Serum availability affects expression of common housekeeping genes in colon adenocarcinoma cell lines: implications for quantitative real-time PCR studies

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Abstract Careful selection of housekeeping genes (HKG) is prerequisite to yield sound qPCR results. HKG expression varies in response to hypoxia but the effect of manipulations of serum availability, a common experimental procedure, remains unknown. Also, no data on HKG expression stability across colon adenocarcinoma lines that would aid selection of normalizers suitable for studies involving several lines are available. Thus, we evaluated the effect of serum availability on the expression of commonly used HKG (ACTB, B2M, GAPDH, GUSB, HPRT1, IPO8, MRPL19, PGK1, PPIA, RPLP0, RPS23, SDHA, TBP, UBC, and YWHAZ) in seven colon adenocarcinoma cell lines (Caco-2, DLD-1, HCT116, HT29, Lovo, SW480, and SW620). Sets of stably expressed line-specific and pan-line HKG were validated against absolutely quantified CDKN1A, TP53, and MDK transcripts. Both serum availability and line type affected HKG expression. UBC was fourfold down-regulated and HPRT1 1.75-fold up-regulated in re-fed HT29 cultures. Line-to-line variability in HKG expression was more pronounced than that caused by altering serum availability and could be found even between isogenic cell lines. PPIA, RPLP0, YWHAZ, and IPO8 were repeatedly highly ranked while ACTB, B2M, UBC, and PGK1 were ranked poorly. Normalization against PPIA/RPLP0/SDHA was found optimal for studies involving various colon adenocarcinoma cell lines subjected to manipulations of serum availability. We found HKG expression to vary, more pronouncedly by line type than growth conditions with significant differences also between isogenic cell lines. Although using line-specific normalizers remains optimal, a set of pan-line HKG that yields good estimation of relative expression of target genes was proposed.

Keywords Housekeeping genes (HKG) · Reference genes · Serum starvation · Serum induction · geNorm · NormFinder

Background

Real-time (quantitative) reverse transcription PCR (RT-qPCR) is frequently employed for unravelling the pathomechanisms of diseases to aid the research on new potential biomarkers and therapeutic strategies (Bustin and Murphy 2013). Normalization against
unregulated genes, called “housekeeping” genes (HKG), is a common way to account for a non-biological variation introduced during sample handling and thus to avoid quantification errors. However, a body of evidence has gathered showing that HKG expression may in fact vary between different tissues or cell lines and change in response to pathology, treatment, or altered environmental conditions (Dheda et al. 2005). Moreover, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the most frequently used normalizer, has been demonstrated to increase over 40-fold in severe sepsis (Cummings et al. 2014) but decrease with ageing (Vigelsø et al. 2015). Concerning cancer, GAPDH confers growth advantage and hence is frequently up-regulated in tumor cells (reviewed in Guo et al. 2013; Ramos et al. 2015). Alterations in HKG expression may be too subtle to affect the results obtained by semi-quantitive methods like end-point PCR or to manifest themselves at protein level. However, standardization against inappropriate HKG may lead to invalid conclusions when much more sensitive assays like quantitative real-time PCR are used as shown by Caradec et al. (2010) demonstrating a false PAR1 up-regulation in LNPCaP cells grown in response to hypoxia following normalization against unstable HKG. Therefore, a necessity of HKG validation for various experimental settings, if RT-qPCR is to be used, is increasingly recognized.

Serum withdrawal, with or without subsequent re-supplementation (serum induction), is a frequently used laboratory procedure, whether it is conducted for creating better defined environment for growing cells, to synchronize their growth, or to study mechanisms involved in stress response, apoptosis and autophagy. It may also serve for establishing an experimental model of conditions associated with nutrient-deprivation, e.g. mimic tumor milieu, where faulty blood vessels inefficiently supply cancer cells not only with oxygen but with nutrients as well (Pirkmajer and Chibalin 2011). Although limitation of oxygen availability occurred to have a profound impact on stability of HKG expression (Caradec et al. 2010), data on the possible effect of serum withdrawal and subsequent induction are scanty. Schmittgen and Zakrzajsek (2000) reported a several-fold increase in GAPDH and ACTB expression, but not that of B2M, in NIH 3T3 fibroblasts upon serum induction while Pirkmajer and Chibalin (2011) observed GAPDH protein level to be decreased in starving primary human myotubes.

Instability of HKG expression has already been demonstrated for normal and cancerous tissue samples obtained from CRC patients (Sørby et al. 2010; Kheirelseid et al. 2010) as well as normal and inflamed bowel of patients with inflammatory bowel disease (Krzystek-Korpacka et al. 2014). However, the issue has not been systematically addressed in colon adenocarcinoma cell cultures yet. Hence, this study was designed to test the effect of growth conditions and line type on expression of fifteen commonly used HKG in order to find relatively stable normalizers to be used in in vitro experiments on colon adenocarcinoma cell cultures involving serum-withdrawal and induction. HKG suitability was verified by comparing the expression of CDKN1A (p21CIP1/WAF1), TP53 (tumor protein p53), and MDK (midkine) calculated using both absolute and relative quantification methods. We found HKG expression to vary, more pronouncedly by line type than growth conditions with significant differences in the expression of some HKG also between isogenic cell lines. Relatively stable line-specific and pan-line HKG were identified. The impact of using inappropriate reference genes ranging from affecting statistical outcome to drawing false conclusions was demonstrated.

Materials and methods

Cell cultures

Seven authenticated human colon adenocarcinoma cell lines (ATCC) were obtained from the Polish Collection of Microorganisms (PCM) of the Institute of Immunology and Experimental Therapy of Polish Academy of Science, Wroclaw, Poland: Lovo (PCM-TC080 = ATCC: CCL-229), HT29 (PCM-TC044 = ATCC: HTB-38), SW620 (PCM-TC046 = ATCC: CCL-227), SW480 (PCM-TC160 = ATCC: CCL-227), HCT116 (PCM-TC161 = ATCC: CCL-247), Caco-2 (PCM-TC017 = ATCC: HTB-37), and DLD-1 (PCM-TC162 = ATCC: CCL-221). Cells were grown on 75 cm² cell culture flasks (BD Bioscience, San Jose, CA, USA) in DMEM/F12 medium (Life Technologies, Carlsbad, CA, USA), supplemented with 10 % FBS (v/v) and 1 % (v/v) L-glutamine-penicillin–streptomycin until 80 % confluence, then harvested using TrypLE Express (Life Technologies), and counted with
atmosphere containing 5 % CO₂. The complete

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counter (Life Technologies). Subsequently, 1 × 10⁶ cells/well were seeded
on plastic 6-well flat bottom culture plates (BD Bioscience), cultured for 24 h at 37 °C in a humidified
atmosphere containing 5 % CO₂. The complete
medium was then replaced with serum-free medium for 24 h and, subsequently, half of the cells received a
new portion of serum-free medium and the other half was re-fed by replacing serum-free medium with a
complete one (supplemented with 10 % FBS). Cells
were harvested at two time points: after 24 and 48 h
following media replacement. Upon termination,
supernatants were removed and cells were scratched
and lysed with 1 ml of TRI Reagent (Sigma-Aldrich,
St. Louis, MO, USA) and stored at
−80 °C until RNA
isolation. For each cell line, two complete sets of cells
cultured in parallel for 24 and 48 h, under both 0 and
10 % FBS were available.

RNA extraction, quantitation and quality
assessment

Cell lysates were centrifuged upon refreezing
(12,000×g, 4 °C, 10 min) and chloroform was added
to the supernatant (0.2 ml per 1 ml of TRI Reagent),
mixed, and centrifuged after 5 min incubation at RT
(12,000×g, 4 °C, 15 min). RNA-containing aqueous
upper phase was collected and passed through gDNA
Eliminator spin columns and then purified using
RNeasy Plus Mini Kit (Qiagen, Hilden, Germany)
according to manufacturer’s instructions. Isolated
RNA was quantified by means of UV spectroscopy
with NanoDrop 2000 (Thermo Scientific, Rockford,
IL, USA), measured in duplicates, and its purity
assessed by calculating ratios of absorbances at 260,
280, and 230 nm. RNA integrity was assessed using the
Experion automated electrophoresis platform
incorporating LabChip microfluidic technology and
Experion RNA StdSens analysis kits (BioRad, Her-
cules, CA, USA). The RNA quality indicator (RQI)
grading RNA from 10 (intact RNA) to 1 (degraded
RNA) was calculated by Experion software for all
samples. Possible presence of inhibitors in each RNA
isolate was tested by calculating RT-qPCR reaction
efficiencies from standard curves prepared by serial
dilutions of respective cDNA samples (fivefold dilu-
tions, 6 point-curve, conducted in duplicates).

cDNA synthesis

1 µg of purified RNA from cell culture samples per
reaction (20 µl) was reversely transcribed using
Maxima First Strand cDNA Synthesis Kit for RT-
qPCR (Thermo Scientific), containing modified
M-MuLV reverse transcriptase, RiboLock™ RNase
inhibitor, and a mixture of oligo (dT)₁₈ and random
hexamer primers, according to the manufacturer’s
protocol: 10 min incubation at 25 °C, 30 min incuba-
tion at 50 °C, and reaction termination by heating
samples at 85 °C for 5 min, all in C1000 termocycler
(BioRad). Negative transcription (no-RT) controls,
devoid of reverse transcriptase, were prepared for all
samples.

RT-qPCR

We evaluated the following HKG: ACTB, B2M,
GAPDH, GUSB, HPRT1, IPO8, MRPL19, PGK1,
PPIA, RPLP0, RPS23, SDHA, TBP, UBC, and
YWHAZ. Full gene names, accession numbers as well
as functions of encoded proteins and the sequences of
specific, intron-spanning primers (designed and tested
for specificity as previously described (manuscript
submitted)) are listed in Table 1. Primers’ efficiencies
(Table 1) were determined with RT-qPCR and a
mixture of DNA templates used in this experiment.

Samples were assessed in three technical replicates
(within the same run) and accompanied by respective no-
RT controls as well as no template control. To minimize
inter-run variation, the same gene was tested in the same
analytical run on different samples; each cDNA was
diluted from stock once, aliquoted, and stored at −80 °C;
all genes were tested on a series of samples within
2–3 days to avoid prolonged storage of diluted cDNA.

All RT-qPCR reactions were conducted with
CFX96 Real-Time PCR system (BioRad) using
SsoFast EvaGreen® Supermix (BioRad), containing
Sso7d-fusion polymerase and EvaGreen dye and the
following cycling conditions: 30 s activation at 95 °C,
5 s denaturation at 95 °C, annealing/extension for 5 s
at 61 °C, 40 cycles, followed by melting step
(60–95 °C with fluorescent reading every 0.5 °C).
Reaction mixture contained 2 µl of diluted 1:10
cDNA, 10 µl of 2 × SsoFast EvaGreen® Supermix,
1 µl of each 10 nM forward and reverse target-specific
primers, and water up to 20 µl.
Additionally, the absolute quantification of three target genes: *CDKN1A* (encoding p21<sub>CIP1/WAF1</sub> protein), *TP53* (encoding tumor protein p53), and *MDK* (encoding midkine, a pro-tumorigenic cytokine) was conducted for comparative purposes. For this, standard curves based on serial tenfold dilutions of *CDKN1A*, *TP53*, or *MDK* transcripts cloned into pJET1.2 plasmid (10<sup>9</sup> to one copy per ml)
(ThermoScientific) were prepared. Mean plasmid DNA concentrations measured with NanoDrop 2000 were 20.6, 26.9, and 11.23 ng/μl, respectively.

Statistical analysis

Technical replicates were averaged prior to any analyses. Expression stability was evaluated using two different statistical approaches, namely by calculating (1) intra- and inter-group variability combined into stability value, derived using NormFinder software version 0.953 (available as MS Excel Add-in at www.mdl.dk/publications/normfinder.htm) (Andersen et al. 2004), and (2) the average pairwise variation of a specific gene as compared with other genes, derived using geNorm utility in qbasePLUS version 2.4 software (Biogazelle BE, Ghent, Belgium) (Vandesompele et al. 2002). NormFinder generates a stability value for each gene, which is a direct measure for the estimated expression variation. It allows ranking genes according to the similarity of their expression profiles with lower values indicative of higher stability. Similarly, GeNorm generates M value for each gene with a lower value representative of increased gene stability across samples. GeNorm M value below 1.5 is arbitrarily suggested to be acceptable expression stability. GeNorm generates also V value, which is a pairwise stability measure to determine the benefit of adding extra reference genes for the normalization process with 0.15 as an arbitrary cut-off.

Data were uploaded as suggested by software designers: in an efficiency-corrected linearized form using the following expression: \( E_{\text{amp}} = 10^{(1/-\text{slope of target standard curve})} \) for NormFinder and as efficiency corrected Cq values for geNorm. Relative expression of target genes (CDKN1A, TP53, and MDK) was calculated using qbasePLUS.

The effect of growth conditions (serum availability or time) on HKG expression in each cell line was tested on relative quantities, log-transformed if necessary, using paired t-test while the impact of line type with Kruskal–Wallis \( H \) test. Relative gene expression in isogenic cell lines was compared using unpaired t-test. Data distribution was tested using Kolmogorov–Smirnov test and homogeneity of variances using Levene’s test. All calculated probabilities were two-tailed and \( p \) values \( \leq 0.05 \) were considered statistically significant. The analyses were performed using MedCalc Statistical Software version 12.7.5 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2013).

Results

All RNA isolates obtained from cell cultures were of very good quality with appropriate purity: mean 260/280 absorbance ratio was 2.07 ± 0.03 and mean 260/230 ratio was 2.03 ± 0.39 and high integrity: mean RQI = 9.4 ± 0.87 (range 7.3–10). Mean efficiency derived from dilution series of resulting cDNA templates was 103.6 ± 3.5 % (range 96.4–109.9 %), mean regression coefficient and slope of respective curves was 0.998 ± 0.002 and 3.239 ± 0.077 (range –3.411 to –3.106).

Effect of serum availability, length of culturing and line type on HKG expression: non-normalized data

To evaluate the potential effect the growth conditions and line type might have upon HKG expression, we calculated inter- and intra-group variability using NormFinder algorithm. Across all evaluated cell lines, the highest inter-group variability was displayed by UBC (commonly down-regulated upon serum re-supplementation) and by HPRT1 and MRPL19 (commonly up-regulated) (Fig. 1a). Subsequently, we compared the relative quantities of these genes in individual cell lines using paired t-test. The analysis showed UBC down-regulation to be statistically significant in HT29 cells (\( p = 0.004 \)) and HPRT1 and MRPL19 up-regulation statistically significant in, respectively, HT29 (\( p = 0.045 \)) and SW480 (\( p = 0.026 \)) cell lines.

The combined effect of line type, length of culturing, and biological replicates on HKG is depicted in Fig. 1b as an intra-group variability calculated by NormFinder. Overall, its magnitude was higher than for alterations in serum availability. The expression of UBC, ACTB, PGKI, B2M, HPRT1, and TBP varied the most, both when serum-starved and serum re-supplemented cultures were examined. Subsequent statistical analysis of relative quantities using Kruskal-Wallis \( H \) test showed significant line-to-line differences in the expression of RPS23 (\( p = 0.008 \)), B2M (\( p < 0.001 \)), GAPDH (\( p = 0.020 \)), GUSB
In expression of RPS23 (p < 0.001), MRPL19 (p = 0.004), PGK1 (p < 0.001), SDHA (p = 0.027), UBC (p < 0.001) and YWHAZ (p = 0.003). While the differences in expression of RPS23, GAPDH, and MRPL19 were limited to one or two cell lines (e.g. GAPDH expression differed significantly in DLD-1 cells as compared to other lines), the pair-wise comparison for UBC or B2M yielded number of significant differences.

Interestingly, even the isogenic cell lines SW480 (primary colon adenocarcinoma) and SW620 (its lymph node metastasis) significantly differed by SDHA and GUSB expression.

All genes were stably expressed overtime except for RPLP0, significantly up-regulated in 48 h cultures of HT29 (p = 0.035) and Lovo (p = 0.032).

Only the variation in the expression of ACTB, TBP, IPO8, and PPIA, induced by growth conditions or line type or both, was not statistically significant when non-normalized relative quantities were analyzed.

Pan-line normalizers

Two popular statistical approaches (NormFinder and geNorm algorithms) were employed to evaluate HKG stability across all cell lines and growth conditions and to select optimal pan-line normalizers. The evaluated genes were ranked from these with the highest stability, indicated by the lowest NormFinder stability value or geNorm M value, to the lowest stability, denoted by the highest scores (Table 2). Although the exact order differed, the same HKG, namely, RPLP0, IPO8, GUSB, YWHAZ, and PPIA, were highly ranked regardless of the algorithm used and the same genes, namely ACTB, B2M, UBC, and PGK1, were found the least stable. GAPDH, the most commonly used reference gene, was middle ranked by both algorithms. However, its scores (stability value and M value, respectively) did not differ from the better ranked HKG by much.

NormFinder found RPLP0 the most stably expressed single HKG, followed by PPIA and IPO8. However, the software suggested RPLP0 and SDHA, the fourth HKG in rank, as an optimal pair of normalizers. As shown by inter-group variability (Fig. 1a), SDHA expression in the present sample set is rather up-regulated upon serum re-supplementation what would compensate RPLP0 down-regulation while the expressions of PPIA and IPO8 tend to be down-regulated as well.

According to GeNorm, under study conditions, the average stability of evaluated HKG was medium with average M value >0.5 but ≤1. Optimal number of genes to be used as normalizers in the studied set of samples was calculated to be three, namely PPIA, RPLP0, and SDHA. As depicted on Fig. 2, there was
significant improvement in normalization based on three than two HKG (GeNorm V2/3 value exceeded arbitrary cut-off of 0.15). In turn, the effect of introducing the fourth gene was insubstantial (GeNorm V3/4 was 0.15).

**Line-specific normalizers**

Using the same approach, we devised line-specific normalizers as well. The resulting geNorm and NormFinder ranking lists were concordant with only small shifts in the positions of specific genes. UBC/PPIA, YWHAZ/RPS23, YWHAZ/B2M, and GAPDH/PPIA pairs were found optimal normalizers, respectively for DLD-1, SW480, HCT116, and Caco-2 lines by NormFinder (Table 3), while GUSB/RPLP0, RPS23/RPLP0, UBC/RPLP0/B2M, and GUSB/YWHAZ by geNorm (Table 4). For HT29, SW620, and Lovo both approaches yielded the same pairs of HKG, respectively, YWHAZ/B2M, YWHAZ/IPO8, and GUSB/YWHAZ.

However, some striking differences in gene stability were found between lines. Regardless the algorithm used, UBC was top-ranked in DLD-1 cells but worst-ranked in HT29. Similarly, stability of YWHAZ was highly ranked in all cell lines except for Caco-2. RPLP0 was generally well-rated except for Caco-2 and Lovo lines, while PGK1 was generally ranked poorly except for HCT116. TBP was one of top-ranked HKG in HT29 but otherwise ranked poorly and IPO8 occupied high positions on SW620 list but last ones on HCT116 list.

As shown in Fig. 3, there were line-to-line differences in their response to serum induction as well, e.g. ACTB was up-regulated in Caco-2 cells and Lovo but down-regulated in DLD-1 while PGK1 was up-regulated in Caco-2 but down-regulated in Lovo. Also the isogenic cell lines differ: B2M was rather down-regulated upon serum re-supplementation in SW480

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**Table 2** Ranking of HKG expression stability across all cell lines grown under serum-free or serum–supplemented conditions calculated using various statistical approaches (in descending order)

| NormFinder stability value* | GeNorm M value† |
|-----------------------------|-----------------|
| RPLP0                       | 0.081 PPIA      |
| PPIA                        | 0.084 RPLP0     |
| IPO8                        | 0.084 SDHA      |
| YWHAZ                       | 0.084 IPO8      |
| RPS23                       | 0.086 GUSB      |
| GUSB                        | 0.088 RPS23     |
| SDHA                        | 0.089 YWHAZ     |
| GAPDH                       | 0.092 GAPDH     |
| MRPL19                      | 0.108 MRPL19    |
| HPRT1                       | 0.124 TBP       |
| TBP                         | 0.126 HPRT1     |
| B2M                         | 0.138 PGK1      |
| PGK1                        | 0.138 B2M       |
| UBC                         | 0.141 UBC       |
| ACTB                        | 0.150 ACTB      |
| RPLP0 and SDHA*             | 0.056 PPIA, RPLP0 and SDHA# |

Data presented as stability values calculated for each HKG using NormFinder or GeNorm algorithms. A set of genes, the combination of which provides increased stability is presented in the last row (stability value of a set is calculated exclusively by NormFinder)

* Norm Finder stability value is a direct measure for the estimated expression variation. Lower values are indicative of higher expression stability

† GeNorm M value indicates gene expression stability across samples with lower values representing increased stability. Arbitrarily, M values <1.5 are indicative of acceptable expression stability

# The improvement of the GeNorm value is not shown for the combination of PPIA, RPLP0, and SDHA
Table 3  Line-specific HKG expression stability in cell lines grown under serum-free or serum–supplemented conditions ranked by increasing stability value calculated with NormFinder software

|           | DLD-1 | HT29 | SW480 | SW620 | HCT116 | Caco-2 | Lovo |
|-----------|-------|------|-------|-------|--------|--------|------|
| YWHAZ     | .005  | .005 | .005  | .005  | .005   | .005   | .005 |
| RPLP0     | .005  | .005 | .005  | .005  | .005   | .005   | .005 |
| MRPL19    | .007  | .007 | .007  | .007  | .007   | .007   | .007 |
| PPIA      | .011  | .011 | .011  | .011  | .011   | .011   | .011 |
| B2M       | .022  | .022 | .022  | .022  | .022   | .022   | .022 |
| HPRT1     | .033  | .033 | .033  | .033  | .033   | .033   | .033 |
| RPS23     | .050  | .050 | .050  | .050  | .050   | .050   | .050 |
| GUSB      | .060  | .060 | .060  | .060  | .060   | .060   | .060 |
| UBC       | .068  | .068 | .068  | .068  | .068   | .068   | .068 |
| IPO8      | .080  | .080 | .080  | .080  | .080   | .080   | .080 |
| PGK1      | .098  | .098 | .098  | .098  | .098   | .098   | .098 |
| SDHA      | .110  | .110 | .110  | .110  | .110   | .110   | .110 |
| ACTB      | .165  | .165 | .165  | .165  | .165   | .165   | .165 |

Data presented as stability values calculated for each HKG using NormFinder. A set of genes, the combination of which provides increased stability is presented in the last row. Lower values are indicative of higher expression stability.

Table 4  Line-specific HKG expression stability in cell lines grown under serum-free or serum–supplemented conditions ranked by increasing GeNorm M value calculated with qbasePLUS software

|           | DLD-1 | HT29 | SW480 | SW620 | HCT116 | Caco-2 | Lovo |
|-----------|-------|------|-------|-------|--------|--------|------|
| YWHAZ     | .005  | .005 | .005  | .005  | .005   | .005   | .005 |
| RPLP0     | .005  | .005 | .005  | .005  | .005   | .005   | .005 |
| MRPL19    | .007  | .007 | .007  | .007  | .007   | .007   | .007 |
| PPIA      | .011  | .011 | .011  | .011  | .011   | .011   | .011 |
| B2M       | .022  | .022 | .022  | .022  | .022   | .022   | .022 |
| HPRT1     | .033  | .033 | .033  | .033  | .033   | .033   | .033 |
| RPS23     | .050  | .050 | .050  | .050  | .050   | .050   | .050 |
| GUSB      | .060  | .060 | .060  | .060  | .060   | .060   | .060 |
| UBC       | .068  | .068 | .068  | .068  | .068   | .068   | .068 |
| IPO8      | .080  | .080 | .080  | .080  | .080   | .080   | .080 |
| PGK1      | .098  | .098 | .098  | .098  | .098   | .098   | .098 |
| SDHA      | .110  | .110 | .110  | .110  | .110   | .110   | .110 |
| ACTB      | .165  | .165 | .165  | .165  | .165   | .165   | .165 |

Data are presented as stability values (M) calculated for each HKG using GeNorm algorithm. A set of genes, the combination of which provides increased stability is presented in the last row (GeNorm does not provide M value for combination of selected genes). Lower values are indicative of increased stability.

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DLD-1

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation

HT-29

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation

SW480

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation

SW620

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation

HCT116

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation

Caco-2

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation

Lovo

up-regulated upon serum re-supplementation

down-regulated upon serum re-supplementation
but up-regulated in SW620, ACTB was rather up-regulated in SW480 but down-regulated in SW620, and TBP was down-regulated in SW480 but its expression was not affected in SW620.

Validation of devised normalizers

In order to validate the devised sets of HKG, we compared relative expression ratios (normalized expressions in serum-induced to serum-starved cultures) obtained using various combinations of reference genes with the one resulting from absolute quantification with a copy number. HKG performance was tested on three target genes, the expression of which was evaluated in 48 h cultures of HT29, Caco-2, and DLD-1 cells. Apart from pan-line normalizers devised by geNorm or NormFinder, we constructed another set consisting of HKG that were not significantly affected by line type or culture growth conditions, that is, ACTB, TBP, IPO8, and PPIA.

As indicated by 6.7-fold and twofold reduction in DNA copy number, CDKN1A and MDK expressions were down-regulated upon serum re-supplementation in HT29 cells, while that of TP53 remained unaffected (Fig. 4a). The same conclusions could be reached whether software-devised line-specific (YWHAZ/B2M) or pan-line (RPLP0/PPIA/SDHA) normalizers were used. Since the overall GAPDH rating (both line-specific and pan-line) was not bad, normalization against this single, commonly used reference gene did not substantially altered study conclusions on target gene expression. However, normalization against the unstable UBC underestimated CDKN1A down-regulation and led to erroneous conclusions on MDK and TP53 up-regulation in response to serum induction.

Despite uniformly poor ratings of ACTB and mediocre/poor of TBP, a set of “unaffected” HKG (ACTB/TBP/IPO8/PPIA) gave an estimation of changes in target gene expression close to the absolute one (Fig. 4a) Similarly, relating MDK expression in Caco-2 cells to ACTB/TBP/IPO8/PPIA (Fig. 4b) did not alter experiment conclusion on lack of MDK regulation upon serum re-supplementation in this particular cell line. It might be explained by relatively low inter-group variability in ACTB and TBP expression in HT29 as compared to other lines (Fig. 3, HT29). In Caco-2 cells, in turn, their variability was high but of similar magnitude and oppositely directed, with ACTB substantially up- while TBP down-regulated (Fig. 3, Caco-2). Hence, the effect of one gene was countered by the other. If ACTB or TBP were used as sole normalizers, MDK would be falsely interpreted as, respectively, down- or up-regulated upon serum induction (Fig. 4b). In DLD-1 cells, ACTB displayed substantial variability that was not countered by TBP (Fig. 3, DLD-1). In such a case, as demonstrated by MDK expression significantly down-regulated by serum re-supplementation (Fig. 4b), software-devised pan-line normalizers were superior. They did not alter experiment conclusion, even though they included genes found significantly affected by growth conditions (RPLP0) or line type (SDHA). On the contrary, normalizing against a set consisting of “unaffected” but poorly ranked genes underestimated the effect so the statistical significance of MDK down-regulation was lost.

Effect of growth condition and line type on HKG expression: validation on normalized data

Statistical analysis on relative quantities (non-normalized) shown line-to-line differences in expression levels of most of the evaluated HKG except for ACTB, TBP, RPLP0, PPIA, and IPO8 to be significant. However, when data were normalized against pan-line normalizers (RPLP0/SDHA/PPIA) to account for non-biological variation (e.g. differences in template load or reaction efficiency), pair-wise comparisons of GAPDH, PGK1, or RPS23 expression did not yield significant differences. The expression of other genes, previously found affected by line type, remained different. Also two isogenic cell lines, SW480 and SW620, significantly differed by their non-normalized GUSB and SDHA expression. To verify this finding, we compared their relative expression normalized against geometric mean of RPLP0, IPO8, and YWHAZ, found optimal by geNorm for SW480 and SW620. Relative GUSB and SDHA expression was up-
regulated in SW620 (line derived from secondary tumor), significantly in case of GUSB (Fig. 5).

To further demonstrate the importance of using validated normalizers, we estimated relative expression of GUSB and SDHA using GAPDH (middle-rated) or ACTB (the worst-ranked) as sole normalizers. While normalization against GAPDH would overestimate the difference in expression, using ACTB as reference would not show any differences in GUSB or SDHA expression between lines.

Using normalized data with line-specific reference genes we also verified the findings on HKG expression being affected by length of culturing and serum availability. The difference in RPLP0 expression between 24 and 48 h cultures of HT29 and Lovo become insignificant ($p = 0.080$ and $p = 0.426$) when normalized against YWHAZ/B2M and YWHAZ/GUSB, respectively. However, the expression of UBC in HT29 cells upon serum induction remained over fourfold down-regulated ($p = 0.011$) following normalization and that of HPRT1—1.75-fold up-regulated ($p = 0.032$). Yet, the twofold increase in MRPL19 transcripts in serum re-supplemented SW480 cells lost significance, whether normalization was based on geNorm (RPLPO/RPS23) or NormFinder (YWHAZ/RPS23) selected pairs of line-specific HKG ($p = 0.145$ and $p = 0.259$, respectively).

Discussion

There is a growing awareness that the expression of housekeeping genes, previously believed to be stable,
may be affected by experimental settings and that normalization against a single, arbitrary chosen HKG may jeopardize the relevance of a study. Thus, it is suggested that quantitative PCR experiments should be preceded by a thorough examination of expression stability of potential HKG under dedicated conditions (Caradec et al. 2010). Manipulating the availability of serum for varying time periods is a common laboratory practice in molecular biology that may serve purposes as different as preparing cells for the proper experiment by increasing homogeneity of culture and uniformity of growing conditions or constitute an experiment per se (Pirkmajer and Chibalin 2011). Although the limited accessibility of nutrients, growth factors, and hormones may potentially affect expression of HKG in a way similar to oxygen deprivation (Caradec et al. 2010), the published data are limited and restricted to fibroblasts and primary cells, entirely depending on serum as a growth factor source (Iyer et al. 2012), while their potential effect on cancer cell lines is unknown. Schmittgen and Zakrzewski (2000) demonstrated that cultured murine fibroblasts grown for 24 h in serum-free medium and subsequently induced with 15 % FBS increased the expression of GAPDH and ACTB several-fold, rendering these genes inappropriate as internal controls for studies involving serum withdrawal and induction. Correspondingly, primary human and rat myotubes as well as human embryonic kidney (HEK)293 cells displayed gradually decreasing GAPDH protein content during 24 h serum withdrawal (Pirkmajer and Chibalin 2011). On the other hand, the excess of glucose in culture media (Liu et al. 2016; Bakhashab et al. 2014) or cell stimulation with growth factors (Tratwal et al. 2014) has been demonstrated to affect HKG stability as well.

GAPDH has been outperformed by other HKG also when normal and cancerous tissues were compared (de Kok et al. 2005; Blanquicett et al. 2002; Dydensborg et al. 2006). However, colon adenocarcinoma cell lines, as demonstrated here by rather low intergroup variability both when assessed combined and individually, do not respond to alterations in serum availability by substantial changes in GAPDH levels. Hence, normalizing against this HKG did not affect the conclusion of our experiments. Yet, with stability of its expression being suboptimal, it could affect the statistical outcome. Of note, preservation of GAPDH expression upon altered conditions has been reported for human umbilical vein endothelial cells (HUVECs) grown under hyperglycemic conditions (Bakhashab et al. 2014), chondrocytes cultured at different temperatures (Ito et al. 2014) or blood cells subjected to radiation (Vaiphei et al. 2015).

Concerning ACTB, its overall intergroup variability was in the current study low but the expression in particular cell lines was affected by alterations in serum availability, discouraging its application in in vitro studies involving serum withdrawal and re-supplementation. However, since the alterations occurred in both directions, ACTB displayed low variability as a pan-line normalizer. Our finding corroborates the observations of other authors on ACTB expression varying considerably with changing experimental conditions or between individuals (Caradec et al. 2010; Kheirelseid et al. 2010; Andersen et al. 2004). On the contrary, ACTB has been found among the most stably expressed HKG in breast cancer cell lines (Liu et al. 2015). We observed that particularly the expression of UBC and HPRT1 in HT29 and MRPL19 in SW480 was significantly altered by changes in serum availability disqualifying them as reference genes, even though UBC (Andersen et al. 2004) and HPRT1 (Sørby et al. 2010) were recommended as suitable normalizers for RT-qPCR studies on tissue specimens from CRC patients.

Caradec et al. (2010) demonstrated on prostate carcinoma cells that great expression variability can be found between cell lines derived from the same tissue. As such, the results obtained for one line should not be easily adopted for the other. Accordingly, we found that the observed fluctuations in HKG expression related to serum availability were surpassed by line-to-line differences in gene stability. Substantiating the notion, we found PGK1 expression to be unaffected by alterations in serum availability in HT29 line. Correspondingly, PGK1 expression was the most stable one after HT29 challenge with probiotic and pathogenic bacteria as reported by Jacobsen et al. (2014). However, concurrently, we found PGK1 to be among the most often up- or down-regulated HKG by serum re-supplementation in other colonic epithelial cell lines. The expression of most of the HKG differed significantly between particular cell lines both when non-normalized data were examined and when a non-biological variation was accounted for. UBC is a striking example how mechanical extrapolation of results obtained for one line to the other can affect
conclusions of the experiment—in our study underestimating the magnitude of CDKN1A down-regulation or demonstrating false up-regulation of MDK (down-regulated) and TP53 (unaltered) upon serum re-supplementation in 48 h HT-29 cultures.

Interestingly, the stability of HKG can very also between isogenic cell lines (derived from the same patient), as demonstrated here for primary colonic adenocarcinoma cells (SW480) and their lymph node metastasis (SW620). The expression of GUSB and SDHA was up-regulated in metastatic cell line as compared to primary one. Also, both lines differ with their response to serum induction with TBP expression down-regulated exclusively in primary SW480, B2M being up-regulated in metastatic but down-regulated in primary adenocarcinoma, and, oppositely, ACTB being down-regulated in metastatic but up-regulated in primary line.

In vitro experiments have usually a complex design; still, it is desirable to limit the number of necessary reference genes. Our results revealed that although using line-specific normalizers remains optimal, it is possible to devise a set of reference genes displaying relatively unaltered expression under study conditions. We started expression stability analysis from statistical evaluation of raw data to exclude from investigation genes obviously regulated under experimental conditions and hence unsuited to serve as normalizers. Similarly to other in vitro experiments, there were several variables in our study that might potentially affect HKG expression: line type, length of culturing, and serum availability. As such, the variability in the expression of only four genes was not found significant in response to at least one of the factors. However, this phenomenon, particularly in case of ACTB and TBP, seems to result from the variability being hard to attribute to any specific factor rather than lack of thereof. As pre-analyses are based on raw data, non-biological variation introduced during sample handling may contribute to observed differences. Accordingly, RPLP0 was no longer found significantly affected by length of culturing when data were normalized against line-specific normalizers. Consequently, normalization against ACTB/TBP/PPIA/IPO8 was suboptimal, failing to show significant down-regulation of MDK in DLD-1 cells, and was outperformed by HKG set devised by dedicated software from among all genes, without any exclusion.

Regardless algorithm used, PP1A, RPLP0, and SDHA were ranked the most stable in the sample set investigated. Normalization against geometric mean of these HKG yielded results similar to these obtained with line-specific reference genes or with absolute quantification, signifying their reliability as normalizers for RT-qPCR studies on multiple colon adenocarcinoma cell lines involving serum withdrawal and induction. RPLP0 has been claimed a suitable reference for human intestinal epithelial cells (Dydensborg et al. 2006). In turn, PP1A has been repeatedly found a suitable normalizer in a number of human studies (Andrusiewicz et al. 2016; Ali et al. 2015; Lemma et al. 2016), also these concerning CRC patients (Sørby et al. 2010; Kheirelseid et al. 2010), but affected by cell stimulation in others (Kaszubowska et al. 2015). IPO8 and GUSB were yet another HKG recommended for CRC studies (Sørby et al. 2010; Blanquicett et al. 2002) and highly ranked in our in vitro study as well. Analyzing HKG in colon and liver tissues from CRC patients with hepatic metastases, Blanquicett et al. (2002) observed that ribosomal HKG displayed the most stable expression while those involved in metabolic pathways were the least stable ones. Substantiating the notion, we and others (Dydensborg et al. 2006; Bakhashab et al. 2014; Jacobsen et al. 2014) demonstrated superior stability of RPLP0 and Bian et al. (2015), Powell et al. (2014), and Ito et al. (2014) that of another ribosomal protein—RPL13A. In turn, PGK1 was one of the least stable genes in our study, although GAPDH, encoding an enzyme involved in the same metabolic pathway, performed well.

Conclusions

Expression of commonly used HKG as well as line response to serum withdrawal and induction differ between colon adenocarcinoma cell lines, though these were derived from the same patient (isogenic cell lines). While normalizing against line-specific reference genes is optional, it is possible to devise common set of HKG, RPLP0/PP1A/SDHA in the sample set investigated, suitable for multiline RT-qPCR studies. GAPDH, the most popular internal control, occurred to be relatively stably expressed and yet normalizing against it may affect statistical outcome of the study. In turn, using ACTB, another frequently used
reference, or adopting without validation genes found stable for other lines may lead to invalid conclusions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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