Selection of Reference Genes for Studies of Human Retinal Endothelial Cell Gene Expression by Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction

Binoy Appukuttan, PhD, **Liam M. Ashander, BS**, **Yuefang Ma, PhD**, and **Justine R. Smith, FRANZCO PhD**

Eye & Vision Health, Flinders University College of Medicine and Public Health, Adelaide, Australia

Abstract

Background—Human retinal endothelial cells are employed increasingly for investigations of retinal vascular diseases. Analysis of gene expression response to disease-associated stimuli by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) is common. However, most reported work does not follow the minimum information for publication of qPCR experiments (MIQE) recommendation that multiple, stably expressed reference genes be used for normalization.

Methods—Two human retinal endothelial cell lines were treated with medium alone or containing stimuli that included: glucose at supraphysiological concentration, dimethyloxalyl-glycine, vascular endothelial growth factor, tumor necrosis factor-α, lipopolysaccharide and *Toxoplasma gondii* tachyzoites. Biological response of cells was confirmed by measuring significant increase in a stimulus-relevant transcript. Total RNA was reverse transcribed and analyzed by commercial PCR arrays designed to detect 28 reference genes. Stability of reference gene expression, for each and both cell lines, and for each and all conditions, was judged on gene-stability measure (M-value) less than 0.2 and coefficient of variation (CV-value) less than 0.1.

Results—Reference gene expression varied substantially across stimulations and between cell lines. Of 27 detectable reference genes, 11–21 (41–78%) maintained expression stability across stimuli and cell lines. Ranking indicated substantial diversity in the most stable reference genes under different conditions, and no reference gene was expressed stably under all conditions of stimulation and for both cell lines. Four reference genes were expressed stably under 5 conditions: *HSP90AB1*, *IPO8*, *PSMC4* and *RPLPO*.

Conclusions—We observed variation in stability of reference gene expression with different stimuli and between human retinal endothelial cell lines. Our findings support adherence to MIQE recommendations regarding normalization in RT-qPCR studies of human retinal endothelial cells.

Corresponding Author: Binoy Appukuttan, PhD, Address: Eye & Vision Health, Flinders University College of Medicine and Public Health, Flinders Medical Centre Room 4E-431, Flinders Drive, Bedford Park, SA 5042, Australia, Telephone: 61-8-8204-6194, binoy.appukuttan@flinders.edu.au.

Keywords

Human; Retina; Endothelial Cell; Reference gene; PCR

Introduction

Diseases that involve the endothelium of the retinal vasculature are leading causes of impaired vision and blindness across the world: diabetic retinopathy; central retinal vein occlusion; retinopathy of prematurity; immune-mediated posterior uveitis; and ocular toxoplasmosis.[1] In these conditions, endothelial cell dysfunction contributes to retinal vascular leakage, neovascularization and/or leukocytic or microbial infiltration. Consequently, human retinal endothelial cells are the subject of multiple *in vitro* studies of basic pathogenic mechanisms. Independent research groups – including our own – isolate primary retinal endothelial cells from human cadaveric eyes[2,3] or purchase cells from commercial sources[4,5] to undertake these studies, which commonly involve analysis by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR).

Since its introduction over 20 years ago as a method for quantifying differences in gene expression between experimental conditions,[6] RT-qPCR has become a standard and common tool in molecular research and diagnostics. Although RT-qPCR is a robust technique, methodological variations may profoundly impact output, with implications for interpretation and reproducibility of results across laboratories. The minimum information for publication of qPCR experiments (MIQE) guidelines were first proposed in 2009, to improve communication in reporting and also provide methodological standards for performing qPCR.[7] One important consideration for compliance with the MIQE guidelines is the selection of endogenous reference genes – previously generically referred to as housekeeping genes – for normalization of mRNA concentrations. The guidelines require reference genes to be constitutively transcribed at the same level in all samples, regardless of source and/or environment, and further, strongly recommend multiple reference genes be employed for any normalization.[7]

To identify reference genes for MIQE-compliant RT-qPCR studies of human retinal endothelial cells, we exposed cell lines derived from retinae of two different human donors to stimuli commonly used to elicit disease-relevant responses: (1) glucose at supraphysiological concentration, which is the central abnormality in diabetes mellitus; (2) hypoxia simulator, dimethyloxalylglycine (DMOG); (3) key regulator of neovascularization, vascular endothelial growth factor (VEGFA); (4) master inflammatory cytokine, tumor necrosis factor (TNF)-α; (5) bacterial component, lipopolysaccharide (LPS); and (6) the cause of ocular toxoplasmosis, *Toxoplasma gondii* tachyzoites. We used PCR array profiling to interrogate expression of 28 reference genes in the cell lines. Our results show that human retinal endothelial cells transcribe a majority of reference genes stably under different conditions, but the exceptions to this finding vary with the stimulus and the cell population.

Materials and Methods

Overview of experimental design

Triplicate human retinal endothelial cell cultures were treated with medium only or one of six molecular or microbial stimuli that included: (1) glucose at supraphysiological concentration; (2) DMOG; (3) VEGFA; (4) TNF-α; (5) LPS; and (6) *T. gondii* tachyzoites. Total RNA was extracted from lysate prepared from each culture, and high quality of the RNA was verified. From every RNA preparation, cDNA was synthesized and separately analyzed by commercial PCR reference gene array. A biological response of cells to each stimulus was confirmed on the same RNA samples by RT-qPCR measurement of a stimulus-relevant transcript: (1) glucose at supraphysiological concentration, interleukin 1β (IL-1β); (2) DMOG, VEGFA; (3) VEGFA, Down syndrome critical region gene 1 (DSCR1); (4) TNF-α, intercellular adhesion molecule 1 (ICAM-1); (5) LPS, ICAM-1; and (6) *T. gondii* tachyzoites, suppressor of cytokine signaling 1 (SOCS1). Two human retinal endothelial cell lines, generated from eyes of two human cadaveric donors, were studied separately.

Endothelial cell and microbial culture

Our method for generating human retinal endothelial cell lines has been published.[1] In summary, endothelial cells were isolated from paired human cadaver retinae (VisionGift, Portland, OR) by enzymatic digestion of tissue and selection with magnetic bead-conjugated anti-human CD31 antibody, and subsequently expanded by transduction with the mouse recombinant amphotropic retrovirus, LXSN16E6E7.[8] These cell lines retain their endothelial phenotype, including expression of endothelial markers and formation of capillary-like tubes on Matrigel,[1] and they have been used in multiple published studies of retinal vascular disease (e.g.[9–11]). Human retinal endothelial cells were cultured in MCDB-131 medium (Sigma-Aldrich, St. Louis, MO; catalogue number M8537), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone-GE Healthcare Life Sciences, Logan, UT) and endothelial growth factors (EGM-2 SingleQuots supplement, omitting FBS, hydrocortisone and gentamicin; Clonetics-Lonza, Walkersville, MD) at 37 °C and 5% CO_2 in air.

GT-1 strain *T. gondii* were maintained in tachyzoite form by serial passage in confluent monolayers of human foreskin fibroblasts in Dulbecco's modified Eagle's medium (DMEM; Catalogue number, 12100; Life Technologies-Gibco, Grand Island, NY), supplemented with 44 mM sodium bicarbonate and 1% FBS, at 37 °C and at 5% CO_2 in air. Plaque assays were performed using human fibroblast monolayers to verify parasite viability for each retinal endothelial cell infection: viability of 15% or greater was required, consistent with published measurements for a natural isolate.[12]

Reagents and proteins

D-Glucose was supplemented to a concentration of 30 mM. Dimethyloxalylglycine (Sigma-Aldrich; catalogue number D3695) was used at a working concentration of 1 mM. Recombinant human VEGFA (VEGF$_{165}$) and TNF-α (both from R&D Systems, Minneapolis, MN; catalogue numbers 293-VE-010 and 210-TA-020) were used at working concentrations of 10 ng/mL and 25 ng/mL, respectively. Lipopolysaccharide from

Escherichia coli 055:B5 (Sigma-Aldrich; catalogue number L6529) was used at a working concentration of 10 µg/mL.

Stimulation of human retinal endothelial cells

Human retinal endothelial cells were grown to confluence in 6-well plates, in modified MCDB-131 medium with 10% FBS and endothelial growth factors. Three monolayers on each plate were treated with one stimulus in fresh medium (i.e., glucose, DMOG, VEGFA, LPS, TNF-α and *T. gondii*: 2.5×10^6 freshly egressed tachyzoites per well) and the three remaining monolayers were treated with fresh medium alone. In the case of the *T. gondii* stimulations, FBS in the medium was reduced to 5%, to minimize effects of serum components on parasite viability. Cell monolayers were incubated for 4 hours at 37 °C and 5% CO_2 in air ahead of RNA extraction, with the exception of cells treated with glucose at high concentration and respective controls; testing 11 potential targets of this stimulus indicated no biological response at 4 hours (data not shown), and therefore incubation was extended to 24 hours.

RNA isolation and reverse transcription

Retinal endothelial cell monolayers were covered with Buffer RLT (Qiagen, Hilden, Germany) containing 0.55 mM β-mercaptoethanol (Sigma-Aldrich), and cell lysates were frozen at −80 °C ahead of RNA extraction. Total RNA was extracted using the RNeasy mini kit (Qiagen), according to the manufacturer's instructions and including the on-column DNase treatment. RNA concentration, purity and quality were determined by nanodrop spectrophotometry (NanoDrop 2000c, Thermo Scientific, Wilmington, DE) and chip-based capillary electrophoresis on the Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany). Only highly pure RNA, with A260/280 ratios greater than 1.8 and RNA integrity numbers over 9.0, was used for qPCR. Reverse transcription was performed using the iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA), with 500 ng RNA template per 20 µl RT reaction mix, yielding 25 ng/µl cDNA. For each RNA template, four 20 µl RT reactions were performed and cDNA was pooled, to minimize inefficiencies in individual RT reactions. This cDNA was diluted 1 in 10 with nuclease-free water, and 4 ul (containing 10 ng of cDNA) was used in subsequent qPCR.

Quantitative real-time polymerase chain reaction

To confirm induction of downstream targets of each stimulation, qPCR was performed on the CFX Connect Real-Time System (Bio-Rad, Hercules, CA) using 4 µL of cDNA, 4 µL of iQ SYBRGreen Supermix (Bio-Rad), 1.5 µL each of 20 uM forward and reverse primers (Supplementary Table 1), and 9 µL of nuclease-free water for each reaction.

Amplification consisted of: a pre-cycling hold at 95 °C for 5 minutes; 40 cycles of denaturation for 30 seconds at 95 °C; annealing for 30 seconds at 60 °C; extension for 30 seconds at 72 °C; and a post-extension hold at 72 °C for 1 second. A melting curve was produced by a 1-second hold at every 0.5 °C between 70 °C and 95 °C to confirm a single PCR amplicon. For each primer set, standard curves were generated with serially diluted amplicon to confirm an efficiency of at least 85%. Amplicon size was confirmed by electrophoresis on 2% agarose gel. Cycle threshold was measured with Cq determination

mode set to regression. Relative expression was calculated in the Gene Expression Analysis module of CFX Manager v3.1 (Bio-Rad), which uses the $2^{-\Delta\Delta Ct}$ method,[13] normalizing to three reference genes that were stable for the specific condition and human donor by the criteria described below. Results corresponding to individual control and stimulated conditions were compared statistically by two-tailed Student's t-test, and a significant difference between conditions was defined as one yielding a *p*-value less than 0.05.

Quantitative real-time polymerase chain reaction array

The qPCR arrays were performed with two PrimePCR Pathway Plates: Reference Genes H96 and Reference Genes Plus H96 (BioRad). Each of these plates contains validated and optimized primer pairs for 14 human reference gene transcripts in triplicate test and control samples (Supplementary Table 2), as well as primer pairs for detection of genomic DNA contamination, and for verifying cDNA quality and performance efficiency of qPCR. The manufacturer's instructions were followed exactly, including the use of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) and 4 μl (10 ng) of cDNA per reaction. The qPCR plates were run on CFX96 Connect Real-Time PCR Detection System, using a pre-programmed thermal cycling protocol consisting of: activation at 95 °C for 2 minutes; 40 cycles of denaturation at 95 °C for 5 seconds; annealing/extension at 60 °C for 30 seconds; and production of a melt curve from 65 °C to 95 °C, in 0.5 °C increments, for 5 seconds at each temperature.

Reference gene expression was evaluated using the Gene Expression Analysis module of CFX Manager v3.1, as described above. The qBase program[14] implemented within this module was used to calculate a gene-stability measure – or M-value – and a coefficient of variation – or CV-value – for each reference gene, by condition and donor. The M-value of a reference gene is the mean pairwise variation – defined as standard deviation of logarithm expression ratios – for that gene, in comparison to other reference genes tested for the same samples.[15] Thus, a relatively low M-value equates with more stable expression. The CV-value represents a comparison of normalized relative quantities of reference gene across samples.[14] Like the M-value, a relatively low CV-value indicates stability of expression. The CV- and M-values for each reference gene in the two retinal endothelial isolates and six conditions were ranked numerically. By applying stringent cut-offs of CV-value less than 0.1 and M-value less than 0.2, stably expressed reference genes were established for each donor and both donors, and for each condition and all conditions.

Results

Comparison of reference gene expression by human retinal endothelial cells treated with molecular or microbial stimuli

Two retinal endothelial cell lines established from paired eyes of two human cadaveric donors were treated with one of six molecular or microbial stimuli that are commonly used to study cell responses in disease: glucose at supraphysiological concentration; DMOG; VEGFA; TNF-α; LPS; and *T. gondii* tachyzoites; or medium alone as control for each treatment. All stimuli elicited significant increases in transcripts encoding known targets over baseline conditions, as determined by RT-qPCR, indicating biological responses by

cells to all treatments: glucose, 2.3- and 1.6-fold increase in IL-1β transcript; DMOG, 7.8- and 18.8-fold increase in VEGFA transcript; VEGFA, 3.9- and 4.5-fold increase in DSCR1 transcript; TNF-α, 21.0- and 75.4-fold increase in ICAM-1 transcript; LPS, 12.5- and 12.7-fold increase in ICAM-1 transcript; and *T. gondii* tachyzoites, 6.4- and 8.6-fold increase in SOCS1 transcript (Figure 1).

Expression of 28 reference genes in stimulated versus control retinal endothelial cells was investigated by qPCR array, using two PrimePCR Pathway Plates, each spotted with primer pairs for 14 human reference gene transcripts in triplicate. The expression of 27 of the 28 reference genes was calculated as fold-difference for each molecular or microbial treatment relative to medium alone, following normalization to: *PSMC4*, *IPO8* and *SDHA* (glucose); *RPL13A*, *YWHAZ* and *G6PD* (DMOG); *HSP90AB1*, *UBC* and *HPRT1* (VEGFA); *HSP90AB1*, *PSMC4* and *ACTB* (TNF-α); *GAPDH*, *GUSB* and *YWHAZ* (LPS); *SDHA*, *ALAS1* and *GUSB* (*T. gondii* tachyzoites). The final reference gene, *HBB*, was removed from this analysis, based on PCR cycle threshold values that exceeded 35, indicating minimal to no expression in human retinal endothelial cells. Comparison of fold-changes revealed substantial differences in reference gene expression across stimulations. For the retinal endothelial cell line generated for donor 1, expression of reference genes varied: 0.5- to 1.1-fold for glucose at supraphysiological concentration, 0.8- to 1.7-fold for DMOG, 0.8- to 1.2-fold for VEGFA; 0.8- to 1.4-fold for TNF-α, 0.9 to 1.5-fold for LPS, and 0.8- to 2.6-fold for *T. gondii* tachyzoites (Figure 2). For the retinal endothelial cell line generated for donor 2, expression of reference genes varied: 0.8- to 1.3-fold for glucose at supraphysiological concentration, 0.04- to 1.7-fold for DMOG, 0.7- to 18.6-fold for VEGFA; 0.7- to 1.5-fold for TNF-α, 0.9- to 1.6-fold for LPS, and 0.8- and 1.3-fold for *T. gondii* tachyzoites (Figure 3). This comparison also revealed differences in reference gene expression between human retinal endothelial cell lines. Largest differences were observed for: *LDHA* under conditions of DMOG stimulation (1.70-fold versus 0.04-fold); *RPLP0* under conditions of VEGFA stimulation (1.0-fold versus 18.6-fold); and *GADPH* under conditions of VEGFA stimulation (0.9-fold versus 4.3-fold), for retinal endothelial cell lines of donors 1 and 2, respectively.

Analysis of stability of reference gene expression in human retinal endothelial cells treated with molecular or microbial stimuli

Expression stability of reference genes in human retinal endothelial cells across stimulated and control conditions was assessed by M- and CV-values. Applying stringent criteria of CV-value less than 0.1 and M-value less than 0.2, we identified reference genes that demonstrated stable expression in retinal endothelial cell lines from the two human donors, under the six different conditions. By these criteria, overall 11–21 of 28 reference genes (41–78%) maintained expression stability following treatments, across stimuli and human retinal endothelial cell lines. For glucose at supraphysiological concentration, 22 and 14 references genes were stable by CV-value, and 25 and 13 references genes were stable by M-value in the two cell lines: 11 reference genes were stable by both criteria in each line (Table 1). For DMOG, 17 and 13 references genes were stable by CV-value, and 13 and 10 references genes were stable by M-value in the two cell lines: 11 reference genes were stable by both criteria in each line (Table 2). For VEGFA, 23 and 17 references genes were stable

by CV-value, and 26 and 17 references genes were stable by M-value in the two cell lines: 17 and 15 reference genes were stable by both criteria (Table 3). For TNF-α, 21 and 16 references genes were stable by CV-value, and 22 and 15 references genes were stable by M-value in the two cell lines: 14 reference genes were stable by both criteria in each line (Table 4). For LPS, 18 and 21 references genes were stable by CV-value in the two cell lines, and 22 references genes were stable by M-value in each line: 21 and 16 reference genes were stable by both criteria (Table 5). For *T. gondii* tachyzoites, 20 and 19 references genes were stable by CV-value, and 18 and 24 references genes were stable by M-value, and in the two cell lines: 17 and 18 reference genes were stable by both criteria (Table 6).

Comparison of stable reference gene expressed in human retinal endothelial cells under different conditions of stimulation and by donor

Ranking of reference genes expressed in human retinal endothelial cells by lowest M- and CV-values for the two cell lines, indicated substantial diversity in the most stably expressed reference genes under different conditions of stimulation (Tables 1–6, summarized in Table 7). For glucose at supra-physiological concentration, the most stably expressed reference genes were *PSMC4*, *IPO8* and *SDHA*. For DMOG, the most stably expressed reference genes were *RPL13A*, *YWHAZ*, *GUSB* and *HPRT1*. For VEGFA, the most stably expressed reference genes were *HSP90AB1*, *UBC* and *HPRT1*. For LPS, the most stably expressed reference genes were *GAPDH*, *GUSB*, *YWHAZ* and *RPLP0*. For TNF-α, the most stably expressed reference genes were *HSP90AB1*, *PSMC4*, and *ACTB*. For *T. gondii* tachyzoites, the most stably expressed reference genes were *SDHA*, *ALAS1* and *GUSB*. Overall, no reference gene was expressed stably under all six conditions of stimulation for both donors. Fourteen reference genes were expressed stably under 3 or more conditions, including: 4 reference genes under 5 conditions (i.e., *HSP90AB1*, *IPO8*, *PSMC4* and *RPLPO*); 6 reference genes under 4 conditions (i.e., *ACTB*, *B2M*, *GUSB*, *RPL30*, *SDHA* and *YWHAZ*); and 6 reference genes under 3 conditions (i.e., *ALAS1*, *GAPDH*, *HPRT1*, *RPL13A*, *RPS18* and *UBC*) (Table 8).

Discussion

Use of multiple stably expressed reference genes is a key implication of the MIQE guidelines. Two surveys conducted since the publication of these recommendations in 2009, reviewing diverse studies that used RT-qPCR in over 1700 articles, indicated normalization procedures were inadequate in the majority of published works.[16] Indeed, the authors of these surveys highlighted "the problem of a huge body of literature that reports conclusions that may be meaningless and will cause research resources to be wasted".

We identified articles published between 2009 and 2015 that described RT-qPCR studies involving human retinal endothelial cells specifically, using the National Library of Medicine of National Institutes of Health PubMed database (Figure 4, Table 9 and Supplementary Table 3). Our search yielded a total of 72 publications: the number of publications increased over time from 2 in 2009 to 23 in 2015. Consistent with current understanding of the pathogenesis of common retinal diseases, investigations most commonly involved treatment of human retinal endothelial cells with: glucose or advanced

glycation end-products; hypoxia, hypoxia mimics or VEGF; and inflammatory stimuli. Overall, studies in just one of the 72 articles employed more than one reference gene, and stability of reference gene expression was reported in only one article also. The most common reference genes used in these works were *ACTB* (32 articles) and *GAPDH* (21 articles). This analysis of the relevant literature indicates that researchers performing RT-qPCR studies involving human retinal endothelial cells often do not follow the MIQE recommendations.

We used PCR array profiling to evaluate the potential application of 28 reference genes in RT-qPCR studies of human retinal endothelial cells. We examined reference gene expression in response to six stimuli, which are commonly used to elicit disease-relevant responses, in two retinal endothelial cell lines separately derived from eyes of two human donors, and we observed variation in expression of reference genes across stimuli and cell lines. Multiple statistical algorithms have been developed to evaluate the stability of reference genes in qPCR; a comparison of different methodologies indicates medium to high correlation.[17] For this reason, and for real-world applicability, we chose to assess reference gene stability with two widely utilized measures – M-value and CV-value – and we set relatively stringent limits. Our investigation identified 10–15 of 28 reference genes (36–54%) expressed stably across different stimulated and control conditions. The group of stably expressed reference genes varied with condition, and no reference gene was stably expressed under every condition, although 4 of the 28 were stable for 5 of 6 conditions (i.e., *HSP90AB1*, *IPO8*, *PSCM4* and *RPLP0*). Of direct relevance to published studies of human retinal endothelial cells, ACTB transcript was stable to treatments with DMOG, VEGFA, TNF-α and LPS, but not glucose and *T. gondii* tachyzoites, while GAPDH transcript was stable to treatments with TNF-α, LPS and *T. gondii* tachyzoites, but not glucose, DMOG and VEGFA.

Two independent groups have evaluated reference gene stability in human retinal endothelial cells under specific conditions. Wei et al[18] stimulated human retinal endothelial cells with polyinosinic:polycytidylic acid, which activates innate immune responses via toll-like receptor 3, and evaluated expression of 10 reference genes by RT-qPCR. The authors identified *HPRT1*, *TBP* and *PGK1* as the most stably expressed reference genes in that setting. Consistently, we observed stability of one or two of the same reference genes in human retinal cells treated with other immune mediators, TNF-α, LPS and *T. gondii*. Xie et al[19] studied expression stability of 14 reference genes in human retinal endothelial cells treated with glucose at supraphysiological concentration and/or hypoxia by RT-qPCR. The investigators found an alternative group of reference genes to be most stably expressed: *TBP*, *PUM1* and *ALAS1*. We also observed ALAS1 transcript level to be unaffected by treatment with highly concentrated glucose. However, none of these three molecules were represented in our list of stable reference genes in cells treated with the hypoxia mimic, DMOG. Reference gene expression has been studied in other endothelial cell populations, including human umbilical vein and brain endothelial cells, treated with metabolic and inflammatory stimuli.[20–22] In each of these studies, different groups of reference genes were stably expressed, including *ACTB*, *ALAS1*, *GAPDH*, *GUSB*, *RPLP0*, *RALBP1*, *RNU6-1* and *TFRC*. Interestingly, *TFRC*, which was represented on the PCR array we used, was not expressed stably under any treatment condition we studied.

Studies in different cell populations have identified a variety of experimental conditions, other than stimulations, that may impact reference gene expression stability. The time over which a stimulation is applied may alter the expression of reference gene transcripts substantially.[23] Confluence of cells in culture may lead to changes in levels of reference genes,[24] and the physical properties of the substrate on which cells are grown may influence reference gene expression.[25] While it is clear that human subjects invariably display a range of responses to the same stimulus, and this is often reflected at the cellular level, in studies of gene expression by human cells, the possibility of inter-individual variation in expression of reference genes is often not considered. Previously we have demonstrated phenotypic differences in human retinal endothelial cell isolates from different donors by molecular profiling, as well as studies of function.[2,26,27] In this current work, we observed differences in the expression stability of reference genes between retinal endothelial cell lines, generated from two human donors, for each of the six disease-associated stimuli that we tested.

Human retinal endothelial cells are being employed increasingly in studies of retinal vascular diseases, and many of these investigations are undertaken with an ultimate goal of developing new treatments. For such research, the importance of accurately normalized RT-qPCR data cannot be understated. Our work provides a guide to reference genes that may be suitable for normalization in RT-qPCR studies of human retinal endothelial cells subjected to different pathological stimuli. More importantly, however, our work highlights the variation in expression stability of reference genes that may be encountered with different stimuli and between human retinal endothelial cell isolates. Our findings provide strong evidence that researchers, who work in this field, should aim to follow MIQE recommendations for normalization of RT-qPCR data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations List

ACTB   Actin beta

ALAS1   Aminoerulinate, delta-synthase 1

B2M   Beta-2-microglobulin

DMEM   Dulbecco's modified Eagle's medium

DMOG   Dimethyloxalylglycine

DSCR1   Down syndrome critical region gene 1

FBS	Fetal bovine serum
G6PD	Glucose-6-phophate dehydrogenase
GAPDH	Glyceraldehyde-3-phophate dehydrogenase
GUSB	Glucuronidase beta
HBB	Hemoglobin, beta
HMBS	Hydroxymethylbilane synthase
HPRT1	Hypoxanthine phosphoribosyltransferase 1
HSP90AB1	Heat shock protein 90kDA (cytosolic), Class B member 1
ICAM-1	Intercellular adhesion molecule 1
IL-1β	Interleukin 1β
IPO8	Importin 8
LDHA	Lactate dehydrogenase A
LPS	Lipopolysaccharide
MIQE	Minimum information for publication of qPCR experiments
NONO	Non-POU domain containing, octamer-binding
p21, Cip1	Cyclin-dependent kinase inhibitor 1A
PGK1	Phosphoglycerate kinase 1
PPIA	Peptidyl isomerase A (cyclophilin A)
PPIH	Peptidyl isomerase H (cyclophilin H)
PSMC4	Proteasome 26S subunit, ATPase 4
PUM1	Pumilio RNA-binding family member 1
RPL13A	Ribosomal protein L13a
RPL30	Ribosomal protein L30
RPLP0	Ribosomal protein, large, P0
RPS18	Ribosomal protein S18
RT-qPCR	Reverse transcription-quantitative real-time polymerase chain reaction
SDHA	Succinate dehydrogenase complex flavoprotein subunit A
SOCS1	Suppressor of cytokine signaling 1
TBP	TATA box binding receptor

TFRC	Transferrin receptor
TNF-α	Tumor necrosis factor α
UBC	Ubiquitin C
VEGFA	Vascular endothelial growth factor
YWHAZ	Tyrosine 3-mono-oxygenase/tryptophan 5-mono-oxygenase activation protein zeta

References

1. Bharadwaj AS, Appukuttan B, Wilmarth PA, et al. Role of the retinal vascular endothelial cell in ocular disease. Prog Retin Eye Res. 2013; 32:102–180. [PubMed: 22982179]

2. Smith JR, Choi D, Chipps TJ, et al. Unique gene expression profiles of donor-matched human retinal and choroidal vascular endothelial cells. Invest Ophthalmol Vis Sci. 2007; 48:2676–2684. [PubMed: 17525199]

3. Browning AC, Halligan EP, Stewart EA, et al. Comparative gene expression profiling of human umbilical vein endothelial cells and ocular vascular endothelial cells. Br J Ophthalmol. 2012; 96:128–132. [PubMed: 22028475]

4. Suarez S, McCollum GW, Bretz CA, et al. Modulation of VEGF-induced retinal vascular permeability by peroxisome proliferator-activated receptor-beta/delta. Invest Ophthalmol Vis Sci. 2014; 55:8232–8240. [PubMed: 25406289]

5. Ye EA, Steinle JJ. miR-146a attenuates inflammatory pathways mediated by TLR4/NF-kappaB and TNFalpha to protect primary human retinal microvascular endothelial cells grown in high glucose. Mediators Inflamm. 2016; 2016:3958453. [PubMed: 26997759]

6. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Res. 1996; 6:986–994. [PubMed: 8908518]

7. Bustin SA, Benes V, Garson JA, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009; 55:611–622. [PubMed: 19246619]

8. Halbert CL, Demers GW, Galloway DA. The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. J Virol. 1991; 65:473–478. [PubMed: 1845902]

9. Ashander LM, Appukuttan B, Ma Y, Gardner-Stephen D, Smith JR. Targeting endothelial adhesion molecule transcription for treatment of inflammatory disease: a proof-of-concept study. Mediators Inflamm. 2016; 2016:7945848. [PubMed: 27293321]

10. Bharadwaj AS, Schewitz-Bowers LP, Wei L, Lee RW, Smith JR. Intercellular adhesion molecule 1 mediates migration of Th1 and Th17 cells across human retinal vascular endothelium. Invest Ophthalmol Vis Sci. 2013; 54:6917–6925. [PubMed: 24022011]

11. Furtado JM, Bharadwaj AS, Chipps TJ, Pan Y, Ashander LM, Smith JR. Toxoplasma gondii tachyzoites cross retinal endothelium assisted by intercellular adhesion molecule-1 in vitro. Immunol Cell Biol. 2012; 90:912–915. [PubMed: 22525368]

12. Khan A, Behnke MS, Dunay IR, et al. Phenotypic and gene expression changes among clonal type I strains of Toxoplasma gondii. Eukaryot Cell. 2009; 8:1828–1836. [PubMed: 19801420]

13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods. 2001; 25:402–408. [PubMed: 11846609]

14. Hellemans J, Mortier G, De Paepe A, et al. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology. 2007; 8:R19. [PubMed: 17291332]

15. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome biology. 2002; 3 RESEARCH0034.

16. Bustin SA, Benes V, Garson J, et al. The need for transparency and good practices in the qPCR literature. Nat Methods. 2013; 10:1063–1067. [PubMed: 24173381]

17. Jacob F, Guertler R, Naim S, et al. Careful selection of reference genes is required for reliable performance of RT-qPCR in human normal and cancer cell lines. PLoS One. 2013; 8:e59180. [PubMed: 23554992]

18. Wei R, Stewart EA, Amoaku WM. Suitability of endogenous reference genes for gene expression studies with human intraocular endothelial cells. BMC Res Notes. 2013; 6:46. [PubMed: 23379847]

19. Xie J, Liu X, Li Y, Liu Y, Su G. Validation of RT-qPCR reference genes and determination of Robo4 expression levels in human retinal endothelial cells under hypoxia and/or hyperglycemia. Gene. 2016; 585(1):135–142. [PubMed: 27041242]

20. Bakhashab S, Lary S, Ahmed F, et al. Reference genes for expression studies in hypoxia and hyperglycemia models in human umbilical vein endothelial cells. G3. 2014; 4:2159–2165. [PubMed: 25193495]

21. Li T, Diao H, Zhao L, et al. Identification of suitable reference genes for real-time quantitative PCR analysis of hydrogen peroxide-treated human umbilical vein endothelial cells. BMC Mol Biol. 2017; 18:10. [PubMed: 28381210]

22. Bennani-Baiti B, Toegel S, Viernstein H, et al. Inflammation modulates RLIP76/RALBP1 electrophile-glutathione conjugate transporter and housekeeping genes in human blood-brain barrier endothelial cells. PLoS One. 2015; 10:e0139101. [PubMed: 26406496]

23. Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods. 2000; 46:69–81. [PubMed: 11086195]

24. Greer S, Honeywell R, Geletu M, et al. Housekeeping genes; expression levels may change with density of cultured cells. J Immunol Methods. 2010; 355:76–79. [PubMed: 20171969]

25. Chen G, Zhao L, Feng J, et al. Validation of reliable reference genes for real-time PCR in human umbilical vein endothelial cells on substrates with different stiffness. PLoS One. 2013; 8:e67360. [PubMed: 23840676]

26. Zamora DO, Riviere M, Choi D, et al. Proteomic profiling of human retinal and choroidal endothelial cells reveals molecular heterogeneity related to tissue of origin. Mol Vis. 2007; 13:2058–2065. [PubMed: 18079679]

27. Pan Y, Appukuttan B, Mohs K, Ashander LM, Smith JR. Ubiquitin carboxyl-terminal esterase L1 promotes proliferation of human choroidal and retinal endothelial cells. Asia Pac J Ophthalmol. 2015; 4:51–55.

Highlights

- We investigated reference gene expression in human retinal endothelial cells

- Gene expression varied substantially across stimulations and between cell lines

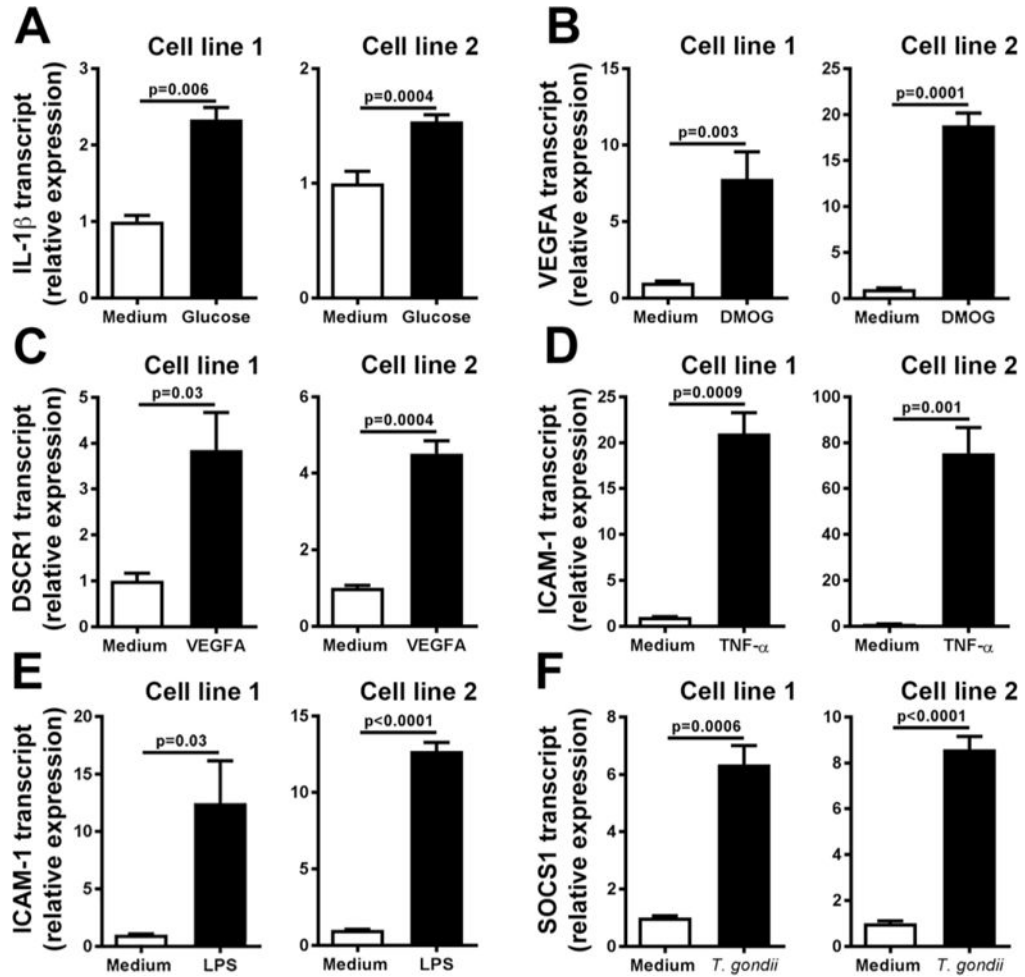- Normalization in RT-qPCR studies of gene expression should follow MIQE guidelines

Figure 1.
Expression of target molecules by human retinal endothelial cell lines following stimulation with: (A) glucose at supraphysiological concentration; (B) dimethyloxalylglycine (DMOG); (C) vascular endothelial growth factor (VEGFA); (D) tumor necrosis factor (TNF)-α; (E) lipopolysaccharide (LPS); and (F) *Toxoplasma gondii* tachyzoites, or medium alone. Three stable reference gene transcripts were applied for normalization of each result: ALAS1, SDHA and UBC (glucose); B2M, RPLP0 and YWHAZ (DMOG, VEGFA and *T. gondii* tachyzoites); ACTB, PPIA and RPLP0 (TNF-α); and ACTB, GAPDH and YWHAZ (LPS). Bars represent mean relative expression, with error bars showing standard error of the mean. n = 3 cultures/condition. Data were analyzed by two-tailed Student's t-test. IL-1β = interleukin 1β; DSCR1 = Down syndrome critical region gene 1 (DSCR1); ICAM-1 = intercellular adhesion molecule 1; SOCS1 = suppressor of cytokine signaling 1.

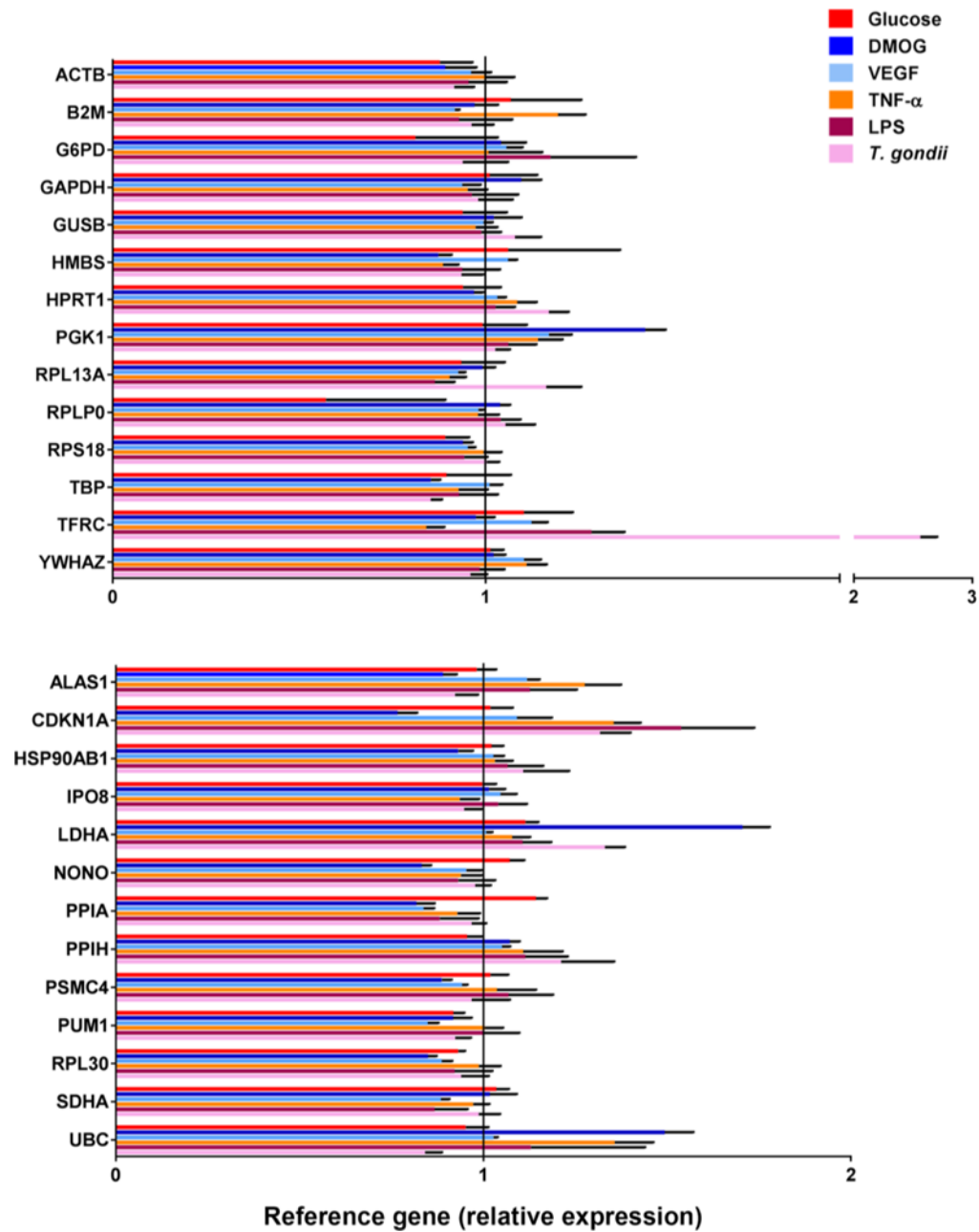**Figure 2.**
Expression of 27 reference genes by human retinal endothelial cell line 1 following treatments with: glucose at supraphysiological concentration; dimethyloxalyl-glycine (DMOG); vascular endothelial growth factor (VEGFA); tumor necrosis factor (TNF)-α; lipopolysaccharide (LPS); and *Toxoplasma gondii* tachyzoites. Three stable reference gene transcripts were applied for normalization of each result: PSMC4, IPO8 and SDHA (glucose); RPL13A, YWHAZ and G6PD (DMOG); HSP90AB1, UBC and HPRT1 (VEGFA); HSP90AB1, PSMC4 and ACTB (TNF-α); GAPDH, GUSB and YWHAZ (LPS); and SDHA, ALAS1 and GUSB (*T. gondii* tachyzoites). Colored bars represent mean expression by stimulated cells, calculated relative to expression by cells exposed to medium

alone. Black error bars show standard error of the mean. n = 3 cultures/condition. Full names of reference genes with abbreviations appear in Supplementary Table 2.
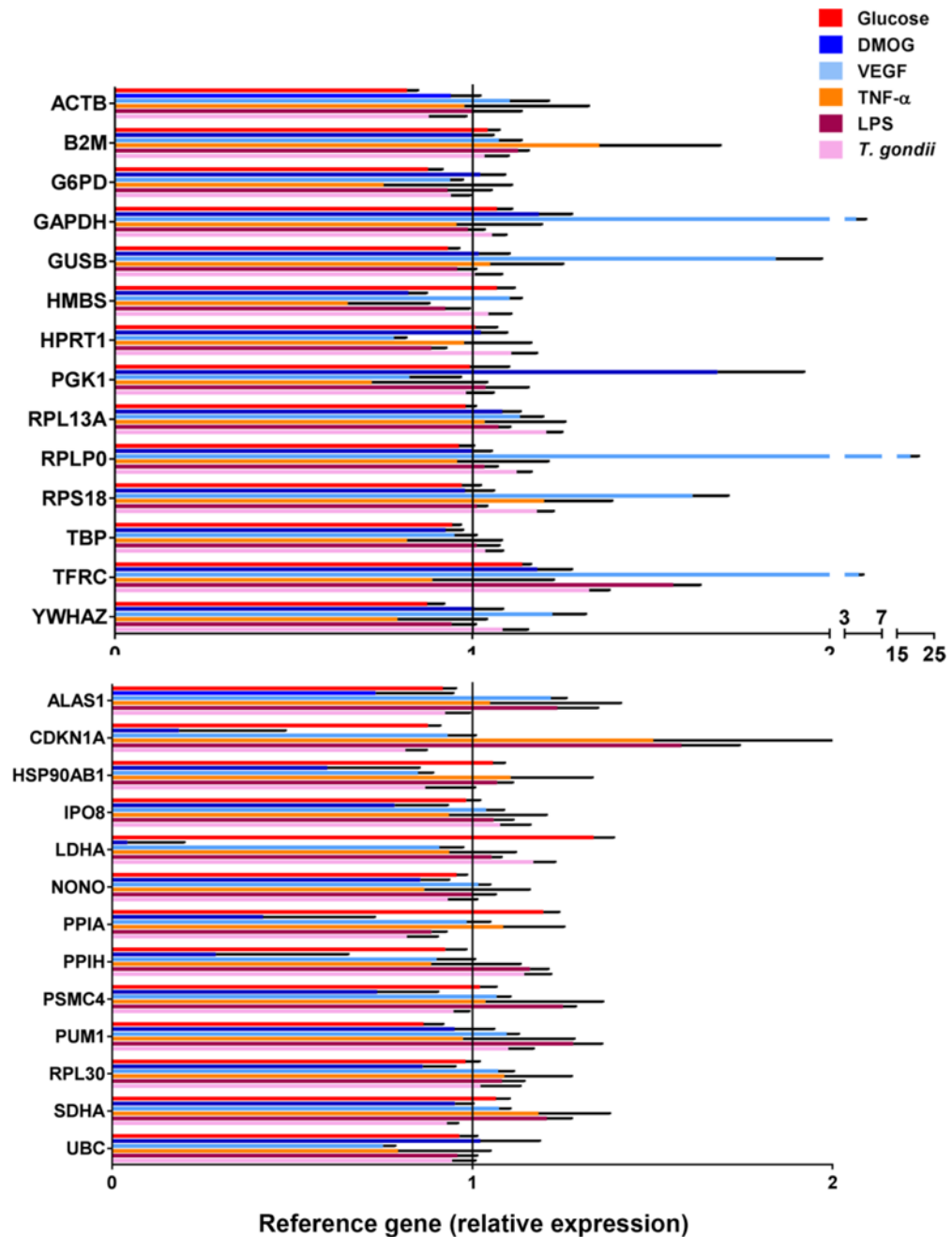
Figure 3.

Expression of 27 reference genes by human retinal endothelial cell line 2 following treatments with: glucose at supraphysiological concentration; dimethyloxalyl-glycine (DMOG); vascular endothelial growth factor (VEGFA); tumor necrosis factor (TNF)-α; lipopolysaccharide (LPS); and *Toxoplasma gondii* tachyzoites. Three stable reference gene transcripts were applied for normalization of each result: PSMC4, IPO8 and SDHA (glucose); RPL13A, YWHAZ and G6PD (DMOG); HSP90AB1, UBC and HPRT1 (VEGFA); HSP90AB1, PSMC4 and ACTB (TNF-α); GAPDH, GUSB and YWHAZ (LPS); and SDHA, ALAS1 and GUSB (*T. gondii* tachyzoites). Colored bars represent mean

expression by stimulated cells, calculated relative to expression by cells exposed to medium alone. Black error bars show standard error of the mean. n = 3 cultures/condition. Full names of reference genes with abbreviations appear in Supplementary Table 2.
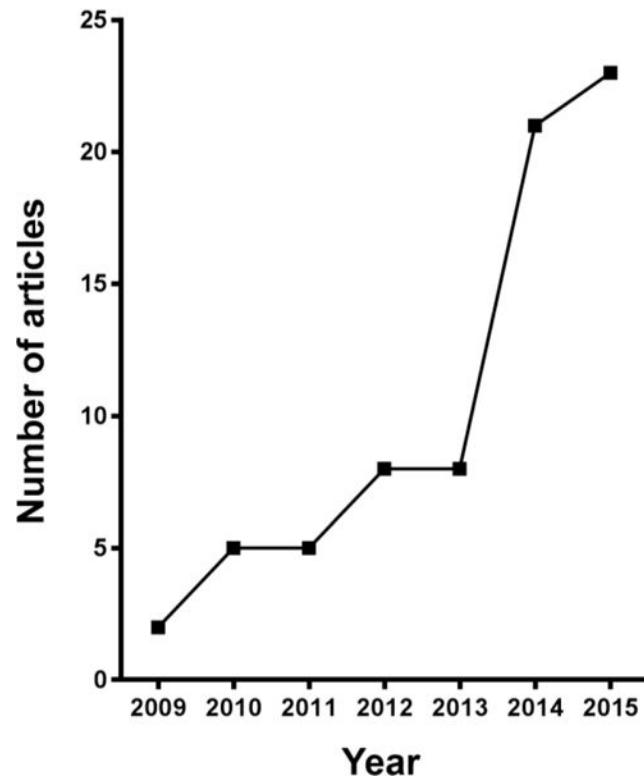
Figure 4.
Number of articles published annually between 2009 and 2015, inclusive, which have reported studies of human retinal endothelial cells using RT-qPCR, as listed in the National Library of Medicine of National Institutes of Health PubMed database (www.ncbi.nlm.nih.gov/pubmed; search date = 28 February, 2017; search text = human retinal endothelial cell; languages filter = English).

Table 1

Reference genes expressed by human retinal endothelial cells following treatment with glucose at supraphysiological concentration. Genes are ordered by coefficient of variation (CV) and gene-stability measure (M) (lowest to highest, indicating decreasing stability), calculated by comparison with control cells treated with medium alone (n = 3 samples/condition). Red indicates reference genes that were stably expressed according to CV-value below 0.1 or M-value below 0.2. Yellow highlight indicates reference genes that were stably expressed according to CV-value below 0.1 and M-value below 0.2, for both cell lines. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Coefficient of variation (CV)				Gene-stability measure (M)			
Cell line 1		Cell line 2		Cell line 1		Cell line 2	
Reference gene	CV	Reference gene	CV	Reference gene	M	Reference gene	M
PSMC4	0.0004	PSMC4	0.0016	RPL13A	0.075	PSMC4	0.0555
RPS18	0.0022	SDHA	0.0052	RPS18	0.075	SDHA	0.0563
RPL13A	0.0055	CDKN1A	0.0091	RPLP0	0.0766	IPO8	0.0573
RPLP0	0.0095	HSP90AB1	0.011	PGK1	0.0769	ALAS1	0.0574
RPL30	0.013	IPO8	0.0171	HPRT1	0.0818	CDKN1A	0.0577
PGK1	0.0143	ALAS1	0.0174	TBP	0.082	HSP90AB1	0.0588
TBP	0.0217	PPIH	0.0365	GUSB	0.088	PPIH	0.069
UBC	0.025	UBC	0.0393	UBC	0.0909	UBC	0.0713
HPRT1	0.0252	NONO	0.0444	IPO8	0.0912	NONO	0.087
IPO8	0.0272	G6PD	0.0501	RPL30	0.0924	RPL30	0.088
SDHA	0.0299	RPL30	0.0547	NONO	0.0925	PUM1	0.1013
GUSB	0.0307	PUM1	0.0648	PSMC4	0.0969	LDHA	0.117
NONO	0.0307	LDHA	0.0721	B2M	0.0976	PPIA	0.1401
HSP90AB1	0.0399	PPIA	0.0895	PPIH	0.1067	RPL13A	0.3229
B2M	0.0489	ACTB	0.1052	ALAS1	0.1111	GUSB	0.3229
PPIH	0.0543	RPS18	0.1167	HMBS	0.1127	HPRT1	0.323
ALAS1	0.0595	TBP	0.119	GAPDH	0.1129	TBP	0.3297
HMBS	0.0659	RPL13A	0.1495	SDHA	0.1151	RPS18	0.3307
GAPDH	0.0661	GUSB	0.1525	HSP90AB1	0.1235	ACTB	0.3384
G6PD	0.0741	HPRT1	0.1529	G6PD	0.1266	PGK1	0.3401
YWHAZ	0.0761	PGK1	0.191	YWHAZ	0.1288	GAPDH	0.3491
CDKN1A	0.0917	RPLP0	0.1961	CDKN1A	0.146	HMBS	0.3789
PUM1	0.1018	GAPDH	0.2042	PUM1	0.1594	B2M	0.3843
TFRC	0.1116	HMBS	0.237	TFRC	0.1737	G6PD	0.3875
ACTB	0.123	B2M	0.2417	ACTB	0.1915	TFRC	0.4147
PPIA	0.1277	TFRC	0.2638	PPIA	0.2189	RPLP0	0.6622
LDHA	0.2047	YWHAZ	1.2083	LDHA	0.3222	YWHAZ	2.7952

Table 2

Reference genes expressed by human retinal endothelial cells following treatment with dimethyloxalylglycine. Genes are ordered by coefficient of variation (CV) and gene-stability measure (M) (lowest to highest, indicating decreasing stability), calculated by comparison with control cells treated with medium alone (n = 3 samples/condition). Red indicates reference genes that were stably expressed according to CV-value below 0.1 or M-value below 0.2. Yellow highlight indicates reference genes that were stably expressed according to CV-value below 0.1 and M-value below 0.2, for both cell lines. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Coefficient of variation (CV)				Gene-stability measure (M)			
Cell line 1		Cell line 2		Cell line 1		Cell line 2	
Reference gene	CV	Reference gene	CV	Reference gene	M	Reference gene	M
YWHAZ	0.0049	HPRT1	0.0064	RPL13A	0.0838	G6PD	0.1106
PPIH	0.0144	RPL13A	0.0084	TFRC	0.0838	RPS18	0.1106
RPL13A	0.016	GUSB	0.0106	B2M	0.0843	YWHAZ	0.1121
TFRC	0.016	HSP90AB1	0.0307	HPRT1	0.085	RPLP0	0.1145
GUSB	0.0183	G6PD	0.0327	YWHAZ	0.0884	GUSB	0.1155
B2M	0.0185	RPS18	0.0379	GUSB	0.0944	HPRT1	0.1174
G6PD	0.0187	YWHAZ	0.0449	G6PD	0.0946	B2M	0.1194
HPRT1	0.02	RPLP0	0.0501	RPS18	0.0995	RPL13A	0.1272
SDHA	0.0225	B2M	0.0576	RPLP0	0.1044	ACTB	0.1277
IPO8	0.0239	ACTB	0.0668	ACTB	0.1321	TBP	0.1401
RPLP0	0.0297	TBP	0.078	GAPDH	0.1459	TFRC	0.2042
RPS18	0.0418	TFRC	0.095	HMBS	0.1481	GAPDH	0.2072
GAPDH	0.0671	GAPDH	0.0977	TBP	0.1706	HMBS	0.2529
ACTB	0.0784	HMBS	0.1621	PUM1	0.2325	PGK1	0.5385
HSP90AB1	0.0845	ALAS1	0.1732	HSP90AB1	0.2325	PSMC4	0.6563
HMBS	0.0928	PSMC4	0.1769	ALAS1	0.2372	IPO8	0.6563
PUM1	0.0956	PPIA	0.2178	PSMC4	0.2382	ALAS1	0.6572
TBP	0.1096	IPO8	0.2215	IPO8	0.2459	NONO	0.6701
ALAS1	0.1169	NONO	0.2817	SDHA	0.2466	RPL30	0.6724
PSMC4	0.1192	RPL30	0.2866	RPL30	0.2588	PUM1	0.718
RPL30	0.1497	PGK1	0.3398	PPIH	0.2712	SDHA	0.7189
NONO	0.1639	PUM1	0.3516	NONO	0.2715	HSP90AB1	0.7208
PPIA	0.1763	SDHA	0.3525	PPIA	0.2855	UBC	0.7749
CDKN1A	0.2211	UBC	0.3994	CDKN1A	0.3463	PPIA	0.8874
UBC	0.2463	PPIH	0.4672	PGK1	0.3955	PPIH	1.1235
PGK1	0.2519	CDKN1A	0.7222	UBC	0.4793	CDKN1A	1.4694
LDHA	0.336	LDHA	1.2184	LDHA	0.5833	LDHA	2.8549

Table 3

Reference genes expressed by human retinal endothelial cells following treatment with vascular endothelial growth factor. Genes are ordered by coefficient of variation (CV) and gene-stability measure (M) (lowest to highest, indicating decreasing stability), calculated by comparison with control cells treated with medium alone (n = 3 samples/condition). Red indicates reference genes that were stably expressed according to CV-value below 0.1 or M-value below 0.2. Yellow highlight indicates reference genes that were stably expressed according to CV-value below 0.1 and M-value below 0.2, for both cell lines. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Coefficient of variation (CV)				Gene-stability measure (M)			
Cell line 1		Cell line 2		Cell line 1		Cell line 2	
Reference gene	CV	Reference gene	CV	Reference gene	M	Reference gene	M
TBP	0.0004	PUM1	0.0048	GUSB	0.0657	PSMC4	0.1376
HSP90AB1	0.0049	SDHA	0.0092	HPRT1	0.0657	IPO8	0.1376
UBC	0.0053	RPL30	0.0105	TBP	0.067	HSP90AB1	0.1381
HPRT1	0.0061	HSP90AB1	0.0112	RPLP0	0.0679	RPL30	0.1384
LDHA	0.0115	PSMC4	0.0135	ACTB	0.0743	SDHA	0.1392
GUSB	0.0117	GUSB	0.0198	RPS18	0.0788	NONO	0.1409
RPLP0	0.0216	RPL13A	0.0203	G6PD	0.0807	PPIA	0.1511
NONO	0.0275	HPRT1	0.0263	HMBS	0.0826	PUM1	0.1517
G6PD	0.0306	IPO8	0.0331	GAPDH	0.0885	UBC	0.1684
HMBS	0.0334	YWHAZ	0.0433	RPL13A	0.0974	YWHAZ	0.1735
ACTB	0.036	RPS18	0.0453	HSP90AB1	0.0997	HPRT1	0.1735
PSMC4	0.0364	NONO	0.048	UBC	0.0997	B2M	0.1769
IPO8	0.0382	B2M	0.0588	LDHA	0.1011	CDKN1A	0.1798
PPIH	0.041	RPLP0	0.0696	B2M	0.1067	RPLP0	0.1817
RPS18	0.0427	PPIA	0.0709	NONO	0.1069	GUSB	0.1837
GAPDH	0.0536	ALAS1	0.0798	YWHAZ	0.1075	RPL13A	0.184
YWHAZ	0.0614	ACTB	0.0915	PSMC4	0.1109	ACTB	0.1963
RPL13A	0.0616	UBC	0.0968	IPO8	0.113	LDHA	0.1993
CDKN1A	0.0676	CDKN1A	0.1096	PPIH	0.1148	RPS18	0.2006
B2M	0.0686	GAPDH	0.1099	TFRC	0.1214	PPIH	0.2074
TFRC	0.0739	HMBS	0.1185	CDKN1A	0.1385	HMBS	0.2205
RPL30	0.0792	LDHA	0.1271	RPL30	0.1394	ALAS1	0.2351
SDHA	0.081	PPIH	0.1331	SDHA	0.141	GAPDH	0.2582
ALAS1	0.0852	G6PD	0.1619	ALAS1	0.1581	G6PD	0.2691
PGK1	0.1029	TFRC	0.1942	PGK1	0.1602	TFRC	0.3528
PUM1	0.11	PGK1	0.2346	PUM1	0.1734	PGK1	0.3679
PPIA	0.1194	TBP	0.4053	PPIA	0.186	TBP	0.6478

Table 4

Reference genes expressed by human retinal endothelial cells following treatment with tumor necrosis factor-alpha. Genes are ordered by coefficient of variation (CV) and gene-stability measure (M) (lowest to highest, indicating decreasing stability), calculated by comparison with control cells treated with medium alone (n = 3 samples/condition). Red indicates reference genes that were stably expressed according to CV-value below 0.1 or M-value below 0.2. Yellow highlight indicates reference genes that were stably expressed according to CV-value below 0.1 and M-value below 0.2, for both cell lines. Full names of reference genes with abbreviations appear in Supplementary Table 2.

| Coefficient of variation (CV) | | | | Gene-stability measure (M) | | | |
| Cell line 1 | | Cell line 2 | | Cell line 1 | | Cell line 2 | |
Reference gene	CV	Reference gene	CV	Reference gene	M	Reference gene	M
RPS18	0.0009	ALAS1	0.0126	ACTB	0.0821	ALAS1	0.1358
PUM1	0.0016	ACTB	0.0179	RPLP0	0.0821	PSMC4	0.1358
HSP90AB1	0.0057	PSMC4	0.0228	GUSB	0.0826	HSP90AB1	0.1382
PSMC4	0.0092	TFRC	0.0232	RPS18	0.0845	PUM1	0.1395
RPLP0	0.0097	HSP90AB1	0.0234	G6PD	0.0886	PPIA	0.1443
RPL30	0.0098	GAPDH	0.0286	GAPDH	0.0895	RPL30	0.1455
G6PD	0.0103	RPLP0	0.0302	TBP	0.1019	LDHA	0.152
ACTB	0.012	PPIA	0.0371	RPL13A	0.1184	IPO8	0.1524
GUSB	0.0141	RPL30	0.0389	HPRT1	0.1233	ACTB	0.1662
SDHA	0.0208	PUM1	0.0396	HMBS	0.1343	GAPDH	0.1662
GAPDH	0.0297	HPRT1	0.044	YWHAZ	0.1376	RPLP0	0.1666
NONO	0.0455	LDHA	0.0677	PGK1	0.159	HPRT1	0.1727
IPO8	0.0478	IPO8	0.0683	PUM1	0.1641	TFRC	0.1753
TBP	0.0482	TBP	0.0818	HSP90AB1	0.1641	PPIH	0.1864
LDHA	0.0523	RPL13A	0.0847	PSMC4	0.1648	SDHA	0.1981
PPIA	0.0529	GUSB	0.0951	RPL30	0.1666	RPL13A	0.1999
HPRT1	0.0622	SDHA	0.098	SDHA	0.1715	TBP	0.2014
RPL13A	0.0668	YWHAZ	0.1053	TFRC	0.1831	NONO	0.2035
PPIH	0.0716	PPIH	0.1065	LDHA	0.1839	GUSB	0.2091
YWHAZ	0.0783	NONO	0.1218	NONO	0.1879	YWHAZ	0.2171
HMBS	0.0811	G6PD	0.1406	IPO8	0.19	G6PD	0.2486
PGK1	0.0975	PGK1	0.1721	PPIA	0.1956	PGK1	0.2841
TFRC	0.1176	UBC	0.1833	PPIH	0.1969	UBC	0.2864
B2M	0.1298	RPS18	0.1891	B2M	0.2022	RPS18	0.3146
ALAS1	0.1705	HMBS	0.2398	ALAS1	0.2853	HMBS	0.3762
CDKN1A	0.2121	CDKN1A	0.2637	CDKN1A	0.3324	CDKN1A	0.4145
UBC	0.2136	B2M	0.2724	UBC	0.3343	B2M	0.4285

Table 5

Reference genes expressed by human retinal endothelial cells following treatment with *Escherichia coli* 055:B5 lipopolysaccharide. Genes are ordered by coefficient of variation (CV) and gene-stability measure (M) (lowest to highest, indicating decreasing stability), calculated by comparison with control cells treated with medium alone (n = 3 samples/condition). Red indicates reference genes that were stably expressed according to CV-value below 0.1 or M-value below 0.2. Yellow highlight indicates reference genes that were stably expressed according to CV-value below 0.1 and M-value below 0.2, for both cell lines. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Coefficient of variation (CV)				Gene-stability measure (M)			
Cell line 1		Cell line 2		Cell line 1		Cell line 2	
Reference gene	CV	Reference gene	CV	Reference gene	M	Reference gene	M
YWHAZ	0.0002	RPLP0	0.0002	GAPDH	0.081	RPS18	0.0807
GUSB	0.0035	PGK1	0.002	YWHAZ	0.081	TBP	0.0807
PSMC4	0.0066	GAPDH	0.0051	GUSB	0.0817	ACTB	0.0823
HSP90AB1	0.0083	RPL30	0.0102	ACTB	0.0858	GAPDH	0.0827
GAPDH	0.0149	RPS18	0.0143	HPRT1	0.0868	GUSB	0.0845
HPRT1	0.0149	TBP	0.0146	RPS18	0.0896	RPLP0	0.0851
LDHA	0.0181	HSP90AB1	0.0191	HMBS	0.0924	PGK1	0.0863
PPIH	0.0229	ACTB	0.0216	RPLP0	0.0934	YWHAZ	0.0922
RPLP0	0.0249	IPO8	0.0248	B2M	0.098	RPL13A	0.1083
IPO8	0.0254	GUSB	0.0266	TBP	0.0982	G6PD	0.1241
ALAS1	0.0305	RPL13A	0.0268	PGK1	0.1052	HMBS	0.1295
UBC	0.0322	LDHA	0.0295	PSMC4	0.1332	HSP90AB1	0.1419
ACTB	0.0365	YWHAZ	0.0381	HSP90AB1	0.1332	RPL30	0.1419
PGK1	0.0381	PPIH	0.0392	IPO8	0.137	IPO8	0.1432
RPS18	0.0451	B2M	0.0617	LDHA	0.1387	LDHA	0.1452
HMBS	0.0494	NONO	0.0645	PPIH	0.1408	B2M	0.1471
PUM1	0.0544	SDHA	0.0664	ALAS1	0.1458	PPIH	0.1529
B2M	0.0556	G6PD	0.074	UBC	0.1474	SDHA	0.165
TBP	0.0558	HMBS	0.0789	PUM1	0.1499	NONO	0.1686
NONO	0.1034	ALAS1	0.0839	RPL13A	0.1668	HPRT1	0.1703
RPL13A	0.1071	PSMC4	0.0933	NONO	0.1826	ALAS1	0.1766
G6PD	0.1096	UBC	0.096	G6PD	0.1847	PSMC4	0.185
RPL30	0.111	PUM1	0.1084	RPL30	0.1894	UBC	0.1967
PPIA	0.1423	HPRT1	0.1094	PPIA	0.2244	PUM1	0.2018
SDHA	0.1536	PPIA	0.1503	SDHA	0.2397	PPIA	0.2574
TFRC	0.1724	CDKN1A	0.255	TFRC	0.2692	CDKN1A	0.4005
CDKN1A	0.2485	TFRC	0.2881	CDKN1A	0.3903	TFRC	0.454

Table 6

Reference genes expressed by human retinal endothelial cells following treatment with GT-1 strain *Toxoplasma gondii* tachyzoites. Genes are ordered by coefficient of variation (CV) and gene-stability measure (M) (lowest to highest, indicating decreasing stability), calculated by comparison with control cells treated with medium alone (n = 3 samples/condition). Red indicates reference genes that were stably expressed according to CV-value below 0.1 or M-value below 0.2. Yellow highlight indicates reference genes that were stably expressed according to CV-value below 0.1 and M-value below 0.2, for both cell lines. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Coefficient of variation (CV)				Gene-stability measure (M)			
Cell line 1		Cell line 2		Cell line 1		Cell line 2	
Reference gene	CV	Reference gene	CV	Reference gene	M	Reference gene	M
GUSB	0.002	ALAS1	0.0051	PSMC4	0.1263	GAPDH	0.0813
RPLP0	0.0096	SDHA	0.009	NONO	0.1263	GUSB	0.0813
PGK1	0.0283	GUSB	0.0092	PPIA	0.1263	HMBS	0.0829
HSP90AB1	0.0403	YWHAZ	0.0094	SDHA	0.1267	YWHAZ	0.0854
RPS18	0.0443	GAPDH	0.01	ALAS1	0.1313	TBP	0.0855
SDHA	0.0465	PSMC4	0.0119	IPO8	0.1339	B2M	0.0864
NONO	0.0484	UBC	0.0144	RPL30	0.1393	HPRT1	0.0924
PSMC4	0.0555	HMBS	0.0171	RPS18	0.147	RPLP0	0.0987
PPIA	0.0555	TBP	0.023	GAPDH	0.147	ALAS1	0.1079
RPL13A	0.0598	NONO	0.0234	B2M	0.1499	PSMC4	0.1079
GAPDH	0.0605	B2M	0.0243	PGK1	0.1506	UBC	0.1084
HPRT1	0.0643	HPRT1	0.0252	YWHAZ	0.1509	SDHA	0.1087
ALAS1	0.0667	RPLP0	0.0345	PUM1	0.153	NONO	0.1124
IPO8	0.0706	RPL30	0.042	RPLP0	0.1589	PGK1	0.1189
B2M	0.0735	PGK1	0.0609	G6PD	0.161	RPL30	0.1234
YWHAZ	0.0757	RPS18	0.0694	HMBS	0.1635	RPS18	0.1297
RPL30	0.0767	HSP90AB1	0.0729	HSP90AB1	0.1653	HSP90AB1	0.1454
PUM1	0.089	IPO8	0.0784	GUSB	0.1666	RPL13A	0.1468
G6PD	0.0909	RPL13A	0.0848	ACTB	0.18	IPO8	0.1476
HMBS	0.0937	G6PD	0.0912	PPIH	0.2069	G6PD	0.1526
PPIH	0.1027	PUM1	0.0933	RPL13A	0.2179	PUM1	0.1609
ACTB	0.1084	PPIA	0.1155	HPRT1	0.223	PPIA	0.1834
UBC	0.1541	CDKN1A	0.1186	UBC	0.2404	CDKN1A	0.1869
TBP	0.1583	PPIH	0.1209	TBP	0.247	PPIH	0.1918
CDKN1A	0.1616	LDHA	0.1363	CDKN1A	0.2597	LDHA	0.2124
LDHA	0.1687	ACTB	0.1391	LDHA	0.2677	ACTB	0.2168
TFRC	0.5818	TFRC	0.1515	TFRC	0.9609	TFRC	0.2364

Table 7

Reference genes that demonstrated stable expression in human retinal endothelial cells following treatment with: glucose at supraphysiological concentration; dimethyloxalylglycine (DMOG); vascular endothelial growth factor (VEGFA); tumor necrosis factor (TNF)-α; lipopolysaccharide (LPS); and *Toxoplasma gondii* tachyzoites, as determined by gene-stability measure below 0.2 and coefficient of variation below 0.1, and ranked according to expression stability. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Rank	Reference gene					
	Glucose	DMOG	VEGF	TNF-α	LPS	T. gondii
1	PSMC4	RPL13A	HSP90AB1	HSP90AB1	GAPDH	SDHA
2	IPO8	YWHAZ, GUSB, HPRT1	UBC	PSMC4	GUSB	ALAS1
3	SDHA	G6PD	HPRT1	ACTB	YWHAZ, RPLP0	GUSB
4	UBC	B2M	GUSB	RPLP0	RPS18, ACTB	PSMC4, GAPDH
5	HSP90AB1	RPS18	PSMC4	PUM1	PGK1	NONO, RPLP0
6	ALAS1	RPLP0	RPLP0, NONO, IPO8	GAPDH	HSP90AB1	YWHAZ
7	RPL30	ACTB	RPL30	RPL30	TBP	B2M
8	NONO	–	RPL13A	PPIA	IPO8	PGK1
9	PPIH	–	SDHA	HPRT1	LDHA	HMBS
10	CDKN1A	–	YWHAZ	LDHA	HMBS	RPS18
11	–	–	ACTB	IPO8	PPIH	RPL30
12	–	–	B2M	SDHA	PSMC4	IPO8
13	–	–	–	RPL13A	B2M	HSP90AB1
14	–	–	–	–	ALAS1	PUM1
15	–	–	–	–	UBC	G6PD

Table 8

Reference genes demonstrating consistent expression stability in human retinal endothelial cells across treatments (ie. glucose at supraphysiological concentration; dimethyloxalyl-glycine (DMOG); vascular endothelial growth factor (VEGFA); tumor necrosis factor (TNF)-α; lipopolysaccharide (LPS); and *Toxoplasma gondii* tachyzoites), as determined by gene-stability measure below 0.2 and coefficient of variation below 0.1. Full names of reference genes with abbreviations appear in Supplementary Table 2.

Reference gene	Condition					
	Glucose	DMOG	VEGF	TNF-α	LPS	*T. gondii*
Stable in 5 conditions						
HSP90AB1	✓	✗	✓	✓	✓	✓
IPO8	✓	✗	✓	✓	✓	✓
PSMC4	✓	✗	✓	✓	✓	✓
RPLPO	✗	✓	✓	✓	✓	✓
Stable in 4 conditions						
ACTB	✗	✓	✓	✓	✓	✗
B2M	✗	✓	✓	✗	✓	✓
GUSB	✗	✓	✓	✗	✓	✓
RPL30	✓	✗	✓	✓	✗	✓
SDHA	✓	✗	✓	✓	✗	✓
YWHAZ	✗	✓	✓	✗	✓	✓
Stable in 3 conditions						
ALAS1	✓	✗	✗	✗	✓	✓
GAPDH	✗	✗	✗	✓	✓	✓
HPRT1	✗	✓	✓	✓	✗	✗
RPL13A	✗	✓	✓	✓	✗	✗
RPS18	✗	✓	✗	✗	✓	✓
UBC	✓	✗	✓	✗	✓	✗

Table 9

Use of reference genes in RT-qPCR studies of human retinal endothelial cells in 72 articles published between 2009 and 2015 on the National Library of Medicine of National Institutes of Health PubMed database (www.ncbi.nlm.nih.gov/pubmed; search date = 28 February, 2017; search text = human retinal endothelial cell; languages filter = English). Some articles reported works that involved multiple treatments. Articles are listed in Supplementary Table 3.

Treatment	Number of articles	Reference genes Identity	Number			Stable	
			>1	1	NR	Yes	NR
Glucose/AGE	25	18S rRNA, ACTB, GAPDH, HPRT1, U6 snRNA	0	24	1	0	25
Hypoxia & hypoxic mimics	6	ACTB, GAPDH	0	5	1	0	6
VEGF	8	ACTB, GAPDH	0	8	0	0	8
Cytokines	12	18S rRNA, ACTB, GAPDH, TBP	0	12	0	0	12
Other proteins	8	18S rRNA, ACTB, GAPDH, HPRT1	0	6	0	0	6
Microbial mimics	3	GAPDH, HPRT1, PGK1, TBP	1	2	0	1	2
Enzymes	2	18S rRNA, HPRT1	0	2	0	0	2
Lipids	2	18S rRNA, GAPDH, PPIA	0	2	0	0	2
Transfection of nucleic acid	8	18S rRNA, ACTB, GAPDH	0	8	0	0	8
Drugs & chemicals	9	ACTB, GAPDH, PPIA	0	9	0	0	9
Shear stress	2	GAPDH	0	2	0	0	2
No treatment	3	ACTA, GAPDH, HPRT1	0	3	0	0	3

Abbreviations: AGE = advanced glycation end-products, VEGF = vascular endothelial growth factor, NR = not recorded. Full names of reference genes with abbreviations appear in Supplementary Table 2.