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Selection of reference genes for studies of human retinal endothelial cell gene expression by reverse transcription-quantitative real-time polymerase chain reaction

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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) < 0.2 and coefficient of variation (CV-value) < 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.

1. 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 (Bharadwaj et al., 2013a). 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

Abbreviations: 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

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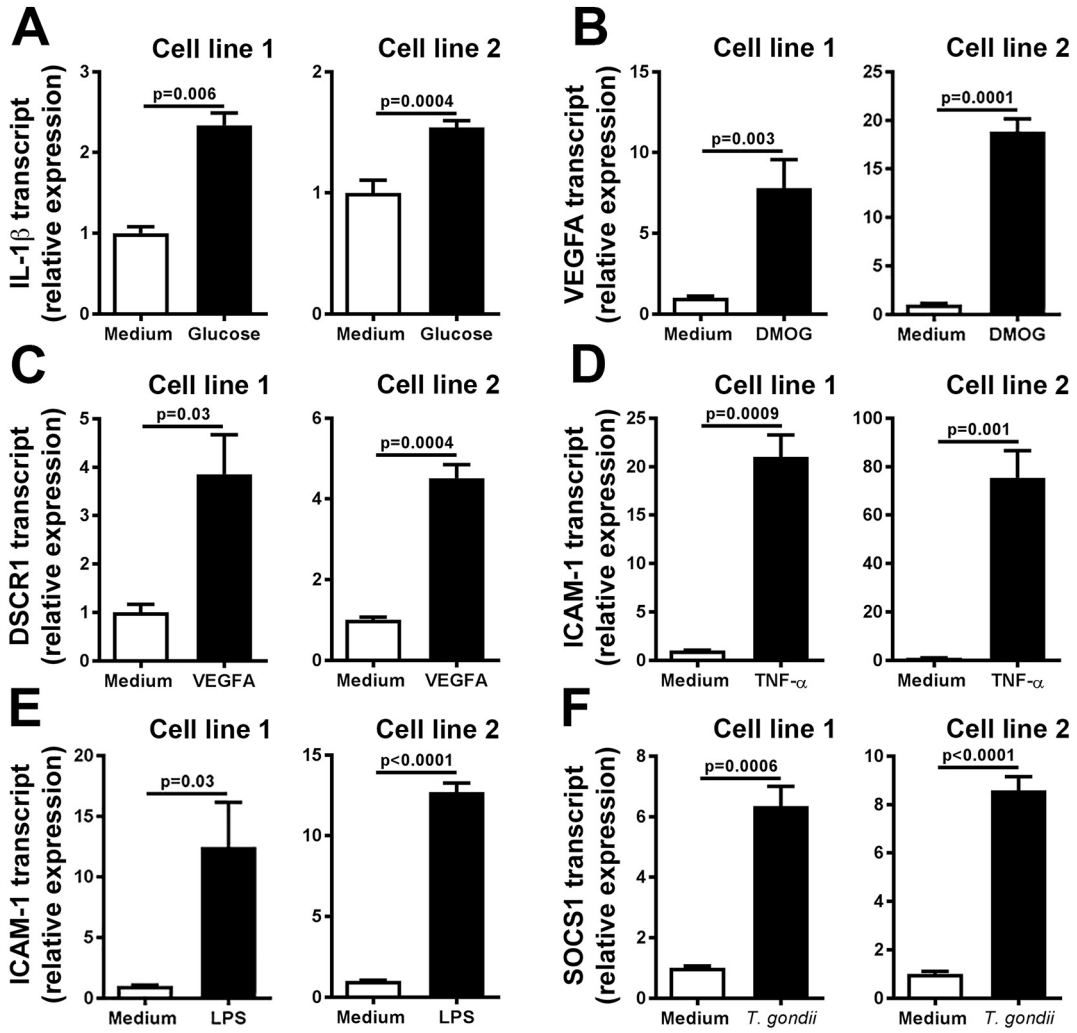

Fig. 1. Expression of target molecules by human retinal endothelial cell lines following stimulation with: (A) glucose at supraphysiological concentration; (B) dimethyloxalylglycine (DMOG); (C) vascular endothelial growth factor (VEGFA); (D) tumor necrosis factor (TNF)-α; (E) lipopolysaccharide (LPS); 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.

studies of basic pathogenic mechanisms. Independent research groups – including our own – isolate primary retinal endothelial cells from human cadaveric eyes (Smith et al., 2007; Browning et al., 2012) or purchase cells from commercial sources (Suarez et al., 2014; Ye & Steinle, 2016) 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 (Heid et al., 1996), 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 (Bustin et al., 2009). 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 (Bustin et al., 2009).

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

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