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**In-vitro stem cell modelling demonstrates a proof-of-concept for excess functional mutant *TIMP3* as the cause of Sorsby Fundus Dystrophy**

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**Running title:** SFD-hiPSC-RPE demonstrate a mutant TIMP3 driven gain of function

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**Abstract**

Sorsby Fundus Dystrophy (SFD) is a rare autosomal dominant disease of the macula that leads to bilateral loss of central vision and is caused by mutations in the *TIMP3* gene. However, the mechanisms by which *TIMP3* mutations cause SFD are poorly understood. Here, we generated human induced pluripotent stem cell-derived retinal pigmented epithelial (hiPSC-RPE) cells from three SFD patients carrying *TIMP3 p.(Ser204Cys)* and three non-affected controls to study disease related structural and functional differences in the RPE. SFD-hiPSC-RPE exhibited characteristic RPE structure and physiology but showed significantly reduced transepithelial electrical resistance associated with enriched expression of cytoskeletal remodelling proteins. SFD-hiPSC-RPE exhibited basolateral accumulation of TIMP3 monomers, despite no change in *TIMP3* gene expression. TIMP3 dimers were observed in both SFD and control hiPSC-RPE, suggesting mutant TIMP3 dimerization does not drive SFD pathology. Furthermore, mutant TIMP3 retained matrix metalloproteinase activity. Proteomic profiling showed increased expression of extracellular matrix proteins, endothelial cell interactions and angiogenesis-related pathways in SFD-hiPSC-RPE. By contrast, there were no changes in VEGF secretion. However, SFD-iPSC-RPE secreted higher levels of monocyte chemoattractant protein 1, platelet-derived growth factor, and angiogenin. Our findings provide a proof-of-concept that SFD patient-derived hiPSC-RPE mimic mature RPE cells and support the hypothesis that excess accumulation of mutant TIMP3, rather than an absence or deficiency of functional TIMP3, drives ECM and angiogenesis related changes in SFD.

**Keywords:** Sorsby fundus dystrophy, human induced pluripotent stem cell, retinal pigment epithelial cell, retinal degeneration, metalloproteinase inhibitor 3 **Introduction**

Sorsby fundus dystrophy (SFD) is a rare, autosomal dominant macular dystrophy caused by mutations in the gene *TIMP3* (metalloproteinase inhibitor 3) and is estimated to affect 1 in 220,000 people [1]. Without genetic testing, SFD patients are often misdiagnosed with either idiopathic choroidal neovascularization or wet age-related macular degeneration (AMD) due to their shared clinical features. However, onset of the disease occurs earlier in SFD patients, usually between the 4th and 6th decade of life [1,2]. Rare variants of *TIMP3* have been identified in genome wide association studies (GWAS) of AMD [3]. However, the causative relationship between *TIMP3* variants and AMD risk alleles remains ambiguous. SFD patients experience bilateral loss of central vision due to atrophy of the retinal pigmented epithelium (RPE) or choroidal neovascularisation (CNV), leading to irreversible photoreceptor loss. Initially, patients show lipid-enriched, drusen-like deposits between the basement membrane of the RPE and the inner-collagenous layer of Bruch’s membrane (BrM) [4]. The RPE and the underlying BrM are the epicentre of SFD development. Together they form the outer blood-retina barrier (BRB) which selectively regulates the transport of nutrients and waste between the retina and the choriocapillaris. A markedly thickened BrM is a key feature observed in SFD patients and is believed to impair diffusion across the BRB and contribute to the accumulation of RPE metabolic waste products and the subsequent formation of sub-RPE deposits [5,6]. Current treatments for SFD patients focus on managing CNV through regular intravitreal vascular endothelial growth factor (VEGF) inhibitor injections to limit choroidal pathology [7].

The family of TIMP proteins are expressed ubiquitously within the body and play a myriad of biological roles through their ability to reversibly inhibit MMPs (matrix metalloproteinases), ADAMs (a disintegrin and metalloproteinase) and ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) [8,9]. *TIMP3* encodes a 24 kDa glycoprotein consisting of two domains: the N-terminal domain is involved in MMP inhibition (and at high levels can induce apoptosis in RPE), whereas the C-terminal domain binds directly to VEGFR2 (KDR), inhibiting VEGF binding and downstream PI3K/Akt and Ras/Raf/ERK signalling in choroidal endothelial cells [1]. Of the four members of the TIMP family, TIMP3 possesses the broadest range of activity, including the ability to inhibit all MMPs and several of the ADAM and ADAMTS family [8]. To date, 18 SFD-causing *TIMP3* mutations have been identified, with the majority occurring at the C-terminus of the protein and resulting in the gain or loss of a cysteine residue. The current hypothesis proposes this odd number of cysteines enables formation of intermolecular disulphide bridges between mutated TIMP3 proteins, resulting in dimers or multimers that are more resistant to turnover/clearance from the ECM, and thus contributes to thickening of BrM [1].

Whilst several studies have shown that mutant TIMP3 retains its MMP inhibitory function, others have reported a loss-of-function phenotype. Thus, it remains unclear how mutations in *TIMP3* affect the resulting protein’s ability to regulate ECM assembly/disassembly (reviewed in [1,2]). Several studies were also unable to confirm dimerization of mutant TIMP3 [10,11]. These inconsistencies are likely due to differences in the *TIMP3* mutation of interest and the cell types used to study them. Of note, not all known SFD *TIMP3* mutations result in a loss or gain of a cysteine, with some mutations generating novel lysine or arginine residues or indeed a premature stop codon. This suggests that alternative structural changes to the TIMP3 protein may underlie disease phenotypes in these patients [1]. Despite being a monogenetic disorder, there is considerable heterogeneity between SFD patients depending on the TIMP3 mutation, and indeed even within families with the same mutation [1,2]. Mouse models of SFD have been unable to successfully recapitulate the phenotypic severity observed in SFD patients. Knock-in mice (*Timp3+/S156C*, *Timp3S156C/S156C*) have been shown to exhibit abnormalities of BrM and the basal microvilli at 8 months of age, compared to 30 months in wild-type littermates; however, electrophysiology demonstrates normal retinal function [12].

The shortcomings of current *in vitro* and animal models necessitate the development of new approaches to elucidate underlying disease mechanisms. Human induced pluripotent stem cells (hiPSCs) enable relevant, patient-specific disease modelling and have provided novel insights into drusen biogenesis in rare retinal dystrophies [13]. In view of the aforementioned limitations, we established hiPSC-RPE cell lines from three TIMP3 p.(Ser204Cys) SFD patients and healthy controls to determine how this mutation alters the structure and function of RPE cells.

**Materials and methods**

Detailed methodology is provided in Supplementary materials and methods.

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**Ethics**

The University of Southampton has ethical approval for SFD hiPSC generation (UK REC reference: 14/LO/1330, 23.06.2014). Participation was voluntary, and patients gave written informed consent. Tampere University has an appropriate licence of The Board for Gene Technology, Finland (022/M/2016, 20.06.2016) and ethical approval of the Ethical Committee of Pirkanmaa Hospital District to derive (R08070, 11.10.2016; R12123, 3.10.2017) and conduct studies with hiPSC lines (R14023, 13.12.2016). The Declarations of Helsinki were adhered to.

**Patients**

After ethical approval and informed consent, skin biopsies were obtained from three SFD patients from Southampton General Hospital, UK: TIMP3 c.610A>T; p.(Ser204Cys), confirmed by genotyping. Two of the three SFD patients were sisters. Skin biopsies were also taken from the unaffected mother of the sisters, who served as a control. In addition, two control hiPSC lines from unaffected individuals were kindly provided by Professor Aalto-Setälä, Tampere University, Finland. All patient information was pseudonymised for analysis.

**Human iPSC-RPE**

Fibroblasts isolated from patient skin biopsies were reprogrammed into human iPSCs using CytoTune-iPS Sendai Reprogramming kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Two clones per patient and one clone per control were further cultured and characterized (Table 1), as described in detail in the Supplementary materials and methods. RPE differentiation was performed using spontaneous differentiation, followed by RPE selection, sequential passaging for purification and expansion, cryopreservation, and final maturation on permeable cell culture inserts (Cat. MCRP24H48, Merck Millipore, Darmstad, Germany) or in 24-well plates (Corning® CellBIND®, Merck Millipore) at passage 4 (P4). Human iPSC-RPE clones (n=3 control, and n=5 SFD) were characterized for expression of cell-specific markers, transepithelial electrical resistance (TEER), polarized secretion of pigment epithelium-derived factor (PEDF) and phagocytic capacity.

**Transmission electron microscopy**

For transmission electron microscopy (TEM) analysis, hiPSC-RPE (n=3 control, n=3 SFD) were cultured on inserts for 71–81 days. Details of sample preparation have been described previously [14].

**RT-qPCR**

Relative *TIMP3* gene expression between control (n=3) and SFD-hiPSC-RPE (n=5 SFD, day 73) was analysed using RT-qPCR using TaqMan Gene Expression Assay HS00165949\_m1 (Applied Biosystems, Thermo Fisher Scientific).

**Western blotting**

Western blotting (WB) was used to investigate the expression of TIMP3 (1:750, ab39184 and ab58804 both from Abcam, Cambridge, UK), Apolipoprotein E (1:1000, ab947, Millipore), Fibulin (1:400, sc-33722, Santa Cruz Biotechnology, Dallas, Texas, USA), and β-Actin (1:1000, sc-47778, Santa Cruz Biotechnology) in hiPSC-RPE (n=3 control, n=5 SFD, 70–76 days) under reducing conditions.

**Enzyme-Linked Immunosorbent Assay (ELISA)**

Secretion of PEDF (DY1177-05, R&D Systems, Minneapolis, Canada), TIMP3 (ab119608, Abcam), and VEGF (DVE00, R&D Systems) by hiPSC-RPE (n=3 control, n=5 SFD) was quantified using enzyme-linked immunosorbent assays (ELISA).

**Collagenase assay**

MMP-inhibitory activity of conditioned medium (CM) of hiPSC-RPE (n=3 control, n=5 SFD) was examined in triplicate using a collagenase activity assay (EnzChek gelatinase/collagenase kit; Thermo Fisher Scientific).

**Sequential window acquisition of all theoretical mass spectra (SWATH-MS) proteomics**

For mass spectrometry proteomics, the hiPSC-RPE were cultured for 70 days on 24-well plates (n=3 control, n=5 SFD and 3–7 replicates/cell line). The cells were initially pelleted and frozen at –80 °C. For MS analysis cell pellets were lysed and total protein concentration of each sample was measured, after which proteins were reduced, alkylated and digested with trypsin as described previously [15,16]. Analysis of the samples was performed using an Eksigent 425 NanoLC coupled with high speed TripleTOF 5600+ mass spectrometer (Ab Sciex, Concord, Canada) using SWATH acquisition as described previously [15,16].

**Human angiogenesis array**
Secretion of angiogenesis-related proteins was analysed in pooled basal culture medium from hiPSC-RPE cells cultured on inserts (n=3 control, n=5 SFD, 2 inserts each) using the Proteome Profiler Human Angiogenesis Array Kit (ARY007, R&D Systems).

**Results**

**Sorsby Fundus Dystrophy patient-derived hiPSC lines**

# Human iPSC lines were generated from three female individuals diagnosed with SFD and carrying *TIMP3* mutation producing p.(Ser204Cys) (previous nomenclature p.(Ser181Cys)) (Table 1). Two of the patients were siblings, the other was unrelated. The mother of the two sisters, who is unaffected with no history of macular degeneration, served as a healthy control, in addition to two healthy, unrelated, age-matched females. Despite anti-VEGF treatment over a 11-year period, SFD pathology progressed, with the development of macular scarring due to ongoing CNV (Figure 1). Two hiPSC clones from each patient were cultured and extensively characterized (Table 1). The removal of the introduced viral vectors and the presence of the disease-causing *TIMP3* missense mutation was confirmed in the hiPSCs. These cells exhibited pluripotency and normal diploid karyotype (46, XY), except for two of the SFD clones (RD01A and RD03B) that showed balanced translocations (supplementary material, Figures S1–S5), which had no effect on hiPSC phenotype.

**SFD-hiPSC-RPE had normal RPE properties and functionality but showed a compromised barrier after 10 weeks in culture**

The hiPSC cells were differentiated into RPE. The protocol and timeline for differentiation is shown in Figure 2A. The cell lines revealed clear clone-specific differences in differentiation capacity, with RD04B failing to produce sufficient pigmented RPE (in five separate differentiation experiments) to warrant further selection and expansion. All other clones produced functional, polarized RPE monolayers on porous inserts (Figure 2). We used a 10-week long-term culture to age the cells and allow phenotypic changes to manifest. Significantly lower (p=0.0095, Mann–Whitney test) average TEER was recorded for SFD-hiPSC-RPE compared to controls (Figure 2C), although clone-specific differences in mean TEER were seen. All cell lines showed intact junctional localization of zonula occludens 1 (ZO1) (Figure 2D) and a normal RPE protein marker profile (supplementary material, Figure S6). All hiPSC-RPE lines demonstrated the capacity to phagocytose photoreceptor outer segments (POS) (Figure 2E), with no difference in the number of attached (total, p=0.7, Mann–Whitney test) or internalised (p=1.0) POS between control and SFD-hiPSC-RPE (Figure 2F). PEDF was secreted in a polarized manner with >4-fold higher secretion to the apical insert compartment compared with the basolateral chamber for both control and SFD-hiPSC-RPE (Figure 2G). The hiPSC-RPE bearing karyotypic translocations (RD01A and RD03B) showed comparable RPE properties to karyotypically normal SFD-hiPSC-RPE cell lines (Figure 2C,G, and supplementary material, Figure 7) and were therefore included in the subsequent analyses.

**SFD-hiPSC-RPE show similar fine structure to controls**

The ultrastructure of hiPSC-RPE monolayers cultured on permeable inserts for 70 days was analysed by TEM. Cells were found to be polarized in a typical apical-basal axis and showed characteristic RPE structures including apical microvilli, melanosomes, basal nuclei and basolateral infolds, as well as sub-RPE deposits (Figure 3A). No difference in cell height (p=0.7477, unpaired, two-tailed *t*-test) (Figure 3A,B), average length of apical microvilli (p=0.9) (Figure 3A,C), or average basal lamina thickness (p=0.6387) (Figure 3D,E) were found between SFD and control-hiPSC-RPE. Sub-RPE deposits with a distinct striated pattern were identified as fibrous long-spacing (FLS) collagen and were observed between RPE and the underlying insert surface in all samples (Figure 3F, arrows). No difference in the number of FLS collagen deposits per cell was observed between SFD and control hiPSC-RPE (p=0.8389) (Figure 3G). The number of melanosomes per cell (open arrow) was also similar between SFD and control hiPSC-RPE (Figure 3H,I) (p=0.6065).

**SFD-hiPSC-RPE retained the capacity to inhibit MMPs but showed basolateral accumulation of TIMP3**

Expression, secretion, accumulation, and functionality of TIMP3 was investigated in hiPSC-RPE lines cultured for 70–84 days on 24-well plates. RT-qPCR analysis showed no difference (p= 0.2500, Mann–Whitney test) in *TIMP3* transcript levels in SFD-hiPSC-RPE compared to controls (Figure 4A). However, WB under reducing conditions showed significantly higher expression levels (8.6-fold difference, p=0.0357) of monomeric TIMP3 in SFD-hiPSC-RPE (21 kDa expected band size for ab39184) (Figure 4B). A larger sized band which was interpreted as 27 kDa glycosylated form, and another 48 kDa band, which was interpreted as a TIMP3 dimer, were found in all cell lines. We observed a similar pattern of significantly increased monomeric TIMP3 expression in SFD-hiPSC-RPE using an alternative TIMP3 antibody (Abcam ab58804) (supplementary material, Figure 8B,C). SFD-hiPSC-RPE showed slightly more TIMP3 glycosylation and dimerization in some of the blots, but the changes were not consistent in all replicate immunoblots (Figure 4B, supplementary material, Figure 8A–C). To investigate differences in the secretion of TIMP3, an ELISA assay was carried out, which showed SFD-hiPSC-RPE lines secreted significantly less TIMP3 compared to controls (p=0.0357) (Figure 4C). An ELISA was also performed on apical and basal conditioned medium (CM) collected from cells cultured on permeable culture inserts. TIMP3 was secreted predominantly via the basolateral surface in both control and SFD-hiPSC-RPE. However, basal secretion was significantly greater compared to apical secretion in SFD-hiPSC-RPE, but not in controls (p=0.0357) (supplementary material, Figure 8D).

The MMP-inhibitory activity of TIMP3 was compared between SFD-hiPSC-RPE and controls. CM was collected from cells cultured in 24-well plates and analysed using an EnzChek collagenase assay. The assay uses quenched, fluorescein-labelled gelatin that is digested by collagenase from *Clostridium histolyticum* leading to increased fluorescence proportional to proteolytic activity. Collagenase activity was moderately inhibited by the CM (containing 50 pg total TIMP3) from both SFD-hiPSC-RPE and control-hiPSC-RPE up to 3 h. However, a significant reduction in collagenase activity was seen in CM from SFD-hiPSC-RPE compared to controls by 20 (p=0.0357) and 26 h (p=0.0357), respectively (Figure 4D, supplementary material, Figure S9).

**Extracellular matrix, cytoskeleton, angiogenesis, and endothelial cell-related pathways and proteins were differentially regulated between control- and SFD-hiPSC-RPE**

A quantitative whole cell proteomic profiling of the hiPSC-RPE was conducted to compare the changes in cellular proteome caused by mutant TIMP3. Out of 2585 candidate proteins, 1638 were included in the analyses after coefficient of variation (CV) filtering, removing proteins with mean CV>30%. Out of the 1638 candidates, 89 proteins were differentially regulated according to fold-change (≥1.5-fold up- or down-regulated) in the SFD-hiPSC-RPE compared to controls (supplementary material, Table S1, Figure S10A). Interestingly, ECM and ECM-remodelling proteins were upregulated, including collagen 1 chains (COL1A1, COL12A1, COL18A1, COL14A1), nidogen 2 (NID2), laminin subunit gamma 1 (LAMC1), and peroxidasin (PXDN). Functional analysis based on the DAVID bioinformatics tool, connected the differentially regulated proteins to ECM, cytoskeleton, cell adhesion, and cell junctions (supplementary material, Figure S10B). A known TIMP3 binding partner EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1, also known as fibulin 3), and a known drusen constituent apolipoprotein E (APOE), were shown to be upregulated and were verified by WB (supplementary material, Figure S10 C–H). TIMP3 (initially filtered out due to variation) was upregulated 1.7-fold in SFD-hiPSC-RPE (supplementary material, Figure S11). Next, a statistical analysis using a two-level nested ANOVA model was performed to compare differences in protein expression between SFD and control-hiPSC-RPE. The model provided a coefficient equivalent to fold-change for each comparison and revealed that 156 proteins were differentially regulated (p<0.05) in SFD-hiPSC-RPE compared to controls (supplementary material, Table S2). These results were used to perform Ingenuity Pathway Analysis which revealed upregulation of pathways associated with endothelial cell functions and interactions, as well as upregulation of cytoskeleton remodelling in the SFD-hiPSC-RPE (Figure 5A, supplementary material, Table S3). Upstream regulators for these pathways such as transforming growth factor beta 1 (TGFβ1), tumour necrosis factor (TNF), and peroxisome proliferator-activated receptor gamma (PPARG) were also identified (Figure 5B, and supplementary material, Table S4). [EdQ: or did you mean S5B ?] As differential regulation of angiogenesis was revealed, secretion of angiogenesis related proteins from the basal media were studied in more detail. VEGF was secreted at similar levels by both control and SFD-hiPSC-RPE (p=0.5714, Mann–Whitney test) (Figure 6A). Further, secretion of 55 angiogenesis-related proteins was studied using a membrane-based antibody array. The same 15 proteins were secreted at detectable levels by both SFD and control-hiPSC-RPE (Figure 6B). Relative quantitation of spot intensities showed SFD-hiPSC-RPE to secrete more monocyte chemoattractant protein 1 (MCP-1/CCL2, 3.1-fold intensity), one of the key chemokines that regulate migration and infiltration of monocytes/macrophages, higher levels of platelet-derived growth factor (PDGF-AA, 1.5-fold intensity), as well as slightly more angiogenin (1.3-fold intensity) (Figure 6C).

**Discussion**

Many aspects of SFD pathophysiology remain poorly understood. Inconsistent results from previous studies have failed to resolve whether mutant TIMP3 dimerizes, retains its MMP inhibitory function and VEGFR2 binding capacity, or induces other yet unknown pathological changes [1,2]. In this study, we generated SFD patient-derived hiPSC-RPE carrying *TIMP3* mutation causing p.(Ser204Cys) to study the underlying biology of the RPE. Long-term culture for 70 d were used to age the RPE, which exhibited polarized monolayers with a characteristic RPE-specific phenotype. Although the clone-specific variations in TEER warrant some caution, the average TEER values in SFD-hiPSC-RPE lines were significantly lower compared to controls. The cell line and clone-specific differences likely represent typical inherent variability between hiPSC lines and clones, similar to those reported in other studies using iPSCs [17] but might also reflect the heterogeneity observed in SFD patients [1]. The proteomic analysis indicated enriched cytoskeletal remodelling, adhesion, and junctional proteins in SFD-hiPSC-RPE, which could explain the lowered TEER of the RPE monolayer. Cytoskeletal remodelling is critical in regulating cellular junctional integrity [18], and alterations to the actin cytoskeleton and microtubules have been shown to have a major impact on RPE and retinal homeostasis in AMD [19]. Although the hiPSC-RPE showed normal architecture with regular polygonal geometry, cell size and shape, and expression of tight junction protein ZO1, it is plausible that TIMP3 dysregulation leads to cytoskeletal reorganization. This could further induce pathological responses in the RPE cells, which warrants further investigation.

At ultrastructural level, sub-RPE deposits were identified in the form of FLS collagen, a polymorphous form of collagen defined by banding pattern periodicity greater than approximately 67 nm [20]. FLS collagen deposits have been observed under the RPE in human post-mortem eyes, suggesting they occur naturally [21]. In a previous study by Galloway *et al* (2017), hiPSC-RPE were used to study drusen biogenesis in three retinal dystrophies, namely Malattia Leventinese (ML), autosomal dominant radial drusen (ADRD), and SFD [13]. TEM imaging revealed an increased number of basal deposits under SFD-hiPSC-RPE from two patients after 90 d of ageing *in vitro* [13]. Contrary to these findings, our study found no significant changes to the number of deposits nor thickness of the basal lamina in SFD-hiPSC-RPE (n=3). It is possible, that the highly permeable cell culture inserts of 1 µm pore size used, or differing culture conditions negatively influenced ECM thickening and accumulations in our study.

Consistent with previous reports, *TIMP3* mRNA expression levels were similar between SFD and control-hiPSC-RPE, suggesting that the mutation causing p.(Ser204Cys) does not affect transcription of the *TIMP3* gene [22,23]. WB analysis confirmed significantly greater quantities of TIMP3 monomer in SFD-hiPSC-RPE lysates compared to controls; however, this did not equate to greater TIMP3 secretion. In fact, secretion of TIMP3 protein was diminished in SFD-hiPSC-RPE, suggesting the protein is retained within RPE cells or the underlying ECM. The presence of TIMP3 dimers has been shown in transfected fibroblast cell lines [24,25] and SFD patient-derived fibroblasts [23] carrying the p.(*Ser204Cys)TIMP3* mutation. However, we observed TIMP3 dimers from both SFD and control hiPSC-RPE lysates, indicating that dimerization is not unique to mutant TIMP3. The WBs also indicated a moderate increase in the abundance of the 27 kDa glycosylated form, although the results were not clear for all WBs due to the glycosylated form being less abundant than the TIMP3 monomer. Differences in TIMP3 glycosylation states for specific SFD mutants p.(Ser179Cys and Ser38Cys) have been reported [10,26]. It has been suggested that the aberrant disulphide bonding of the mutated TIMP3 could alter its binding affinity for C-terminal binding partners such as pro-MMPs, which in turn could influence glycosylation of the TIMP3 protein [10].[EdQ: please check that your meaning has been retained]

Results from the enzyme assay confirmed that mutant TIMP3 retains its MMP inhibiting capacity, demonstrated by a reduction in collagenase activity on a gelatin substrate in the presence of CM from SFD-hiPSC-RPE. Of note, from 20 h onwards, collagenase activity in the presence of SFD CM was significantly lower compared to controls, suggesting mutant TIMP3 exhibits greater MMP inhibition compared to its WT counterpart. MMP-2 and MMP-9 are key MMPs implicated in BrM dysregulation [27-29]. [EdQ: please check. Are you referring to your data from using the EnZcheck assay? This was used to assess generic collagenase/gelatinase activity in your biological samples and does not ‘utilize type IV collagenase’. Did you use the standard kit or optional ‘DQ-collagen IV’ substrate ? Please note that ‘....DQ collagens and DQ gelatin can be digested by proteases other than gelatinases and collagenases’ ] If mutant TIMP3 retains its ability to inhibit MMPs, or even exhibits increased inhibition as our data suggests, the retention of active, mutant TIMP3 in RPE and BrM may enable continuous MMP inhibition and subsequent increase in ECM synthesis (reduced ECM turnover). This theory is consistent with BrM thickening as a key hallmark of SFD pathology [30].

Proteomic profiling indicated enriched expression of many ECM proteins in SFD-hiPSC-RPE, consistent with increased adhesiveness of the mutant TIMP3 to the ECM [25]. One such protein was EFEMP1 (Fibulin 3), a known TIMP3 binding partner. Mutations in *EFEMP1* lead to the rare macular autosomal dominant disease ML, which shares striking similarities with SFD and AMD [31]. EFEMP1 is an extracellular glycoprotein expressed in the basement membranes of epithelial and endothelial cells, including the BrM. EFEMP1 stimulates the expression of TIMP3 but inhibits expression of MMP-2 and MMP-9 [32]. Mutated EFEMP1 has been shown to misfold and accumulate within RPE cells and contribute to drusen formation in ML [33-35]. EFEMP1 also has a role in regulating angiogenesis [36]. Our results suggest possible accumulation of TIMP3–EFEMP1 complexes [37] which could further promote inhibition of MMPs and contribute to the build-up of toxic deposits, augment angiogenesis, and cause chronic activation of the alternative complement pathway [38,39]. This theory is supported by the data from our IPA data that identified endothelial cell migration and angiogenesis as key pathways associated with the 156 significantly differentially-expressed proteins observed in SFD hiPSC-RPE, relative to controls. TGFβ, TNF and PPARG are upstream regulators that were identified by IPA. TGFβ and TNF are known positive regulators of many cellular processes including angiogenesis, whilst the negative regulator PPARG is a nuclear receptor transcription factor that plays an important role in the control of ocular pathophysiological processes such as anti-angiogenesis, inflammation, and oxidative stress response [40].

Moreover, SFD-hiPSC-RPE secreted increased levels of monocyte chemoattractant protein 1 (MCP1/CCL2), platelet-derived growth factor (PDGF-AA) and angiogenin. MCP1/CCL2 is linked to both inflammation and angiogenesis and is released from Müller glia and the RPE under stress conditions to attract microglia/macrophages expressing chemokine receptor 2 (CCR2) to sites of retinal damage [41]. MCP1/CCL2 polymorphisms have been linked to AMD [42,43]. Furthermore, MCP1/CCL2 has been shown to attract microglia to amyloid-β plaques in the brain and mediate their clearance in Alzheimer's disease [44]. Thus, MCP1/CCL2 may also play an important role in clearance of sub-RPE deposits, inflammation, and angiogenesis in SFD.

The C-terminal domain of TIMP3 directly binds to VEGFR2, inhibiting the binding of VEGF and the downstream signalling that regulates choroidal angiogenesis [45,46]. Our results revealed no difference in the basal secretion of VEGF by SFD-hiPSC-RPE compared to controls, suggesting that SFD-RPE continue to secrete VEGF to the underlying choroid normally. However, it is possible that accumulation of mutant TIMP3 protein within/under RPE may restrict the amount of bioavailable TIMP3 reaching the choroid, enabling VEGF-mediated proliferation and migration of endothelial cells to continue unchecked. It remains unclear whether mutant TIMP3 may be less effective at inhibiting VEGF binding to VEGFR2, favouring angiogenesis; however, the available data in this area remains contradictory [26,47,48]. Differentiation of endothelial cells from SFD-hiPSCs, and their co-culture with SFD-hiPSC-RPE could be used to generate a patient-specific *in vitro* 3D model that mimics the RPE-choroidal interface, providing new insights into SFD pathobiology [49,50].  Furthermore, extrapolation of these findings to an hiPSC-RPE model of AMD, or with gene corrected isogenic control lines pose interesting future research directions.

Here, we have generated SFD patient-derived hiPSC-RPE carrying the *TIMP3 p.(Ser204Cys)* mutationand through extensive characterisation have confirmed they are consistent with mature RPE cells. The findings from this study offer novel insights into the molecular mechanisms underpinning SFD and suggest that accumulation and retention of functional mutant TIMP3 protein within the BrM may promote ECM thickening and choroidal angiogenesis, which drives SFD pathophysiology.

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**Author contributions statement**

HH was mainly responsible for the cell culture, designing and performing the experiments, analysing, and interpreting data, and writing the manuscript. JMD made a major contribution in performing experiments, analysing and interpreting data, and writing the manuscript. DRGC participated in cell culture and optimized methods. JS contributed to cell culture and DNA extraction of hiPSC-RPE cell lines. AJC contributed to project design and management**.** JN conducted the bioinformatics for the proteomics and generated related images. JM, AJ, UA, and HU carried out the proteomics analyses and helped in data analyses. KK participated in study design and funding, JAR participated in study design, and data interpretation. HS and AJ contributed to study design, data interpretation, and provided financial and administrative support. AL organised collection of donor patient samples. All authors were involved in writing the manuscript and had final approval of the submitted and published versions.

**SUPPLEMENTARY MATERIAL ONLINE**

**Supplementary materials and methods**

**Figure S1.** SFD-hiPSC characteristics

**Figure S2.** Characterization of the SFD-hiPSC clones RD01A and RD01C

**Figure S3.** Characterization of the SFD-hiPSC clones RD03A and RD03B

**Figure S4.** Characterization of the SFD-hiPSC clone RD04B and control-hiPSC clone RD08A (Control 1)

**Figure S5.** Characterization of the Control-hiPSC lines UTA.04311.WTs (Control 2) and 10211.EURCCs (Control 3)

**Figure S6.** RPE characteristics

**Figure S7.** The hiPSC-RPE bearing karyotypic translocations (RD01A and RD03B) showed similar RPE properties as the other SFD-hiPSC-RPE lines

**Figure S8.** TIMP3 expression and secretion

**Figure S9.** EnzCheck collagenase assay results shown for each cell line individually

**Figure S10.** Proteomic profiling of SFD-hiPSC-RPE comparing to control-hiPSC-RPE

**Figure S11.** TIMP3 was initially filtered out of the analysed data during stringent CV filtering

**Table S1.** Differentially expressed proteins according to fold change in SFD-hiPSC-RPE/Control-hiPSC-RPE

**Table S2.** Differentially expressed proteins according to statistical significance (LMER linear mixed effects model) in SFD‐hiPSC‐RPE/Control‐hiPSC‐RPE

**Table S3.** IPA pathway analysis for diseases and biological functions for the significant proteins with p‐value <0.05

**Table S4.** Upstream regulators of the disease associated pathways

**[EdQ: please provide Tables S1–S4 in editable Word or Excel format (separate or combined into a single file)]**

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References [51-57] are cited only in the supplementary material.

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**Table 1.** Human iPSC lines and characteristics.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Cell lineformal name | Status | TIMP3status | Age when sampled (yr) | Clone | Karyotype | hiPSC markers | Pluripotency | Presence of viral vectors | RPE |
| RD01\*  | SFD | p.Ser204Cys | 39 | RD01A | 46, XXt(X;4)(p11;p15) | Pos  | EB | Neg | Yes^ |
|  |  |  |  | RD01C | 46, XX | Pos | EB | Neg | Yes^ |
|  |  |  |  |  |  |  |  |  |  |
| RD03  | SFD | p.Ser204Cys | 48 | RD03A | 46, XX | Pos | EB Teratomas | Neg | Yes^ |
|  |  |  |  | RD03B | 46, XXt(2;4)(p23;p1?2) | Pos | EB Teratomas | Neg | Yes^ |
|  |  |  |  |  |  |  |  |  |  |
| RD04\*  | SFD | p.Ser204Cys | 42 | RD04A | 46, XX | Pos | EBTeratomas | Neg  | Yes^ |
|  |  |  |  | RD04B | 46, XX | Pos | EB | Neg  | No |
|  |  |  |  |  |  |  |  |  |  |
| Control1\*\* RD08 | Healthy |  | 70 |  | 46, XX | Pos | EB | Neg  | Yes^^ |
| Control2 UTA.04311.WTs | Healthy |  | 46 |  | 46, XX | Pos | EB | Neg  | Yes^^ |
| Control3 10211.EURCCs | Healthy |  | 58 |  | 46, XX | Pos | EB | Neg  | Yes^^ |

\* Siblings; \*\* unaffected mother of the two siblings. Abbreviations: SFD Sorsby Fundus Dystrophy; p, passage; Pos, positive; EB, embryoid body; Neg, negative; ^n=5 for SFD-hiPSC-RPE, ^^n=3 for control-hiPSC-RPE

**Table and figure legends**

# Figure 1. Retinal fundoscopy and optical coherent tomogram (OCT) images of SFD patient RD04 over a 11-year period. (A) Retinal fundus image showing early choroidal neovascularization (CNV) superior to the fovea\* and normal subfoveal OCT (B) Retinal and OCT images of left eye in 2020 showing progression to disciform scar formation and macular scarring due to progression of CNV. (C) OCT image of right eye in 2009 showing disciform scar and abnormal macula. (D) OCT image of right eye in 2020 showing increase in scar size due to ongoing CNV. Progressive macular scarring occurred in each eye despite repeated therapy with bilateral intravitreal bevacizumab over the 11-year period.

**Figure 2. Human iPSC-RPE showed characteristic RPE phenotype.** (A) Study outline and timetable for cell culture as well as main analyses shown. mo=months, p=passage. (B) Differential interference contrast (DIC) images of the hiPSC-RPE monolayers showing morphology and mosaic pigmentation pattern for the three control lines and the three karyotypically normal SFD lines. Scale bar 20 µm, valid for all images. (C) Scatter dot plots for TEER values measured from separate inserts for each cell line, mean value shown by line and presented above of each dataset, n=separate differentiation experiments. Box blots showing average TEER values for control and SFD-hiPSC-RPE. Whiskers for min and max values with line for median. Mean shown with +. (D) x–y maximum intensity projections (MIP) of confocal z-stacks shown for ZO1 IF labelling. Scale bar 10 µm valid for all images. (E) Confocal z-stack images 4 h after feeding porcine POS and IF labelling with anti-rhodopsin. White arrows indicate internalized POS (green). Phalloidin detected to visualize cells. Scale bar 10 µm, valid for all images. (F) Bar chart showing quantification of average number of POS/frame, n= number of cell lines with three replicate confocal frames (20x magnification) each. Error bars indicate SD. G) Ratio of apical to basolateral secretion of PEDF. Each dot represents the ratio measured from one insert of a single differentiation experiment. Box plots combining the data for control and SFD. Red line at y=1 indicates equal secretion to both apical and basal sides. The secreted concentrations are presented in supplementary material Figure S6.

**Figure 3.** **RPE ultrastructure analyses by TEM. (**A) Cultured hiPSC-RPE from SFD and control patients exhibited characteristic RPE structures. No significant differences were observed between SFD and control hiPSC-RPE cells with regards to (B) cell height (C) apical microvilli length, (D,E) basal laminar area, (F,G) the number of sub-RPE FLS collagen deposits (arrow) per cell and (H,I) the number of melanosomes (open arrow) per cell. Scale bar 2 µm.

**Figure 4. TIMP3 expression, secretion, and functionality.** (A) RT-qPCR assessment of *TIMP3* transcript levels for all of the hiPSC-RPE lines, individually and combined, to compare expression between control and disease lines. Dots represent technical replicates. Mean fold-change is shown on a logarithmic scale. Dotted lines mark two-fold difference in expression; n=number of cell lines with 3 technical replicates for each. RD08A control set equal to one. (B) A representative image of WB analysis for TIMP3 protein expression showing accumulation of the (21 kDa/24 kDa) monomer, expression of the glycosylated form (27 kDa), and the dimer (48 kDa) in the SFD-hiPSC-RPE. Relative expression levels were measured densitometrically, normalized to β-actin. Error bars show SD, \*p=0.0357. (C) Box plots for TIMP3 secretion analysed in CM using ELISA. Median value indicated with a line. (D) EnzCheck collagenase assay assessing the effect of the p.(Ser204Cys) mutant on TIMP3’s ability to inhibit MMPs. The general MMP inhibitor 1,10-phenanthroline (+inhibitor) or CM from hiPSC-RPE containing 50 ng TIMP3, showed a decrease in fluorescence relative to no inhibitor, indicating MMP inhibition. Error bars show SD.

**Figure 5. Proteomic profiling revealed upregulation of angiogenesis related pathways and downstream regulators in SFD-hiPSC-RPE comparing to controls.** (A) Enriched *diseases and biological functions* for the significantly (p<0.05) differentially expressed proteins according to IPA pathway analysis. Heat map displays the general terms (excluding cancer-related terms), which have either highly increased >1.5 (red) or decreased <-1.5 (green) activation z-score. On the left, more specific terms and on the right, the terms are grouped under more general categories. (B) Selected upstream regulators of the enriched pathways and associated proteins with increased (red) and decreased (green) expression, as well as predicted effect of the regulator to protein expression. TGFβ and TNF, known positive regulators of angiogenesis, and negative regulator PPARG and their interactions to protein expression are shown.

**Figure 6.** **Secretion of angiogenesis related proteins**. (A) Basal VEGF secretion levels measured by ELISA for control-hiPSC-RPE (n=3 cell lines, 5 inserts each) and SFD-hiPSC-RPE (n=5 cell lines, 3-4 inserts each). (B) Human Angiogenesis Array membranes showing basal secretion of 15 angiogenesis-related proteins and (C) densitometric relative quantification of the spot intensities for SFD-hiPSC-RPE compared to control.