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Note

Validation of reference genes for qPCR studies on Caco-2 cell differentiation

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Abstract

Validation of reference gene expression stabilities is a prerequisite for reliable normalization of qPCR data. The present study assessed the variation of six reference genes (*ACTB*, *GAPDH*, *B2M*, *HPRT1*, *SDHA*, *YWHAZ*) in Caco-2 cells under the influence of different growth supports and cultivation periods. Genes were ranked according to their stability using the geNorm software. To verify the influence of reference gene selection, *ALPI* gene expression during differentiation was quantified using the most or the least stable reference gene for normalization. Experimental conditions significantly affected the expression levels of reference genes. Whereas *GAPDH* and *ACTB* were revealed as most stable genes, *SDHA* was the least stable one. The extent of *ALPI* gene expression was significantly changed by the selection of the reference gene. This study provides a basis for qPCR studies related to both the differentiation process of Caco-2 cells and the elucidation of cell behaviour influenced by surface modifications.

Keywords: Caco-2; Differentiation; Cell-surface interaction; qPCR; Normalization; Reference genes; geNorm

1. Introduction

Caco-2 colon adenocarcinoma cells represent a well established *in vitro* model for the human intestinal epithelium. In this regard, the Caco-2 cell line is used for biopharmaceutical purposes such as transport, toxicity, or proliferation studies and is also recognized by the US FDA as part of bioequivalence waiver processes. Upon confluency, they are known to differentiate to an intestinal phenotype indicated by morphological and functional parameters [1]. In recent studies, it was shown that extracellular matrix proteins as well as surface hydrophobicity strongly influenced proliferation and differentiation indicated by promotion or suppression of certain marker proteins [2,3]. These results suggested that cells might be programmed by growing on surfaces with specific characteristics. A future challenge in biomaterial sciences would be to tailor materials able to stimulate the development of a certain phenotype.

For the investigation of gene expression levels, real-time quantitative RT-PCR (qPCR) is becoming the method of choice due to its sensitivity, efficiency, and robustness [4]. However, accurate normalization of qPCR data, which is required to control for the experimental error introduced during the multistage process of isolating and processing RNA, represents a major challenge for this method [5]. To date, the most popular approach is normalizing to a stable internal control gene – often referred to as reference or housekeeping gene. Due to the potential regulation of those reference genes [6–8], careful validation of their expression stabilities during experimental conditions is required for the acquisition of biologically meaningful data [5,9].

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The aims of the present study were, first, to quantitatively compare mRNA levels of six commonly used reference genes including beta actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta-2-microglobulin (*B2M*), hypoxanthine phosphoribosyl-transferase I (*HPRT1*), succinate dehydrogenase complex, subunit A (*SDHA*), and tyrosine 3-monooxygenase/tryptophane 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) in Caco-2 cells under the influence of different matrices and second, to evaluate appropriate reference genes for qPCR studies dealing with Caco-2 cell differentiation. Further, alkaline phosphatase (*ALPI*) gene expression – known to be up-regulated during the course of Caco-2 cell differentiation [10] – was normalized using both the most and the least stable reference genes to demonstrate the importance of reference gene validation.

2. Materials and methods

2.1. Cell culture and sample collection

Caco-2 cells (ATCC, USA, passage numbers 50–66) were grown to confluency in 75 cm^2 tissue culture flasks (Costar, USA) using RPMI-1640 medium (Sigma, USA), containing 10% fetal calf serum (Biochrom AG, Germany), 4 mM L-glutamine, and 150 μg/ml gentamycin in a humidified 5% CO_2/95% air atmosphere at 37 °C. The experimental setup for the qPCR study included 4 different surfaces and three differentiation levels. 20,000 Caco-2 cells/cm^2 were seeded on 25 cm^2 polystyrene tissue flasks (Greiner bio-one, Austria), on Matrigel™ Basement Membrane Matrix (BD Biosciences, USA) coated tissue flasks (20 μg/cm^2), on microscopic glass slides (Assistent, Germany) purified by treatment with piranha-solution (70% H_2SO_4/30% H_2O_2), and on surface modified ETC01-slides (Easy-To-Clean) developed by INM. The ETC01-slides are characterized by hydrophobic as well as lipophobic surface structures as described previously [3]. Preconfluent cells were harvested on day 3, early-postconfluent cells on days 10, and late-postconfluent cells on day 21, respectively, using Tryple™ Select (Gibco, Austria). Each of these 12 study groups consisted of three identically treated biological replicates.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated using the NucleoSpin® RNA II Kit (Macherey-Nagel, Germany) and quality-controlled on agarose gels. The quantity of extracted RNA was determined using the Quant™-It RiboGreen® reagent (Molecular Probes, USA). Total RNA (2 μg) was transcribed using the StrataScript® First-Strand Synthesis System (Stratagene, USA).

2.3. Reference gene selection and primer optimization

Out of different functional classes, six reference genes (*ACTB*, *GAPDH*, *B2M*, *HPRT1*, *SDHA*, *YWHAZ*) (Metabion, Germany) were selected to reduce the possibility that these genes might be co-regulated (Table 1). Primers for *GAPDH* were designed using AlleleID 2.01 software (Premier Biosoft, USA). All other primer sequences were used as described by Vandesompele et al. [9]. Optimal primer concentrations for each reference gene were identified within a range of 50–900 nM.

2.4. Real-time qPCR

All qPCRs were performed in 25 μl reaction mixtures containing 1 μl cDNA (diluted 1:10), 12.5 μl Brilliant® SYBR® Green QPCR Master Mix (Stratagene, USA), primer pairs as listed in Table 1, and nuclease-free water to 25 μl. Each biological replicate was run in duplicate on a Mx3000P® QPCR system. Thermocycling conditions consisted of an initial polymerase activation step at 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, at 55 °C for 1 min, and at 72 °C for 1 min. The specificity of ampli-

Table 1
Reference genes and their primer sequences used for qPCR analyses

Symbol	Function	Primer sequence	nM	Efficiency	Amplicon length (bp)
ACTB	Cytoskeletal structural protein	fw: CTGGAACGGTGAAGGTGACA rv: AAGGGACTTCCTGTAACAATGCA	300 100	1.94 ± 0.01	140
GAPDH	Oxidoreductase in glycolysis and gluconeogenesis	fw: GGAGTCCACTGGCGTCTTCAC rv: GAGGCATTGCTGATGATCTTGAGG	300 600	1.95 ± 0.01	165
B2M	Beta-chain of major histocompatibility complex class I molecules	fw: TGCTGTCTCCATGTTTGATGTATCT rv: TCTCTGCTCCCCACCTCTAAGT	300 900	2.01 ± 0.04	86
HPRT1	Purine synthesis in salvage pathway	fw: TGACACTGGCAAAACAATGCA rv: GGTCCTTTTCACCAGCAAGCT	300 900	1.93 ± 0.03	94
SDHA	Electron transporter in the TCA cycle and respiratory chain	fw: TGGGAACAAGAGGGCATCTG rv: CCACCACTGCATCAAATTCATG	300 900	2.00 ± 0.02	86
YWHAZ	Signal transduction by binding to phosphorylate serine residues	fw: ACTTTTGGTACATTGTGGCTTCAA rv: CCGCCAGGACAAACCAGTAT	600 600	1.98 ± 0.01	94