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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

fication was verified by melting curve analysis (55–95 °C). The results were analysed using the MxPro™ real-time QPCR software. The crossing point of the amplification curve with the threshold represented the cycle threshold (C_t). PCR efficiencies (E) for each primer pair were derived from standard curves ($n = 3$). The reactions were run in duplicate and no-template controls were included.

2.5. Data presentation and calculations

Results were exported to Microsoft® Excel and GraphPad Prism® for further analyses. For each reference gene the distribution of the expression levels (C_t values) under the 12 experimental conditions was displayed as Box and Whiskers plots. Data within each experiment were normalized to the average level of mRNA expression (C_t value) of the respective control group using the relationship $E^{-\Delta C_t}$. Statistics were performed using one-way ANOVA with post hoc Dunnett tests comparing each study group with the control group ($p < 0.05$). To identify the most stable and the optimal number reference genes, the geNorm application software [9] was used. The ranking of the candidate reference genes is based on their M value, the mean pairwise variation for a gene compared with all other tested control genes. Genes with the lowest M values have the most stable expression and vice versa. The optimal number of reference genes is determined by calculating the pairwise variations V_n/V_{n+1} between two sequential normalization factors (NF_n and NF_{n+1}). A large variation means that the added gene has a significant effect and should preferably be included for calculation of reliable normalization factor. Values below 0.15 indicate that a further reference gene is not required for accurate normalization [9].

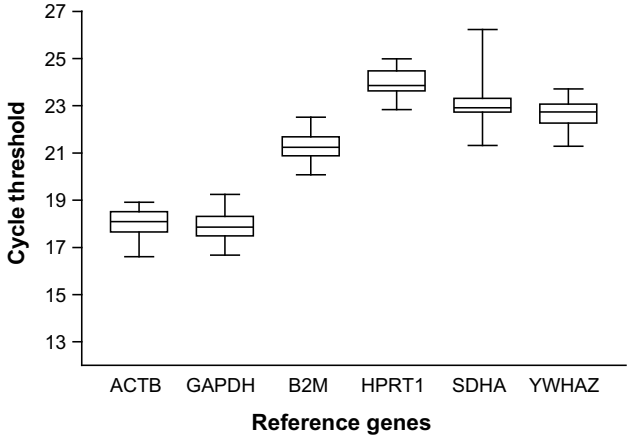

Fig. 1. Expression levels of candidate reference genes. qPCR cycle threshold values for six reference genes in Caco-2 cells grown on different matrices (polystyrene, Matrigel-coated polystyrene, glass, and ETC01-slides) for various culture periods (3, 10, and 21 days). Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers) for 12 different culture conditions ($n = 70$).

2.6. Validation of selected reference genes

To examine the importance and the influence of reference gene selection, the *ALPI* gene expression of Caco-2 cells grown on polystyrene and Matrigel-coated polystyrene for 3 and 21 days was quantified. Both the most and the least stable reference genes evaluated by the geNorm software (*SDHA*) were used for normalization of the *ALPI* mRNA levels. The samples grown on polystyrene for 3 days were defined as the calibrator sample. Fold changes were subjected to a Student's t-test ($p < 0.05$) to determine significant differences between study groups.

3. Results and discussion

For drug evaluation processes based on the Biopharmaceutics Classifications Systems (BCS) the characterization

Table 2
Relative gene expression levels of Caco-2 cells grown on different matrices

Experimental condition		mRNA relative to control group (polystyrene 3 days postseeding) [means ± SD]					
		ACTB	GAPDH	B2M	HPRT1	SDHA	YWHAZ
3 days postseeding polystyrene	Polystyrene	1.00	1.00	1.00	1.00	1.00	1.00
	coated	**1.61 ± 0.14**	**1.66 ± 0.12**	**1.75 ± 0.19**	**1.68 ± 0.11**	1.46 ± 0.06	**2.05±0.36***
	Glass	0.98 ± 0.22	1.03 ± 0.28	1.40 ± 0.29	1.16 ± 0.22	0.97 ± 0.42	1.09±0.22
	ETC01	**0.73 ± 0.04**	0.86 ± 0.14	1.01 ± 0.15	0.92 ± 0.10	0.93 ± 0.08	1.02±0.08
10 days postseeding	Polystyrene	**0.74 ± 0.22**	0.87 ± 0.03	**2.23 ± 0.52***	**1.38 ± 0.40**	0.87 ± 0.60	1.44 ± 0.55
	Coated polystyrene	**0.62 ± 0.13**	1.01 ± 0.19	**1.77 ± 0.34**	0.96 ± 0.22	1.15 ± 0.19	1.18 ± 0.40
	glass	0.81 ± 0.19	1.33 ± 0.41	**3.29 ± 0.91***	1.32 ± 0.40	**3.03 ± 0.61***	**1.78 ± 0.49**
	ETC01	**0.53 ± 0.07**	**0.63 ± 0.27**	**1.96 ± 0.36**	1.00 ± 0.13	0.56 ± 0.50	0.71 ± 0.19
21 days postseeding	Polystyrene	**0.54 ± 0.11**	**0.50 ± 0.05**	**2.36 ± 0.42***	**0.55 ± 0.09**	1.24 ± 0.16	0.82 ± 0.19
	Coated polystyrene	**0.50 ± 0.07**	**0.55 ± 0.09**	**2.31 ± 0.38***	**0.58 ± 0.09**	1.28 ± 0.21	0.88 ± 0.18
	Glass	**0.46 ± 0.04***	**0.62 ± 0.04**	**2.46 ± 0.24***	**0.58 ± 0.07**	1.31 ± 0.17	0.63 ± 0.04
	ETC01	**0.58 ± 0.11**	**0.65 ± 0.17**	**2.44 ± 0.30***	**0.66 ± 0.13**	**1.68 ± 0.41**	0.90 ± 0.14

mRNA levels of Caco-2 cells grown on different matrices 3, 10, and 21 days postseeding relative to the control group (polystyrene 3 days postseeding) (Means ± SD, $n = 6$; value of one represents average level of mRNA expression in control group).
Boldfaced values $p < 0.05$.
* $p < 0.05$ and change >2-fold.

and standardization of Caco-2 cells on the genetic and enzymatic level as well as a good correlation between laboratories are a prerequisite. Therefore, the availability of validated reference genes for differentiating Caco-2 cells can improve data interpretation in qPCR studies involving this cell line.

3.1. Transcription profiling of the reference genes

Fig. 1 gives an overview of the distribution of the C_t values from each evaluated gene (*ACTB*, *GAPDH*, *B2M*, *HPRT1*, *SDHA*, *YWHAZ*) under the different culture conditions (incubation time and underlying matrix). The expression levels ranged from 17.88 (*GAPDH*) to 23.95 (*HPRT1*). However, this approach does neither consider qPCR efficiencies nor dependencies to the experimental conditions [5].

Table 2 presents the expression relative to the control group (polystyrene for 3 days). A significant variability ($p < 0.05$) of most reference gene expression levels depending on both the cultivation period and the growth support could be demonstrated. After a growth period of 10 days a significant regulation of *ACTB* and *B2M* was detectable. After 21 days all reference genes were regulated ($p < 0.05$) – except for *SDHA* and *YWHAZ*. A significant up-regulation of all reference genes except for *SDHA* was also observable for Matrigel-coated polystyrene on day 3. A 2-fold change in expression level indicating biological meaningfulness [6] was exceeded in the case of *YWHAZ* on Matrigel-coated polystyrene on day 3, in the case of *B2M* after 10 days of incubation on polystyrene and glass, and after 21 days following seeding on all surfaces. Generally, Matrigel-coated polystyrene and ETC01 induced a stronger tendency towards modulation already from the beginning of culture. By contrast, polystyrene and glass – two commonly used materials in cell culture – regulated gene expression levels to a pronounced extent just after longer incubation periods.

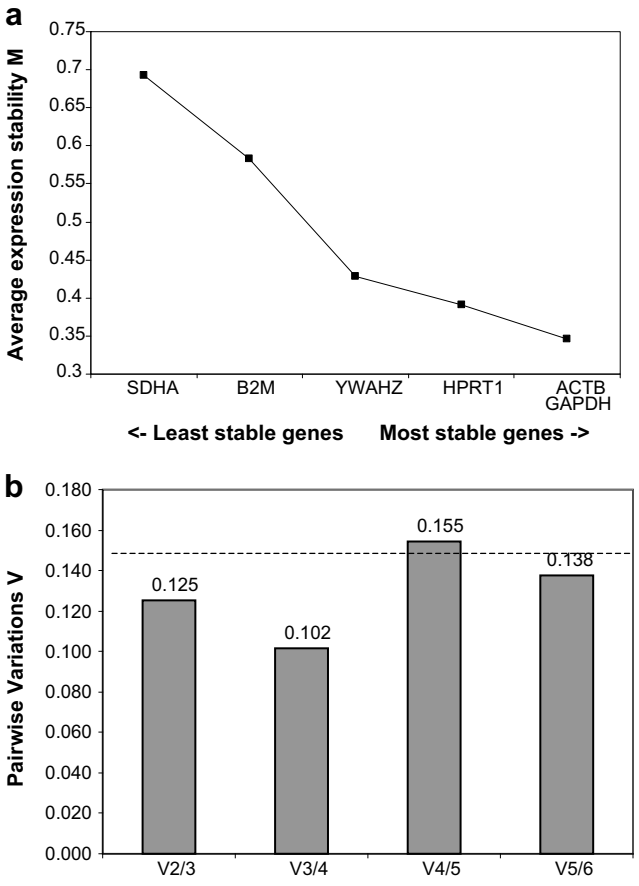

Fig. 2. geNorm output charts. Gene expression stability of the six candidate reference genes analysed by the geNorm program [9]. (a) Average expression stability value (M) of the control genes during stepwise exclusion of the least stable control genes, plotted from least stable (left) to most stable (right). (b) Determination of the optimal number of control genes for normalization calculated on the basis of the pairwise variation (V) analysis. V values below 0.15 threshold indicate no need to include further housekeeping genes for the calculation of a reliable normalization factor.

3.2. GeNorm analysis

To select appropriate reference genes and to define the optimal number of reference genes needed for accurate normalization the geNorm software was used [9]. Considering all data collectively, Fig. 2a shows that during Caco-2 differentiation on different surfaces expression stabilities decreased in the order *ACTB*, *GAPDH*, *HPRT1*, followed by *YWAHZ*, *B2M*, and *SDHA*. As shown in Fig. 2b, the $V_{2/3}$ value yielded 0.125. Since the default cut-off value was set to 0.15, the consideration of a third reference gene would not improve accurate normalization [9]. For comparative studies dealing with surface triggered differentiation of Caco-2 cells, we therefore recommend the

Table 3
Ranking of reference genes suggested by geNorm (each surface treated individually)

	Polystyrene	Matrigel-coated polystyrene	Glass	ETC01
Most stable genes	**ACTB/GAPDH**	**GAPDH/HPRT1**	**GAPDH/HPRT1**	**ACTB/YWAHZ**
↕	**HPRT1**	ACTB	YWAHZ	**HPRT1**
	YWAHZ	YWAHZ	ACTB	GAPDH
	SDHA	SDHA	B2M	B2M
Least stable genes	B2M	B2M	SDHA	SDHA

The genes are ranked in order of their expression stability cultivated on particular surfaces for 3, 10, and 21 days. The two most stable reference genes cannot be ranked because of the required use of ratios for gene expression stability.
Boldfaced genes display the optimal number of reference genes suggested by geNorm.

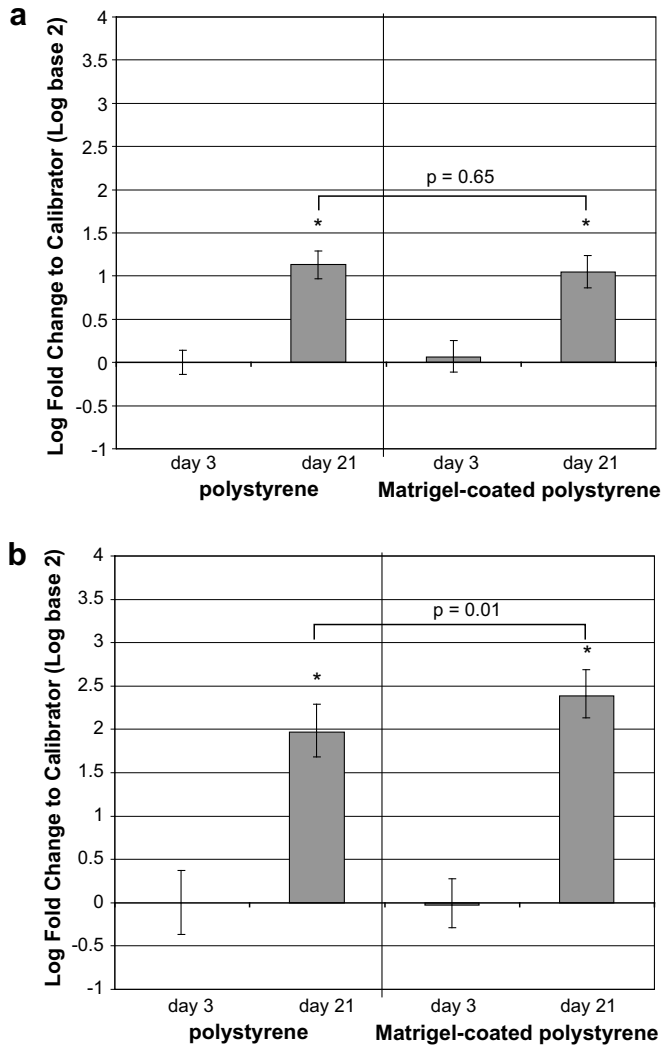

Fig. 3. Validation of reference genes. Log-fold change of *ALPI* gene expression in Caco-2 cells grown on polystyrene or Matrigel-coated polystyrene normalized to (a) SDHA and (b) *GAPDH*, found as the least and the most stable reference genes in geNorm analysis, respectively (means ± SD, $n = 6$). All data are efficiency corrected and related to a calibrator sample (polystyrene 3 days postseeding). Significant differences of fold changes related to day 3 of the particular surface are marked with asterisks ($p < 0.05$). The brackets indicate the statistical difference (p-value) between the surfaces on day 21.

selection of at least one reference gene out of *ACTB* and *GAPDH*. For the fine measurement of small differences in expression levels, the geometric mean of multiple reference genes could be of advantage as suggested by Vandesompele et al. [9].

To estimate the impact of different surfaces, respective *M* values were calculated for each surface separately (Table 3). Generally, the results did not vary to a high extent. The reference genes *B2M* and *SDHA* displayed a very low gene expression stability under the influence of each surface. Regarding polystyrene, we could demonstrate that during the course of Caco-2 cell differentiation *ACTB*, *GAPDH*, and *HPRT1* had the three most stable expression profiles. The set of suitable reference genes suggested by geNorm for Matrigel-coated polystyrene as well as for glass were *GAPDH* and *HPRT1*, and that for ETC01-slides *ACTB*, *YWAHZ*, and *HPRT1*. Due to these similarities it might be hypothesized that *ACTB*, *GAPDH*, and *HPRT1* were appropriate also for studies on further matrices, not included in the present report.

3.3. Validation of selected reference genes

Moreover, to point out the importance of careful reference gene selection, *ALPI* gene expression levels were determined using the least or the most stable reference genes, i.e. *SDHA* and *GAPDH*, respectively. A significant up-regulation of *ALPI* gene expression ($p < 0.05$) up to day 21 was detectable on polystyrene as well as on Matrigel-coated polystyrene regardless of the reference gene used (Fig. 3a and b). However, when normalizing to non-stable *SDHA* no significant difference between polystyrene and Matrigel-coated polystyrene on day 21 was revealed ($p = 0.65$). In contrast, when using a stable validated *GAPDH* as a reference gene a statistically significant difference between the matrices could be observed ($p = 0.01$). Furthermore, as compared to *SDHA*-normalized data (Fig. 3a), *GAPDH*-normalized *ALPI* levels on day 21 (Fig. 3b) were 1.8-fold higher in the case of polystyrene and 2.6-fold higher in the case of Matrigel-coated polystyrene.

In conclusion, this report underlines the importance of using validated reference genes for data normalization in studies on Caco-2 cells. The use of a wrong reference gene might introduce an unpredictable error and significantly affect the study's outcome by over- or underestimating mRNA expression and thus obscure relevant findings. Based on the presented results, we recommend the use of *GAPDH* or *ACTB* as internal controls for the normalization of marker gene expression levels in qPCR studies on native Caco-2 cell differentiation.

Acknowledgments

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