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Normalizing genes for real-time polymerase chain reaction in epithelial and nonepithelial cells of mouse small intestine

Fengchao Wang, Junping Wang, Dengqun Liu, Yongping Su *

Institute of Combined Injury, State Key Laboratory of Trauma, Burns and Combined Injury, College of Preventive Medicine, Third Military Medical University, 30 Gaotanyan Road, Shapingba District, Chongqing 400038, China

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ABSTRACT

Gene expression studies in intestinal epithelial and stromal cells are a common tool for investigating the mechanisms by which the homeostasis of the small intestine is regulated under normal and pathological conditions. Quantitative real-time PCR (qPCR) is a sensitive and highly reproducible method of gene expression analysis, with expression levels quantified by normalization against reference genes in most cases. However, the lack of suitable reference genes for epithelial cells with different differentiation states and nonepithelial tissue cells has limited the application of qPCR in gene expression studies of small intestinal samples. In this study, 13 housekeeping genes, ACTB, B2M, GAPDH, GUSB, HPRT1, HMBS, HSP90AB1, RPL13A, RPS29, RPLP0,PPIA, TBP, and TUBA1, were analyzed to determine their applicability for isolated crypt cells, villus cells, deepithelialized mucosa, and whole mucosa of the mouse small intestine. Using geNorm and NormFinder software, GUSB and TBP were identified as the most stably expressed genes, whereas the expressions of the commonly used reference genes GAPDH, B2M, and ACTB, and ribosomal protein genes RPL13A, RPS29, and RPLP0 were relatively unstable. Thus, this study demonstrates that GUSB and TBP are the optimal reference genes for the normalization of gene expression in the mouse small intestine.

The intestinal mucosa can be divided into the epithelium, lamina propria, and muscularis mucosa. The complex cross talk between the epithelial cells and nonepithelial cells plays important roles in maintaining intestinal homeostasis and in the progress of diseases such as sepsis [1–3]. In addition, the small intestinal epithelium is divided spatially and morphologically into the crypt or proliferative unit and the villus, the differentiation and functional unit [4,5]. Different levels of gene expression are the causes and effects of cell proliferation and lineage-specific differentiation [4,6]. Many models based on wild-type and transgenic mice have been constructed to investigate the mechanism involved in intestinal homeostasis under normal and pathological conditions [3,7–9]. Evaluating the expressions of target genes in the villus and crypt epithelial cells and the nonepithelial cells usually are important subjects for these investigations.

Quantitative PCR (qPCR)[1] is widely used for the quantification of steady-state mRNA levels because it is sensitive and accurate [10]. In qPCR, gene expression measurements require a normalization strategy to allow meaningful comparisons to be made across biological samples [10,11]. Compared with tissue counts, cell counts, or total RNA measurements, endogenously expressed control genes provide more precise normalization standards [12].

GAPDH and ACTB are the most popular reference genes used in intestine-related studies [3,9,13–16], including comparing the gene expression along the crypt–villus axis [17]. However, based on an in vitro model of intestinal epithelial differentiation, it was found that expressions of GAPDH and actin are unstable during the enterocytic differentiation process [18]. In addition, the reports based on microarray data suggested that the expression of actin and cytoplasmic ribosomes in villus enterocytes is different than that in crypt enterocytes in mouse and human small intestinal tissues [6,19]. New evidence also indicates that ribosomal protein genes exhibit important proliferation-dependent variations in their mRNA expression [20]. Besides the uncertainty involved in selecting reference genes during the epithelial differentiation process, there is also no identified reference gene for use in the comparison of intestinal epithelial cells and corresponding nonepithelial cells in the mouse model.

In this study, we used an improved method to isolate and enrich mouse villus units and crypt units separately and simultaneously produced deepithelialized intestinal mucosa. The gene expression levels of 13 commonly used housekeeping genes (HKGs), ACTB, B2M, GAPDH, GUSB, HPRT1, HMBS, HSP90AB1, RPL13A, RPS29,

* Corresponding author. Fax: +86 023 68752009.
E-mail address: suyp2003@yahoo.com.cn (Y. Su).

[1] *Abbreviations used:* HKGs, housekeeping genes; NRT, no reverse transcription; qPCR, quantitative real-time polymerase chain reaction; RT-PCR, real-time PCR.

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RPLP0, PPIA, TBP, and TUBA1, were investigated in the villus, crypt, deepithelialized mucosa, and intact whole mucosa. The RT-PCR results were subsequently analyzed with two algorithms, geNorm [21] and NormFinder [22], to select the best candidate reference gene for the analysis of gene expression in the mouse small intestinal mucosa.

Materials and methods

Animals

Twelve male C57BL/6 mice (18–22 g) were obtained from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The mice were caged in an environmentally controlled room with alternating 12 h dark/light cycles and access to food and water ad libitum. All animal experiments were performed with the approval of the Animal Ethics Committee of the Third Military Medical University, People's Republic of China.

Isolation and enrichment of villus and crypt units

The epithelium from the crypt–villus axis was isolated using a modified Neil Flint method [23]. Briefly, for one isolation, four mouse small intestinal tracts were removed from the abdominal cavity. The intestinal tracts were then flushed twice with ice-cold Ca,Mg-free Hanks' buffered salt solution and everted. The duodenum and jejunum compartments were cut into 3- to 5-mm segments and suspended in 25–30 ml of chelating buffer (27 mM trisodium citrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 0.5 mM dithiothreitol, 55 mM D-sorbitol, 44 mM sucrose, 5 mM EDTA, 5 mM EGTA) and incubated at 4 °C for 30 min. Crypt and villus units were continuously released from the lamina propria with gentle shaking on ice. The crypt and villus units in the supernatant were separately enriched according to their different sedimentation rates (30–50 s for the first sedimentation of the villus portion, and about 5 min for the crypt units). Pure villus units were obtained by pipetting the villus enrichment and repeating the sedimentation, and the jointed crypt units were eliminated. The deepithelialized intestinal mucosa was collected until no new villus or crypt units were released. The enriched crypt and villus units and the deepithelialized mucosa were mounted onto slides and observed by microscopy.

Histological analysis

The crypt units, villus units, deepithelialized tissue, and intestinal tissue were fixed with formaldehyde for 24 h, dehydrated with increasing concentrations of ethanol, and then embedded in paraffin wax. The deparaffinized sections (5 μm) were stained with hematoxylin and eosin (HE) and observed by microscopy.

RNA isolation and quality control

The freshly isolated villus and crypt units were pelleted (50- to 100-μl volume), and then the pellets, deepithelialized intestinal mucosa, and whole intestinal mucosa (50–100 mg) were dissolved in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) by inverting or with a homogenizer. The total RNA was isolated according to the manufacturer's protocol. An additional RNase-free DNase1 (Qiagen, Hilden, Germany) treatment step was included. The concentration of the isolated RNA and the 260/280 absorbance ratio were measured with a Beckman DU-640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The integrity of the RNA samples was confirmed by electrophoretic assessment of the 28S/18S ratio on a 1.3% TBE/agarose gel; the ratios for all the samples used in the study were >1.5. The concentration of RNA was adjusted to 1 μg/μl with nuclease-free water. The total RNA (2 μg) was primed with oligo(dT)15 (Promega, Beijing, China) and reverse-transcribed to cDNA using M-MLV reverse transcriptase (Invitrogen) in a total volume of 20 μl, according to the manufacturer's instructions. No reverse-transcription (NRT) controls were prepared in parallel.

Real-time PCR

Oligonucleotide primers were designed with Primer 5 software from the sequences obtained from the GenBank database. Thirteen commonly used reference genes, ACTB, B2M, GAPDH, GUSB, HMBS, HPRT1, PPIA, RPL13A, HSP90AB1, RPS29, RPLP0, TBP, and TUBA1 (Table 1), and three target genes, myelocytomatosis oncogene (MYC), sucrase-isomaltase gene (SI), and Toll-like receptor 4 gene (TLR4), were used. All the amplicons (except that for TLR4) spanned at least one intron (Table 2). NRT controls were used to eliminate the potential impact of genome contamination on the test for TLR4 expression. SYBR Green real-time PCR was run in triplicate in 96-well reaction plates with the iQ5 machine (Bio-Rad). Blank controls were included in parallel for each master mix. The PCR volume was 15 μl, containing 2× Bio-Rad SYBR Green PCR master mix, 250 nM forward and reverse primers, and 1 μl of 1:10 diluted cDNA. The cycling conditions were as follows: initial template denaturation at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55–60 °C (the annealing temperature of each primer pair was optimized by thermal gradient PCR) for 15 s, and elongation at 72 °C for 15 s. These cycles were followed by a melting-curve analysis, ranging from 56 to 95 °C, with temperature increases in steps of 0.5 °C every 10 s. Only cycle threshold (C_t) values of <35 were used to calculate the PCR efficiency from the given slope generated with the iQ5 system software V2.0, according to the equation: PCR efficiency = $(10^{[-1/\text{slope}]} - 1) \times 100$. All PCR assays displayed efficiencies between 90% and 102% (Table 3, Supplementary Figs. 1 and 2).

Statistical analysis

All data are expressed as means ± SD. Using SPSS (version 14.0; SPSS, Chicago, IL, USA), one-way analysis of variance (ANOVA) followed by least-squares differences (LSD) was used for multiple group comparisons. A probability (P) value of <0.05 was considered significant. The results were analyzed with the iQ5 software (Bio-Rad) and Microsoft Excel. Relative quantification was performed using the ΔC_t method. The stability of the housekeeping genes was evaluated with the freely available geNorm (http://med-

Table 1
Candidate reference genes evaluated.

Gene symbol	Genbank Accession No.	Gene name
ACTB	NM_007393	Actin, beta
B2M	NM_009735	Beta-2-microglobulin
GAPDH	NM_008084	Glyceraldehyde-3-phosphate dehydrogenase
GUSB	NM_010368	Beta-glucuronidase
HMBS	NM_013551.2	Hydroxymethylbilane synthase
HPRT1	NM_013556	Hypoxanthine-guanine phosphoribosyltransferase1
HSP90AB1	NM_008302	Heat shock protein 90 alpha (cytosolic), class B member 1
PPIA	NM_008907	Peptidylprolyl isomerase A
RPLP0	NM_007475.4	Ribosomal protein, large, P0
RPL13A	NM_009438	Ribosomal protein L13A
RPS29	NM_009093.1	Ribosomal protein S29
TBP	NM_008907	TATAA-box binding protein
TUBA1	NM_011653.2	Tubulin, alpha 1A