Cell-autonomous lipid-handling defects in Stargardt iPSC-derived retinal pigment epithelium cells

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SUMMARY

Stargardt retinopathy is an inherited form of macular degeneration caused by mutations in gene ABCA4 and characterized by the accumulation of lipid-rich deposits in the retinal pigment epithelium (RPE), RPE atrophy, and photoreceptor cell death. Inadequate mechanistic insights into pathophysiological changes occurring in Stargardt RPE have hindered disease treatments. Here, we show that ABCA4 knockout and induced pluripotent stem cell-derived RPE (STGD1-iRPE) from patients with Stargardt differentiate normally but display intracellular lipid and ceramide deposits reminiscent of the disease phenotype. STGD1-iRPE also shows defective photoreceptor outer segment (POS) processing and reduced cathepsin B activity—indicating higher lysosomal pH. Lipid deposits in STGD1-iRPE are lowered by increasing the activity of ABCA1, a lipid transporter, and ABCA4 ortholog. Our work suggests that ABCA4 is involved in POS and lipid handling in RPE cells and provides guidance for ongoing gene therapy approaches to target both RPE and photoreceptor cells for an effective treatment.

INTRODUCTION

Stargardt disease (STGD1; MIM: 248200) is a recessive inherited retinal degeneration that affects 1 in 10,000 people in the US and is currently not treatable (Briggs et al., 2001; Molday et al., 2009). Clinical features of STGD1 include slower dark adaptation and loss of central and color vision, suggesting that the disease affects both rod and cone photoreceptors (PRs). STGD1 is caused by mutations in gene ABCA4, a member of the ATP-binding cassette (ABC) superfamily of transporters (Briggs et al., 2001). Over 800 mutations distributed across different functional domains of ABCA4 have been associated with variable ocular phenotypes. Some genotypes result in rapid PR degeneration, leading to an end-stage disease that looks like advanced retinitis pigmentosa, whereas other genotypes result in slower disease progression with pisciform flecks and central vision loss (Quazi et al., 2012; Schindler et al., 2010; Webster et al., 2001). This suggests different roles of ABCA4 protein domains in disease onset and progression. Previously, ABCA4 was shown to be selectively expressed in the disc of PR outer segments (POSs), where it is thought to work as a lipid flippase and flip N-retinylidene-phosphoethanolamine (N-ret-PE), located inside the PR disc, to the cytoplasmic side of POSs (Molday et al., 2009). It is thought that in STGD1 PRs, N-ret-PE accumulates, driving the formation of A2PE—a precursor to A2E and lipofuscin (Boyer et al., 2012). RPE cells phagocytose POS as part of their normal diurnal activity (Bharti et al., 2006). In STGD1 RPE, POS discs full of A2PE are phagocytosed by RPE cells, and the phosphatidyl moiety of A2PE is removed by RPE lysosomal enzymes to produce a non-digestible material A2E (Boyer et al., 2012; Molday and Zhang, 2010; Sparrow et al., 2003). Over time, the accumulation of A2E inside RPE cells leads to RPE atrophy (Parmar et al., 2018; Sparrow et al., 2010). Furthermore, RPE atrophy in Abca4−/− mice is associated with elevated lysosomal pH, accumulation of cholesterol and lipid metabolites like ceramide, increased complement activation, and autophagy downregulation. These changes in the RPE are thought to result in loss of RPE functional capacity to support PRs, leading to PR cell death and vision loss (Kaur et al., 2018; Liu et al., 2008; Radu et al., 2011; Toops et al., 2015).

Several recent observations suggest that in the RPE STGD1 disease pathology may occur independently of the previously known mechanism from PRs: (1) ABCA4 is...
expressed on mouse RPE plasma membrane (Lenis et al., 2018); (2) overexpression of a complement regulator in STGD1 mouse RPE corrects atrophy despite bisretinoid accumulation (Lenis et al., 2017); (3) complement dysregulation triggered by the buildup of autofluorescence material leads to complement-mediated cellular death in 12-month cultured STGD1-induced pluripotent stem cell-derived RPE (iRPE) cells (Radu et al., 2021); (4) overexpression of Abca4 solely in the RPE of STGD1 mouse rescues some of the disease pathology (Lenis et al., 2018); and (5) variable disease phenotype associated with mutations affecting different ABCA4 functional domains suggests different roles of ABCA4 protein domains in RPE and PR cells (Ahn et al., 2000; Curtis et al., 2020; Quazi et al., 2012).

Here, we developed monolayer cultures of STGD1-iRPE cells that demonstrate key RPE features, including cobblestone and polarized morphology, RPE-specific gene signature, and physiological electrical responses (May-Simera et al., 2018; Miyagishima et al., 2016; Sharma et al., 2019). We show that ABCA4 loss of function (LOF) in STGD1-iRPE (ABCA4 knockout and patient iRPE) triggers a cell-autonomous (without the presence of STGD1 POS) disease phenotype in RPE cells, including intracellular lipid deposits and defective POS digestion. Furthermore, defective POS digestion in ABCA4 LOF is restored by lysosome-acidifying drugs. Lipid deposits in STGD1-iRPE can be reduced by activating ABCA1 and increased by knocking down the expression of ABCA1, another member of the ABC superfamily involved in lipid homeostasis. Our work shows that STGD1-iRPE represents a physiologically relevant in vitro disease model for STGD1 disease and that ABCA4 LOF triggers STGD1 POS-independent disease phenotype in RPE cells. Lipid metabolism defects in STGD1-iRPE suggest a potential therapeutic mechanism for STGD1 disease via lipid-lowering drugs, including modulation of ABCA1 activity.

**RESULTS**

**ABCA4 is localized on the apical membrane of human RPE cells**

Polarization of RPE is achieved by the formation of tight junctions that allow the segregation of distinct sets of receptors and channels on its apical and basolateral membranes (Lehmann et al., 2014). To identify apical and basolateral selective cell-surface markers, primary human fetal RPE (hfRPE) cells were matured for 6 weeks on semi-permeable Transwell membranes as described previously (Maminishkis et al., 2006). Apical and basolateral proteins were differentially captured using the cell-surface-capturing (CSC) technique performed selectively on apical or basal sides of RPE cells, using a previously published protocol (Boheler and Gundry, 2018; Khristov et al., 2018; Yan et al., 2018) (Figure S1A). This technique uses oxidation and biotinylation of glycan moieties present on membrane proteins and lipids. Biotinylated glycopeptides are enriched using streptavidin beads following restricted proteolysis of membrane proteins. Enzyme peptide-N-glycosidase F cleaves between the innermost N-acetylglucosamine and asparagine residues and deglycosylated peptides are released and analyzed by high-mass-accuracy mass spectrometry (Boheler and Gundry, 2018) (Figure 1A). 1,850 proteins were identified on the RPE surface using CSC technology (Figure S1B; Table S1). 485 proteins were present predominantly on the RPE apical surface, 411 on the basal surface, and 964 on both the apical and basal sides of RPE cells (Figure S1B; Table S1). To further validate these results, we confirmed the presence of known RPE proteins on the apical and basolateral membranes, including known apical proteins like clusters of differentiation (CD) receptor CD59 (Yang et al., 2009), transient receptor potential cation channel subfamily M member 1 (TRPM1) (Zhao et al., 2015), and known basal proteins like CD63 (Otsuki et al., 2021) and COL6A5 (Sadada et al., 2020) (Table S1). One finding of this CSC analysis was the identification of ABCA4 on the apical membrane of RPE. Membrane localization of ABCA4 was further confirmed by the western blot of the membrane and the cytoplasmic fraction of hfRPE and iRPE cells (Figure 1B).

Furthermore, specific apical membrane localization was confirmed by colocalization with a well-known apical membrane marker Na⁺/K⁺ ATPase and not with a basal membrane marker, collagen IV (Figure 1C). Immunogold labeling, using transmission electron microscopy (TEM) of iRPE monolayers, further confirmed apical membrane localization in wild-type iRPE cells on apical processes and membrane in between processes (Figures 1D, S1C, and S1D). Combined, our hfRPE and iRPE data support recently published mouse RPE data that ABCA4 is expressed in both human and mouse RPE cells (Lenis et al., 2018).

**Normal maturation of ABCA4 knockout and iRPE cells from patients with STGD1**

The presence of ABCA4 in the apical membrane of human RPE cells suggested that this protein may play a role in RPE physiology and STGD1 disease pathogenesis. To discover ABCA4 function in RPE cells and disease pathophysiology, we generated ABCA4 knockout (KO) induced pluripotent stem cells (iPSCs) using the CRISPR-Cas9 approach. Two independent guide RNAs were used to target exon 1 of the ABCA4 gene (Figure S2A). Resultant targeted clones were screened for out-of-frame deletions. Two independent clones (C1 and C2) that resulted in out-of-frame deletions on both alleles were selected for further analysis (Figure S3A). Both clones of ABCA4+/− iPSCs and the
corresponding isogenic control iPSC line displayed normal karyotype (Figures S2B-S2D) and showed comparable expression of pluripotency markers OCT4 and SSEA4 (Table S2). ABCA4−/− iPSCs were differentiated into RPE (ABCA4−/−-iRPE) cells and matured on Transwells using our previously published protocol (Sharma et al., 2019).

qRT-PCR, western blot, and immunostaining confirmed the null phenotype with lacking ABCA4 mRNA and protein expression in ABCA4−/−-iRPE cells (Figures 2A, 2B, and S2E-S2K). ABCA4−/−-iRPE and isogenic control cells expressed similar levels of developmental (MITF, PAX6, DCT, TYRP1, TYR, GPNMB) and mature (RLBP1, ALDH1A3, BEST1, RPE65, CLDN19, EZRIN, MFRP) RPE markers, as confirmed by RNA sequencing (RNA-seq) analysis (Sharma et al., 2019) (Figure S2L), suggesting no significant differences in the ability of mutant cells to mature into RPE cells. Immunostaining of iRPE monolayers for maturity markers RPE65 and EZRIN further confirmed the RNA-seq data (Figures 2C-2H).

TEM of iRPE monolayers showed normal polarized RPE features like abundant apical processes, apically located melanosomes, tight junctions between neighboring cells, and basally located nuclei in ABCA4−/−-iRPE (Figures 2I-2K). Scanning electron microscopy (SEM) also suggested the formation of normal and fully confluent apical processes in ABCA4−/−-iRPE similar to isogenic control-iRPE (Figures 2L-2N). Transepithelial potential (TEP), a difference in membrane potential of apical and basolateral membranes, shows the differential expression of ion channels on either membrane and suggests functional polarization and maturation of the RPE monolayer (May-Simera et al., 2018; Miyagishima et al., 2016). Compared with the control RPE monolayer (TEP of 2.56 ± 0.99 mV), both ABCA4−/−-iRPE samples had similar TEPs (1.65 ± 0.30 and 0.56 ± 0.19 mV, respectively; Figures 2O and S2M-S2O). Furthermore, compared with the isogenic control, ABCA4−/−-iRPE did not show any difference in the hyperpolarization responses induced by decreasing the apical potassium concentration from 5 to 1 mM or the depolarization response induced by apical ATP application. These two physiological stimuli mimic the subretinal space changes upon a dark-to-light transition (Joseph and Miller, 1991) (Figures 2O and S2M-S2O; Table S3).
Figure 2. Comparable maturation of Stargardt patient, ABCA4 knockout (KO), and control iPSC-derived RPE (iRPE) cells

(A and B) qRT-PCR (A) and western blot (B) analysis of ABCA4 expression in iRPE with CRISPR-Cas9-mediated ABCA4 KO (n = 3 for each hfRPE, iPSCs, isogenic control, ABCA4+/C0/C0 clone 1 [C1], and ABCA4+/+/C0/C0 clone 2 [C2]). In (A), ABCA4 mRNA levels relative to the isogenic control are shown. In (B), hfRPE and wild-type iRPE served as positive controls, whereas iPSCs served as negative control. β-tubulin was used as the loading control.

(C–H) Immunostaining of mature RPE markers (RPE65, green) (C–E) and EZRIN (green) (F–H), ZO-1 (red), and DAPI (blue) in isogenic control, and ABCA4+/C0/C0 clone 1 and 2 iRPE (n = 3 for each line).

(I–K) Transmission electron micrographs (TEM) of isogenic control and ABCA4+/C0/C0 clone 1 and 2 iRPE cells show polarized cells with apical processes, apically located melanosomes, tight junctions, and basally located nuclei (n = 3 for each line).

(L–N) Scanning electron microscopy (SEM) showing apical processes in iRPE cells.

(O) Transepithelial potential (TEP) response in iRPE. Resting-state TEP and change in TEP in response to apical 1 mM K+ or 100 μM ATP are shown (n = 3 for each isogenic control, ABCA4+/+/C0/C0 clone 1, and ABCA4+/+/C0/C0 clone 2).

(P) SNP genotyping analysis of patient cells showing homozygosity for the ABCA4 c.6088C>T mutation (n = 3, for each unaffected control [red] and patient [blue]). Squares indicate no-template controls (NTCs).

(Q and R) ABCA4 expression in the patient-iRPE by qRT-PCR (Q) and western blot (R) analyses (n = 3, for each hfRPE, iPSCs, unaffected control, and patient). iPSCs are used as the negative control, and hfRPE and unaffected control iRPE are used as a positive control for droplet digital PCR (dd-PCR) and western blot.

(S–V) TEM (S and T) and SEM (U and V) images of RPE derived from an unaffected control and patient iPSCs (n = 3 for each line). n represents independent samples from each group.
To complement the analysis of Abca4−/−-iRPE, we also generated STGD1 patient-iRPE. To avoid potentially confounding effects of diverse ABCA4 mutations, we chose cells from patients with a homozygous mutation (C>T in exon 44 at 6,088 bp position) that leads to a non-sense-mediated decay of mutant ABCA4 mRNA (Singh et al., 2006). iPSCs were derived from skin fibroblasts isolated from patients with Stargardt and an unaffected control. Both iPSC lines expressed pluripotency markers OCT4, TRA1-81, and SSEA4 (Table S2) and were karyotypically normal (Figures S3 A and S3B). The patient iPSC line was confirmed to be homozygous for a single nucleotide C-to-T substitution (C.6088C>T) (Figure S3C), resulting in the creation of a premature stop codon (i.e., Arg2030Stop) (Singh et al., 2006). Patient-iRPE were genotyped using mutation-specific TaqMan probes to verify that the patient cells contained the ABCA4 mutation (Figure 2P). Droplet digital PCR and western blot analyses confirmed that mRNA decay and loss of ABCA4 protein (Figures 2Q and 2R). Similar to Abca4−/−-iRPE, patient and unaffected control-iRPE cells expressed similar levels of developmental (MITF, PAX6, DCT, TYRP1, TYR, GPNMB) and mature (RLBP1, ALDHI1A3, BEST1, RPE65, CLDN19, EZRIN, MFRP) RPE markers, as confirmed by RNA-seq analysis (Sharma et al., 2019) (Figure S3D), suggesting there are no defects in the ability of mutant cells to mature into RPE cells. TEM of patient iRPE monolayers revealed normal apical processes, apically located melanosomes, tight junctions between neighboring cells, and basally located nuclei (Figures 2S and 2T). SEM further confirmed comparable apical processes in the patient and healthy-iRPE (Figures 2U and 2V). Again, similar to Abca4−/−-iRPE, patient-iRPE did not show any difference in resting transepithelial resistance (TER), TEP, or response to reduced apical potassium concentration from 5 to 1 mM compared with control cells (Figures S3E and S3F). Overall, these results confirmed that the ABCA4 LOF does not affect the ability of iPSCs to differentiate into mature and functional RPE cells. A comprehensive single-cell-resolution morphometric analysis of iRPE lines was performed to determine potential changes in iRPE cell shape metrics caused by ABCA4 LOF (Ortolan et al., 2022). STGD1-iRPE did not show any significant differences compared with control-iRPE on four different morphometric features that define compact packing of epithelial cells in a sheet—cell area, aspect ratio, hexagonality, and the number of neighbors (Figures S3G–S3J).

**Stargardt pathophysiology is replicated cell autonomously in STGD1-iRPE**

STGD1 pathophysiology in RPE cells is thought to be induced by A2PE-laden POS from Abca4-mutant rod PRs phagocyted by RPE cells (Radu et al., 2011). Here, we asked whether there was a cell-autonomous (independent of STGD1 POS) functional defect in the ability of STGD1-iRPE (Abca4−/− C1 and C2 and patient-iRPE) to process wild-type (WT) POS. Previously, POS feeding to patient RPE has been used to induce disease phenotype in cells (Miyagishima et al., 2021). STGD1-iRPE and corresponding controls (isogenic and unaffected) were fed with WT POS for 7 days, and the accumulation of intracellular lipid deposits was evaluated using lipid dye (BODIPY493/503). iRPE monolayer stained for BODIPY shown in cross-sections (compare Figures 3A–3C with 3D–3F, arrowheads point to sub-RPE deposits) and en face view (compare Figures 4A–4C with 4D–4F, see inset for higher magnification) revealed 2- to 3-fold higher intracellular RPE accumulation of lipids in POS-treated STGD1-iRPE compared with control cells (Figure 3G; p < 0.0001). These results confirm that WT POS feeding is sufficient to induce intracellular lipid deposits in STGD1-iRPE.

Previously, RPE cells in Abca4−/− mice have been shown to accumulate cholesterol (Toops et al., 2015). These defects have been attributed to PR-derived A2E-induced cholesterol metabolism defects in the RPE. We asked if cholesterol accumulation was also an autonomous cell phenotype in STGD1-iRPE. Filipin, a cholesterol-staining dye, showed 3- to 4-fold higher cholesterol accumulation in POS-fed STGD1-iRPE compared with control cells (compare Figures 3H–3J with 3K–3M; p < 0.0001). Ceramide (a lipid metabolite) accumulation at the RPE’s apical side is a hallmark feature of the pigmented Abca4−/− mouse model (Kaur et al., 2018). Immunostaining for ceramide confirmed a 2- to 3-fold increase in apical ceramide in STGD1-iRPE when exposed to WT POS (compare Figures 3O–3Q and 3R–3U). Combined, these data support our hypothesis of STGD1-POS-independent, cell-autonomous functional defects in STGD1-iRPE to process WT POS. Furthermore, this work showed that ABCA4 KO and patient-iRPE cells represent a physiologically relevant in vitro disease model for STGD1.

Previously, complement signaling has been suggested to enhance the disease phenotype in Abca4−/− mouse eyes (Radu et al., 2011). We asked if STGD1-iRPE responds similarly to activated complement signaling. STGD1-iRPE was treated with 5% complement-competent human serum (CC-HS) or complement-incompetent HS (CI-HS) for 48 h based on a protocol developed previously in our lab (Sharma et al., 2021). Activated complement challenge led to a 3- to 4-fold increase in intracellular lipid deposits in STGD1-iRPE compared with a 2-fold increase seen in control-iRPE (compare Figures S4G–S4I and S4J–S4M; insets show higher magnification of lipid deposits; p < 0.0001). Similarly, activated complement treatment led to higher ceramide accumulation in STGD1-iRPE than control-iRPE (compare Figures S4N–S4P and S4Q–S4S).
Figure 3. Stargardt disease pathophysiology is replicated in STGD1-iRPE using wild-type POS

(A–F) Representative cross-sections of iRPE monolayers grown on Transwell membrane, unfed (A–C) or POS fed for 7 days (D–F), and labeled for lipid deposits with BODIPY (green, white arrowheads) and F-ACTIN (red).

(G) Quantification of BODIPY-stained lipid deposits, p < 0.001 for POS-fed isogenic control versus C1 and C2 and control versus patient (n = 10, for each isogenic control, ABCA4/C0/C0/C1, ABCA4/C0/C0/C2, unaffected control, and patient).

(H–M) En face views of representative filipin (blue)-stained lipid deposits in unfed (H–J) or POS-fed for 7 days (K–M) STGD1-iRPE (I, J, L, and M) compared with the control (H and K).

(N) Quantification of filipin-stained lipid deposits, p < 0.001 for POS-fed isogenic control versus C1 and C2 and control versus patient-iRPE (n = 10, for each isogenic control, ABCA4/C0/C0/C1, ABCA4/C0/C0/C2, unaffected control, and patient).

(O–T) En face views of ceramide immune stained (ceramide species 16 and 24, red) in unfed (O–Q) and POS-fed for 7 days (R–T) STGD1-iRPE (P, Q, S, and T) compared with the control cells (O and R). F-actin is labeled in green.

(U) Quantification of ceramide intensity signal, p < 0.001 for POS-fed isogenic control versus C1 and C2 and control versus patient (n = 12, for each isogenic control, ABCA4/C0/C0/C1, ABCA4/C0/C0/C2, unaffected control, and patient). The control image represents isogenic control for ABCA4/C0/C0-iRPE and unaffected control for the patient-iRPE. n represents independent samples from each group.
Quantification of ceramide signal showed a 3-fold increase in CC-HS-treated STGD1-iRPE compared with CI-HS-treated cells (Figure S4T; p < 0.0001).

**Defective POS processing in STGD1-iRPE**

Increased lipid and ceramide accumulation upon POS feeding in STGD1-iRPE suggested defective lipid metabolism and defective POS digestion in these cells (Bosch et al., 1993; Mazzoni et al., 2014). To determine if STGD1-iRPE is defective in POS digestion, we fed Stargardt and control-iRPE with pHrodo dye-labeled WT POS (10 POS per RPE cell) based on previous protocols and analyzed samples by flow cytometry (Mao and Finnemann, 2013; May-Simera et al., 2019). Since pHrodo dye only fluoresces inside lysosomes, a comparative analysis of its signal at 4 and 24 h after POS feeding helped distinguish between POS uptake (4 h signal) and POS digestion (24 h signal). STGD1-iRPE cells showed a similar ability to uptake POS as control cells at the 4 h time point (Figure S5A) but showed a 50%–70% reduced POS digestion rate compared with control cells at the 24 h time point (Figures S5A and 4A; p < 0.001). POS digestion is mediated in the endo-lysosomal compartments (Bosch et al., 1993; Deguchi et al., 1994). We analyzed the cathepsin B activity using the Magic Red substrate to determine if the POS digestion defect is due to dysfunctional lysosomes. The Magic Red substrate fluoresces red upon cleavage by active enzymes, indicating lysosomal pH (Lu et al., 2018). STGD1-iRPE cells showed reduced cathepsin B activity, suggesting elevated lysosomal pH, compared with the control-iRPE (Figures 4B–4E), similar to the known effect of drug hydroxychloroquine on increasing lysosomal pH (Figures 4B and 4F–4H) (Halcrow et al., 2021). These results are consistent with previously reported data from Abca4 –/– mouse RPE cells (Liu et al., 2008) and suggest that ABCA4 mutation in RPE cells disrupts lysosomal activity, likely contributing to inefficient POS digestion—causing lipid deposits in RPE cells. Over time, these lipid deposits may contribute to RPE atrophy leading to PR degeneration.

Elevated lysosomal pH is associated with several degenerative diseases (Colacurcio and Nixon, 2016). Drugs that lower lysosomal pH are used as potential therapies for lysosome-defect-associated diseases (Bonam et al., 2019). To test whether lowering lysosomal pH in STGD1-iRPE improved POS digestion, we used the drug CGS 21680 HCL-A2, known to decrease lysosomal pH by stimulating A2A adenosine or β-adrenergic receptors (Liu et al., 2008). β-adrenergic and A2A adenosine are G protein-coupled receptors whose activation stimulates adenylate cyclase and increases intracellular cAMP, resulting in the acidification of lysosomes (Liu et al., 2008). An increase in cathepsin B activity suggested that CGS 21680 HCL-A2 lowered the lysosomal pH of STGD1-iRPE (Figures 4I–4L). This reduction of lysosomal pH improved the POS digestion rate of STGD1-iRPE cells by 30%–50% compared with untreated cells (Figure 4M; p < 0.0001). These results were further confirmed by rhodopsin western blot after POS feeding (Figure S5B). STGD1-iRPE showed a higher accumulation of rhodopsin after 24 h of POS feeding compared with control-iRPE, suggesting reduced POS digestion. This accumulation was ameliorated by lowering lysosomal pH using CGS 21680 HCL-A2. Overall, this work showed that higher lysosomal pH likely causes defective phagosomal processing in STGD1-iRPE and contributes to disease pathogenesis.

**Correction of lipid handling rescues disease phenotype in STGD1-iRPE**

To discern if ABCA4 indeed participates in the lipid-handling pathway in RPE cells, we focused on the ABCA4 ortholog ABCA1, which regulates lipid transport in RPE cells and shares 64.5% sequence homology with ABCA4 (Storti et al., 2019). We asked if modulating ABCA1 expression would change the lipid accumulation phenotype in STGD1-iRPE cells, suggesting that the two ABC proteins work through a common lipid-handling pathway. ABCA1 expression was knocked down (KD) using lentivirus-mediated delivery of specific short hairpin RNA (shRNA) targeting ABCA1 mRNA. Expression downregulation of ABCA1 was confirmed by qRT-PCR (Figure S5C). ABCA1 KD in STGD1-iRPE exacerbated CC-HS-induced lipid deposits (compare Figures 5A–5F and 5G–5L). Quantification of BODIPY493/503 signal showed a 1.5- to 2-fold increase in CC-HS-treated ABCA1KD-STGD1-iRPE compared with non-targeting shRNA CC-HS-treated STGD1-iRPE (Figure 5M). In contrast to ABCA1 KO, activation of its expression via an LXR agonist, GW3965 (Xu et al., 2013), reduced lipid accumulation in CC-HS-treated STGD1-iRPE cells (compare Figures 5A–5F and 5N–5S). Quantification of BODIPY493/503 signal showed a 1.5- to 2-fold reduction in CC-HS + GW3965-treated STGD1-iRPE compared with the CC-HS + vehicle-treated STGD1-iRPE (Figure 5T; p < 0.001; Figure S5D, and three-dimensional [3D] images in Figures S5E–S4H). These data further supported the hypothesis that the lipid-handling defects seen in STGD1-iRPE are induced cell autonomously in these cells. Furthermore, the loss of ABCA1 function has an additive effect on this cell-autonomous phenotype in Stargardt cells, suggesting that, similar to ABCA1, ABCA4 works in the RPE lipid-handling pathway. It also provided a mechanism for developing potential therapeutics for STGD1.

**DISCUSSION**

Our work provides an in vitro model for improved understanding of STGD1 pathogenesis, a rare inherited retinal...
degeneration with no current treatment. Here, we cell-autonomously replicated part of Stargardt’s pathophysiology, including increased intracellular lipid and ceramide deposits and defective POS digestion, in patients and ABCA4 KO-iRPE. Our work provides direct evidence that the loss of ABCA4 function in human RPE contributes to ...
Figure 5. Manipulation of ABCA1 levels in STGD1-iRPE alters lipid deposits

(A–F) BODIPY (green) staining for lipid deposits in non-targeting shRNA ABCA4−/− iRPE (B, C, E, and F) and isogenic control iRPE (A and D) after complement-incompetent human serum (CI-HS) (A–C) and complement-competent human serum (CC-HS) (D–F) treatment. Inserts in (D–F) show higher magnification of the BODIPY signal.

(G–L) Activated complement-induced lipid deposits in ABCA1 knockdown (KD)/ABCA4 wild type (WT) (G and J) versus ABCA1 KD/STGD1-iRPE (H, I, K, and L) in CC-HS-treated (compare insets J–L) cells (J–L) compared with CI-HS treated cells (G–I).

(M) Boxplot showing quantification of BODIPY* signal in CC-HS-treated ABCA1KD-STGD1-iRPE compared with CC-HS-treated STGD1-iRPE, p < 0.01 and p < 0.05 (n = 7, for each non-targeting shRNA isogenic control, non-targeting shRNA ABCA4−/− C1, non-targeting shRNA ABCA4−/− C2, ABCA1 KD/isogenic control, ABCA1 KD/ABCA4−/− C1, ABCA1 KD/ABCA4−/− C2).

(N–S) BODIPY (green) lipid staining in STGD1-iRPE cells treated CC-HS + vehicle (N–P) versus CC-HS + GW3965 (Q–S).

(T) Boxplot showing quantification of BODIPY* signal in CC-HS + GW3965-treated STGD1-iRPE compared with the CC-HS + vehicle-treated STGD1-iRPE, p < 0.01 p < 0.001, and p < 0.0001 (n = 6, for each isogenic control, ABCA4−/− C1, ABCA4−/− C2, unaffected control, and patient). n represents independent samples from each group.
STGD1 cellular phenotypes, in addition to its known function in PRs. The significance of our approach relies on a previously underappreciated role of ABCA4 in human RPE cells.

Previously, lipofuscin, cholesterol, and ceramide deposits were reported in RPE of patients with Stargardt and Abca4−/− mice, where they were thought to be caused by Stargardt POS-mediated and A2E-induced lipid metabolism defects in RPE cells (Birnbach et al., 1994; Kaur et al., 2018; Toops et al., 2015). Here, we recapitulated lipid, cholesterol, and ceramide deposits in STGD1-iRPE without exposure to Stargardt POS or A2E. Our work suggests a Stargardt POS-independent pathway leading to lipid accumulation in STGD1-iRPE. We identified two stimuli, WT POS and activated complement, that triggered lipid deposits in STGD1-iRPE. WT POS-mediated lipid deposits in STGD1-iRPE are likely associated with defective maturation of phagolysosomes and reduced POS digestion. This hypothesis is consistent with our observation of reduced cathepsin B activity, suggesting a lower lysosomal function in STGD1-iRPE.

It is, however, not clear if, in STGD1-iRPE, phagolysosomal dysfunction is primarily caused by ABCA4 LOF or indirectly by ceramide accumulation, as has been suggested previously (Kaur et al., 2018). In the RPE, excess ceramide alters membrane dynamics and results in aberrant endosome biogenesis, impaired autophagosome trafficking, and mitochondrial fragmentation (Kaur et al., 2018; Tan et al., 2020). In addition, ceramide’s role in phagolysosomal maturation was shown previously (Pathak et al., 2018). We speculate that the feedback loop of initial phagolysosomal maturation defects in STGD1-iRPE leads to inefficient POS digestion and lipid processing, causing ceramide and other lipid accumulation in diseased RPE that further enhances STGD1 pathogenesis. All these phenotypes may be further exaggerated by A2PE-laden STGD1-POS that STGD1-iRPE would phagocyte in the patient eyes.

Our data demonstrate that ABCA1 KD exacerbates the cellular phenotype of STGD1-iRPE and that LXR agonist GW3965 (known to increase ABCA1 expression [Hussein et al., 2015; Xu et al., 2013]) reduces lipid deposits in STGD1-iRPE. These data further support our hypothesis that ABCA4 LOF disrupts lipid homeostasis in RPE cells and adds to the growing list of lipid-handling surface proteins in RPE lipid homeostasis cells. However, our data do not provide evidence that, similar to ABCA1, ABCA4 is a lipid transporter (Rivera et al., 2000). It is, however, not surprising that RPE cells may depend on multiple lipid-handling ABC transporters; a given RPE cell phagocytoses 7%–10% of OSs of up to 30 PRs every day for its lifetime and has to metabolize all those lipids present in POS (Young and Bok, 1969). Therefore, it is feasible that the three ABC transporters (ABCA1, ABCA4, ABCG1) are involved at different stages of POS uptake, transport, and digestion, and in the event that one or the other transporter is non-functional, it leads to lipid metabolic defects (Storti et al., 2019). Overall, these data support the notion that strategies of reducing lipid accumulation, including LXR agonists used in the clinic (ClinicalTrials.gov: NCT02922764), and improved lysosomal function in the RPE, provide promising therapeutic approaches for STGD1 (Bonam et al., 2019; Travis et al., 2007).

Here, we show that STGD1-iRPE recapitulates key disease phenotypes. The STGD1-iPSCs developed here offer a renewable and reproducible source of cells to produce RPE and retinal organoids. There are significant efforts in the eye community to develop cocultures of RPE and retinal organoids such that POS-RPE interaction can be further studied in vitro. These iPSC-derived Stargardt tissues will allow an in-depth understanding of disease pathogenesis and help decipher the relative contribution of RPE and PRs in this disease. Currently, there is limited availability of Stargardt iPSC lines (Claassen et al., 2019; Jennings et al., 2020; Riera et al., 2019). To overcome this barrier, the National Eye Institute has initiated the STGD1-iPSC banking program from patients with broad ABAC4 mutations. These cells will be available to the community for mechanistic and genotype-phenotype studies.

Over 800 mutations are known to affect ABCA4 function and are associated with a broad spectrum of Stargardt’s disease phenotypes (Quazi et al., 2012). The patient sample used in our study led to non-sense-mediated RNA decay leading to no protein. Similar results were noted with CRISPR-Cas9-mediated ABCA4 KO iRPE generated in our study. It is, however, enticing to speculate that different mutations affecting varied ABCA4 domains may affect RPE and PRs differently—suggesting that ABCA4 may have slightly different functions in the two cell types. A genotype-phenotype study with iPSCs derived RPE and PRs with such different mutations may help further clarify this hypothesis. Overall, our work provides additional insights into the current understanding of STGD1 disease pathophysiology and suggests that ongoing gene therapy approaches need to target both RPE and PR cell types for longer-term efficacy.

**EXPERIMENTAL PROCEDURES**

**Generation, characterization, and differentiation of hiPSCs**

Detailed procedures and information on the generation, characterization, and differentiation of hiPSC are provided in the supplemental experimental procedures.

**Cell culture iPSC-RPE differentiation**

iPSCs were differentiated into RPE (iRPE) using a previously published protocol (Sharma et al., 2019). Detailed protocol for
differentiation is provided in the supplemental experimental procedures.

**Protein/lipid staining and immunoblotting**

Immunostaining of iRPE monolayers was performed as previously described (Sharma et al., 2019). For immunoblotting, lysates were made using radioimmunoprecipitation assay (RIPA) lysis buffer with 1% phosphatase and protease inhibitors (Thermo Fisher Scientific). Detailed procedures are provided in the supplemental experimental procedures.

**Phagocytosis assay/flow cytometry**

Detailed procedures of phagocytosis assay of iRPE monolayers are provided in the supplemental experimental procedures.

**SEM and TEM**

Detailed protocols for SEM and TEM are provided in the supplemental experimental procedures.

**RNA purification and RNA-seq analysis**

Detailed procedures for RNA purification and RNA-seq analysis are provided in the supplemental experimental procedures.

**Statistical analysis**

The data were presented as boxplots. The statistical analysis was performed using GraphPad Prism software (La Jolla, CA, USA). Adjusted p values of *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 suggested significance. Detailed procedures are provided in the supplemental experimental procedures.

**DATA AND CODE AVAILABILITY**

The bulk RNA-seq data generated in this study have been deposited in the GEO database under GEO: GSE198362.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.stemcr.2022.10.001](https://doi.org/10.1016/j.stemcr.2022.10.001).

**AUTHOR CONTRIBUTIONS**

Conceptualization, K.B. and M.F.; methodology, K.B., M.F., I.M., and K.R.B.; investigation, M.F., D.B., V.K., P.J.S., M.A.-A., M.C., R.V., Q.W., A.M., F.B., I.M., S.M., D.R.R., R.L.G., K.R.B., and R.V.; formal analysis, M.F., R.V., and D.M.; project administration, M.F.; writing – original draft, M.F. and D.B.; writing – review & editing, M.F. and K.R.B.; funding acquisition, K.B.; resources, K.B.; supervision, K.B.

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**CONFLICT OF INTERESTS**

The authors declare no competing interests.

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