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Reference gene selection for real-time RT-PCR in regenerating mouse livers

Kohei Tatsumi [a], Kazuo Ohashi [b,*], Sanae Taminishi [a], Teruo Okano [b], Akira Yoshioka [a], Midori Shima [a]

[a] Department of Pediatrics, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan
[b] Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

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ABSTRACT

The liver has an intrinsic ability to undergo active proliferation and recover functional liver mass in response to an injury response. This regenerative process involves a complex yet well orchestrated change in the gene expression profile. To produce accurate and reliable gene expression of target genes during various stages of liver regeneration, the determination of internal control housekeeping genes (HKGs) those are uniformly expressed is required. In the present study, the gene expression of 8 commonly used HKGs, including GAPDH, ACTB, HPRT1, GUSB, PPIA, TBP, TFRC, and RPL4, were studied using mouse livers that were quiescent and actively regenerating induced by partial hepatectomy. The amplification of the HKGs was statistically analyzed by two different mathematical algorithms, geNorm and NormFinder. Using this method, PPIA and TBP gene expression found to be relatively stable regardless of the stages of liver regeneration and would be ideal for normalization to target gene expression.

The process of liver regeneration is a crucial intrinsic event by which the liver is able to recover from a loss of functional hepatic mass following injuries due to either surgical resection, or toxic, chemical or viral-based challenges [1,2]. The molecular events that are involved in the liver regeneration process are very complex, and the altered gene expressions ultimately orchestrate the integration of these distinct pathways to promote the regenerative biological response. Multiple studies have elucidated potential mechanistic pathways that may be involved in the process of liver regeneration, but many aspects of this phenomenon in terms of its gene expression profiles and its associated functional phenotypes remain to be further elucidated.

Among currently available methods to analyze gene expression profiles, reverse transcriptase coupled to real-time polymerase chain reaction (real-time RT-PCR) has recently been shown to be more efficient and reliable compared to the other methods [3]. To accurately quantify gene expression, one method is to normalize the target unknown gene expression level to an endogenously expressed reference gene(s), which are frequently housekeeping genes (HKGs). The ideal HKGs should be expressed at a constant level regardless of the liver regeneration status, otherwise the normalization using particular HKGs will lead to erroneous gene expression profiles of the target gene of interest. Due to the rapid and differential phenotypic changes during liver regeneration process, it is crucial to determine HKGs that remain unaltered throughout the regenerative time period, which generally is terminated by day 5 [2].

Statistical algorithms such as geNorm [4] and NormFinder [5] have been previously developed to evaluate the suitability of reference HKGs for use as a normalization marker following quantitative RT-PCR data in a given set of biological samples. Using these methods of statistical analysis, various HKGs have been recently assessed to determine their level of expression under specific conditions [6,7].

In the present study, we induced liver regeneration in mice using the most commonly used experimental procedure to promote hepatocyte proliferation, known as the 2/3 partial hepatectomy (PHx) [8]. Using this model of liver regeneration, the gene expression levels of 8 commonly used HKGs (GAPDH, ACTB, HPRT1, GUSB, PPIA, TBP, TFRC, and RPL4) and 2 liver-expressing target genes were investigated in quiescent and actively proliferating livers at different time points following PHx. The RT-PCR results were subsequently analyzed by two computer programs, geNorm and NormFinder, to select the best candidate reference gene during the liver regenerative process. To our knowledge, the present study describes the first systematic study to examine a series of HKGs to determine their utility as reference markers to evaluate the expression levels of target genes in regenerating mouse livers.

* Corresponding author. Fax: +81 3 3359 6046.
E-mail address: ohashi@abmes.twmu.ac.jp (K. Ohashi).

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Materials and methods

Animals. A total of 25 female wild-type C57BL/6 mice, 10–12 weeks old, were used in this study. Experimental protocols were developed in accordance with the guidelines outlined by our local animal committee located at Nara Medical University. Mice were placed in cages within a temperature-controlled room having a 12-h light/dark cycle (8:00 AM lights on/8:00 PM lights off).

2/3 Partial hepatectomy (PHx). The stimuli for liver regeneration was induced by a 2/3 partial hepatectomy on the C57BL6 wild-type mice ($n = 25$) as described previously [8–11]. The hepatectomy was performed at within a specified time window (between 8:00 and 10:00 AM) in order to minimize the circadian rhythm variations that may influence the speed and peak of the regenerative activity [12]. The liver lobes that were removed from each mouse at the time of hepatectomy were used as control liver samples in the quiescent state ($n = 25$). The mice were sacrificed at different time points (1, 2, and 5 days after hepatectomy; $n = 9$, 8, and 8, respectively) at which time the remainder of the liver lobes were harvested to determine the gene expression at different temporal points of liver regeneration.

RNA isolation and quality controls. Total RNA was extracted from each liver sample using the RNeasy Mini Kit (QIAGEN, Hilden, Germany). DNaseI was used to digest and remove genomic DNA contamination. The RNA concentration of each sample was measured at a wavelength of 260 nm (A260). The purity of extracted total RNA was determined by the A260/A280 ratio. The real-time RT-PCR analyses were only performed on samples that had A260/A280 ratios between 1.9 and 2.1. The integrity of RNA samples was confirmed by electrophoresis on a 1% agarose gel.

Reverse transcription (RT) coupled quantitative real-time PCR. Total RNA (1 μg) was reverse-transcribed using oligo $d(T)_{16}$ primers and Omniscript RT Kit (QIAGEN). First-strand cDNA samples were subjected to quantitative PCR amplification using the PRISM 7700 Sequence Detector (Applied Biosystems Japan, Tokyo, Japan). Each of the liver cDNAs was determined for the expression levels of 8 commonly used housekeeping genes (HKGs) and two target genes as shown in Table 1. TaqMan probes and primers were chosen from the TaqMan Gene Expression Assay (Applied Biosystems) (Table 1). All of the PCR cycling conditions were 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. For quantification of gene expression, the cDNAs derived from pooled normal mouse livers were used to generate the reference standard curves.

Statistical analysis and determination of appropriate HKGs by geNorm and NormFinder. Significant differences between the non-normalized gene expression levels of quiescent and regenerating liver samples were analyzed by two-tailed Mann–Whitney *U*-test using Excel with ystat2006 software (Igakutosyosyuppan, Tokyo, Japan). $P < 0.05$ was considered significant. For stability comparisons of candidate reference genes, two additional validation software programs, geNorm (http://medgen.ugent.be/~jvdesomp/genorm/) [4], and NormFinder (http://www.mdl.dk/publicationsnormfinder.htm) [5] were used.

Results

RNA quality control

The mean A260/280 ratio of the extracted RNA of the 25 quiescent and 25 regenerating livers averaged 2.02 ± 0.09 (ranging from 1.95 to 2.10), reflecting the purity and protein-free nature of the RNA. The RNA integrity was characterized by the 28 S/18 S ratio on a 1% agarose gel, and the ratios of all samples were >1.0.

Non-normalized expression levels of candidate reference genes

Non-normalized gene expression levels of 8 candidate HKGs were quantified, and the data was reported as comparative ratio to the day 0 samples. As shown in Fig. 1, the expression levels of GAPDH, ACTB, GUSB, PPIA, and TFRC were significantly higher at days 1 and/or 2 after the hepatectomy, and tended to decrease back towards basal levels by day 5. The *P* values from day 1, 2, and 5 compared to the day 0 values are as follows: GAPDH: 0.007 (day 1), 0.195 (day 2), and 0.46 (day 5), ACTB: 0.053, 0.0004, and 0.649, GUSB: 0.158, 0.004, and 0.051, PPIA: 0.028, 0.0002, and 0.125, and TFRC: 0.758, 0.006, and 0.074. HPRT1 expression was also significantly upregulated at day 2, but the upregulated levels persisted through to day 5. The *P* values of day 1, 2, and 5 compared to the day 0 values were 0.065, 0.001, and 0.017, respectively. In marked contrast, expression levels of TBP and RPL4 genes did not show any significant differences between day 0 with the other days 1, 2, and 5; the *P* values were as follows: TBP: 0.644, 0.23, and 0.35; and RPL4: 0.073, 0.249, and 0.258, respectively. These indicated that the gene expression levels of TBP and RPL4 were constant during the liver regeneration regardless of the time point after partial hepatectomy.

Statistical validation of an appropriate reference gene by geNorm and NormFinder

To analyze and provide a rank order of the 8 candidate HKGs in the quiescent and regenerating liver samples, we utilized geNorm

Table 1
Description of housekeeping gene (HKG) and target gene primers used in the quantitative RT-PCR assay

Symbol	Gene name	Function	Assay ID	Amplicon length (bp)
Housekeeping genes				
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	Mm99999915_g1	107
ACTB	Actin, beta	Cytoskeletal structural protein	Mm00607939_s1	115
HPRT1	Hypoxanthine phosphoribosyltransferase	Purine synthesis in salvage pathway	Mm03024075_m1	131
GUSB	Glucuronidase, beta	Glycoside hydrolases cleaving glucuronides	Mm00446953_m1	76
PPIA	Peptidylprolyl isomerase A	Catalyzing the *cis-trans* isomerization of proline imidic peptide bonds in oligopeptides and accelerating the folding of proteins	Mm02342430_g1	148
TBP	TATA box binding protein	General RNA polymerase II transcription factor	Mm00446973_m1	73
TFRC	Transferrin receptor	Carrier protein for transferrin	Mm00441941_m1	66
RPL4	Ribosomal protein L4	Component of the 60S subunit of ribosome	Mm00834993_g1	129
Target genes				
OTC	Ornithine carbamoyltransferase	Enzyme catalyzing the reaction of urea cycle	Mm00493267_m1	102
AAT	α1-antitrypsin	Glycoprotein functioning as serum trypsin inhibitor	Mm00522856_m1	58

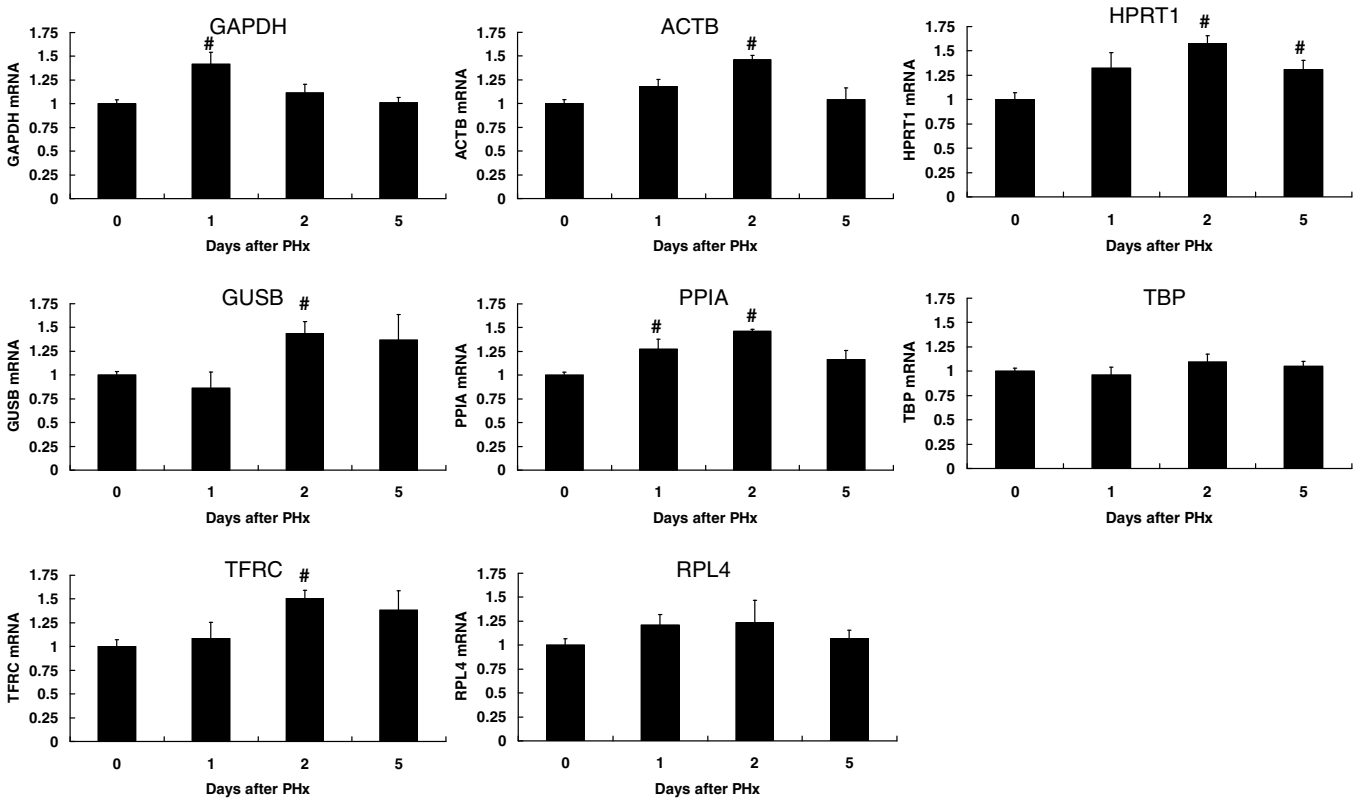

Fig. 1. The expression levels of 8 candidate HKGs in mouse livers. Non-normalized expression levels of the 8 HKGs of mouse livers under quiescent and regenerating status. The liver lobes removed at the time of hepatectomy were used as liver samples under quiescence status (day 0, $n = 25$). The remaining liver lobes obtained 1, 2, or 5 days after hepatectomy were used as samples to study the different stages of regeneration ($n = 9$, 8, and 8, respectively). The data was expressed as a comparative ratio to the day 0 samples, and represented as the mean ± SEM. $^{\#}P < 0.05$ vs day 0.

software. The geNorm is a statistical algorithm that was designed to determine the measure of stability (M) for all of the candidate genes 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 [4].

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 time in the quiescent and regenerating livers obtained at days 1, 2, and 5 after the hepatectomy procedure. As a result, the ranking of gene expression stability value (M) of tested HKGs were as follows; GUSB > TFRC > RPL4 > HPRT1 > ACTB > GAPDH > PPIA, and TBP. This rank order data analysis indicated that PPIA and TBP were the most stable HKGs making them ideal for our quantitative analyses due to their lack of change during the liver regenerative time line (Fig. 2A).

In some cases, the normalization with two or more stable internal control reference genes may be required. Therefore, pairwise variations were calculated using geNorm for each data set to determine the optimal number of internal reference genes needed for normalization. In brief, the normalization factors (NF) were calculated for the most stable control genes (i.e., the lowest M value) and then other reference genes with the next lowest M values were added in a stepwise manner. Subsequently, pairwise variations of NF_n and NF_{n+1} were calculated, reflecting the effect of including additional ($n + 1$) genes [4]. From these analyses, two genes with most stable expression (TBP and PPIA) were found to be optimal for reliable normalization with a pairwise variation cut-off value of 0.15 (Fig. 2B). Below this cut-off value, there was no need to include an additional HKG, because the inclusion of a third gene had no significant effect on the pairwise variation.

Next, we used NormFinder, which was another algorithm to calculate the most stable HKGs in our set of 8 candidate HKGs [5]. The

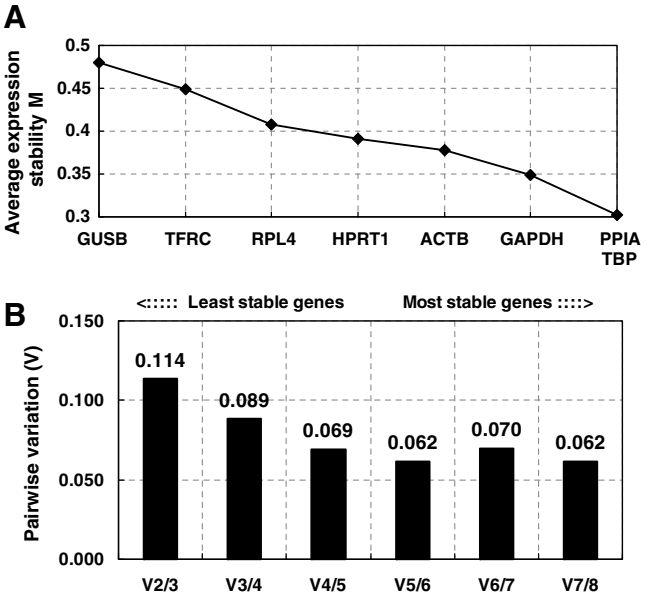

Fig. 2. (A) Average expression stability values (M) of 8 candidate housekeeping genes (HKGs). HKGs were graphed to illustrate the average gene expression stability (M) on the Y-axis, and its associated ranking from least to most stable expression (X-axis) as calculated by geNorm ($n = 50$ liver samples). Lower M value of average expression stability indicates more stable expression. (B) Determination of the optimal number of control genes for normalization. The optimal number of control genes for normalization was calculated on the basis of a pair-wise variation (V) analysis.

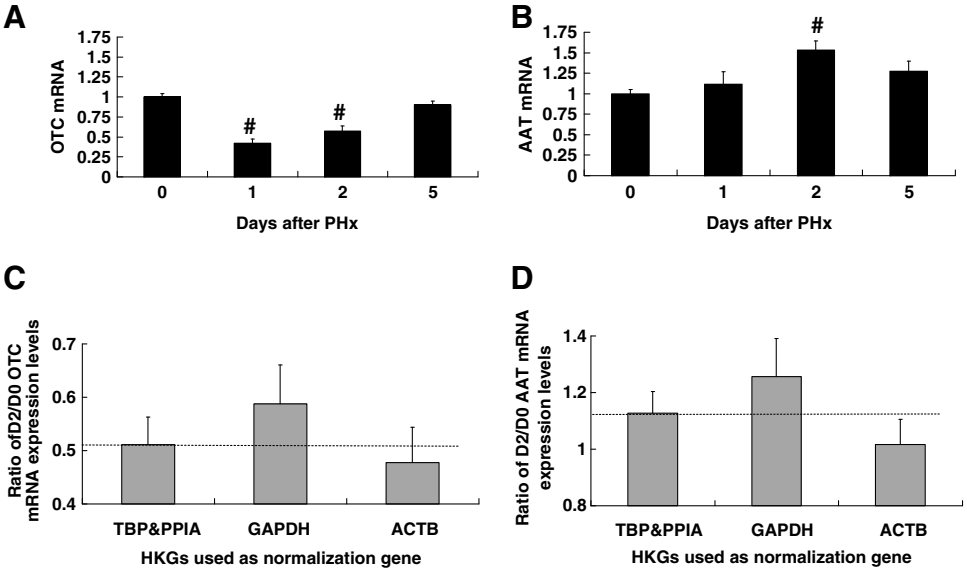

Fig. 3. Expression levels of two target genes, ornithine carbamoyltransferase (OTC) and α1-antitrypsin (AAT). (A and B) Non-normalized expression of two target genes transcribed in mouse livers during states of quiescence and active regeneration post-hepatectomy. The liver lobes removed at the time of hepatectomy were used as quiescent liver samples (day 0, $n = 25$). The remaining liver lobes were obtained 1, 2, or 5 days after hepatectomy and used as the proliferative liver samples ($n = 9$, 8, and 8, respectively). Data was expressed as a ratio to the day 0 liver samples and were represented as the mean ± SEM. $^{\#}P < 0.05$ vs day 0. (C and D) The gene expression levels of OTC and AAT were normalized to three different sets of HKGs (the geometric mean of PPIA and TBP, GAPDH, and ACTB). The normalized gene expression values were shown for the day 2 liver samples relative to the corresponding expression values at day 0 ($n = 8$). Data was represented as the mean ± SEM.

NormFinder was designed to calculate the stability by using the combined estimate of intra- and intergroup expression variations of the analyzed genes. The calculated stability values of the 8 candidate HKGs were as follows: GAPDH = 0.172; ACTB = 0.138; HPRT1 = 0.132; GUSB = 0.199; PPIA = 0.088; TBP = 0.118; TFRC = 0.167; and RPL4 = 0.142. Based on these values, the NormFinder program validated the findings with the geNorm algorithm, in which the most stable single gene was PPIA, and the best combination of the HKGs was PPIA and TBP.

Expression levels of target gene influenced by the choice of a normalization gene(s)

The expression profile of target genes can be markedly influenced depending on the choice of the normalization gene(s). The expression levels of the two target genes, ornithine carbamoyltransferase (OTC) and α1-antitrypsin (AAT), both of which are synthesized by liver, were assessed. We determined the non-normalized mRNA expression profile of OTC and AAT in the same liver samples used in the determination of the HKG expression profile. The expression of OTC genes was shown to be significantly suppressed 1 and 2 days after hepatectomy (Fig. 3A), whereas the AAT gene expression was upregulated 2 days after hepatectomy ($P < 0.05$ vs quiescent status) (Fig. 3B). The mRNA expression levels of the liver samples 2 days after hepatectomy were normalized against three different sets of HGK, specifically GAPDH, ACTB and the geometric mean of PPIA and TBP. Subsequently, the normalized day 2 values were divided by the normalized liver values obtained at day 0. As shown in Fig. 3C and D, though not significantly, both OTC and AAT mRNA levels that were normalized by the geometric mean of PPIA and TBP were lower than levels normalized by GAPDH, and higher than those normalized by ACTB. These results indicate that depending on which particular HKG was used in the normalization, the expression profile of the target gene can be influenced and produce different levels of gene expression.

Discussion

The appropriate choice of individual or a group of reference genes, in general endogenously expressed housekeeping genes (or HKGs), is critical in the quantification of gene expression profiles under different conditions, such as liver regeneration. In the present study, we examined 8 candidate HKGs that were selected in terms of their broad use in previously published studies using the liver and other type of tissues. The expression levels of the candidate HKG were assessed in quiescent or regenerating mouse livers at various stages of proliferation. The present results were based on geNorm and NormFinder analyses, which confirmed that the expression levels of the PPIA and TBP reference genes were the most stable among the 8 candidates regardless of the cell cycle status of the livers.

In the first 24 h following a partial hepatectomy in mice, hepatocyte DNA synthesis is initiated. Over the next 24 h period, the DNA synthesis phase become synchronized and peaks, and then slowly returns back to normal levels by day 5 after the hepatectomy. At day 5, the remnant liver lobes have undergone sufficient hyperplasia to return the liver weight back to its original pre-hepatectomy weight [2,13]. This rapid process from initiation to completion of the regeneration event prompted us to assess a panel of HKGs using liver samples harvested at day 1, 2, and 5 after the hepatectomy procedure.

In this present study, mRNA expression levels were upregulated in 6 out of 8 HKGs analyzed during the active phase of liver regeneration (days 1 and/or 2). The upregulation of these particular HKGs demonstrated that they likely play a vital role in the events related with DNA synthesis, cell division and hepatic growth. As an example, GAPDH and ACTB genes, which have been widely applied for the normalization of gene expression analysis of various types of tissues, showed significant increases in the mRNA expression at day 1 and/or 2 after hepatectomy (Fig. 1). The temporal upregulation of these two HKGs in the liver regeneration process were consistent with previous reports [14–16]. We have recently

showed that human hepatocytes undergoing active proliferation following transplantation into mouse liver exhibited higher GAPDH mRNA expression compared to normal human livers [17]. These data taken together, suggest that these two genes may not be appropriate reference genes to normalize target gene expression, particularly in the context of liver regeneration. Therefore, the identification of suitable internal reference genes that are not affected by a given experimental condition is critically important to accurately analyze expression levels of target genes. To address this problem, we utilized two different algorithms, geNorm and Normfinder [4,5], to select an optimal reference gene from a panel of candidate HKGs in quiescent and regenerating mouse livers. As a result, we found that the PPIA and TBP mRNA were most stably expressed, and both programs concluded that a combination of the two genes were the most appropriate for normalization of unknown target genes to determine their expression profiles in liver regeneration (Fig. 1). From examining the non-normalized RPL4 gene expression (Fig. 1), the values were found to be stable regardless of the time point. However, analyzing the expression data in a comprehensive manner using the two software programs demonstrated that the RPL4 reference gene was not stable. This is one example in which non-normalized data is only one method to demonstrate the potential reliability as a reference gene, but that other comparative analyses are needed, such as the geNorm and NormFinder, to further validate the suitability of selecting appropriate HKGs.

PPIA (peptidylprolyl isomerase A), also called as cyclophilin A, forms a ternary complex with cyclosporin A and the calcium-calmodulin-activated serine/threonine-specific protein phosphatase calcineurin [18]. This gene has been recently reported to be preferred over other commonly used HKGs following its analysis in various cell lines and tissues [19]. The other stably expressed reference gene, TBP (also known as TATA box binding protein), is a protein required by all three eukaryotic RNA polymerases to correctly initiate the transcription of ribosomal, messenger, small nuclear and transfer RNAs [20]. The usefulness of TBP gene has also been validated in other sets of experimental conditions [21]. Although there is a strong possibility that other more suitable reference genes other than the ones presently analyzed in our study, we have confirmed that PPIA and TBP genes gave us reliable and stable gene expression compared to other more commonly used HKGs, including ACTB or GAPDH (Fig. 3C and D).

The elucidation of liver regeneration events are further supported by recent studies in which hepatocyte-based approaches were developed, including liver tissue engineering [10,11,22], hepatocyte transplantation [23], hepatocyte propagation [17], and hepatic differentiation from stem cells [24]. Taken together, the results from the current study where we validated merit of normalizing target gene expression with the combined expression values of PPIA and TBP genes should serve as a viable method to accurately quantify gene expression profiles related with liver regeneration.

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