

Monocarboxylate Transporter 1 (MCT1) Mediates Succinate Export in the Retina

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PURPOSE. Succinate is exported by the retina and imported by eyecup tissue. The transporters mediating this process have not yet been identified. Recent studies showed that monocarboxylate transporter 1 (MCT1) can transport succinate across plasma membranes in cardiac and skeletal muscle. Retina and retinal pigment epithelium (RPE) both express multiple MCT isoforms including MCT1. We tested the hypothesis that MCTs facilitate retinal succinate export and RPE succinate import.

METHODS. We assessed retinal succinate export and eyecup succinate import in short-term ex vivo culture using gas chromatography–mass spectrometry. We tested the dependence of succinate export and import on pH, proton ionophores, conventional MCT substrates, and the MCT inhibitors AZD3965, AR-C155858, and diclofenac.

RESULTS. Succinate exits retinal tissue through MCT1 but does not enter the RPE through MCT1 or any other MCT. Intracellular succinate levels are a contributing factor that determines if an MCT1-expressing tissue will export succinate.

CONCLUSIONS. MCT1 facilitates export of succinate from retinas. An unidentified, non-MCT transporter facilitates import of succinate into RPE.

Keywords: monocarboxylate transporter, succinate, retina, retinal pigment epithelium (RPE)

Succinate has a unique role in the eye. It is a valuable metabolic fuel, and evidence supports a model where succinate produced by fumarate respiration and exported from the retina is oxidized by the neighboring retinal pigment epithelium (RPE).¹ Succinate is also implicated in retinal pathology. In a model of O₂-induced retinopathy, succinate accumulates in the retina and drives angiogenesis via succinate receptor 1 (SUCNR1) signaling on retinal ganglion cells.² SUCNR1 deficiency in mice promotes premature subretinal dystrophy, and SUCNR1 variants are associated with age-related macular degeneration in a human population.³ Under both normal and pathological circumstances, succinate mediates its effects in a non-cell-autonomous manner. However, the transporter used by succinate to exit or enter any ocular cell type has not yet been identified.

Monocarboxylate carrier 1 (MCT1) is the most ubiquitously expressed of the Slc16 family and is best known for transporting lactate, pyruvate, and other monocarboxylates.^{4–6} Recently, MCT1 has been shown to facilitate succinate export in exercising muscle and ischemic heart, and the ability for MCT1 to transport this non-canonical substrate has been validated using *Xenopus* oocytes injected with MCT1 cDNA.^{7,8} Because retina and the RPE both express MCT1 in addition to other MCT isoforms, we set out to test if MCT1 might also be responsible for facilitating retinal succinate export and/or RPE succinate import and whether additional MCTs also transport succinate in the eye.

METHODS

Animals

All experiments used 2- to 5-month-old male and female wild-type C57BL6/J mice. These mice were housed at an ambient temperature of 25°C, with a 12-hour light cycle and ad libitum access to water and normal rodent chow. Experiments using animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Ex Vivo Metabolite Uptake/Export

In all ex vivo metabolic analysis experiments, mice were euthanized by awake cervical dislocation, and retinas and/or eyecups were dissected in Gibco Hank's Balanced Salt Solution (HBSS; 14025-076; Thermo Fisher Scientific, Waltham, MA, USA). Tissue was then incubated in Krebs–Ringer Bicarbonate (KRB) Buffer (formulations used are specified below) supplemented with 5-mM glucose and U-¹³C-succinic acid (CLM-1571-0.1; Cambridge Isotope Laboratories, Tewksbury, MA, USA) or U-¹³C-malate (CLM-8065; Cambridge Isotope Laboratories) as indicated in each figure. For experiments using KRB buffer, the buffer was pre-equilibrated at 37°C, 21% O₂, and 5% CO₂ prior to incubations, and the incubations were carried out at those conditions. For experiments where pH was modulated and KRM buffer was used, the buffer was pre-equilibrated at 37°C, and room oxygen and incubations were carried out under those conditions.



For the determination of metabolite uptake or export rates, the incubation media were sampled at three time points (typically 0, 20, and 40 minutes), and export or uptake was confirmed to be in the linear range. Retinas were incubated in 200 μ L and eyecups in 100 μ L over this range of time. Inhibitors used were AZD3965 (19912; Cayman Chemical, Ann Arbor, MI, USA), AR-C155858 (HY-13248; MedChemExpress, Monmouth Junction, NJ, USA), and diclofenac sodium salt (70680; Cayman Chemical). Ethanol was used as a solvent for AZD3965, dimethylsulfoxide (DMSO) was used as a solvent for AR-C155858, and separate experiments were done using both DMSO and ethanol as a solvent for diclofenac. For control experiments, an equal volume of vehicle alone was included.

Buffer Formulations

KRB buffer was used in all experiments, except for those shown in Figures 1E and 3E (98.5-mM NaCl, 5.1-mM KCl, 1.2-mM KH_2PO_4 , 1.2-mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 2.7-mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 20.8-mM HEPES, and 25.9-mM NaHCO_3). Krebs-Ringer MOPS (KRM) buffer was used in the experiments shown in Figures 1E and 3E (98.5-mM NaCl, 5.1-mM KCl, 1.2-mM KH_2PO_4 , 1.2-mM $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 2.7-mM $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$, 10-mM HEPES, and 15-mM MOPS). KRB buffer equilibrates to pH 7.4 at 37°C in a 5% CO_2 incubator. The KRM buffer was adjusted to the desired pH using HCl at 37°C in room O_2 .

Metabolite Extraction

Metabolites were extracted from tissue using 80% MeOH, 20% H_2O supplemented with 10- μ M methylsuccinate (M81209; Sigma-Aldrich, St. Louis, MO, USA) as an internal standard to adjust for any metabolite loss during the extraction and derivatization procedures. The extraction buffer was equilibrated on dry ice, and 150 μ L was added to each sample. Tissues were then disrupted by sonication and incubated on dry ice for 45 minutes to precipitate protein. Proteins were pelleted at 17,000g for 30 minutes at 4°C. The supernatant containing metabolites was lyophilized at room temperature until dry and stored at -80°C until derivatization. The pellet containing protein was resuspended by sonication in radioimmunoprecipitation assay buffer (150-mM NaCl, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50-mM Tris, pH 8.0), and the amount of protein was determined by a bicinchoninic acid (BCA) assay (23225; Thermo Fisher Scientific). Media samples were directly added to 90% MeOH supplemented with 10- μ M methylsuccinate and immediately lyophilized, then stored at -80°C until derivatization.

Metabolite Derivatization

Lyophilized samples were first derivatized in 10 μ L of 20-mg/mL methoxyamine HCl (226904; Sigma-Aldrich) dissolved in pyridine (270970; Sigma-Aldrich) at 37°C for 90 minutes and subsequently with 10 μ L *N*-tert-butyltrimethylsilyl-*N*-methyltrifluoroacetamide (394882; Sigma-Aldrich) at 70°C for 90 minutes.

Gas Chromatography–Mass Spectrometry

Metabolites were analyzed on an Agilent 7890/5975C gas chromatography–mass spectrometry system (Agilent Tech-

nologies, Santa Clara, CA, USA) using selected ion monitoring methods described in a previous report.⁹ Peaks were integrated in MSD ChemStation (Agilent Technologies), and correction for natural isotope abundance was performed using IsoCor software.¹⁰ Corrected metabolite signals were converted to molar amounts by comparing metabolite peak abundances in samples with those in a “standard mix” containing known quantities of metabolites run along each individual experiment. These known metabolite concentrations were used to generate a standard curve that allowed for metabolite quantification.

Lactate Assay

Lactate levels were quantified using an enzymatic lactate dehydrogenase (LDH) assay.¹¹ Prior to each assay, fresh assay buffer was prepared consisting of 6.5 mL glycine buffer (0.6-M glycine, 0.5-M hydrazine, pH 9.2), 13 mL water, 0.325 mL L-lactate dehydrogenase (L-LDH; (830 U/mg, L2500; Sigma-Aldrich) and 200 μ L 250-mM NAD^+ (16077; Cayman Chemical). Then, 45 μ L of assay buffer was added to 5 μ L of experimental incubation medium in a 96-well plate, and samples were incubated at 37°C to allow the LDH reaction to go to completion and deplete the entirety of the sample lactate. Subsequently, NADH absorbance was monitored in a BioTek plate reader (Agilent Technologies) at 340 nm, and known lactate standards run alongside each experiment were used to convert absorbance values to lactate concentrations for each sample.

Single-Cell Transcriptomics

The expression of Slc16a family members in retinal cell classes from mouse and human was assessed using Seurat 3.1.5, and dotplots were generated using ggplot2 3.3.2. Single-nucleus RNA sequencing (RNA-seq) data representing three distinct 5-week-old C57Bl/6J mouse retinas were obtained using the Chromium Next GEM Single Cell 3' Library & Gel Bead Kit v3.1 (1000128; 10x Genomics, Pleasanton, CA, USA) and were sequenced using an Illumina NovaSeq SP 100-cycle flow cell at the Northwest Genomics Center at the University of Washington. The single nucleus RNA-seq data from adult human retina and accompanying analysis are described in Thomas et al. and are available through the Gene Expression Omnibus database with accession number GSE183684.¹²

Statistical Analysis

Statistical tests were performed in Prism 9 (GraphPad, San Diego, CA, USA). The tests used for the experiments are indicated in the figures. Briefly, Welch's *t*-test was used in instances where two groups were compared, and a one-way ANOVA followed by the recommended correction for multiple comparison was used when multiple conditions were compared. The *n* values indicated in each figure caption represent biological replicates (e.g., *n* = 3 indicates that three rate measurements were obtained from three different retinas, rather than three rate measurements made from the same retina). Retinas and eyecups from the same mouse were always used in different experimental conditions and thus were considered biological replicates.

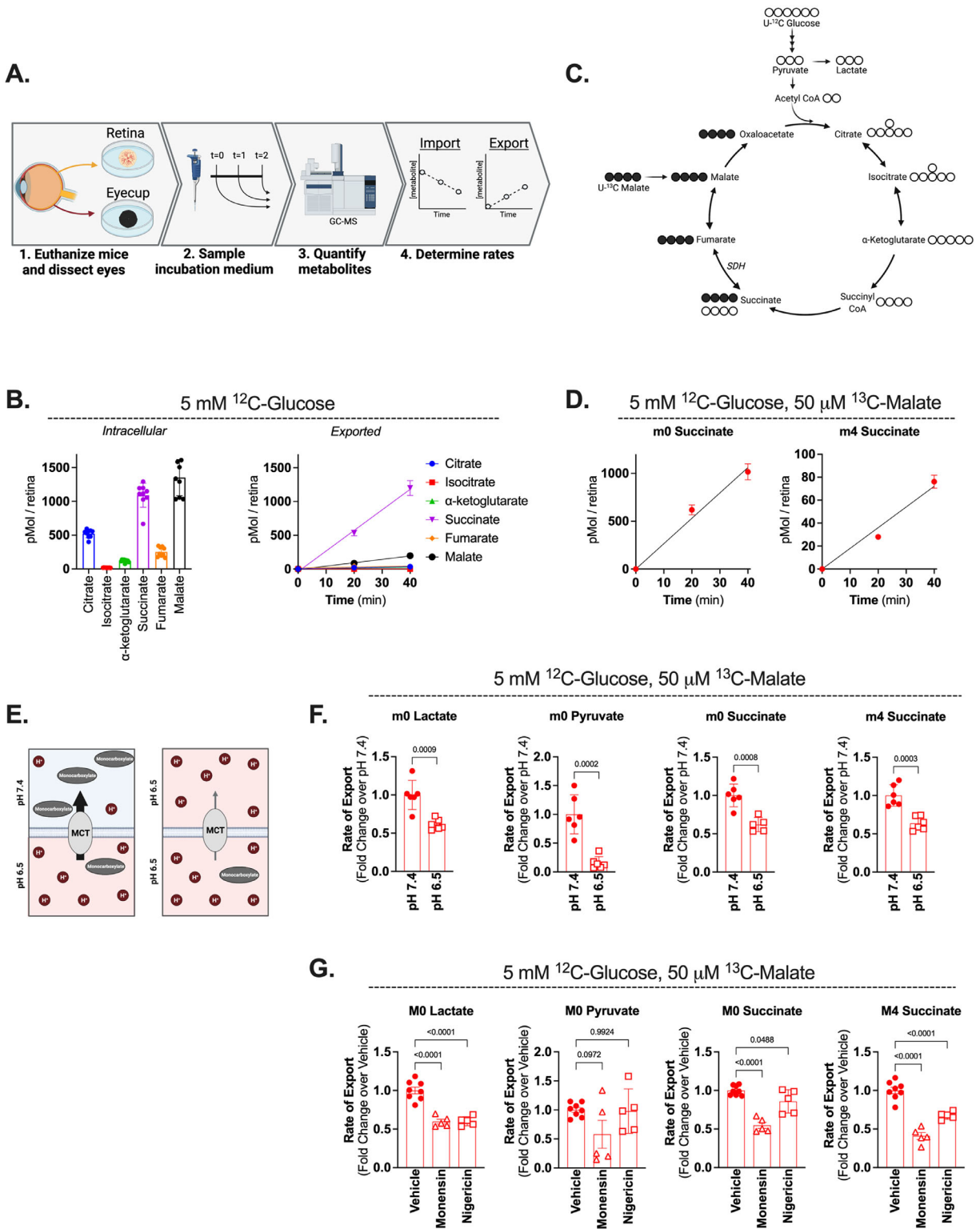


FIGURE 1. H^+ dependence of succinate export in retinas. (A) Experimental workflow for determination of metabolite export and import rates. (B) Intracellular levels of TCA cycle metabolites from freshly dissected retinas (*left*, $n = 9$) and rate at which they are exported when retinas are incubated in 5 mM glucose (*right*, $n = 6$). (C) Diagram showing the isotopologues of succinate that are made by metabolism of $U-^{12}C$ -glucose and $U-^{13}C$ -succinate. *Open circles* represent ^{12}C ; *filled circles* represent ^{13}C . (D) Rates of m0 and m4 succinate export by retinas incubated in 5-mM ^{12}C -glucose and 50 μM ^{13}C -Malate ($n = 6$ retinas). (E) Representation of how pH gradients can influence MCT activity. (F) Rate of export of the canonical MCT substrate m0 lactate and m0 pyruvate, as well as m0 succinate and m4 succinate, from retinas incubated in 5-mM ^{12}C -glucose and 50- μM ^{13}C -Malate at pH 7.4 and pH 6.5. The P values were determined using Welch's t -test ($n = 6$ retinas). (G) Rate of export of m0 lactate, m0 pyruvate, m0 succinate, and m4 succinate from retinas in the presence of 100- μM monensin or 100- μM nigericin. The P values were determined using one-way ANOVA ($n = 6$, vehicle; $n = 6$, monensin; $n = 6$, nigericin).

RESULTS

Retinal Succinate Export Is Proton Dependent

Succinate is an abundant tricarboxylic acid (TCA) cycle metabolite in retinas, and retinas in short-term ex vivo culture export a molar amount of succinate equivalent to their entire intracellular succinate pool approximately every 40 minutes (Figs. 1A, 1B).¹ As previously reported, retinas also have exceptionally high intracellular lactate levels and export a large quantity of pyruvate and lactate (Supplementary Fig. S1A).^{13–15}

To evaluate the role of MCTs in retinal succinate export, MCT activity must be reduced. Retinas are exceptionally glycolytic so reducing MCT activity could block flux through glycolysis and indirectly affect intracellular succinate production rather than its release. To control for this potentially confounding factor, we supplied retinas with 5-mM ¹²C-glucose and 50- μ M ¹³C-malate in all experiments testing the role of MCTs in succinate export in this section (Figs. 1C, 1D). Including this tracer quantity of ¹³C-malate allows for quantification of succinate produced both by oxidative (m0 succinate) and by reductive (m4 succinate) TCA cycle activity.¹ Production of m4 succinate does not require acetyl coenzyme A and thus should not depend on glycolytic flux, which we validated (Supplementary Figs. S1B–S1D). So, a decrease in both m0 and m4 succinate export can be attributed to specific inhibition of MCT-mediated succinate transport rather than a change in intracellular succinate production.

MCT-dependent substrate transport depends on pH because it transports protons along with each anionic substrate. Lowering pH on the *trans* (in this case extracellular) side of a MCT decreases its ability to export anions, as it must now transport protons against a concentration gradient (Fig. 1E). We measured succinate export in retinas incubated in medium adjusted to pH 7.4 or 6.5 and observed that lower extracellular pH reduced export of m0 and m4 succinate, as well as the canonical MCT substrates lactate and pyruvate (Fig. 1F). We treated retinas incubated in pH 7.4 medium with H⁺ ionophores (the H⁺/Na⁺ ionophore monensin or the H⁺/K⁺ ionophore nigericin) to diminish any proton gradient that may normally drive MCT-mediated export in retinas. Rates of m0 lactate, m0 pyruvate, m0 succinate, and m4 succinate export all were diminished by the ionophores (Fig. 1G). The high concentration of Na⁺ rela-

tive to K⁺ in KRB buffer might contribute to the increased effectiveness of monensin relative to nigericin.

Retinas Express MCT1, MCT2, and MCT4

We queried single-cell RNA-seq databases to assess the cell-type specificity of MCT family member expression in the retina. In the mouse retina, Slc16a1 (MCT1) expression is most robust in the photoreceptors and Müller glia. Expression of Slc16a3 (MCT4) is limited throughout the retina, and several inner retinal cell types express Slc16a7 (MCT2) (Fig. 2A). Single-cell transcriptomics performed using human retinas roughly mirror this expression pattern (Fig. 2B). In general, these results correlate with reports of MCT1 and MCT2 protein expression in the retina. MCT1 immunoreactivity is seen throughout the retina, and studies performed using rod-specific Basigin-knockout mice indicate that rods express a higher proportion of MCT1 compared to the inner retina, whereas MCT2 and MCT4 immunoreactivity appears to be restricted to the inner retina.^{16–18} However, retinal MCT4 expression was reduced ~75% in rod-specific Basigin-knockout retinas, indicating that rods may express a large proportion of the MCT4 protein in retinas, a finding that was not reflected in the transcriptomics analysis.¹⁷

MCT1 Contributes to Succinate Export in Retinas

We tested several MCT isoform-specific inhibitors to determine if any might reduce retinal succinate export (Table). The dual MCT1/MCT2 inhibitors AR-C155858 and AZD3965 (Figs. 3A, 3B) slowed m0 and m4 succinate export in a dose-dependent manner. These effects could be mediated through MCT1, MCT2, or a combination of both. The dual MCT1/MCT4 inhibitor diclofenac (Fig. 3C) also slowed both m0 and m4 succinate export. In addition to inhibiting MCT1 and MCT4, diclofenac also inhibits cyclooxygenase (COX) 1 and 2. However, we found that aspirin did not alter export of succinate, lactate, or pyruvate in retinas, indicating that the reduction of succinate export exhibited by retinas incubated in diclofenac occurs via a COX1/2-independent pathway (Fig. 3D).

To date, only the ability of MCT1 to transport succinate in an ex vivo system (*Xenopus* oocytes expressing MCT1 cDNA) has been demonstrated.⁷ Given this, a reasonable interpretation of these results is that MCT1 transports a

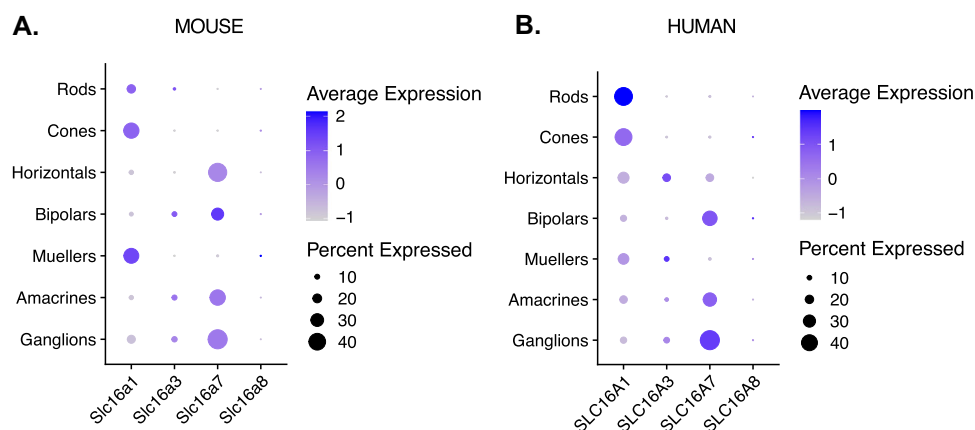


FIGURE 2. Expression of Slc16a family members in retinal cell types. Single-cell transcriptomics analysis of adult mouse (A) and human (B) retinas. Slc16a1, MCT1; Slc16a3, MCT4; Slc16a7, MCT2; Slc16a8, MCT3.

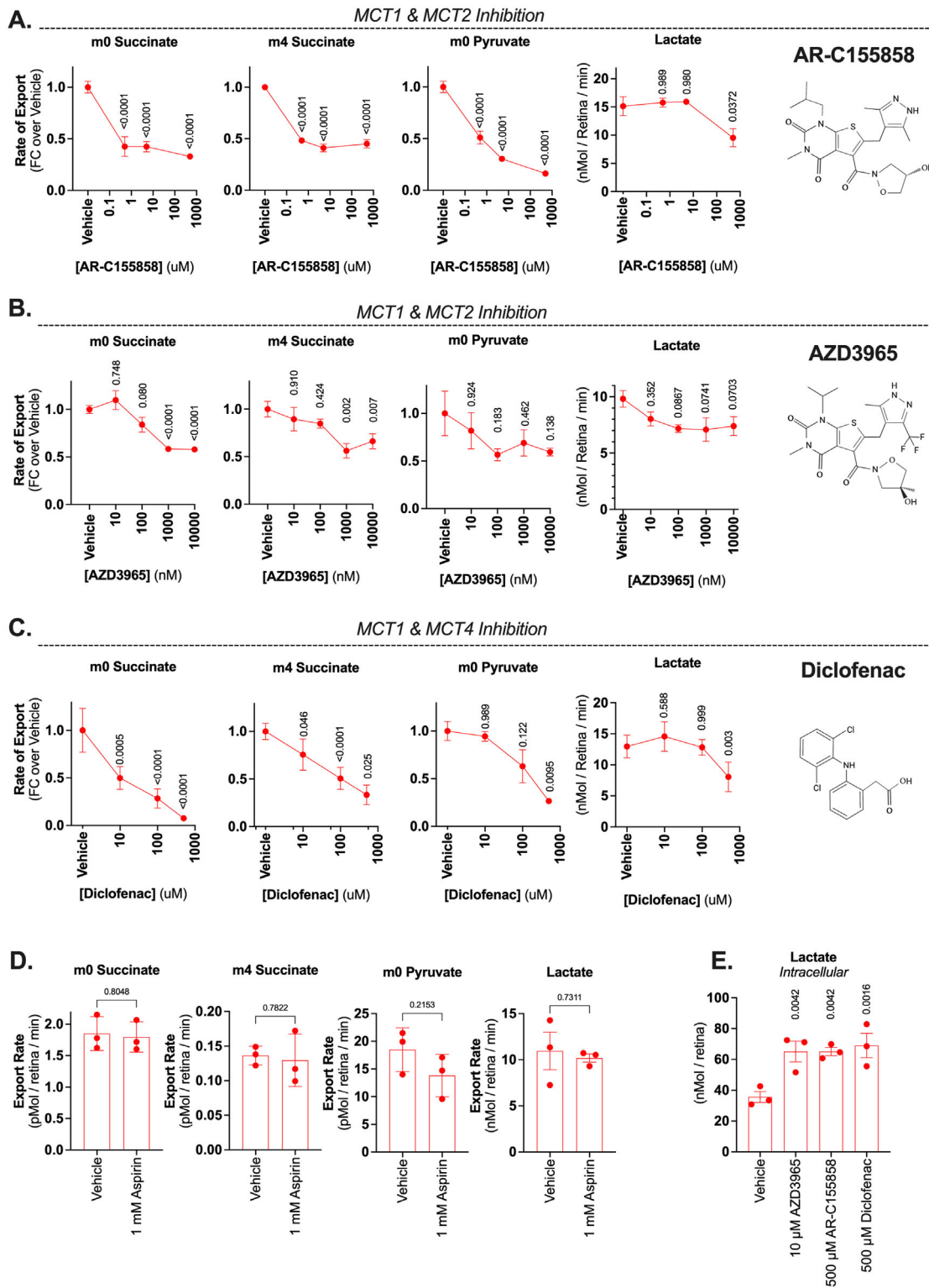


FIGURE 3. Effect of MCT inhibition on succinate export in retinas. Influences of various MCT inhibitors on retinal export of m0 succinate, m4 succinate, lactate, and m0 pyruvate. Rates were determined by sampling incubation media from retinas incubated in 5-mM 12 C-glucose and 50- μ M 13 C-malate. The *P* values shown above each point were calculated using ordinary one-way ANOVA (all conditions compared to vehicle) followed by Dunnett's correction for multiple comparisons. **(A)** AR-C155858 ($n = 5$, 0 nM; $n = 3$, 500 nM; $n = 5$, 5 μ M; $n = 5$, 500 μ M). **(B)** AZD3965 ($n = 9$, 0 nM; $n = 3$, 10 nM; $n = 9$, 100 nM; $n = 6$, 1 μ M; $n = 9$, 10 μ M). **(C)** Diclofenac ($n = 6$, 0 nM; $n = 3$, 10 μ M; $n = 6$, 100 μ M; $n = 3$, 500 μ M). **(D)** Influence of 1-mM aspirin on the retinal export of m0 succinate, m4 succinate, and lactate. The *P* values shown above each point were calculated using Welch's *t*-test ($n = 3$ retinas per condition). **(E)** Intracellular lactate levels in retinas incubated at the highest concentration of each inhibitor for 40 minutes. The *P* values shown above each point were calculated using ordinary one-way ANOVA (all conditions compared to vehicle) followed by Dunnett's correction for multiple comparisons ($n = 3$ retinas per condition).

TABLE. Reported Kinetic Parameters for Various MCT Inhibitors

Inhibitor	MCT1	MCT2	MCT3	MCT4	Refs.
AZD3965	3.2 nM (K_i)	20 nM (K_i)	No inhibition	No inhibition*	Curtis et al. ²⁵
AR-C155858	2.3 nM (K_i)	10 nM (K_i)	n.d. [†]	Minimal inhibition‡	Ovens et al. ²⁰
Diclofenac§	1.45 μ M (IC ₅₀)	n.d.	n.d.	0.14 μ M (IC ₅₀)	Sasaki et al., ²¹ Renner et al. ²⁶

* n.d. indicates no data for the effectiveness of an inhibitor for that MCT has been reported.

† No inhibition observed up to 10 μ M.

‡ No inhibition observed up to 1 μ M but some inhibition observed at 10 μ M.

§ The K_i for diclofenac in a mixed population of MCT1, MCT2, and MCT4 is 20 μ M.

portion of the succinate exported by retinas, whereas other MCTs also may possibly contribute. This is supported by the observation that photoreceptors have been reported to be responsible for exporting over half of the succinate released by retinas and that MCT1 is the predominant MCT expressed on rods and cones in mouse retinas (Fig. 2A).¹

The MCT inhibitors we tested suppressed pyruvate export more effectively than lactate export (Figs. 3A–3C). We quantified intracellular lactate levels in retinas after a 40-minute incubation in the highest concentration of each inhibitor and found that intracellular lactate levels roughly doubled in the presence of all MCT inhibitors (Fig. 3E). This build-up of intracellular lactate could contribute to the sustained lactate export exhibited by retinas incubated in AZD3965, as it is a competitive inhibitor.¹⁹ However, AR-C155858 and

diclofenac both appear to be non-competitive inhibitors, so their effectiveness should not depend on intracellular lactate concentration.^{20,21} Instead, non-ionic diffusion of the free acid form of lactate across the plasma membrane could be responsible for a portion of the sustained lactate export in the presence of the non-competitive MCT inhibitors. The free diffusion of lactate is reported to be faster than that of pyruvate, and the build-up of intracellular lactate (coupled with the already exceptionally high intracellular lactate concentrations in retinas) could further facilitate this process.^{22–24}

Eyecup Succinate Import Is Not MCT Mediated

Unlike retina tissue, eyecup explants do not export succinate and instead measurably deplete it from the incubation

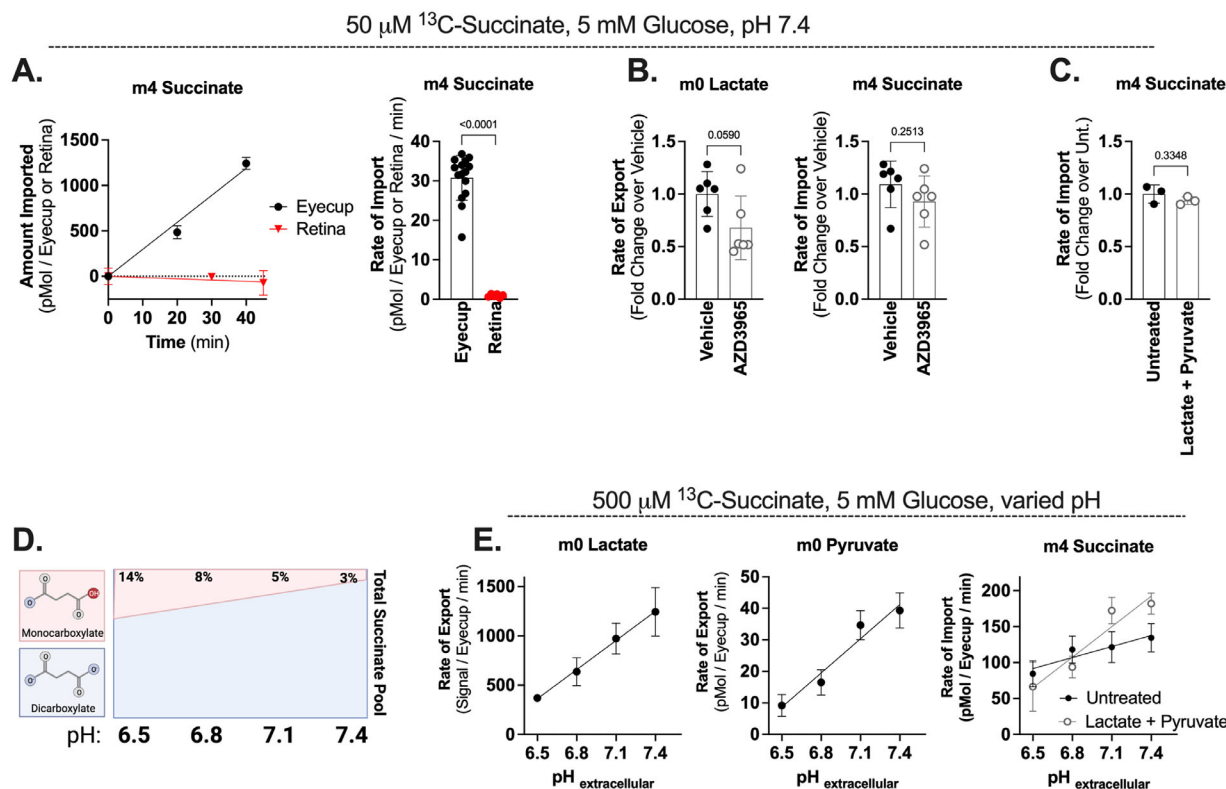


FIGURE 4. MCTs do not import succinate in eyecups. (A) Depletion of succinate from the incubation media by eyecups ($n = 14$) and retinas ($n = 6$) incubated in 5-mM ¹²C-glucose and 50- μ M ¹³C-succinate. The linearity of import over time is shown on the left, and calculated rates of import are shown on the right. (B) Rate of succinate import in the presence of 100 μ M AZD3965. The P values were determined by two-tailed Welch's t -test ($n = 6$ eyecups for each condition). (C) Rate of succinate import in the presence of 15-mM lactate and 1-mM pyruvate. The P values were determined by a two-tailed Welch's t -test ($n = 3$ eyecups for each condition). (D) Graphical representation of how pH can influence the fraction of monocarboxylate succinate. The ratio of monocarboxylate to dicarboxylate succinate was determined using the Henderson–Hasselbalch equation and a pKa value of 5.69. (E) Rate of succinate import by eyecups supplied with 5-mM ¹²C-glucose and 500- μ M ¹³C-succinate, in the presence or absence of 15-mM lactate and 1-mM pyruvate ($n = 6$ untreated eyecups at each pH; $n = 3$ lactate + pyruvate eyecups at each pH).

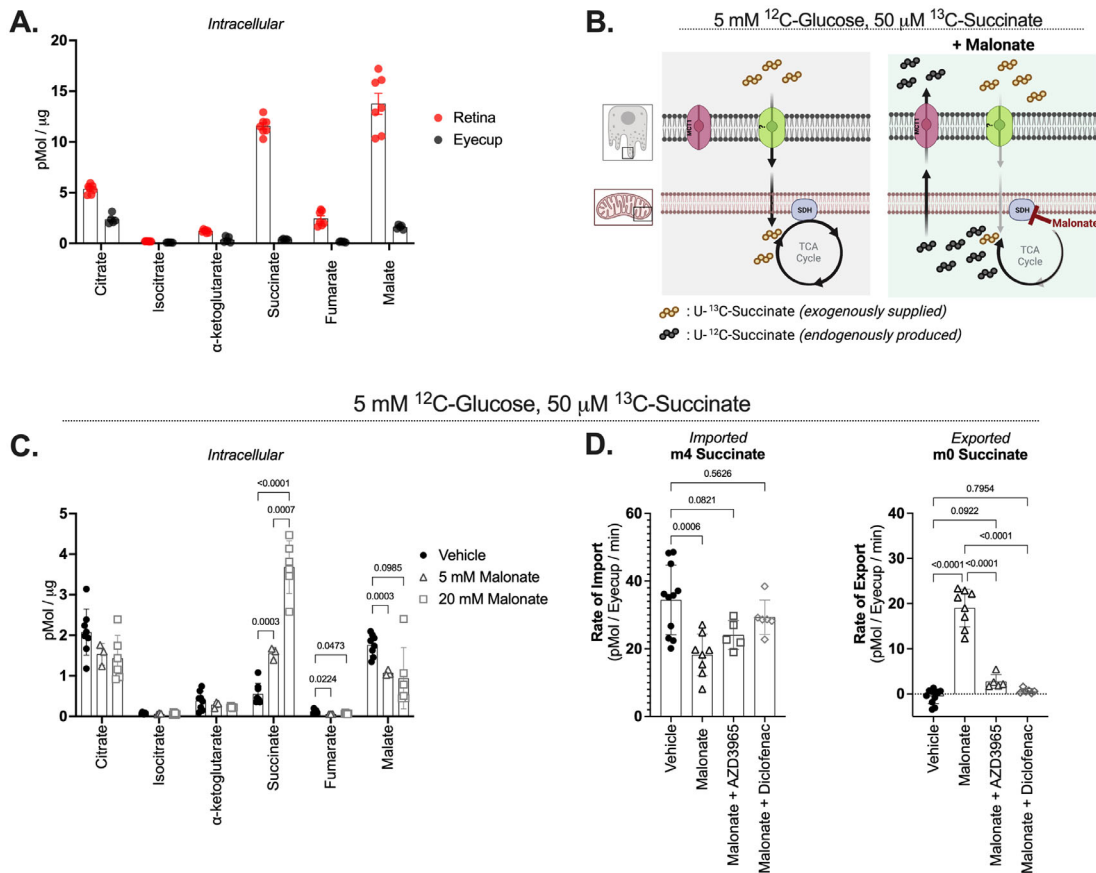


FIGURE 5. Eyecups can be induced to export succinate via MCT1. (A) Total intracellular metabolites per microgram dry protein in freshly dissected retinas and eyecups ($n = 7$ retinas and $n = 5$ eyecups). (B) Influences of malonate on succinate dynamics in eyecups. (C) Intracellular metabolite levels per microgram dry tissue in eyecups incubated in 0-, 5-, or 20-mM malonate for 40 minutes. The P values were determined using ordinary one-way ANOVA followed by Dunnett's correction for multiple comparisons ($n = 8$, vehicle; $n = 3$, 5 mM; $n = 6$, 20 mM). (D) Rates of m4 succinate import and m0 succinate export from eyecups incubated with 5-mM malonate, 5-mM malonate + 100-nM AZD3965, or 5-mM malonate + 100-μM diclofenac. The P values were determined using one-way ANOVA followed by Dunnett's correction for multiple comparisons ($n = 11$, vehicle; $n = 8$, malonate; $n = 5$, malonate + AZD3965; $n = 6$, malonate + diclofenac).

media (Fig. 4A).^{1,27} Eyecup tissue is composed of sclera, choroidal endothelial cells, and RPE cells. MCT distribution on RPE cells is polarized, with MCT3 localized to the basal RPE surface and robust MCT1 expression on the apical processes.^{16,28,29} Because apical processes are in direct contact with the succinate-exporting retina, we hypothesized that MCT1 may be responsible for both retina succinate export and RPE succinate import.

We first tested if MCT1 is responsible for eyecup succinate import by measuring import of 50-μM U-¹³C-succinate from pH 7.4 incubation medium in the presence of the MCT1 inhibitor AZD3965 (Fig. 4B). AZD3965 did not inhibit succinate import; however, it is possible that MCT3 maintains succinate uptake when MCT1 is inhibited. We were unable to identify a well-characterized MCT3 inhibitor, so we tested if we could outcompete U-¹³C-succinate import with an excess of lactate and pyruvate (substrates for both MCT1 and MCT3 that would act as competitive inhibitors) (Fig. 4C). Lactate and pyruvate did not alter the rate of succinate import.

We next considered that eyecups could express a high-affinity, non-MCT succinate transporter and that MCTs might engage in succinate import only under specific conditions (such as when pH is low and/or when extracellular succinate concentrations are higher). Lowering pH in *cis* with succinate could modulate MCT-mediated succinate trans-

port in two ways: (1) as shown previously in Figure 1D, a high proton gradient can drive transport, and (2) at acidic pH, a greater fraction of succinate exists as a MCT-transportable monocarboxylate (Fig. 4D).⁷ With this in mind, we tested if we could engage measurable MCT-mediated succinate import in eyecups by acidifying the extracellular pH and increasing the extracellular succinate concentration (Fig. 4E). As expected, the rates of lactate and pyruvate export decreased as extracellular pH was acidified and MCTs began to export these substrates against an increasingly steep proton gradient. However, the rate of succinate import also decreased as pH acidified, indicating that the steeper proton gradient was ineffective at enhancing succinate import.

It is possible that low pH suppresses mitochondrial succinate oxidation and that an increase in MCT-mediated succinate transport is masked by a decrease in succinate oxidation at acidic pH. To control for this, we tested if the MCT substrates lactate and pyruvate might be able to outcompete a greater fraction of succinate uptake as the pH acidified (Fig. 4E). Even at the lowest pH, lactate and pyruvate had no significant influence on succinate import. This indicates that an unidentified succinate transporter and not MCT1 or MCT3 is the primary succinate importer in eyecups.

Eyecups Can Be Induced to Export Succinate Via MCT1

Despite expression of MCT1 by both retinas and eyecups, retinal explants export succinate but eyecup explants do not. In fact, MCT1 can be found on red skeletal muscle, cardiac muscle, red blood cells, liver, kidney cortex and tubule cells, adipose tissue, cerebral neurons and glia, and pancreatic acinar cells, but retina and pancreas are the only tissues reported to date that export succinate under basal conditions.^{29–32} We next sought to determine if we could identify a metabolic difference between MCT1-expressing tissues in the eye that engages succinate export.

In other tissues that can be induced to export succinate (such as exercising muscle or ischemic heart tissue), succinate export is accompanied by a rise in intracellular succinate levels and intracellular acidification.^{7,8,33} We compared intracellular succinate levels between retina and eyecup tissue and saw that retinas contain approximately 20-fold more succinate per microgram protein compared to eyecups (Fig. 5A).

To test if increasing eyecup succinate levels was sufficient to induce succinate export, we treated eyecups with the SDH inhibitor malonate and observed a dose-dependent increase in intracellular succinate levels (Figs. 5B, 5C). When we sampled incubation media from eyecups incubated with malonate, we observed that import of exogenous succinate (m4) was significantly reduced and that eyecups began to export endogenous succinate (m0) (Fig. 5D). This succinate export was effectively shut down by the MCT1 inhibitors AZD3965 and diclofenac (Fig. 5D). The nearly complete inhibition of malonate-induced m0 succinate export by AZD3965 in eyecups indicates that MCT3 is not able to transport succinate, as AZD3965 has no inhibitory effect on MCT3 even well above the concentration we used.²⁵ Taken together, these results show that a high intracellular succinate concentration is at least one factor that contributes to a tissue exporting succinate through MCT1 (Fig. 5B).

DISCUSSION

The goal of this study was to understand how succinate is transported between cells in an eye. Our results indicate that MCT1 can be added to the model of succinate transit in the retinal ecosystem as the retinal succinate exporter but not as the RPE succinate importer.

Our previous report described evidence that photoreceptors are major contributors to retinal succinate export. Retinas from *Aipl1*^{-/-} animals (in which photoreceptors have degenerated) export less succinate per microgram of protein compared with wild-type retinas.¹ This is supported by the high expression of MCT1 on rods and cones (Fig. 2).¹⁷ It is possible that Müller glia contribute to the remainder of retinal succinate export, as they also appear to express high levels of MCT1 (Fig. 2). Reported expression of MCT4 protein on rods (which comprise ~97% of photoreceptors in the mouse retina), combined with the enhanced effectiveness of the MCT1/MCT4 inhibitor diclofenac relative to the MCT1/MCT2 inhibitors used, indicates that MCT4 might also contribute to retinal succinate export.^{17,34} However, the lack of commercially available MCT4 inhibitors that do not also inhibit MCT1 makes it difficult to test this hypothesis directly.³⁵

Determining why MCT1 plays no significant role in importing succinate under physiological *ex vivo* condi-

tions in both retinas and eyecups is important to understanding the biological implications of circulating succinate. The current understanding of MCT activity states that, at equilibrium, there will be a balance among the $[H^+]_{out}$, $[monocarboxylate]_{out}$, $[H^+]_{in}$, and $[monocarboxylate]_{in}$.⁶ A recent report has illustrated the added influence pH has on modulating the amount of MCT-transportable succinate due to its physiologically relevant pKa values (Fig. 4D).⁷ Based on this, we reasoned that we might detect MCT1-mediated succinate import in eyecups if we enhanced it by lowering the extracellular pH and supplying a higher concentration of extracellular succinate (Fig. 4E). However, this did not enhance succinate import. Perhaps an unidentified succinate importer has a significantly higher affinity for succinate than MCT1 does, and import of succinate into eyecups may not be the rate-limiting step for overall succinate oxidation. In this scenario, any change to the small amount of succinate that could be imported by MCT1 would not affect the overall process of succinate depletion.

Even though retinas express copious MCT1, they appear to be incapable of using it as a succinate importer. Retinas incubated in U-¹³C-succinate do not deplete measurable amounts from the media (Fig. 4A), and intracellular downstream intermediates are ¹³C-labeled by succinate only to a minor degree.^{1,36} Because a small fraction of downstream intermediates can be labeled by exogenous succinate in retinas, MCT1 may be able to import a small amount of succinate. However, because supplying retinas with as much as 100 mM succinate does not stimulate significant O₂ consumption, it is unlikely that the small amount of MCT1-mediated succinate import in retinas is biologically relevant.²⁷

Considering that for a MCT at equilibrium, $[H^+]_{out}$, $[monocarboxylate]_{out} = [H^+]_{in}$, $[monocarboxylate]_{in}$, the likely explanation for the observed unidirectional MCT1 activity in retinas is that they have both high intracellular succinate levels (Fig. 5A) and an unusually acidified cytosol. In vivo pH measurements made from a cat eye indicate that the pH of the retina was ~7.1, whereas the pH of the choroid (near the RPE) was ~7.4.³⁷ A retina maintains an unusually high rate of aerobic glycolysis relative to the RPE (and most other tissues), which likely contributes to retinal acidification.^{11,38} This may also explain why most MCT1-expressing tissues do not export succinate under basal conditions. Because most tissues do not rely on aerobic glycolysis and fumarate respiration to the same extent that the retina does, they may lack the requisite highly acidified cytosol and high intracellular succinate levels required to engage MCT1-mediated succinate transport.

Like healthy retina tissue, MCT1 overexpressing cancers also relies on aerobic glycolysis and converts a large fraction of the glucose consumed to lactate.³⁹ Succinate has recently been identified as a metabolite exported by cancer cells and that promotes metastatic activity through SUCNR1 signaling.⁴⁰ Determining if this succinate also is exported via MCT1 will allow it to be targeted for inhibition by existing MCT1-specific chemotherapy drugs.

By identifying the retinal succinate exporter, we hoped we would be able to harness it to study the role of succinate exchange in the retinal ecosystem by enhancing or decreasing its expression. However, the canonical role MCTs play as lactate and pyruvate transporters is fundamental to retinal health. Ocular MCT function is required for maintaining vision; global loss of Basigin (which is required to traffic MCT1, 3, and 4) results in blindness, and RPE-specific

Basigin knock-down results in electroretinogram defects.^{16,17} These defects are thought to be driven by the widespread metabolic dysregulation caused by a build-up of lactate, which means it would be difficult to identify specific defects driven by loss of succinate transport. However, this general strategy may still be pursued when the RPE succinate importer has been identified.

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