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Research paper

Identification and validation of reference genes for quantitative RT-PCR analysis of retinal pigment epithelium cells under hypoxia and/or hyperglycemia

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ABSTRACT

Retinal pigment epithelium (RPE) cell-based gene expression studies performed under hypoxia and/or hyperglycemia show huge potential for modeling cell responses in diabetic retinopathy, retinopathy of prematurity and other retinal diseases. However, normalization of gene expression on RPE cells under those conditions has commonly been done using either GAPDH or β-actin as reference genes without any validation of their expression stability. Therefore, we aimed to establish a suitable set of reference genes for studies on RPE cells cultured under both normal culturing glucose and atmospheric oxygen tension (normoxia, 21%), under a low oxygen tension (hypoxia, 1%), under a high glucose growth medium (25 mmol/l) and under the combination of the two changed conditions above for distinct time points taking together from 24 h to 7 days. Quantitative real-time PCR (qRT-PCR) was applied on RNA obtained from a cell line, ARPE-19. Stability of 14 commonly used reference genes was assessed and ranked according to their stability values using the geNorm and NormFinder softwares with the aim to find the most stable expressed gene under all conditions. Our findings confirm that HPRT1, GUSB and PPIA are the most suitable reference genes for RPE cell gene expression experiments subjected to hypoxia and/or hyperglycemia. To emphasize the importance of selecting the most stably expressed reference genes for obtaining reliable results, mRNA expression levels of hypoxia induced factor-1α were analyzed vs the best reference genes, the worst ones and the most commonly used ones. These reference genes gave the most reliable normalization for comparative analyses of gene transcription under those conditions.

1. Introduction

Retinopathy related to blood retinal barrier, not limited to diabetic retinopathy (DR), retinal detachment, retinal vascular occlusion and retinopathy of prematurity, may lead to irreversible damage to the precise photosensitive elements of retina and is a major cause of low vision even blindness in different groups of people. Multiple pathogenic changes have been identified to explain the mechanisms of these diseases' progression. Among them, hypoxia with/without hyperglycemia is considered to lead to and participate in the pathogenesis of these diseases. The environmental stress and its consequent responses in retina may cause damages on retinal vascular cells and retinal pigment epithelium (RPE) cells. In turn, the cells change their functions and secretion patterns to make adaption (Wangsa-Wirawan and Linsenmeier, 2003; Strauss, 2005; Simo and Hernandez, 2015).

RPE, a monolayer of highly specialized cells located between retinal photoreceptors and choroid, plays a central role in retinal homeostasis by forming outer blood retinal barrier and supporting function of photoreceptors and could be activated by the milieu changes (Strauss, 2005). In response to hypoxia, expression of hypoxia induced factor-1α (HIF-1α) in RPE cells is upregulated, which leads to increased secretion of vascular endothelial growth factor (VEGF) and other growth factors and cytokines that are relating to cell proliferation and migration, blood retinal barrier damage and neovascularization, and eventually visual impairment (Witmer et al., 2003; Sall et al., 2004). To investigate the molecular events and assess the effect of new potential therapeutic agents on involved retina, in vitro RPE cells cultured under relevant conditions and time intervals that can reflect the clinical situations are crucial.

For determining the alteration of gene expression under different culturing conditions in RPE cells, quantitative real-time PCR (qRT-PCR)

Abbreviation: DR, diabetic retinopathy; RPE, retinal pigment epithelium; HIF-1α, hypoxia induced factor-1α; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Rn18s, 18S ribosomal RNA; ACTB, actin, beta; ALAS1, 5′-aminolevulinate synthase 1; B2M, beta-2-microglobulin; GUSB, glucuronidase, beta; HPRT1, hypoxanthine phosphoribosyl transferase 1; HMBS, hydroxymethylbilane synthase; PPIA, peptidyl prolylisomerase A; PUM1, pumilio RNA binding family member 1; RPL29, ribosomal protein L29; RPLP0, ribosomal protein, large, P0; TBP, TATA box binding protein; TFRC, transferrin receptor; RIN, RNA integrity number.

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is often a good method, due to its specific, sensitive and rapid characters. However, accurate and reliable gene expression detection with this method is mainly depended on reference genes by which the genes of interest are to be normalized. Reference genes, also known as housekeeping genes, should express steadily in tissues or cells irrespective of the experimental conditions and their responses to any experimental conditions will lead to erroneous results (Dheda et al., 2005; Bakhashab et al., 2014). Traditionally, reference genes are chosen from genes involved in metabolism such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or structural genes such as beta-actin (ACTB). However, under hypoxia and/or hyperglycemia, metabolic pathways are always changed and cytoskeleton genes might be modulated when the cells are proliferating or disrupted, resulting in uselessness of these genes for normalization purposes (Fink et al., 2008). Currently, most of the gene expression studies concerning RPE cells preconditioned by hypoxia and/or hyperglycemia, use either GAPDH or ACTB without assessing the stability of reference genes selected for quantification. Therefore, in the present study, the most suitable and reliable reference genes for RNA expression analysis in ARPE-19 cells (a cell line of human RPE cells) preconditioned under normoxia, hypoxia and/or hyperglycemia were identified from 14 commonly used housekeeping genes. With the information from the above analyses, the hypoxia and/or hyperglycemia modulation of mRNA expression of HIF-1α was also investigated.

2. Materials and methods

2.1. Cell culture

The human retinal pigment epithelial cell line (ARPE-19) was obtained from the American Type Culture Collection (ATCC, USA) and cultured following the instructions from the ATCC. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Hyclone, China) containing 10% fetal bovine serum (Gibco, America) and the medium was renewed every other day. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. The cells were passaged by trypsinization every 3–4 days.

2.2. Hypoxia and/or hyperglycemia studies

The ARPE-19 cells were cultured in a sealed, anaerobic workstation (Ruskin Technologies, UK), where the hypoxic condition (1% O_2, 94% N_2 and 5% CO_2), temperature (37 °C), and humidity (90%) were kept constant (Tian et al., 2015). As the hypoxic situation in retina is usually a relatively long course compared with acute myocardial infarction in which situation cell death happens in 20 min to 4 h, three longer time courses in hypoxia – 24 h, 48 h and 72 h – were chosen.

The cells were cultured either with a euglycemic glucose concentration of 5.5 mM and in normoxia as a control or with hyperglycemic glucose concentration of 25 mM for 3 days, 5 days and 7 days to mimic the long course and uncontrolled blood glucose in diabetic patients.

For the combined hyperglycemia and hypoxia study, the cells were cultured for 3 days under hyperglycemic condition and then in hypoxia for 24 h to mimic the increased blood glucose followed by tissue hypoxia in middle stage of diabetic retinopathy.

2.3. Total RNA isolation and cDNA synthesis

Total RNA from ARPE-19 cells in different conditions of distinct course was extracted using Animal Total RNA Isolation Kit (Beijing Dingguo, China) according to the manufacturer's instructions. RNA concentration and purity were determined by NanoDrop 2000c Spectrophotometer (Thermo Scientific, USA). Ribosomal RNA band integrity was evaluated by conventional 1% agarose gel electrophoresis and Agilent Bioanalyzer with RNA 6000 Nano Kit (Agilent, USA) (Imbeaud et al., 2005; Schroeder et al., 2006; Taylor et al., 2010; Bruge et al., 2011).

For cDNA synthesis, 300 ng total RNA from each sample was reverse transcribed into cDNA using a PrimeScript RT reagent kit Perfect Real Time (Takara Bio, China) in a 20 μl reaction volume, according to the manufacturer's instructions. All the cDNA samples were stored at −20 °C until qPCR analyses.

2.4. Quantification of mRNA

The PCR mixture contained 1 μl diluted cDNA, 10 μM gene-specific primer (forward and reverse mixed together) and 10 μl of 2× Fast SYBR Green Master Mix (Roche Diagnostics, Switzerland) in a total volume of 20 μl. Amplification was performed in 96-well optical reaction plates (Roche Diagnostics, Switzerland) on LightCycler 480 (Roche Diagnostics, Switzerland) using the following program: 94 °C for 3 min to activate polymerase, 40 cycles at 94 °C for 20 s, 57 °C for 20 s and 72 °C for 20 s; melting curve analysis was performed after every run by heating up to 95 °C to monitor presence of unspecific products. Two negative controls were included in each assay run, with water instead of template. Three replicates for each biological replicate were performed.

Primers were designed and checked with Primer Premier 5.0 (Premier, CA) and NCBI primer BLAST tool and synthesized by Sangon Company (Shanghai, China) except for HIF-1α, RPLP0 and TFRC, which were chosen from the literature (Bakhashab et al., 2014; Tian et al., 2015). Primer sequences are listed in Table 1.

2.5. Data analysis

To analyze the results, the geNorm (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) softwares were applied according to the developers' recommendations as published. These bioinformatics packages calculate stability values according to which the genes were ranked.

The mRNA expression of HIF-1α in hypoxia and/or hyperglycemia treated cells was calculated relative to the expression in control cells (in both normoxic and euglycemic situations), according to the delta–delta Ct method ($2^{-\Delta\Delta Ct}$) using the most and least stable reference genes found, as well as the most commonly used GAPDH and ACTB (Schmittgen and Livak, 2008). Meanwhile, relative quantification of the target gene expression normalized to more than one reference gene was determined. In this method, target gene expression was normalized to the geometric mean expression of the best combination of stably expressed reference genes analyzed by the software (Wang et al., 2010).

3. Results

3.1. RNA quality and primer efficiency

RNA was isolated from ARPE-19 cells subjected to different culture conditions and the purity was measured using a NanoDrop Spectrophotometer (NanoDrop Technologies, USA). The A260/280 ratio of RNA samples was 1.81 ± 0.05 on average. RNA integrity evaluated by conventional 1% agarose gel electrophoresis showed that 28S:18s was around 2 and by Agilent Bioanalyzer showed the RNA integrity number (RIN) of all the samples were around 9.5, which indicated a good quality of RNA. Application efficiency of all primers was from 1.91–2.10 with $R_2 > 0.93$, and one single peak was obtained in every amplification reaction in melting curve, confirming that all the primers worked specifically.

3.2. Expression profiles of candidate reference genes

qRT-PCR was performed and Ct (cycle threshold) values were obtained and compared to assess the variations in mRNA expression levels for each reference gene. The Ct is defined as the number of cycles that is