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Selection of reference genes for quantitative real-time reverse transcription-polymerase chain reaction in concanavalin A-induced hepatitis model

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ABSTRACT

Quantitative real-time reverse transcription-polymerase chain reaction (Q-PCR) has become an indispensable technique for accurate determination of gene expression in various samples. In mice, intravenous injection of concanavalin A (ConA) leads to acute hepatitis and liver injury. Functional studies based on this model have provided insights for understanding the mechanisms of liver injury. However, no data have been reported to validate reference genes during the progression of ConA-induced hepatitis (CIH). In this study, IκBα and C/EBPβ messenger RNA (mRNA) levels were examined using Q-PCR with ACTB as the reference gene after ConA injection. However, we got inconsistent results with previous reports determining IκBα and C/EBPβ mRNA expression levels. The results indicate the necessity for stability analysis of candidate reference genes in the CIH model. geNorm, NormFinder, and BestKeeper software analysis indicates that ACTB is the most unstable gene during CIH progression among the 10 reference genes tested, whereas RPLP0 or HPRT1 is the most stable one. This study demonstrates that some of the commonly used reference genes are inadequate for normalization of Q-PCR data due to their expression instability. Furthermore, this study validates HPRT1 and RPLP0 as appropriate reference genes for Q-PCR analysis in the CIH model.

To determine messenger RNA (mRNA)[1] expression levels of target genes between different biological samples, it is necessary to select a reference gene as a normalization control. In each case, the normalization procedure is designed to allow investigators to obtain an accurate measurement of gene expression levels across different samples. Moreover, selection of the reference gene(s) in animal models is extremely important, because of the notable variance of tissue samples compared with cell line samples [1–3].

Concanavalin A (ConA)-induced hepatitis (CIH) in mice is a widely used animal model for the investigation of immune-mediated acute liver injury as well as subsequent liver regeneration. The hepatitis model can be induced by intravenous injection of ConA via the tail [4–6]. After ConA injection, T and NKT cells are activated [7,8] and then produce various inflammatory cytokines and chemokines such as TNF-α, IFN-γ, IL-4, and MCP-1 [9]. These factors recruit and activate more immune cells that would attack hepatocytes and lead to severe liver injury [10].

Based on reports related to this model, quantitative real-time reverse transcription-polymerase chain reaction (Q-PCR) is the most widely used technique for quantifying the expression levels of cytokines, chemokines, and many other molecules. To validate the successful establishment of the CIH model, several genes related to hepatic immune response and liver regeneration were checked. Genes analyzed in this study are listed in Table 1. To date, ACTB has frequently been used as the reference gene for various hepatitis models, including CIH [11], lipopolysaccharide (LPS)-induced hepatitis [12], and partial hepatectomy-induced hepatitis [13]. Thus, ACTB was used here as the reference gene at the beginning of Q-PCR analysis. We found that IFN-γ [14], iNOS [14,15], and SOCS3 [9] mRNA were significantly elevated at different time points, consistent with previous reports. Also, elevated COX2

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[1] *Abbreviations used:* mRNA, messenger RNA; ConA, concanavalin A; CIH, ConA-induced hepatitis; Q-PCR, quantitative real-time reverse transcription–polymerase chain reaction; LPS, lipopolysaccharide; HKG, housekeeping gene; SLAC, Shanghai Laboratory Animal Company; PBS, phosphate-buffered saline; H&E, hematoxylin and eosin; cDNA, complementary DNA; SE, standard errors; CP, crossing point; Ct, threshold cycle; CV, coefficient of variance expressed as a percentage of the CP.

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Table 1
Description of housekeeping gene and target gene primers used in Q-PCR assay.

Symbol and official symbol[a]	Full name	Entrez gene ID	Gene function	Real-time primer sequences
Housekeeping genes				
ACTB (Actb)	β-Actin	11461	Formation of major component of the cytoskeleton	F: 5′ GGCTGTATTCCCCTCCATCG R: 5′ CAGTTGGTAACAATGCCATGT
TUBA (Tuba1a)	Tubulin α1A	22142	Microtubules of the eukaryotic cytoskeleton	F: 5′ CCAGGGCTTCTTGGTTTTCC R: 5′ CGCTCAATGTCGAGGTTTCT
B2M (B2m)	β2 Microglobulin	12010	Association with the major histocompatibility complex class I heavy chain	F: 5′ TTCTGGTGCTTGTCTCACTGA R: 5′ CAGTATGTTCGGCTTCCCATTC
GAPDH (Gapdh)	Glyceraldehyde-3-phosphate dehydrogenase	14433	Enzyme in an important energy-yielding step in carbohydrate metabolism	F: 5′ TGACCACAGTCCATGCCATC R: 5′ GACGGACACATTGGGGGTAG
GUSB (Gusb)	Glucuronidase β	110006	Enzyme in the stepwise degradation of glucuronic acid-containing glycosaminoglycans	F: 5′ CACCTGCCGGATCACGATT R: 5′ GATTCCTCACCGATGTCTTGC
HPRT1 (Hprt1)	Hypoxanthine guanine phosphoribosyl transferase 1	15452	Enzyme in conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate	F: 5′ TCAGTCAACGGGGGACATAAA R: 5′ GGGGCTGTACTGCTTAACCAG
RPLP0 (Rplp0)	Ribosomal protein, large P0	11837	Protein synthesis	F: 5′ GAAACTGCTGCCTCACATCCG R: 5′ CTGGCACAGTGACCTCACACG
TBP (Tbp)	TATA box binding protein	21374	Composed of transcription factor IID with TBP- associated factors	F: 5′ GCTCTGGAATTGTACCGCAG R: 5′ CTGGCTCATAGCTCTTGGCTC
TFRC (Tfrc)	Transferrin receptor	22042	Uptake of iron-loaded transferrin into cells	F: 5′ GTTTCTGCCAGCCCCTTATTAT R: 5′ GCAAGGAAAGGATATGCAGCA
Target genes				
C/EBPβ (Cebpb)	CCAAT/enhancer binding protein β	12608	Formation of a homodimer to CCAAT/enhancer regulatory region	F: 5′ TGGACAAGCTGAGCGACGAG R: 5′ TGTGCTGCGTCTCCAGGTTG
COX2 (Ptgs2)	Prostaglandin-endoperoxide synthase 2	18126	Synthase nitric oxide as a biological mediator in several processes	F: 5′ TGAGCAACTATTCCAAACCAGC R: 5′ GCACGTAGTCTTCGATCACTATC
IκBα (Nfkbia)	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor α	18035	NF-κB inhibitor by trapping it in the cytoplasm	F: 5′ TGAAGGACGAGGAGTACGAGC R: 5′ TTCGTGGATGATTGCCAAGTG
IFN-γ (Ifng)	Interferon gamma	15978	Cytokine in innate and adaptive immunity	F: 5′ ATGAACGCTACACACTGCATC R: 5′ CCATCCTTTTGCCAGTTCCTC
iNOS (Nos2)	Nitric oxide synthase 2, inducible	18126	Synthesize nitric oxide as biological mediator in several biological processes	F: 5′ GTTCTCAGCCCAACAATACAAGA R: 5′ GTGGACGGGTCGATGTCAC
SOCS3	Suppressor of cytokine signaling 3	12702	Inducible cytokine with negative regulators of cytokine signaling	F: 5′ ATGGTCACCCACAGCAAGTTT R: 5′ TCCAGTAGAATCCGCTCTCCT

Note. F, forward primer; R, reverse primer.
[a] Symbols are the gene symbols used in this article. Official symbols (in parentheses) are gene symbols from http://www.informatics.jax.org/mgihome/nomen.

mRNA expression was observed, and this is a downstream molecule of the activated NF-κB pathway. However, C/EBPβ and IκBα mRNA expression levels appeared to be inconsistent with previous reports when ACTB was used as the reference gene [15,16]. To find a reasonable explanation for the inconsistent results we obtained, the suitability of ACTB as the reference gene was called into question. Unfortunately, no report was found to have compared and validated candidate reference genes in the CIH model.

In this study, using Q-PCR, the expression levels of 10 well-recognized housekeeping genes (HKGs) were checked for the first time at different time points after ConA injection. Then their expression stability was tested using geNorm, NormFinder, and BestKeeper software. According to the software analysis results, the most stable HKGs among the 10 candidate reference genes were recommended for Q-PCR analysis in the CIH model.

The stability analysis results provide evidence for appropriate reference gene selection in the CIH model and make it possible to provide more reliable results of quantifying mRNA expression levels of target genes. Furthermore, the results make it possible to obtain more reasonable analysis of target gene functions in CIH.

Materials and methods

Animal treatment

C57BL/6 male mice, 8–10 weeks of age, were purchased from Shanghai Laboratory Animal Company (SLAC, Shanghai, China). Mice were housed in the animal facilities of the Shanghai Institute of Endocrine and Metabolic Diseases, Shanghai Jiao-Tong University School of Medicine, under pathogen-free conditions according to the institutional animal care and use committee guidelines. Mice were fed ad libitum a standard laboratory chow diet provided by SLAC.

CIH model

Mice (4 or 5 per group) were injected via the tail vein with a single dose (15 mg/kg body weight) of ConA (Vector Laboratories, Burlingame, CA, USA) to produce the hepatitis model. In control-treated animals, only the carrier solution (phosphate-buffered saline [PBS]) was injected. Mice were injected at 8:00 am, and liver tissues were removed at different time points after neck dislocation. For each animal, the left lobe of the liver was collected for histological purposes and the right lobe was collected for Q-PCR and DNA extraction.

Histology

For observation of hepatic morphological changes during CIH, livers were removed after neck dislocation and fixed in 4% phosphate-buffered paraformaldehyde (Sigma, St. Louis, MO, USA) and then embedded in paraffin. Tissue sections (5 μm) were prepared, stained with hematoxylin and eosin (H&E, Baso Diagnostics, Taipei, Taiwan), and examined under Olympus BX51 light microscopy with an Olympus DP71 digital camera (Tokyo, Japan). Images were analyzed with Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). A total of 10 tissue sections were analyzed for each animal.

RNA extraction and reverse transcription

Total RNA was isolated from liver tissues using the standard TRIzol method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). All RNA samples were examined as to their concentration and purity. RNA purity was measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Based on the absorbance ratio at 260/280 nm (mean ± standard deviation = 1.93 ± 0.04), all RNA samples were pure and protein free. RNA was stored at −80 °C before use. First-strand complementary DNA (cDNA) synthesis was performed for each RNA sample using the Promega Reverse Transcription System (Madison, WI, USA). Oligo dT was used to prime cDNA synthesis.

Quantitative real-time RT-PCR

Q-PCR was performed for determination of hepatic mRNA expression of related genes during CIH. Q-PCR was performed by using TaKaRa SYBR Master Mix (Shiga, Japan) on LightCycler 480 (Roche, Basel, Switzerland). PCR conditions included an initial holding period at 95 °C for 5 min, followed by a two-step PCR program consisting of 95 °C for 5 s and 60 °C for 30 s for 50 cycles. Data were collected and quantitatively analyzed using LightCycler 480 software (release 1.5.0). Primers for those genes were selected according to PrimerBank (http://pga.mgh.harvard.edu/primerbank), except for C/EBPβ and RPLP0 from Refs. [17,18], and the sequences are listed in Table 1. Relative quantitation analysis of gene expression data was conducted according to the $2^{-\Delta\Delta Ct}$ method [19].

Hepatic DNA fragmentation analysis

For semiquantitative determination of DNA fragmentation, the pattern of low-weight DNA was analyzed on agarose gel electrophoresis. Fragmented DNA was isolated using a genomic DNA extraction kit according to the manufacturer's instructions (Beyotime, Hangzhou, China). The eluants containing DNA pellets were subsequently electrophoresed on a 2% agarose gel, and ethidium bromide staining was performed. A DL2000 molecular weight marker was used for gel electrophoresis (TaKaRa). Gel images were captured with the Syngene G:BOX Imaging System (Cambridge, UK).

Statistical analysis of gene expression and software determination of appropriate HKGs

For stability comparisons of candidate reference genes, three validation software programs—geNorm (http://medgen.ugent.be/~jvdesomp/genorm) [20], NormFinder (http://www.mdl.dk/publicationsnormfinder.htm) [21], and BestKeeper (http://gene-quantification.com/bestkeeper.html) [22]—were used according to their original publications. All of the results are expressed as means ± standard errors (SE). Statistical comparisons between two groups were made using Student's *t* test after analysis of variance. The level of significance was set at $\alpha = 0.05$. All of the tests were two-sided.

Results

ConA treatment induces hepatitis and hepatocellular apoptosis

Hepatic changes were examined histologically and biochemically at different time points after ConA injection. Consistent with a previous report [23], serious necrotic phenotypes were observed in livers at 8 and 24 h after ConA injection, whereas the necrotic phenotype nearly disappeared at 72 h (Fig. 1A). Simultaneously, based on the genomic DNA fragmentation analysis [16], hepatocytes demonstrated obvious apoptosis at 8 h as compared with the control group, and even more serious at 24 h, whereas the apoptosis decreased markedly at 72 h (Fig. 1B). Thus, both the his-

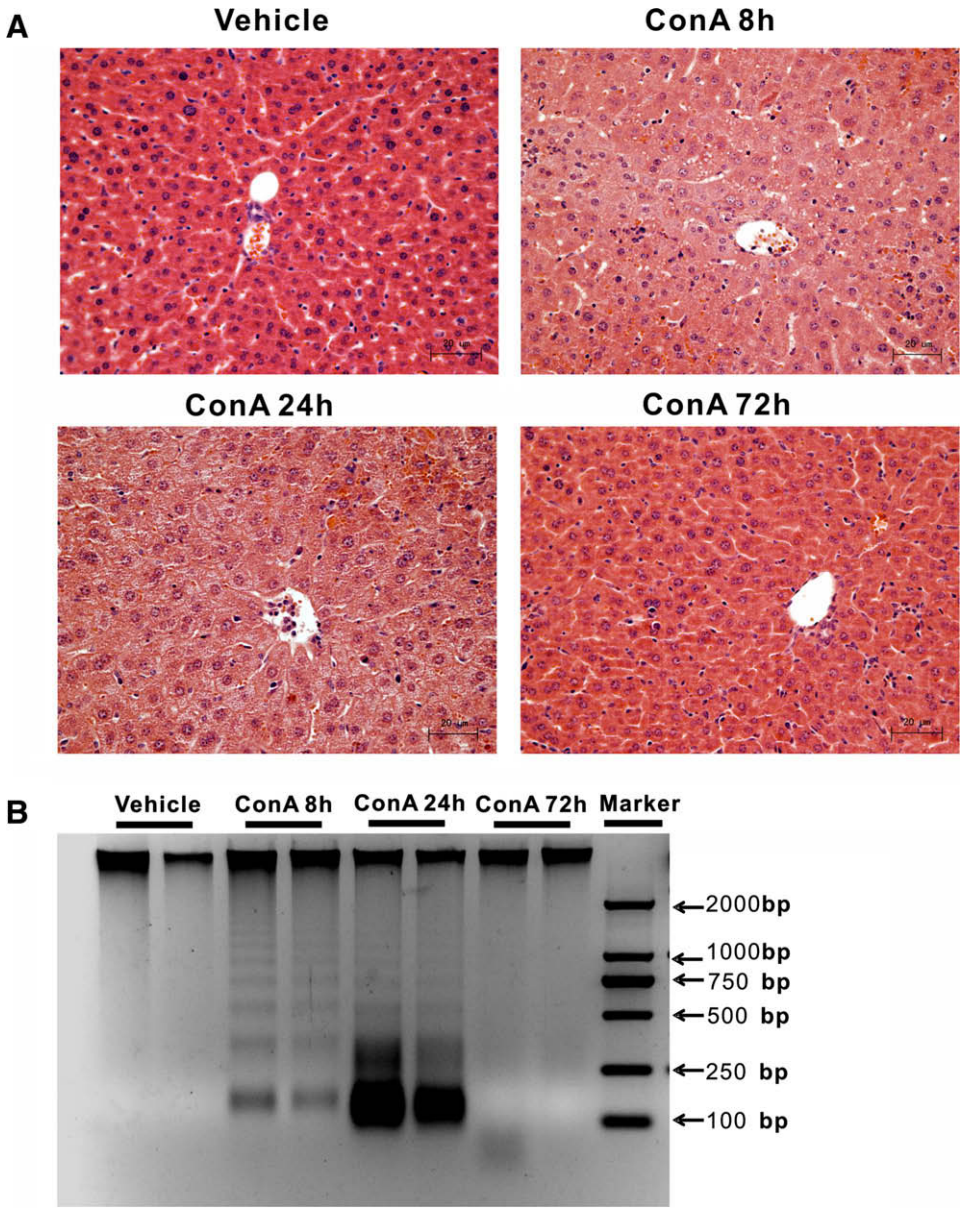

Fig. 1. ConA-induced acute liver injury. Four groups of C57BL/6 mice (n = 4 or 5) received a ConA (15 mg/kg) injection via the tail vein. (A) H&E staining of liver sections acquired from ConA-injected mice in a quiescent condition (0 h) and 8, 24, and 72 h after ConA injection. Original magnification, 400×. (B) Hepatic DNA fragmentation analysis after ConA injection. Genomic DNA from ConA-treated liver tissues at quiescent (0 h) and 8, 24, or 72 h after injection was extracted and run on agarose gel electrophoresis and is shown with black–white color reversed.

tological and DNA fragmentation analyses in Fig. 1 indicate that a typical form of hepatitis is induced by ConA in this study.

Induction of IFN-γ, iNOS, SOCS, and COX2 expression after ConA injection

To further study the expression profiles of specific genes during CIH, mRNA levels of those genes were determined using Q-PCR. ACTB was previously selected as a reference gene for normalization of mRNA expression levels in several mouse liver injury models, including the CIH model [11,24]. Therefore, ACTB was used as the reference gene at the beginning of our study. It is known that IFN-γ and iNOS are two of the most important inflammatory molecules induced on NF-κB activation, and both of them are the most commonly used markers of ConA-induced liver injury [14,15]. Fig. 2A and B show that IFN-γ and iNOS mRNA were significantly induced after ConA injection. COX2 and SOCS3 are also recognized as markers of inflammatory response on NF-κB activation in certain mouse models [25,26]. Their mRNA expression levels were also checked after ConA injection, as shown in Fig. 2C and D. Similarly, the mRNA levels were significantly elevated during CIH. Based on the Q-PCR analysis of selected genes shown in Fig. 2, successful induction of hepatitis by ConA is further confirmed in this study.

Unsuitability of ACTB and GAPDH as reference genes for Q-PCR analysis in CIH model

We are interested in the functions of C/EBPβ and IκBα during CIH progression. C/EBPβ plays a very important role in the pathogen-induced inflammatory response. It binds to the promoters of target genes and initiates transcription on stimulation [25]. Furthermore, C/EBPβ is increased dramatically after ConA injection

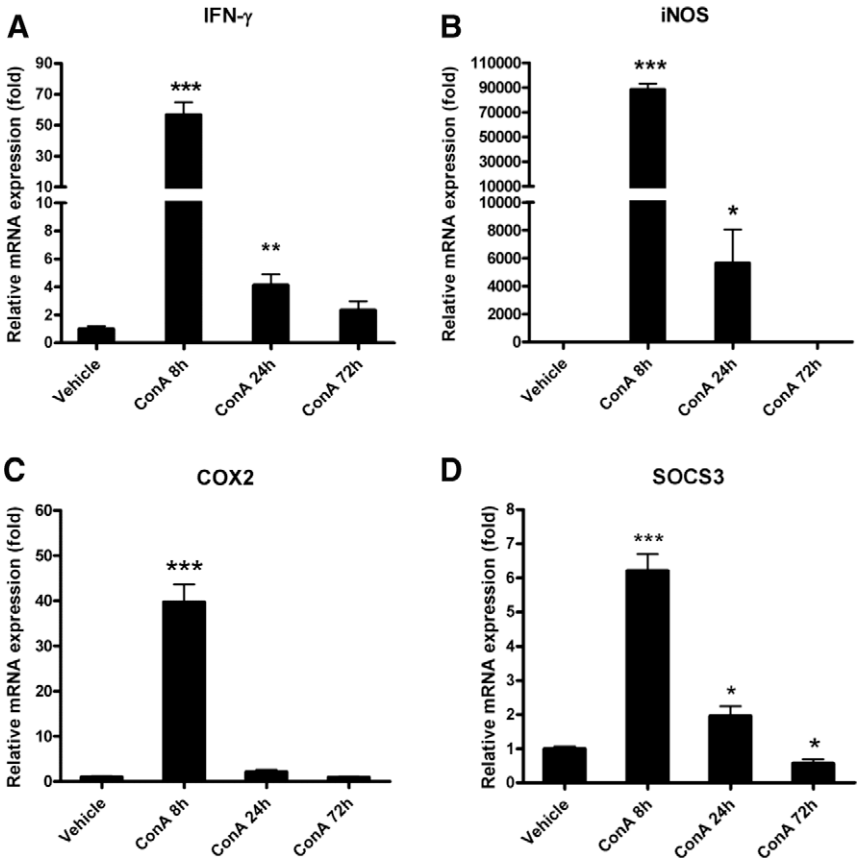

Fig. 2. Relative mRNA expression of genes after ConA injection. Shown are relative INF-ã (A), iNOS (B), COX2 (C), and SOCS3 (D) mRNA expression levels at indicated time points after ConA injection with ACTB as the reference gene for normalization. Relative quantitation analysis of gene expression data was conducted according to the $2^{-\Delta\Delta Ct}$ method. Data are expressed as ratios to the control liver samples and are represented as means ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

and is involved in hepatocellular proliferation when liver damage occurs [16]. IκBα is an inducible gene for feedback repression of NF-κB activation. When NF-κB target genes are induced, the mRNA expression level of IκBα will increase subsequently [15]. Surprisingly, as shown in Fig. 3A and B, both C/EBPβ and IκBα expression levels were not increased but rather significantly reduced. The result we obtained is inconsistent with previous reports [15,16].

We then asked what may have contributed to the reduced mRNA expression levels of C/EBPβ and IκBα, as shown in Fig. 3A and B. One of the considerations is the selection of an appropriate reference gene for the CIH model. To settle this point, we went though previous reports and found that ACTB mRNA expression level had significantly changed in a mouse partial hepatectomy model [27,28]. Therefore, we checked the mRNA expression levels of two frequently used reference genes, ACTB and GAPDH, in the samples above. The mRNA expression levels at different time points were calculated relative to the control group without normalization by reference genes. Surprisingly, as Fig. 3C and D indicate, ACTB and GAPDH were significantly changed during CIH progression. Those results suggest that ACTB and GAPDH, although widely used, may be unsuitable as reference genes in the CIH model.

Selection of HKGs as candidate reference genes and determination of their non-normalized expression levels

To guide selection of appropriate reference genes for the CIH model, 10 of the most widely used HKGs, including ACTB and GAPDH, were checked for mRNA expression profiles at different time points during CIH. The basic information about those genes is listed in Table 1. Because the $2^{-\Delta\Delta Ct}$ method was used in this study, amplification efficiencies of the 10 HKGs were analyzed according to previous reports [29]. The result is shown in Supplementary Fig. 2 (see Supplementary material). Non-normalized expression levels of the 8 candidate reference genes are shown in Fig. 4, with ACTB and GAPDH already displayed in Fig. 3C and D. The relative mRNA expression levels of those genes indicate that RPLP0, HPRT1, and TRFC are relatively stable in mRNA expression levels across different time points during CIH.

Statistical validation of appropriate reference genes by using geNorm, NormFinder, and BestKeeper

To analyze the expression stability of the 10 candidate reference genes in the liver samples (Table 1), geNorm, NormFinder, and BestKeeper programs were used [3]. geNorm is a statistical algorithm and was designed to determine the measure of stability (*M*) for all of the candidate genes. The measure is based on the geometric averaging of multiple control genes as well as the mean pairwise variation of a gene from all other control genes in a given set of samples [20,28]. The geNorm program relies on the principle that the expression ratio of two ideal internal control genes is identical in all of the samples regardless of the experimental condition. The genes with the lowest *M* values will be considered to have the most stable expression across the quiescent (0 h) and injured livers obtained at 8, 24, and 72 h after ConA injection. As a result, the ranking of gene expression stability (*M*) values of tested HKGs was as follows; ACTB > TBP > GUSB > B2M > TFRC > TUBA > GAPDH > PPIA > RPLP0 and HPRT1 (Fig. 5A). Analysis of the rank order data indicates that ACTB is the most unstable HKG, whereas RPLP0 and HPRT1 rank as the most stable HKGs. Therefore, RPLP0 and

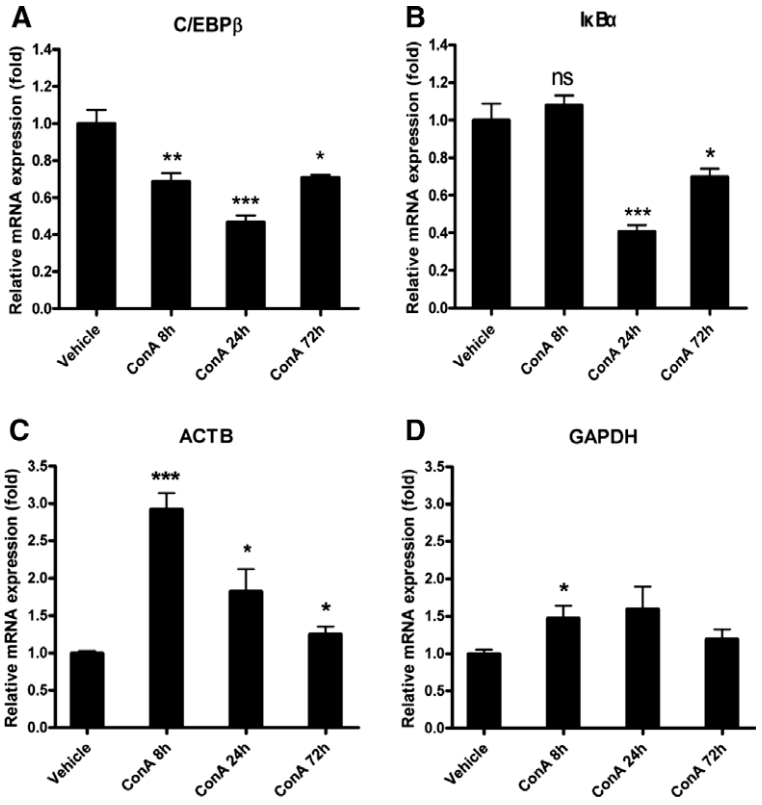

Fig. 3. Relative mRNA expression levels of C/EBPâ, IêBá, ACTB, and GAPDH after ConA injection. (A and B) Relative C/EBPâ (A) and IêBá (B) mRNA expression levels at indicated time points after ConA injection with ACTB as the reference gene for normalization. (C and D) Relative ACTB (C) and GAPDH (D) mRNA expression levels at different time points after ConA injection without normalization with reference gene. Relative quantitation analysis of gene expression data was conducted according to the $2^{-\Delta\Delta Ct}$ method. Data are expressed as ratios to the control liver samples and are represented as means ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

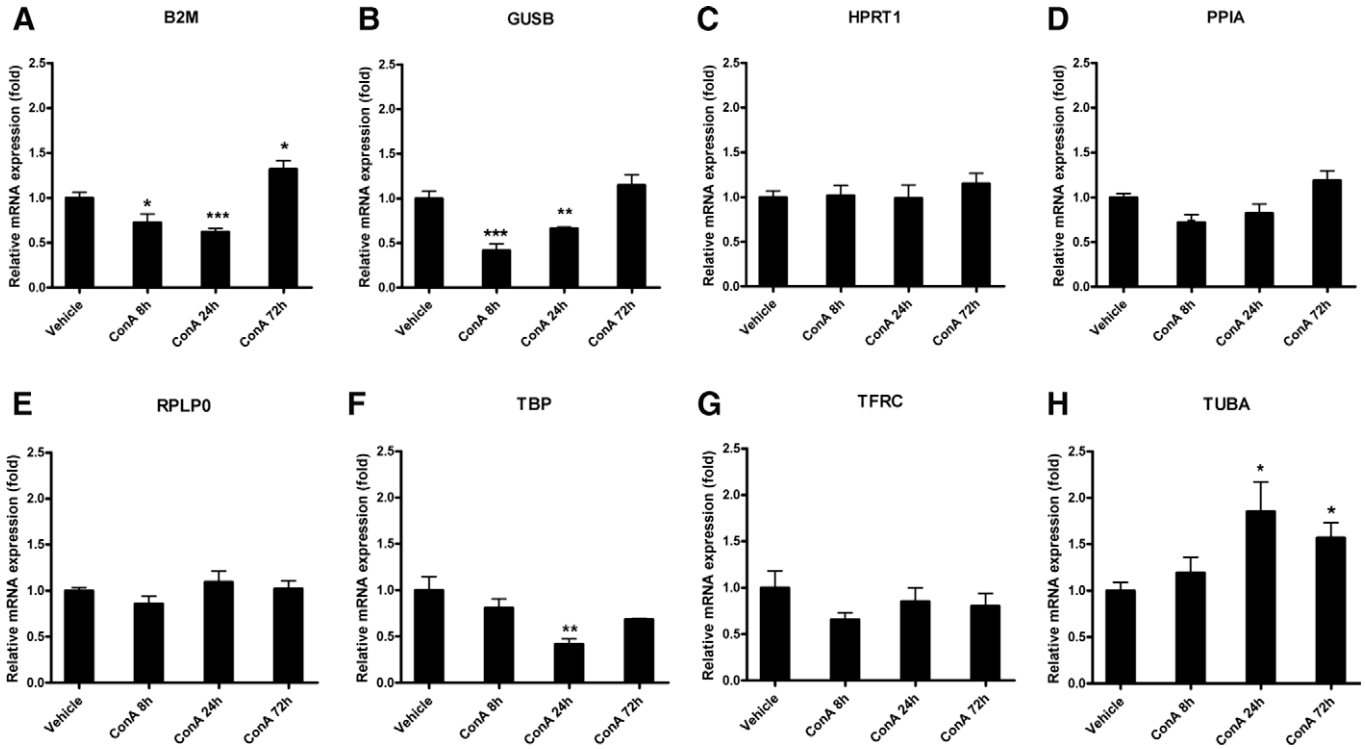

Fig. 4. Relative mRNA expression levels of selected HKGs at different time points after ConA injection. Shown are mRNA expression levels of B2M (A), GUSB (B), HPRT1 (C), PPIA (D), RPLP0 (E), TBP (E), TFRC (F), and TUBA (G) at different time points after ConA injection relative to control group without normalization. Relative quantitation analysis of gene expression data was conducted according to the $2^{-\Delta\Delta Ct}$ method. Data are expressed as ratios to the control liver samples and are represented as means ± SE. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

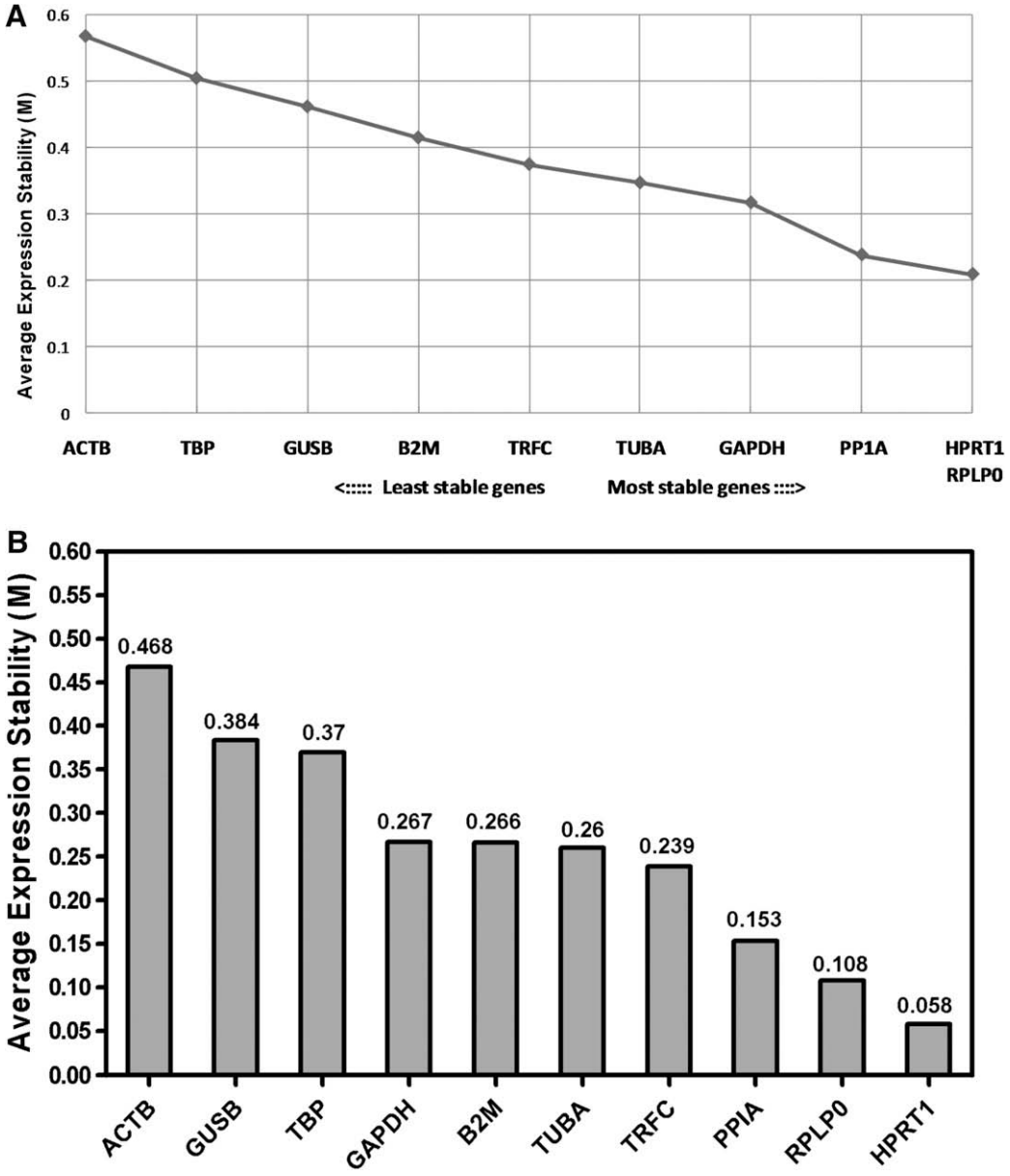

Fig. 5. Software analysis of expression stability for the 10 HKGs. (A) geNorm analyses of average expression stability values (*M*) of the 10 HKGs indicated above. (B) NormFinder analysis of average expression stability values (*M*) of the 10 HKGs indicated above. HKGs were graphed to illustrate the average gene expression stability on the *y* axis and its associated ranking from least to most stable expression (*x* axis) as calculated by geNorm and NormFinder (n = 19 liver samples). Lower *M* values of average expression stability indicate more stable expression.

HPRT1 are ideal for the quantitative analysis due to their expression stability during the liver injury and regenerative time line.

NormFinder was also used for ranking the 10 HKGs in the CIH model. NormFinder was designed to calculate stability by using the combined estimation of intra- and intergroup expression variations of the analyzed genes [21,28]. The calculated stability values of the 10 HKGs are shown in Fig. 5B. Based on these values, the NormFinder program validates the findings of the geNorm algorithm, where the most unstable gene was ACTB, the most stable single gene was HPRT1, and the best combination of HKGs was RPLP0 and HPRT1, although the ranking of the other seven genes differs from that of the geNorm result.

For further validation and a more detailed understanding of the software analysis data of the ranking, BestKeeper was applied to determine the best-suited HKG. All data processing by BestKeeper is based on the crossing point (CP) or threshold cycle (Ct) basis. Because CP or Ct is normally distributed, and a parametric test can be performed in correlation and regression analysis, the use of the CP or Ct seems to be the best estimator for the expression levels. Besides, BestKeeper may provide more detailed information from the analysis. The result of BestKeeper analysis is listed in Table 2. Based on the CV (coefficient of variance expressed as a percentage of the CP) level value analysis described in Refs. [22,30], ACTB remained the most unstable HKG, whereas RPLP0 and HPRT1 were ranked the most stable HKGs, a finding that is relatively consistent with the other two software results mentioned above. Yet the ranking of the other seven genes differed from the geNorm and NormFinder results. However, RPLP0 ranked as the most stable single gene instead of HPRT1, as compared with the NormFinder result.

Besides, the time course experiment of the Q-PCR analysis needs a complementary analysis as described previously [31]. The detailed complementary analysis is shown in Supplementary Fig. 1 and Supplementary Table 1 (see Supplementary material).

Table 2
Detailed expression stability analysis of housekeeping genes by BestKeeper software.

Gene	ACTB	B2M	GUSB	TUBA	GAPDH	PP1A	TBP	TRFC	HPRT1	RPLP0
n	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00	19.00
geo Mean (CP)	18.97	16.10	25.61	20.40	17.50	17.86	26.76	24.10	22.64	18.83
ar Mean (CP)	18.98	16.11	25.62	20.41	17.51	17.8	26.77	24.10	22.65	18.84
Min (CP)	17.81	15.29	24.70	19.26	16.53	17.15	25.59	23.09	22.12	18.20
Max (CP)	19.78	16.72	27.08	21.10	18.01	18.58	28.03	24.77	23.17	19.36
Std dev. (±CP)	0.57	0.42	0.52	0.40	0.33	0.33	0.43	0.39	0.27	0.20
CV (% CP)	2.99	2.59	2.03	1.97	1.90	1.85	1.62	1.61	1.18	1.08
Min (*x*-fold)	−2.23	−1.76	−1.88	−2.21	−1.97	−1.64	−2.26	−2.01	−1.44	−1.56
Max (*x*-fold)	1.76	1.54	2.76	1.62	1.42	1.64	2.41	1.60	1.44	1.44
Std dev. (±*x*-fold)	1.48	1.34	1.43	1.32	1.26	1.26	1.35	1.31	1.20	1.15

Note. Shown are descriptive statistics of 10 HKGs based on their crossing point (CP) values. *n*, number of samples; geo Mean (CP), geometric mean value of the CP; ar Mean (CP), average mean value of the CP; Min (CP) and Max (CP), extreme values of CP; Std dev. (±CP), standard deviation of the CP; CV (% CP), coefficient of variance expressed as a percentage on the CP level; Min (*x*-fold) and Max (*x*-fold), extreme values of expression levels expressed as absolute *x*-fold over or under regulation coefficient; Std dev. (±*x*-fold), standard deviation of the absolute regulation coefficients.

Taken together, the software analysis results indicate that ACTB ranked as the most unstable HKG, whereas HPRT1 and RPLP0 ranked as the most stable HKGs. Therefore, ACTB might not be a good choice for use as a reference gene, whereas HPRT1 and RPLP0 may serve well as the reference genes in the CIH model.

mRNA expression levels of target genes are influenced by the choice of normalization gene(s)

The expression profiles of target genes can be markedly influenced depending on the choice of the normalization gene(s) [1]. To indicate the influence of different reference genes on determination of gene expression levels, C/EBPβ and IκBα expression levels were sequentially normalized with the 10 HKGs. As shown previously in Figs. 2 and 3A,B, mRNA expression levels of several HKGs were significantly changed at 8 h after ConA injection. So, expression levels at 8 h after ConA injection were analyzed in this part. The expression levels of C/EBPβ and IκBα were normalized to the 10 different HKGs shown in Fig. 6A and C, respectively. As shown in Fig. 6A, the C/EBPβ mRNA expression level was calculated relative to the control group without normalization to a reference gene (the second bar), which was assumed to be the most accurate result of analyzing the expression level. Subsequent bars represented the different mRNA expression levels of C/EBPβ normalized by different HKGs. As shown in Fig. 6A, when ACTB or GUSB was used as the reference gene, the relative mRNA expression level of C/EBPβ was significantly different compared with the non-normalized data. Similarly, as shown in Fig. 6C, the relative mRNA expression levels of IκBα were significantly different compared with the non-normalized data when ACTB, GAPDH, PPIA, TRFC, or GUSB was used for normalization. However, as shown in Fig. 6A and C, relative mRNA expression levels of C/EBPβ and IκBα were relatively equal to the non-normalized expression levels when HPRT1 or RPLP0 was used. Finally, RPLP0 was used as the reference gene for determination of C/EBPβ and IκBα mRNA expression levels at different time points, as shown in Fig. 6B and D. Those data indicate that some HKGs, especially ACTB and GUSB, are inappropriate as the reference gene for determination of relative mRNA expression levels in the CIH model, whereas HPRT1 and RPLP0 are suitable for this purpose.

Discussion

Hepatitis is a potentially life-threatening liver disease. Mouse hepatitis models provide valuable insights in attenuating and curing the disease. CIH in mouse is a model developed during recent years and is used with increasing interest to investigators of liver disease [32]. Gene expression profiles at mRNA and protein levels during hepatitis progression are extremely valuable for functional analysis. Q-PCR analysis provides great convenience and accuracy in determination of target gene mRNA expression during the progression of CIH. However, selection of reference genes could significantly contribute to the result obtained.

Although validations of candidate reference genes in other models have been reported recently [19,28,33], no publication has reported the validation of reference genes in the CIH model. To date, both ACTB and GAPDH are commonly used as reference genes in most disease models. Furthermore, the application of ACTB or GAPDH as a reference gene is reinforced in accumulating publications [11,24]. Nevertheless, suitability validation of ACTB and GAPDH is necessary from a scientific perspective.

As to this study, ACTB was used as the reference gene for determination of C/EBPβ and IκBα mRNA expression levels at first. However, inconsistent results from previous reports were obtained, as mentioned above and as shown in Fig. 2. The inconsistent results led to the further study to validate the stability of HKGs in this study. As shown in Table 1, 10 widely used HKGs were selected as candidate reference genes. Surprisingly, Figs. 3 and 4 show that the mRNA expression levels of several genes were significantly increased compared with the control group after ConA injection when calculated without normalization to a reference gene. As further shown in Fig. 5 and Table 2, the analysis of the Q-PCR data by geNorm, NormFinder, and BestKeeper indicate that ACTB and GAPDH were not the appropriate reference genes for the CIH model. Despite the differentially ranked stability of those HKGs, Figs. 4 and 5 and Table 2 show that RPLP0 and HPRT1 were the most stable among them. Therefore, they are recommended as the appropriate reference genes for Q-PCR analysis in the CIH model.

As shown in Fig. 5, results from geNorm and NormFinder analysis recommended the combination of HPRT1 and RPLP0 as the normalization method for the CIH model. However, it seems that the combined normalization strategy would not be of wide application. The use of a single reference gene was described in most publications, and a single appropriate reference gene could give results without significant difference compared with results obtained from the combined reference genes. However, if the combined normalization strategy is used according to the validated data, it will surely provide the most accurate result, especially when we are analyzing complex or scarce samples [19].

Based on the results from software analysis above, either RPLP0 or HPRT1 can be selected as the reference gene. However, RPLP0 rather than HPRT1 is recommended in this study based on the following considerations. First, BestKeeper analysis shows that RPLP0 was more stable than HPRT1 during CIH. Secondly, previous validating reports showed that expression stability of RPLP0 was higher than HPRT1 in the mouse liver partial hepatectomy model [28]. Third, RPLP0 has been in wide use, as described in recent publications, especially articles on energy metabolism [17]. Finally, RPLP0 expression at the mRNA level, as well as at the protein level,

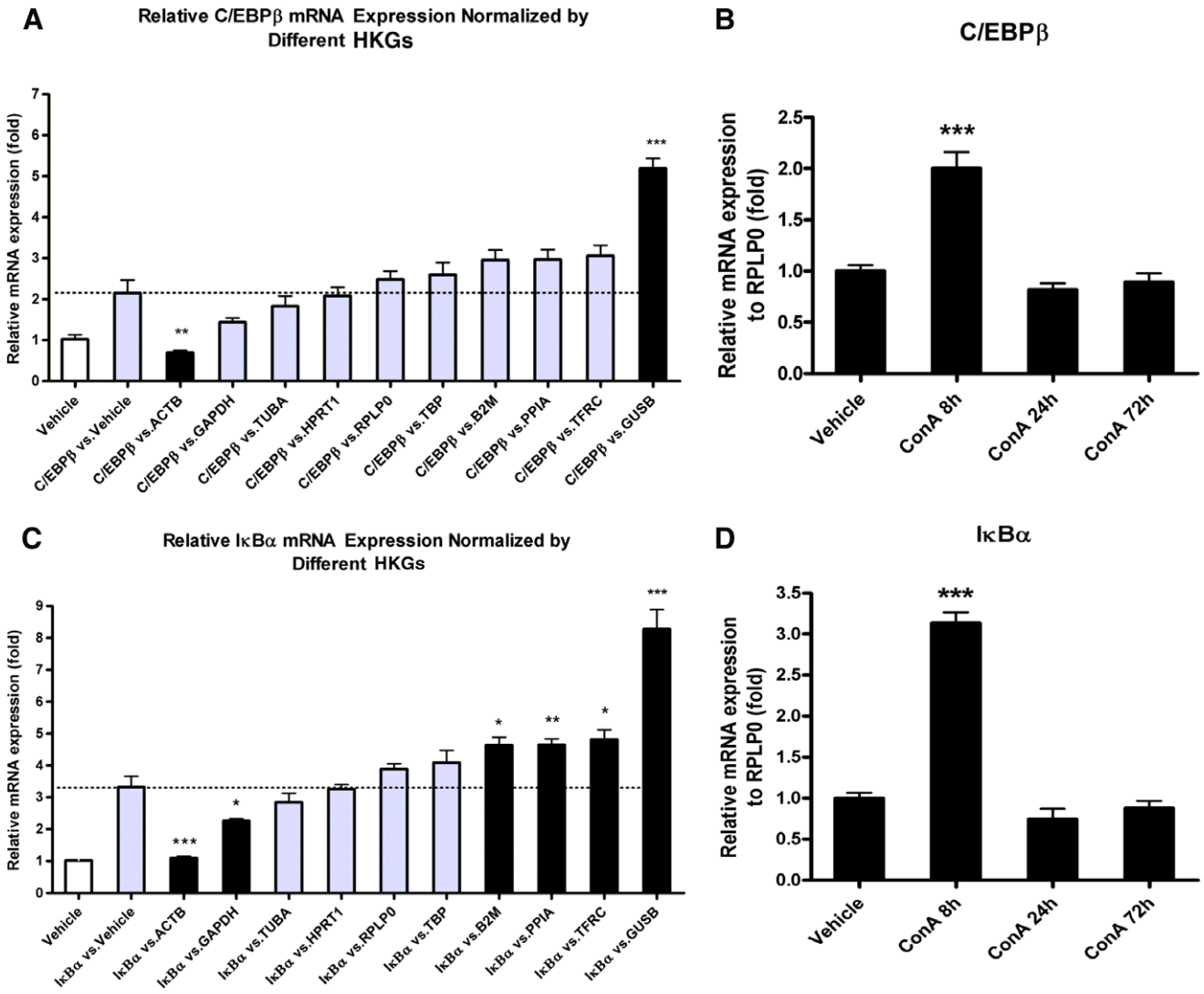

Fig. 6. Contribution of reference gene selection to determination of target gene mRNA expression levels. (A and C) Relative mRNA expression levels of C/EBPâ (A) and IêBá (C) at indicated time points after ConA injection with normalization by the 10 different reference genes indicated above. (B and D) Relative mRNA expression levels of C/EBPâ (B) and IêBá (D) at indicated time points after ConA injection normalization by RPLP0. Data are expressed as ratios to the control liver samples and are represented as means ± SE. Statistical analysis was made between the normalized values by different HKGs and the non-normalized value. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

showed insignificant variation among tissues [34]. It is acknowledged that liver is the key organ linking stress, inflammation, and metabolism [35,36]. Therefore, RPLP0 can be a first choice for a reference gene in the CIH model.

Taken together, results from the current study indicate that ACTB and GAPDH are inappropriate as the reference gene, although they are widely used in practice. Furthermore, RPLP0 is recommended as an appropriate reference gene among the 10 HKGs in this study. Application of RPLP0 as the reference gene will afford convenience as well as accurate results for investigators who are dealing with the mouse CIH model.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2010.02.007.

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