi G, Kang XJ, Srinivas SP. Reevaluation of Cl-/HCO3- exchange in cultured bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci.* 1998;39:2713-2722.
 39. Liu C, Cheng Q, Nguyen T, Bonanno JA. Knockdown of NBCe1 in vivo compromises the corneal endothelial pump. *Invest Ophthalmol Vis Sci.* 2010;51:5190-5197.
 40. Wigham C, Hodson S. Bicarbonate and the trans-endothelial short circuit current of human cornea. *Curr Eye Res.* 1981;1:285-290.
 41. Wigham CG, Hodson SA. Physiological changes in the cornea of the ageing eye. *Eye.* 1987;1(pt 2):190-196.
 42. Deora AA, Philp N, Hu J, Bok D, Rodriguez-Boulan E. Mechanisms regulating tissue-specific polarity of monocarboxylate transporters and their chaperone CD147 in kidney and retinal epithelia. *Proc Natl Acad Sci U S A.* 2005;102:16245-16250.
 43. Kimura K, Teranishi S, Kawamoto K, Nishida T. Protection of human corneal epithelial cells from hypoxia-induced disruption of barrier function by hepatocyte growth factor. *Exp Eye Res.* 2010;90:337-343.
 44. Rosnoblet C, Peanne R, Legrand D, Foulquier F. Glycosylation disorders of membrane trafficking. *Glycoconj J.* 2013;30:23-31.
 45. Adamsons I. Irreversible corneal decompensation in patients treated with topical dorzolamide. *Am J Ophthalmol.* 1999;128:774-776.
 46. Konowal A, Morrison JC, Brown SV, et al. Irreversible corneal decompensation in patients treated with topical dorzolamide. *Am J Ophthalmol.* 1999;127:403-406.
 47. Tanimura H, Minamoto A, Narai A, Hirayama T, Suzuki M, Mishima HK. Corneal edema in glaucoma patients after the addition of brinzolamide 1% ophthalmic suspension. *Jpn J Ophthalmol.* 2005;49:332-333.
 48. Wirtitsch MG, Findl O, Heinzl H, Drexler W. Effect of dorzolamide hydrochloride on central corneal thickness in humans with cornea guttata. *Arch Ophthalmol.* 2007;125:1345-1350.
 49. Zhao JC, Chen T. Brinzolamide induced reversible corneal decompensation. *Br J Ophthalmol.* 2005;89:389-390.