

## Human Umbilical Tissue-Derived Cells Rescue Retinal Pigment Epithelium Dysfunction in Retinal Degeneration

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### ABSTRACT

Retinal pigment epithelium (RPE) cells perform many functions crucial for retinal preservation and vision. RPE cell dysfunction results in various retinal degenerative diseases, such as retinitis pigmentosa and age-related macular degeneration (AMD). Currently, there are no effective treatments for retinal degeneration except for a small percentage of individuals with exudative AMD. Cell therapies targeting RPE cells are being developed in the clinic for the treatment of retinal degeneration. Subretinal injection of human umbilical tissue-derived cells (hUTC) in the Royal College of Surgeons (RCS) rat model of retinal degeneration was shown to preserve photoreceptors and visual function. However, the precise mechanism remains unclear. Here, we demonstrate that hUTC rescue phagocytic dysfunction in RCS RPE cells in vitro. hUTC secrete receptor tyrosine kinase (RTK) ligands brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), and glial cell-derived neurotrophic factor (GDNF), as well as opsonizing bridge molecules milk-fat-globule-epidermal growth factor 8 (MFG-E8), growth arrest-specific 6 (Gas6), thrombospondin (TSP)-1, and TSP-2. The effect of hUTC on phagocytosis rescue in vitro is mimicked by recombinant human proteins of these factors and is abolished by siRNA-targeted gene silencing in hUTC. The bridge molecules secreted from hUTC bind to the photoreceptor outer segments and facilitate their ingestion by the RPE. This study elucidates novel cellular mechanisms for the repair of RPE function in retinal degeneration through RTK ligands and bridge molecules, and demonstrates the potential of using hUTC for the treatment of retinal degenerative diseases. *STEM CELLS* 2016;34:367–379

### SIGNIFICANCE STATEMENT

Retinal degeneration is a leading cause of blindness. There is currently no effective treatment. Retinal pigment epithelium (RPE) play a pivotal role in retinal preservation. The phagocytic function of the RPE is crucial for the integrity of the light-sensing photoreceptors, thus vision. We previously reported that subretinal injection of human umbilical tissue-derived cells (hUTC) in a rodent model of retinal degeneration preserved photoreceptors and vision with an unknown mechanism. Here, we report that hUTC can rescue RPE phagocytic dysfunction through the release of receptor tyrosine kinase ligands and bridge molecules. This study identifies novel mechanisms of a cell-based therapy for correcting the RPE dysfunction in retinal degeneration, suggesting that hUTC may provide utility in treating patients with retinal degenerative diseases, such as AMD and retinitis pigmentosa.

### INTRODUCTION

The retinal pigment epithelium (RPE) is a monolayer of pigmented epithelial cells that reside in the outer retina between the photoreceptors and Bruch's membrane. It performs many functions vital for retinal preservation [1]. RPE cell dysfunction plays a central role in various retinal degenerative diseases [2–5]. An important function of the RPE is to phagocytize the daily-shed

photoreceptor outer segments (OS), which is crucial for photoreceptor survival and function and, thus, vision [6]. Disruption of RPE phagocytosis results in retinitis pigmentosa and rod/cone dystrophy [2, 7]. Individuals that carry mutations in the *Mertk* gene are affected with retinal degenerative disease that likely results from defective outer segment phagocytosis by the RPE [2, 8–14]. Retinal aging has a significant impact on RPE phagocytosis. For example, RPE

phagocytosis reduced dramatically in aged rats [15]. Increased iron accumulation in the RPE of aged animals markedly reduced their phagocytic activity [16]. Therefore, it has been indicated that one of the driving forces of RPE dysfunction is an age-dependent phagocytic and metabolic insufficiency of postmitotic RPE cells [17]. With aging, a decline in the RPE phagocytic activity results in the build-up of toxic by-products [18–20]. Among them is lipofuscin which is generated during aging mainly by the incomplete decomposition of phagocytized material from photoreceptor OS in RPE [21–24]. Excessive lipofuscin accumulation is characteristic for RPE aging in the human eye and for various blinding diseases, including age-related macular degeneration (AMD) [25, 26]. Circumstantial evidence shows oxidative stress and free radical damage to the RPE underlie the pathogenesis of AMD by impairing RPE phagocytic function [16, 27–30].

Cell therapies targeting RPE are being developed for the treatment of retinal degeneration. Current preclinical and clinical studies with various cell types are focusing on either replacement or protection of RPE in the diseased retina [31–34]. We reported previously that subretinal injection of human umbilical tissue-derived cells (hUTC) in the Royal College of Surgeons (RCS) rat, a well-established model of retinal degeneration, preserved photoreceptors and visual function apparently through an unknown trophic mechanism [35]. In this study, we investigated the mechanisms of hUTC on retinal and visual preservation in the RCS rat model of retinal degeneration. The rats exhibit a progressive and postnatal loss of vision because of a failure of the RPE cells to ingest shed photoreceptor OS. The phagocytic defect is due to an inherited recessive mutation in the gene expressed in the RPE that encodes the receptor tyrosine kinase (RTK) Mer (MerTK) [36], a member of the RTK superfamily [37]. It has been shown that the underlying function of MerTK in OS ingestion by the RPE is regulation of the cytoskeleton reorganization apparently through activation of phosphoinositide 3 (PI3) kinase and Rho GTPases [38–40]. Both signaling modules are also commonly activated by the signaling of the RTK superfamily [41–43]. Basic fibroblast growth factor (bFGF), a RTK ligand of the FGF receptor family, was shown to rescue the phagocytic activity of cultured RCS RPE cells [44]. These lead to our hypothesis that hUTC may rescue phagocytosis in the RCS RPE through secretion of RTK ligands that bind to the RTKs in the RCS RPE and activate their downstream signaling modules shared by MerTK to compensate for the loss of the MerTK function.

It has been indicated that RPE cells use a molecular mechanism for OS phagocytosis that is highly similar to mechanisms used by other phagocytic cells for clearance of apoptotic cells [45]. Isolated OS possess externalized phosphatidylserine (PS) whose blockade or removal reduces their binding and engulfment by the RPE [46]. The bridge molecules milk-fat-globule-epidermal growth factor 8 (MFG-E8), growth arrest-specific 6 (Gas6), protein S, and thrombospondins (TSPs) all bind to externalized PS on apoptotic cell surfaces [47]. MFG-E8 can then be recognized by the  $\alpha v\beta 3$  and  $\alpha v\beta 5$  integrins through its RGD motif [48, 49], Gas6 and protein S by the RTKs of the Axl, Tyro3, and Mer family [50], and the TSPs by CD36 in conjunction with integrins  $\alpha v\beta 3$  and  $\alpha v\beta 5$  [51]. Similarly, the RPE membrane receptors, integrin  $\alpha v\beta 5$ , MerTK, and CD36 participate in OS recognition and internalization through secreted MFG-E8, Gas6, and protein S by the RPE [45]. Gas6 and protein S are also the

RTK ligands for MerTK and have been shown to promote phagocytosis in the RPE [52]. Therefore, we hypothesized that hUTC may also rescue OS phagocytosis by the RCS RPE through the secretion of bridge molecules which serve as opsonins to facilitate OS internalization.

Here, we show that hUTC rescued the phagocytic defect in the RCS RPE cells in vitro. Using RNA-Sequencing (RNA-Seq), we identified gene expression of multiple RTK ligands and bridge molecules in hUTC and gene expression of the corresponding RTK and bridge molecule receptors in the RCS RPE cells. hUTC secreted the RTK ligands brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), and glial cell-derived neurotrophic factor (GDNF), as well as the bridge molecules MFG-E8, Gas6, TSP-1, and TSP-2. Recombinant human proteins for these factors dose-dependently increased phagocytosis in the RCS RPE cells. The role of the hUTC-secreted factors in promoting phagocytosis was confirmed by siRNA-mediated gene silencing in hUTC. Further, hUTC-derived bridge molecules bound to isolated OS and enhanced their phagocytosis by the RCS RPE. These findings demonstrate novel mechanisms of a cell based-therapy for the correction of RPE phagocytic dysfunction, and indicate an alternative therapeutic strategy that may be beneficial for the treatment of retinal degenerative diseases with RPE dysfunction and photoreceptor degeneration.

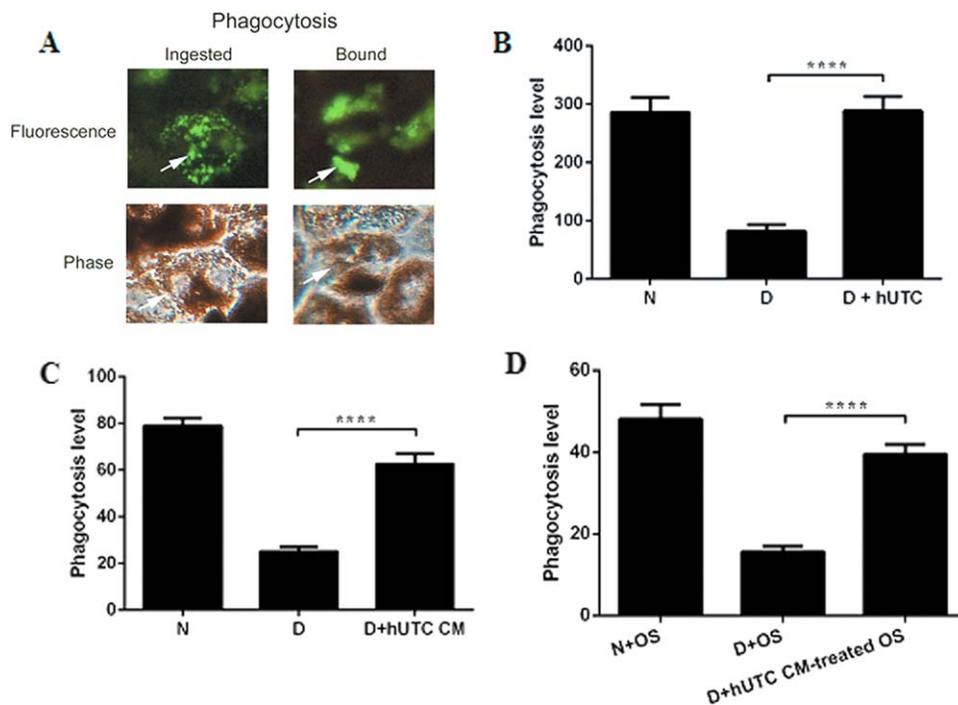
## MATERIALS AND METHODS

Detailed descriptions of the following Materials and Methods are available in Supporting Information Materials and Methods: preparation of hUTC conditioned medium (CM), human recombinant proteins and enzyme linked immunosorbent assay (ELISA) kits, and antibodies for immunofluorescence.

### Cells and Cell Culture

hUTC were isolated, cultured, and cryopreserved as described previously [35]. Briefly, human umbilical cords were obtained with donor consent following live births from the National Disease Research Interchange (Philadelphia, PA). Tissues were minced and enzymatically digested. After almost complete digestion with a Dulbecco's modified Eagle's medium (DMEM)-low glucose (Lg) (Invitrogen, Carlsbad, CA) medium containing a mixture of 50 U/ml collagenase (Sigma, St. Louis, MO), the cell suspension was filtered through a 70- $\mu$ m filter, and the supernatant was centrifuged at 350g. Isolated cells were washed in DMEM-Lg a few times and seeded at a density of 5,000 cells per square centimeter in DMEM-Lg medium containing 15% (v/v) fetal bovine serum (FBS) (Hyclone, Logan, Utah) and 4 mM L-glutamine (Gibco, Grand Island, NY). When cells reached approximately 70% confluence, they were passaged using TrypLE (Gibco, Grand Island, NY). Cells were harvested after several passages and banked.

The RPE cells were obtained from 6- to 10-day-old pigmented RCS rats (*rdy/p+*) and normal pigmented congenic control rats (*rdy+/p+*) as described [44]. All procedures using animals were conducted in accordance with The Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. The anterior part of the eye was removed anterior to the limbus. The retina was gently removed and the eye cup was incubated in dispase (Roche Diagnostics, Mannheim, Germany) for 20–30 minutes. The RPE sheets were carefully removed, suspended in



**Figure 1.** Effect of hUTC on phagocytosis in the RCS rat RPE cells. **(A):** Fluorescent assay of photoreceptor OS phagocytosis by normal rat RPE. Cultured normal rat RPE ( $5 \times 10^4$  cells) were subjected to OS phagocytosis by feeding fluorescein isothiocyanate (FITC)-labeled OS ( $5 \times 10^6$ ) to them. The panel on the right shows the microscopic images of the RPE (arrow, phase) and un-phagocytized, bound OS (arrow, fluorescence) at 1 hour. The OS were mostly in aggregates, appearing indistinct, and bound but not ingested by the RPE at this time. The panel on the left shows the microscopic images of the RPE (arrow, phase) and phagocytized OS (arrow, fluorescence) at 10 hours. The OS appeared as discreet, intracellular phagosomes at this time. Magnification,  $250\times$ . **(B):** The RCS RPE cells were cocultured with hUTC plated in transwells or **(C)** incubated with the hUTC conditioned medium (CM) for 24 hours and then subjected to phagocytosis assay. A significantly increased phagocytosis was observed in the RCS RPE cocultured with hUTC or incubated with the hUTC CM. **(D):** The photoreceptor OS were incubated with the hUTC CM for 24 hours and then fed to the RCS RPE cells for phagocytosis assay in the absence of the hUTC CM. Phagocytosis of the hUTC CM-treated OS by the RCS RPE was significantly restored. Data represent the mean  $\pm$  SEM ( $n = 3$ ). \*\*\*\*,  $p < 0.0001$ . Abbreviations: CM, conditioned medium; N, normal RPE; D, dystrophic RCS RPE; OS, outer segments.

Minimum Essential Medium containing 20% (v/v) FBS and penicillin 200 U/ml/strep 200  $\mu$ g/ml, triturated with trypsin treatment, and plated out in 24-well plate. The cells were cultured at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ .

### Isolation of Photoreceptor OS and Phagocytosis Assay

Eyes were obtained from 2- to 8-week-old Long Evans rats several hours after light onset. Retinas were isolated, homogenized with Polytron (8 mm generator) or a Dounce glass homogenizer, layered on top of 27%–50% linear sucrose gradient, and centrifuged at 38,000 rpm in SW41 rotor (240,000g) for 1 hour at  $4^\circ\text{C}$ . The top two OS bands were collected, diluted with Hanks' balanced saline solution, and centrifuged at 7000 rpm in HB-4 rotor (8000g) for 10 minutes to pellet the OS.

The fluorescent vital assay of OS phagocytosis was performed as described [44] with modifications. Briefly,  $5 \times 10^4$  primary rat RPE cells were plated out on a 12-mm circular glass cover slip in the 24-well culture dish and cultured for at least 1 week before usage for phagocytosis assay. There was not much growth and the cell density remained quite constant in RPE cultured this way. Isolated rat OS were fluorescently stained with fluorescein isothiocyanate (FITC).  $5 \times 10^6$  FITC-OS was fed to the RPE cultures and incubated for 8 to 13 hours. The RPE cells were then vigorously washed four times with agitation to remove the uningested OS and fixed with 2% paraformaldehyde. Phagocytosis

level was quantitated microscopically by counting the number of internalized OS in 10 representative fields, each field being  $0.021 \text{ mm}^2$  in area, at  $250\times$  magnification with the appropriate filters. Microscopically, early in incubation the uningested, bound fluorescent OS appear blurry and randomly distributed on the RPE. Later, the ingested OS appear smaller, sharp, and intracellular in distribution and are clearly distinguishable [53], as shown in Figure 1A.

### RNA-Seq

hUTC were seeded at 5,000 cells per square centimeter and grown in DMEM-Lg medium containing 15% (v/v) FBS (Hyclone, Logan, UT) and 4 mM L-glutamine (Gibco, Grand Island, NY) for 24 hours followed by a medium change to DMEM:F12 medium containing 10% (v/v) FBS and grown for another 48 hours. The cells were then collected for total RNA extraction and DNA removal using the Qiagen RNeasy extraction and on-column DNase kit (Qiagen, Valencia, CA). The integrity and quantity of RNA in the samples were determined using the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Library preparation and sequencing were performed by Expression Analysis Inc., Durham, NC. RNA libraries were prepared using Illumina's TruSeq RNA-Seq Sample Prep kit following the manufacturer's instructions, and sequenced with Illumina's

HiSeq 2000. Sequencing reads were mapped to the reference human genome (GRCh37 patch8) using the software ArrayStudio version 6.1. Fragments per kilobase of transcript per million mapped reads (FPKM) was used to calculate gene expression.

### siRNA Knockdown

The On-TARGETplus human siRNA-SMARTpools directed against human *BDNF*, *HGF*, *GDNF*, *MFG-E8*, *Gas6*, *TSP-1*, and *TSP-2*, as well as ON-TARGETplus Non-targeting pool (scrambled siRNA pool) were purchased from GE Dharmacon (Lafayette, CO). Twenty five nanomoles of each siRNA pool was incorporated into hUTC respectively, using the DharmaFECT transfection reagent (GE Dharmacon, Lafayette, CO).

### Immunofluorescence

A  $10 \times 10^6$  OS was incubated for 24 hours at 37°C in 1 ml of hUTC CM, 1 ml of control medium, or 1 ml of control medium containing 124 ng/ml MFG-E8, 8.75 ng/ml Gas6, 1.2 µg/ml TSP-1, or 238 ng/ml TSP-2. The OS were pelleted, washed, and embedded in Tissue-Tek O.C.T compound (Sakura Finetek USA, Inc., Torrance, CA). A cryostat (Leica CM1950, Leica Microsystems, Inc., Buffalo Grove, IL) was used to obtain 10 µm serial sections. The sections were transferred to glass slides for the immunofluorescence staining. Circled spots with the OS pieces were treated with a blocking buffer (10% (v/v) goat serum, 1% (v/v) BSA, and 0.1% (v/v) Triton X 100 in phosphate-buffered saline [PBS]) for 1 hour at room temperature and then double stained with Alexa Fluor 568 conjugated anti-rhodopsin antibody and Alexa Fluor 488 conjugated anti-MFG-E8, anti-Gas6, anti-TSP-1, anti-TSP-2, or mouse IgG<sub>2A</sub> or IgG<sub>2B</sub> isotype control antibody for 2 hours at room temperature. After washing three times with PBS, the sections were mounted in Vectashield mounting media (Vector Laboratories, Inc., Burlingame, CA) and evaluated with a Zeiss Photomicroscope III (Carl Zeiss, Oberkochen, Germany) equipped with epifluorescence. Images were captured with a Kodak 290 digital camera and analyzed using Kodak Microscopy Documentation System 290 Photoshop image analysis software (Eastman Kodak, Rochester, NY). Images were made at 250 × magnification with the appropriate filters.

### Statistical Analysis

Statistical significance was assessed by unpaired two-tailed Student's *t*-test. A  $p < 0.05$  was considered statistically significant. All statements of variability are for SEM unless noted otherwise.

## RESULTS

### hUTC Rescue RCS RPE Phagocytosis

The photoreceptor degeneration and visual loss in RCS rats are caused by a defect in RPE phagocytosis [36]. To assess whether hUTC have an effect on RCS RPE phagocytosis, we isolated RPE cells from normal and RCS rat eyes and cultured them in vitro for phagocytosis assay, as shown in Figure 1A. Untreated RPE cells from normal congenic control rat eye were used as control to show the normal level of phagocytosis. Defective phagocytosis in the RCS RPE was almost completely restored to the level of normal RPE when they were cocultured with hUTC (Fig. 1B) or treated with hUTC CM (Fig. 1C). Moreover, phagocytosis of OS by the RCS RPE was rescued when the cells were fed with OS pre-incubated with

hUTC CM and subjected to phagocytosis in the absence of hUTC CM (Fig. 1D). These results demonstrate that hUTC secrete specific factors to promote RPE phagocytosis.

### Gene Expression Analysis in hUTC and RCS RPE

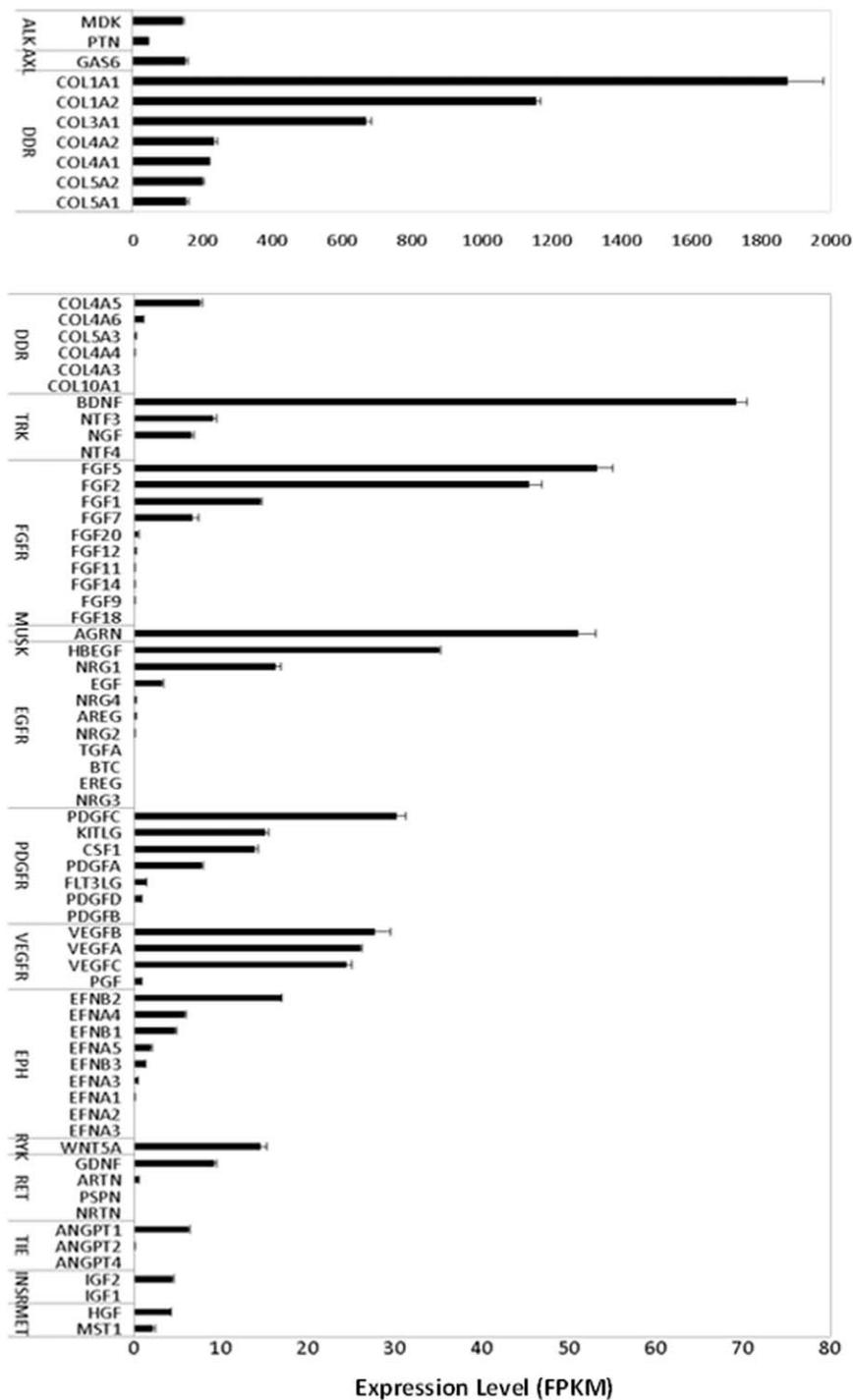
We hypothesized that RTK ligands and bridge molecules may play a role in mediating the effect of hUTC on the phagocytosis rescue in the RCS RPE. To assess whether hUTC express the genes encoding RTK ligands and bridge molecules, we performed RNA-Seq-based transcriptome profiling of hUTC. Gene expression of multiple RTK ligands for 15 RTK subfamilies was detected (Supporting Information Table S1). The expression level of the RTK ligand genes for each RTK subfamily was sorted and graphed based on the values of FPKM (Fig. 2; Supporting Information Table S1). The gene expression of bridge molecules, *MFG-E8*, *Gas6*, *protein S*, *TSP-1*, and *TSP-2*, was also detected (Supporting Information Table S2). We then proceeded to examine the presence of gene expression for the corresponding RTK subfamilies and receptors for the bridge molecules in the RCS RPE. The RTK superfamily can be grouped into 20 subfamilies based on the kinase domain sequences [37]. Gene expression of 18 out of 20 RTK subfamilies was detected in the RCS RPE (Supporting Information Table S3). Among them are the 15 RTK subfamilies corresponding to the RTK ligand genes expressed in hUTC (Fig. 2). Gene expression of receptors reported for bridge molecule binding [45] were also detected (Supporting Information Table S4) in the RCS RPE, including integrin  $\alpha\beta3$ ,  $\alpha\beta5$ , *Axl*, *Tyros3*, *Mertk*, and *CD36*.

### hUTC Secrete RTK Ligands and Bridge Molecules

Due to the large number of RTK ligand genes expressed by hUTC, we selectively measured in hUTC CM the presence of 10 RTK ligands that have relatively high gene expression level and correspond to 10 RTK subfamilies, using ELISA. These ligands include BDNF, HGF, GDNF, platelet-derived growth factor type C (PDGF-C), basic fibroblast growth factor (FGF2), ephrin-B2, heparin-binding EGF (HB-EGF), vascular endothelial growth factor (VEGF)-A, agrin, and Gas6 (Supporting Information Table S5).

The concentrations of BDNF, HGF, GDNF, PDGF-C, and Gas6 in hUTC CM were  $405.0 \pm 1.7$  pg/ml,  $131.7 \pm 1.9$  pg/ml,  $52.8 \pm 1.8$  pg/ml,  $17.1 \pm 2.6$  pg/ml, and  $70.6 \pm 2.1$  pg/ml, respectively. They were undetectable in the control medium, except for a trace amount of PDGF-C (0.3 pg/ml) (Fig. 3A). The levels of FGF2, ephrin-B2, HB-EGF, and VEGF-A were undetectable in both the hUTC CM and the control medium. The concentration of agrin ( $55.0 \pm 8.0$  pg/ml) in the hUTC CM was similar to that of the control medium ( $63.0 \pm 7.8$  pg/ml), suggesting that it comes mainly from the serum in the medium.

We also measured the concentrations of the bridge molecules MFG-E8, protein S, TSP-1, and TSP-2 in the hUTC CM by ELISA (Fig. 3B). hUTC secreted high levels of MFG-E8 ( $15.5 \pm 0.1$  ng/ml), TSP-1 ( $151.8 \pm 0.9$  ng/ml), and TSP-2 ( $8.8 \pm 0.3$  ng/ml). TSP-1 ( $4.0 \pm 0.3$  ng/ml) and negligible levels of TSP-2 ( $0.01 \pm 0.1$  ng/ml) were detected in the control medium, while MFG-E8 was undetectable. Protein S was not detected in both the hUTC CM and control medium (data not shown). Taken together, these results demonstrate that hUTC secrete bridge molecules we identified by RNA-Seq and some, but not all, of the RTK ligands we selected. The RTK ligands and bridge molecules detected in the CM are secreted from hUTC and not from serum in the medium.

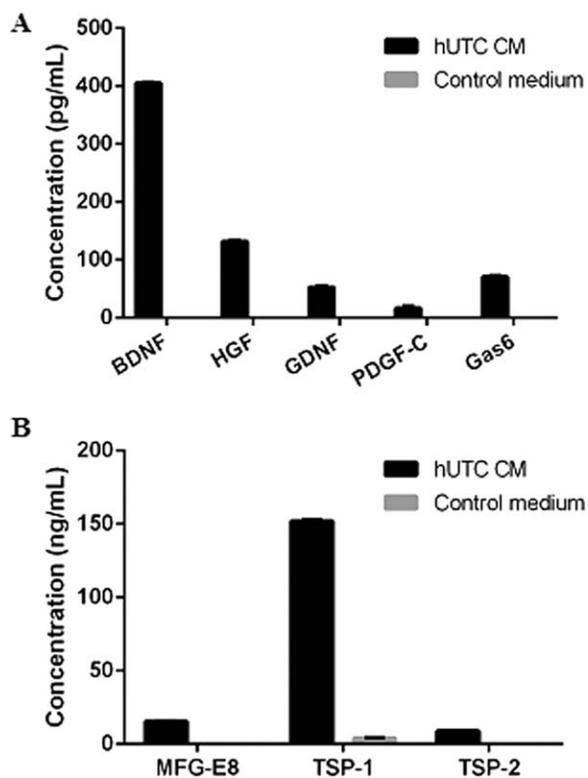


**Figure 2.** Gene expression level of RTK ligands identified in hUTC. Total mRNAs were extracted from hUTC and RNA-Seq was performed to identify and quantify the RTK ligands gene expression in hUTC. The identified RTK ligands were sorted based on the corresponding RTK subfamilies and ranked according to their fragments per kilobase of transcript per million mapped reads value. Gene expression of multiple RTK ligands for 15 RTK subfamilies was detected in hUTC. Abbreviation: RTK: receptor tyrosine kinase; FPKM, fragments per kilobase of transcript per million mapped reads.

**Human Recombinant RTK Ligands and Bridge Molecules Rescue Phagocytosis in RCS RPE**

To investigate whether the RTK ligands and bridge molecules have an effect on the RCS RPE phagocytosis, we treated the RCS RPE cells in two different ways and performed phagocytosis assays. For a functional test of RTK ligands, we incubated

the RCS RPE with recombinant human BDNF, HGF, or GDNF individually for 24 hours and then performed phagocytosis assay with the addition of OS. The RCS RPE incubated with the hUTC CM was used as a positive control. For a functional test of bridge molecules, isolated OS were incubated with each of the recombinant human MFG-E8, Gas6, TSP-1, and



**Figure 3.** Concentrations of RTK ligands and bridge molecules in hUTC conditioned medium (CM) and control medium. The hUTC CM and control medium were prepared as described in the Supporting Information Materials and Methods. The levels of the RTK ligands (A) and bridge molecules (B) were measured by enzyme linked immunosorbent assay (ELISA). Data shown are the mean  $\pm$  SEM ( $n=2$  for thrombospondin [TSP]-1 and TSP-2 ELISAs;  $n=3$  for all the other ELISAs). Abbreviations: CM, conditioned medium.

TSP-2, respectively, for 24 hours and then fed to the RCS RPE cells for phagocytosis assay. The OS incubated with the hUTC CM was used as a positive control. The minimum doses used for each RTK ligand and bridge molecule were the same as what they were found in the hUTC CM by ELISA. BDNF, HGF, and GDNF dose-dependently increased the phagocytosis level in the RCS RPE cells, with the effect of HGF being the strongest even at the lowest dose. When applied at higher concentrations, BDNF, HGF, and GDNF were able to completely rescue phagocytosis in the RCS RPE (Fig. 4A–4C). Similar dose-response effects were observed with the bridge molecule MFG-E8, Gas6, TSP-1, and TSP-2 (Fig. 4D–4G). These results show that recombinant RTK ligand and bridge molecule proteins can mimic the effect of the hUTC CM and restore RCS RPE phagocytosis, suggesting that these factors could be involved in the hUTC-mediated phagocytosis rescue in the RCS RPE.

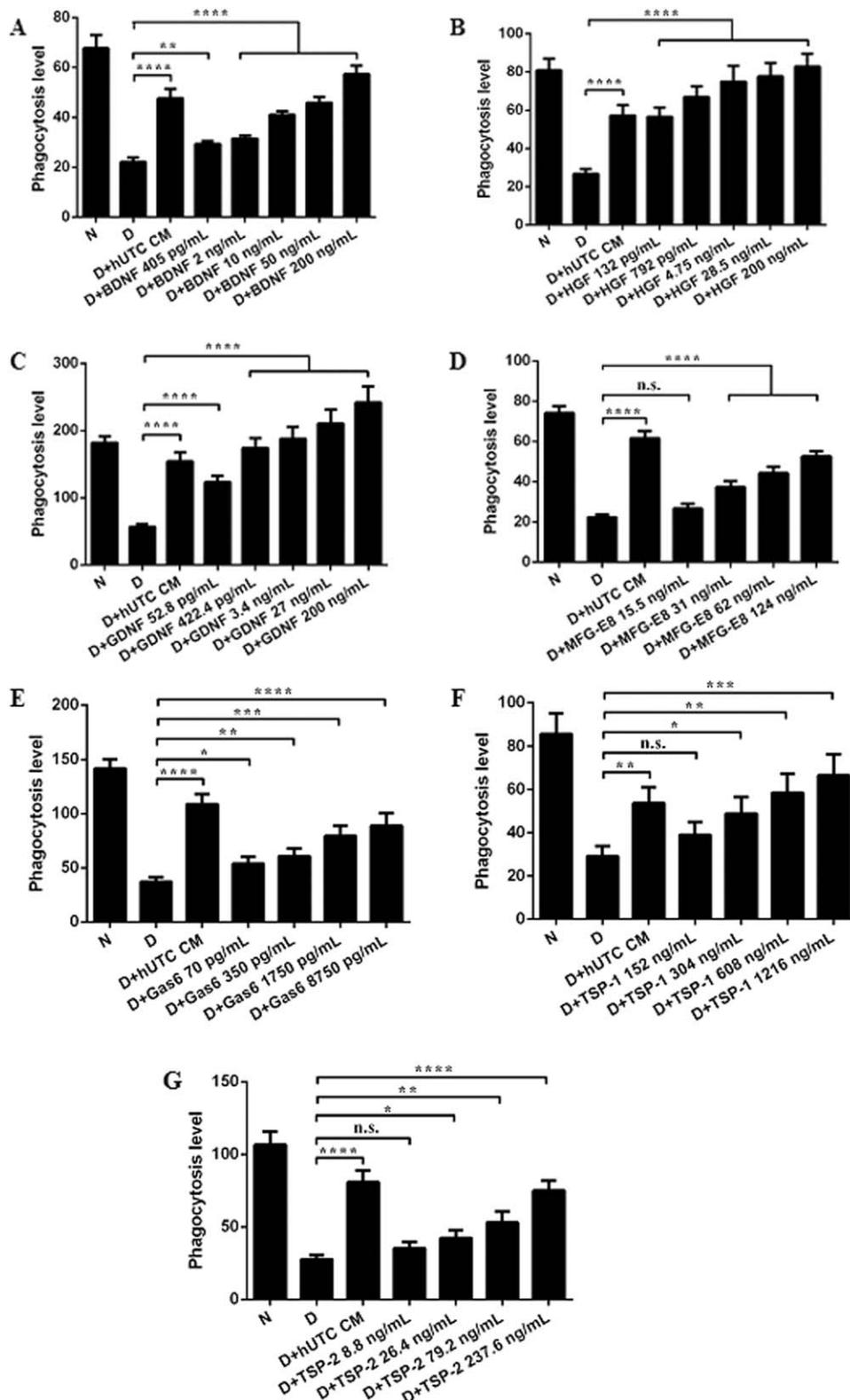
#### hUTC-Secreted RTK Ligands and Bridge Molecules Are Necessary for the hUTC-Mediated Phagocytosis Rescue

To test whether the hUTC-secreted RTK ligands and bridge molecules are required for the hUTC-induced phagocytosis rescue in the RCS RPE, we knocked down individually *BDNF*, *HGF*, *GDNF*, *MFG-E8*, *Gas6*, *TSP-1*, and *TSP-2* in hUTC by siRNA mediated gene silencing. A scrambled siRNA pool that does not target any genes was used as the knockdown

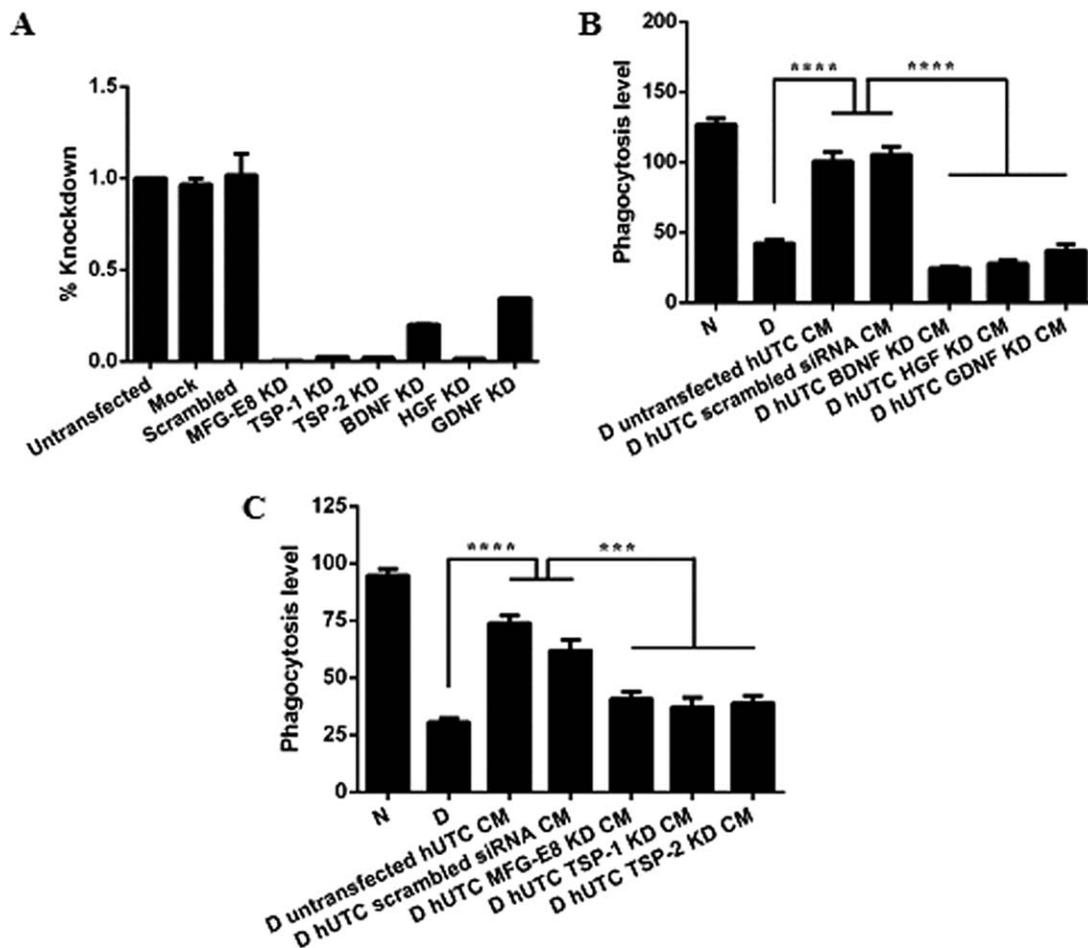
(KD) control. The knockdown efficiency of each factor was examined by measuring the level of each factor in the cell culture supernatants collected from hUTC transfected with the siRNA (Fig. 5A). Mock or scrambled siRNA transfection had no effect on the hUTC secretion of these factors. siRNA targeting *MFG-E8*, *TSP-1*, *TSP-2*, and *HGF* yielded almost 100% knockdown efficiency. The 80% and 65% knockdowns were observed for *BDNF* and *GDNF*, respectively (Fig. 5A). siRNA targeting *Gas6* in hUTC did not work (data not shown). CM was produced from the siRNA-transfected hUTC and applied to the RCS RPE to identify the effects of RTK ligands and bridge molecule knockdown. The RCS RPE were cultured with the CM produced from hUTC transfected with siRNA targeting *BDNF*, *HGF*, or *GDNF* (Fig. 5B), or were fed with the OS pretreated with CM produced from hUTC transfected with siRNA targeting *MFG-E8*, *TSP-1*, or *TSP-2* (Fig. 5C). CM prepared from untransfected and scrambled-siRNA-transfected hUTC were used as the knockdown control CMs. Individual knockdown of each of the RTK ligands completely abolished the effect of the hUTC CM on the phagocytosis rescue compared to that of the knockdown control CMs (Fig. 5B), suggesting that the effects of the RTK ligands are tightly dependent on each other and are not redundant. Knocking-down of each of the bridge molecules significantly decreased the phagocytosis of OS by the RCS RPE (Fig. 5C). These results indicate that the RTK ligands and bridge molecules are required for the hUTC-mediated phagocytosis rescue in the RCS RPE.

#### hUTC-Derived Bridge Molecules Bind to OS

To determine how the bridge molecules contribute to the effect of hUTC on phagocytosis rescue, we next examined whether they can bind to OS. The OS were first incubated with individual recombinant human bridge molecule protein, hUTC CM, or control medium for 24 hours. The OS were then spun down, washed, and embedded. The blocks were sectioned and transferred to slides for immunofluorescence staining as described in Materials and Methods section. The OS particles had a dust-like appearance because of the fragmentation that occurred during their isolation and often formed small aggregates (Fig. 6). Therefore, we performed dual staining for each individual bridge molecule and rhodopsin to assure the OS were being evaluated. Rhodopsin is the visual pigment localized in the photoreceptor OS and is a hallmark for OS staining [54]. Figure 6A shows the rhodopsin-stained (Alexa Fluor 568 conjugated, red) particles, which were preincubated with individual recombinant human bridge molecules, also stained positively with each of the four bridge molecule antibodies (Alexa Fluor 488 conjugated, green), but not with the Alexa Fluor 488 conjugated mouse IgG<sub>2A</sub> or IgG<sub>2B</sub> isotype control antibody. Similar results were obtained for the OS incubated with the hUTC CM (Fig. 6B), whereas no staining for any of the bridge molecules was observed for the control medium-incubated OS (Fig. 6C). The specificity of the anti-rhodopsin antibody was confirmed by double staining of the OS particles with Alexa Fluor 568 conjugated anti-rhodopsin antibody and Alexa Fluor 488 conjugated mouse IgG2b,  $\kappa$  isotype control antibody. The OS was stained positively only with anti-rhodopsin antibody (Fig. 6D). Taken together, our results demonstrate that bridge molecules MFG-E8, Gas6, TSP-1, and



**Figure 4.** RTK ligands and bridge molecules promote phagocytosis in the RCS rat RPE cells. **(A–C):** The RCS RPE cells were incubated with recombinant human BDNF (A), HGF (B), or GDNF (C) for 24 hours, and then subjected to phagocytosis assay. The RCS RPE cells incubated with hUTC conditioned medium (CM) was used as a positive control for the assay. BDNF, HGF, or GDNF dose-dependently increased the phagocytosis in the RCS RPE cells. **(D–G):** The photoreceptor OS were incubated with recombinant human MFG-E8 (D), Gas6 (E), TSP-1 (F), or TSP-2 (G) for 24 hours and then fed to the RCS RPE cells for phagocytosis assay in the absence of the hUTC CM. The OS preincubated with the hUTC CM was used as a positive control for the assay. Phagocytosis of OS by the RCS RPE cells was rescued in a dose-dependent manner by MFG-E8, Gas6, TSP-1, or TSP-2. Data represent the mean  $\pm$  SEM ( $n = 3$ ). \*\*\*\*,  $p < 0.0001$ ; \*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ . Abbreviations: CM, conditioned medium; N, normal RPE; D, dystrophic RCS RPE.



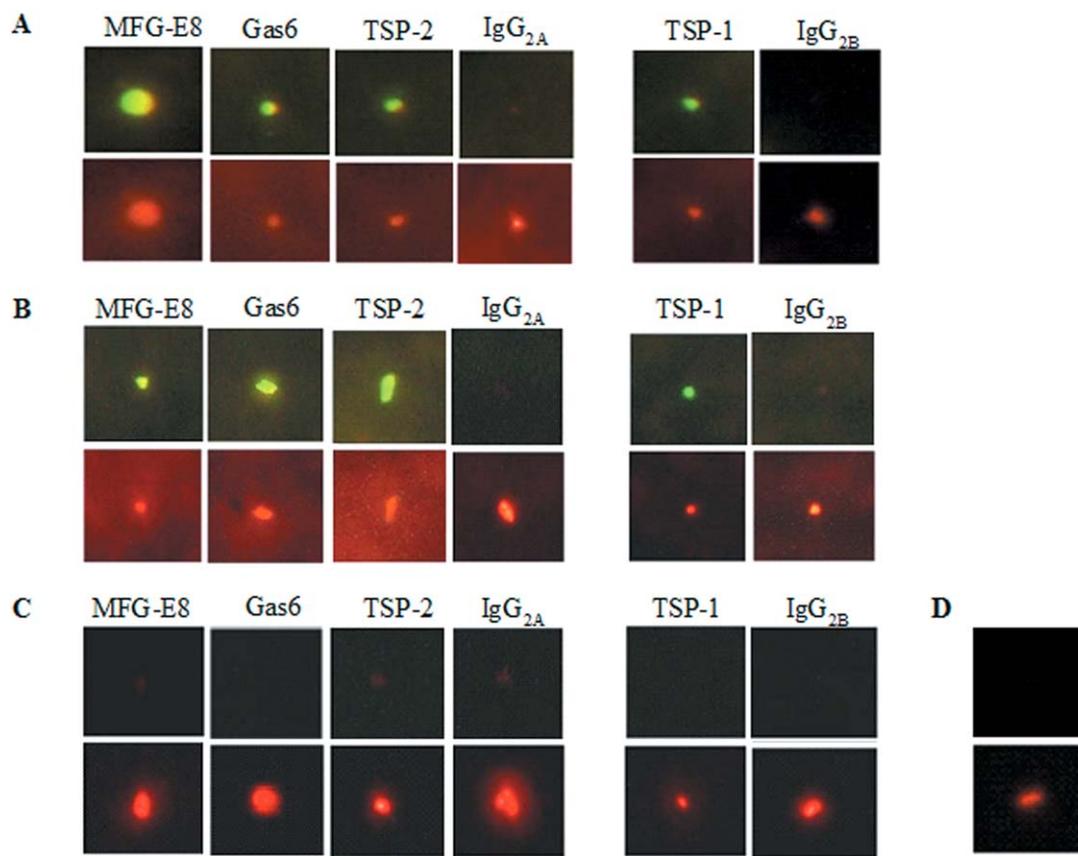
**Figure 5.** RTK ligands and bridge molecules are required for hUTC-induced phagocytosis rescue in the RCS rat RPE cells. **(A):** Enzyme linked immunosorbent assay (ELISA) of the cell culture supernatants collected from the untransfected hUTC and hUTC transfected with siRNA. **(B):** Expression of the RTK ligands *BDNF*, *HGF*, and *GDNF* was silenced by the siRNA transfection of hUTC. The knockdown (KD) hUTC conditioned medium (CM) was harvested. The RCS RPE were incubated with the KD hUTC CM for 24 hours and then subjected to phagocytosis assay. The effect of hUTC on the RCS RPE phagocytosis was significantly abolished when *BDNF*, *HGF*, or *GDNF* was knocked down. **(C):** Expression of bridge molecules *MFG-E8*, *TSP-1*, and *TSP-2* was silenced in hUTC by the siRNA transfection. The KD hUTC CM was harvested. The RCS RPE were fed with the photoreceptor OS pre-incubated with the KD hUTC CM for 24 hours and subjected to phagocytosis assay. Knocking-down of *MFG-E8*, *TSP-1*, or *TSP-2* significantly reduced the hUTC-mediated OS phagocytosis rescue in the RCS RPE. The CM prepared from untransfected and scrambled siRNA transfected hUTC were used as controls for (A–C). Data represent the mean  $\pm$  SEM,  $n = 3$  for (B) and (C),  $n = 6$  for untransfected, mock and scrambled siRNA transfected hUTC CM ELISA (A). \*\*\*\*,  $p < 0.0001$ , \*\*\*,  $p < 0.001$ , \*\*,  $p < 0.01$ , \*,  $p < 0.05$ . Abbreviations: CM, conditioned medium; N, normal RPE; D, dystrophic RCS RPE; KD, knockdown.

*TSP-2* in the hUTC CM colocalize with rhodopsin on the OS, suggesting that they bind to the OS.

## DISCUSSION

We have demonstrated that hUTC rescue the phagocytic function of RPE isolated from the RCS rat, a well-established model of retinal degeneration. Because RPE phagocytosis is crucial for photoreceptor survival and function [6], the effect of phagocytosis rescue by hUTC could well play a role in photoreceptor preservation, as we observed previously in the RCS rat injected with hUTC [35]. Isolated RCS RPE exhibit restored phagocytosis when they are cocultured with hUTC or incubated with the hUTC CM. This suggests that hUTC secrete specific factors that promote phagocytosis. Gene expression analysis shows that

hUTC express genes of multiple RTK ligands and bridge molecules whose receptors are present in the RCS RPE. Among the selected RTK ligands measured by ELISA, we detected relatively high levels of *BDNF*, *HGF*, *GDNF*, and *Gas6* in hUTC CM. *Gas6* is also a bridge molecule reported in apoptotic and RPE phagocytosis. We identified *BDNF*, *HGF*, and *GDNF* as the critical hUTC-secreted RTK ligands that rescue phagocytosis in the RCS RPE, which may be responsible for the photoreceptor and vision preservation observed in the hUTC-injected RCS rat. In the eye, *BDNF*, *HGF*, and *GDNF* are known for their neurotrophic effect, possibly by an anti-apoptotic mechanism, on photoreceptor survival [55–58] and are mainly produced by the RPE cells [55]. Here, we show for the first time that *BDNF*, *HGF*, and *GDNF* promote phagocytic function of RPE cells, thereby providing an alternative mechanism for photoreceptor survival as a consequence of RPE function restoration. It has been shown that



**Figure 6.** hUTC-secreted bridge molecules bind to the photoreceptor outer segments (OS). Immunofluorescence (IF) staining of OS incubated with individual recombinant human MFG-E8 (124 ng/ml), Gas6 (8.75 ng/ml), TSP-1 (1.2  $\mu$ g/ml), or TSP-2 (238 ng/ml) (**A**), or hUTC conditioned medium (CM) (**B**), or control medium (**C**) for 24 hours with subsequent double IF staining of rhodopsin with Alexa Fluor 568 conjugated anti-rhodopsin antibody and of bridge molecules with Alexa Fluor 488 conjugated anti-MFG-E8, anti-Gas6, anti-TSP-1, anti-TSP-2, mouse IgG2A, or mouse IgG2B isotype control antibody. The rhodopsin-stained OS particles also stained positively with each of the recombinant bridge molecule proteins (A) or the secreted bridge molecules present in the hUTC CM (B). No staining for any of the bridge molecules was observed for control medium-incubated OS (C). (**D**): The specificity of anti-rhodopsin antibody was confirmed by double IF staining of OS with Alexa Fluor 568 conjugated anti-rhodopsin antibody and Alexa Fluor 488 conjugated mouse IgG2b,  $\kappa$  isotype control antibody. Upper panel (A–D), bridge molecule and isotype control antibody staining; lower panel (A–D), rhodopsin antibody staining. Magnification,  $\times 250$  with the appropriate filters.

RPE factor secretion changes in response to damage or injury and can be involved in the pathogenesis of retinal diseases [1, 6]. Aberrant growth factor production has been linked with AMD [59, 60]. It is likely that the RPE secretion of some trophic factors vital for photoreceptor viability and function may decrease with aging and in retinal degenerative diseases. RPE degeneration could result in a further decrease of trophic factor release. In this scenario, hUTC could serve as a source of supply for BDNF, HGF, and GDNF to the diseased retina for both photoreceptor survival and RPE function repair.

RTKs and their ligands play an important role in the control of most fundamental cellular processes including the cell cycle, cell migration, cell metabolism and survival [61]. However, their role in phagocytosis has been unclear. The effect of RTK ligands on phagocytosis has rarely been reported. It is only known that basic FGF rescued the phagocytic defect in the RCS RPE cells [44]. BDNF enhanced the phagocytic activity of cultured macrophages and iris pigment epithelial cells [62, 63]. GDNF increased the phagocytosis ability of primary rat microglia [64]. HGF- $\beta$ , a proteolytic cleavage fragment of HGF,

enhanced phagocytosis by macrophages [65]. We demonstrate for the first time that RTK ligands BDNF, GDNF, and HGF rescue the phagocytic defect in the RCS RPE cells which lack MerTK protein. The mechanism of how these factors promote phagocytosis needs further investigation. It was shown that OS challenge activates focal adhesion kinase (FAK) in the RPE [66]. FAK is a cytoplasmic tyrosine kinase that colocalizes with integrins at focal contacts, and is a key component of the signal transduction pathways triggered by integrins [67]. In RPE-mediated clearance, FAK signals bi-directionally between MerTK and  $\alpha v \beta 5$  integrin. Inhibition of FAK diminishes MerTK phosphorylation and impairs OS phagocytosis [66], suggesting that MerTK is a target of FAK signaling. The RPE cells from the RCS rat bind OS normally [68] and retain similar FAK activation as wild-type Long Evans rat RPE in response to OS challenge [66], but ingestion does not occur. It is possible that FAK-mediated MerTK activation is disrupted in the RCS RPE due to the absence of MerTK protein, resulting in the loss of downstream signaling driving phagocytosis. Cumulative evidence indicates that the underlying function of MerTK in OS ingestion by the RPE is the regulation of the cytoskeleton

through activation of PI3 kinase and Rho GTPases [38–40], both of which are also common signaling modules shared by the RTK signaling [61]. It has become clear that PI3 kinases and Rho GTPases are involved in the regulation of the actin cytoskeleton [42, 69]. PI3 kinase and Rac1, a member of Rho GTPases, has been shown to be required for OS internalization by the RPE cells [38, 40]. Therefore, hUTC-secreted BDNF, HGF, and GDNF could bind and activate their cognate RTKs, which in turn compensate for the function of MerTK by regulating the cytoskeleton reorganization for phagocytosis. In addition, receptors for BDNF, HGF, and GDNF may also serve as surrogate for MerTK downstream of FAK, leading to the phagocytosis rescue. Our results show that knocking-down BDNF, HGF, and GDNF individually in hUTC completely abolished the phagocytosis rescue, suggesting the effect of these factors are dependent on each other and are not redundant.

We show that the phagocytosis in the RCS RPE cells is also rescued when the cells are fed with hUTC CM pretreated-OS and cultured in the absence of the hUTC CM during the phagocytosis assay. This suggests that some factors in the hUTC CM may serve as opsonins for OS to facilitate their internalization. Indeed, we identified bridge molecules MFG-E8, TSP-1, and TSP-2 as the critical hUTC-secreted factors that promote phagocytosis using siRNA-mediated gene silencing. Due to the failure of siRNA gene knockdown for *Gas6*, we were unable to confirm the role of *Gas6* in the hUTC-mediated effect. We show that hUTC-secreted MFG-E8, *Gas6*, TSP-1, and TSP-2 all bind to OS and recombinant human proteins of these factors dose-dependently increase the phagocytosis of OS by the RCS RPE. This could be an explanation for the hUTC-mediated phagocytosis rescue in the RCS RPE when they are fed with the hUTC CM-treated OS in the absence of hUTC CM. It is interesting that recombinant human *Gas6* alone restores phagocytosis in the RCS RPE which lack MerTK. *Gas6* is a cognate ligand for MerTK, but is also a ligand for the other two members, Axl and Tyro 3 of the Axl RTK subfamily which is also known as TAM receptors [70]. For apoptotic cell phagocytosis, *Gas6* is involved in linking PS exposed on apoptotic cells to TAM receptors on the phagocytes [47]. These receptors appear important for clearance of apoptotic cells in the eye, testes, thymus, and other tissues [70, 71]. The effect of *Gas6* on phagocytosis in the RPE is thought to be dependent on MerTK [72]. The role of Axl and Tyro 3 in RPE phagocytosis has not been reported. It is plausible that the phagocytic stimulatory effect of *Gas6* we found on the RCS RPE is mediated through Tyro 3 and Axl. The gene expression of both receptors is present in the RCS RPE.

The bridge molecule binding sites on the OS are not clear, but could be associated with externalized PS which was reported to be possessed by the OS [46]. We demonstrate that recombinant human MFG-E8 and *Gas6*, as well as those secreted by hUTC, bind to isolated OS. This is in line with the findings from other researchers who showed purified bovine MFG-E8, or recombinant murine MFG-E8, and recombinant human *Gas6* bind to OS [46, 72, 73]. The binding of TSPs to OS and their role in RPE phagocytosis have never been reported, but have been indicated in apoptotic cell phagocytosis wherein they bridge between the exposed PS and the receptor complex of CD36 and integrin  $\alpha v\beta 3$  and  $\alpha v\beta 5$  [74]. We demonstrate for the first time that the recombinant

human TSP-1, TSP-2, and the ones secreted by hUTC bind to the OS, and have stimulatory effect on phagocytosis in the RCS RPE. These results reveal a novel role of TSPs in RPE phagocytosis. The intracellular mechanism of how hUTC-secreted bridge molecules promote phagocytosis remains to be investigated. RPE membrane receptors integrin  $\alpha v\beta 5$  and CD36, previously shown to participate in OS recognition and internalization by the RPE [45], could be involved in the process.

In the eye, the RPE plays many important roles and is essential for retinal preservation and visual function [1]. Impairment of normal RPE functions is known to result in retinal degeneration and loss of vision [2–5]. Phagocytosis of shed OS is a key function of RPE to maintain the survival and excitability of photoreceptors for light transduction [6]. Disruption of the RPE phagocytosis causes retinal dystrophy in rodents and in humans [2, 36]. Mutations in *Mertk* gene have been shown to be associated with retinal degenerative diseases in humans likely caused by the defective RPE phagocytosis [2, 8–14]. Therefore, hUTC could be beneficial for the treatment of this class of patients with RPE phagocytic defect.

The exact mechanisms that cause AMD remain unclear and the etiology of AMD involves multiple factors such as aging, genetic predisposition, environmental elements, oxidative stress, and inflammation [75]. Because AMD is a multifactorial disease with largely unknown pathogenesis, it is unclear if a reduction in RPE phagocytosis is causative in AMD pathology. Drusen are subretinal pigment epithelial deposits that are characteristic of AMD [76]. It is not known if drusen are the primary cause for the degeneration of RPE in AMD, but they do ultimately affect RPE health due to impaired transport across Bruch's membrane [77]. How drusen are formed in AMD remains elusive. RPE dysfunction and failure of phagocytosis result in debris accumulation in the intracellular and extracellular space [21, 23, 24, 76, 78]. Some believe that drusen may be unphagocytized debris that translocates from the apical to sub-RPE region [79]. In this sense, promoting RPE phagocytosis may reduce drusen formation by reducing debris accumulation and translocation to sub-RPE region. The phagocytosis of OS involves formation of the phagosome and its fusion with a lysosome followed by digestion of ingested materials [80]. Excessive accumulation of lipofuscin in the RPE cells, a key feature of the aging retina and of various retinal degenerations including AMD [25, 26], is believed to be caused by incomplete digestion of OS in the RPE, a defect in a step in the phagocytic function [21–24]. A well-established cytotoxic constituent of lipofuscin is a di-retinal conjugate A2E which can cause RPE damage [20]. Thus, reduced phagocytic function in RPE with aging [15, 16] and possibly in AMD may contribute to lipofuscin accumulation due to the insufficient degradation of phagocytized OS by lysosome. In this scenario, increasing phagocytic function may prevent lipofuscin formation and its toxic effect on RPE cells by a more efficient digestion of the ingested OS. Additionally, RPE phagocytosis may promote RPE cell survival. Photoreceptor outer segments have the highest level of docosahexaenoic acid (DHA) in the body [81]. It was reported that RPE phagocytosis can convert phagocytized DHA to neoroprotectin D1, an anti-apoptotic molecule that promotes the survival of RPE and photoreceptors against oxidative stress [81–83]. Taken together, promoting

RPE phagocytic function could contribute to the survival of both photoreceptors and RPE cells in retinal degenerative diseases.

Cell therapy targeting RPE holds great promise for the treatment of retinal degenerative diseases and falls into two types: cell replacement therapy and trophic therapy [31, 32]. Cell replacement therapy delivers RPE cells differentiated from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) to replace the damaged native cells in order to restore retinal function [84–86]. The future use of these cells may be hindered by obstacles, such as limited cell source, ethical debate, and immunological issues [87–89]. In the trophic therapy, the transplanted cells remain undifferentiated and secrete trophic factors that induce the degenerating resident tissue to self-restore [34]. Studies of several trophic cell therapies in both animal models and human clinical trials have been reported. Ciliary neurotrophic factor (CNTF), delivered by encapsulated cell intraocular implants, appeared to slow the progression of vision loss in geographic atrophy of AMD [90] probably through its neurotrophic effect. Autologous bone marrow-derived stem cells were shown to protect cone photoreceptors in mouse models of retinal degeneration by stabilizing the degenerating blood vessels. However, the mediators for this effect in mice are not clear [91]. Our results demonstrate novel trophic mechanisms of hUTC in restoring RPE function in retinal degeneration, which is mediated through the secretion of multiple factors and their stimulatory effect on RPE phagocytosis. These findings provide insight into the complex mode of action of a cell therapy and indicate a potential therapeutic strategy for the treatment of retinal degenerative diseases with RPE dysfunction and photoreceptor degeneration. Molecular crosstalk may exist between integrin and RTKs that amplifies intracellular signals for OS binding and internalization leading to the rescue of phagocytosis. Investigations are in progress to address these questions.

## CONCLUSIONS

In summary, this study identifies novel mechanisms of a cell-based therapy for correcting the RPE phagocytic dysfunction in retinal degeneration through RTK ligands and bridge molecules, suggesting that hUTC may provide utility in treating patients with retinal degenerative diseases, such as retinitis pigmentosa and AMD. The results shown here indicate a potential new therapeutic approach for the treatment of retinal degeneration.

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## AUTHOR CONTRIBUTIONS

J.C.: conception, study design, experiment design and performance, preparation and provision of study materials, collection and assembly of data, data analysis and interpretation, manuscript writing, revision and editing; C.M. and W.A.: experiment performance and data collection; X.Y.: collection and assembly of data, data analysis and interpretation; J.L.: experimental design and performance, data collection, data analysis and interpretation; S.S.-M.: conception; I.R.H.: conception, financial support, final approval of manuscript; G.I.: collection and assembly of data, data analysis and interpretation, manuscript editing.

## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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