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ORIGINAL ARTICLE

Validation of reference genes for estimating wound age in contused rat skeletal muscle by quantitative real-time PCR

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Abstract Gene expression profiling by quantitative real-time PCR (RT-qPCR) is a valuable tool in forensic science for estimating the age of a wound. To accurately assess gene expression levels over time in injured tissue, the genes used as internal reference standards must be carefully validated for transcriptional stability. This study examined the transcriptional stability of nine potential reference genes (β-actin, GAPDH, RPL32, PGK1, SDHA, RPL13, HPRT, Tbp, and Ywhaz) in contused rat skeletal muscle by RT-qPCR. The raw Ct values were determined for each candidate gene at different time points following contusion, and the data were analyzed by the NormFinder, geNorm, and BestKeeper validation programs. The reference genes RPL13 and RPL32 were the most stably expressed genes in contused skeletal muscle, whereas PGK1 was the least stable. The commonly used reference genes β-actin and GAPDH appeared to be too unstable for normalization of RT-qPCR expression profiling in contused muscle. The reference genes RPL13 and RPL32 were also the best combination for multianalysis. The use of RPL13 and RPL32 as internal standards may improve the accuracy of gene expression studies aimed at determining the age of early wounds in forensic investigations.

Keywords Reference genes · Data normalization · Contused skeletal muscle · Quantitative real-time PCR · Forensic science

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Introduction

Quantitative methods for mRNA expression profiling, like real-time quantitative PCR (RT-qPCR), have become standard techniques in many fields. In addition to quantification, other advantages of RT-qPCR over older methods include enhanced sensitivity, larger dynamic range, and the potential for high throughput [1]. The RT-qPCR technique is now widely used in forensic medicine [2, 3]. There are limitations to this technique, however, including the inherent variability of RNA expression, RNA instability, the need for high-quality starting material, variability in the efficiencies of enzymes and primers, and improper normalization and analysis techniques that can all lead to misinterpretation of results [1, 4]. Consequently, it is especially important that an accurate method of normalization is chosen to control for these confounding variables. Various normalization strategies have been suggested to help decrease the variability observed in RT-qPCR, including the use of internal standard reference genes, normalization to starting material quantity, and the use of external control nucleotides of known quantity [1, 5]. While there are pitfalls to all normalization approaches, the use of internal reference genes has proven to be most accepted because these endogenous genes are expression and amplified under the same experimental conditions as the gene(s) of interest [6, 7]. An ideal reference gene should exhibit stable expression when exposed to the same experimental protocol as the genes under study. In order to select the best reference genes, the programs NormFinder [8], GeNorm [9], and BestKeeper [10] are commonly used.

In recent years, some cytokine and enzyme mRNAs have been studied to estimate the age of injuries in contused skeletal muscle [11, 12]. There has not been a rigorous

statistical analysis of reference gene stability in skeletal muscle after injury, however, so the validity of these results remains in question. In the present study, we assessed the expression stability of nine potential reference genes following contusion of skeletal muscle. Stability was assessed in control rats and at two post-contusion time points for nine candidate reference genes [13]: β-actin, glyceraldehydes-3-phosphate dehydrogenase (GAPDH), ribosomal protein L32 (RPL32), cGMP-dependent protein kinase 1 (PGK1), succinate dehydrogenase complex subunit A (SDHA), ribosomal protein L13 (RPL13), hypoxanthine phosphoribosyl transferase 1 (HPRT), TATA box binding protein (Tbp), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta polypeptide (Ywhaz).

Materials and methods

Experimental design

Twelve Sprague Dawley male rats, 10 to 12 weeks old and weighing between 250 and 300 g, were used in this study. All procedures were approved by the Animal Center of Shanxi Medical University. The rats were divided into a control group (*n*=4) and 6- and 12-h post-contusion groups (*n*=4 each).

Animals were kept under a 12-h light–dark cycle with free access to food and water. After the rats were anesthetized with ethyl ether and the right posterior limb was shaved, a depilatory agent (Nair, Carter–Wallace, Inc., New York, NY) was applied to remove residual hair. Subsequently, the rats were placed on a foam bed, and a 250-g weight was allowed to fall 150 cm through a clear Lucite guide tube onto the right posterior limb [14]. After injury, the rats were returned to cages and fed commercial rat food and tap water ad libitum.

The experimental procedures were based on the "Principles of Laboratory Animal Care" (NIH publication no. 85–23, revised 1985) and were conducted in accordance with the Guideline for Animal Experimentation established by our university.

Tissue and total RNA preparation

At 6 and 12 h after contusion, the three groups of rats were sacrificed with a lethal dose of pentobarbital (350 mg/kg by intraperitoneal injection). Muscle samples (approximate 100 mg) were dissected from the right posterior limbs, cut into two parts, and immediately frozen in liquid nitrogen.

Total RNA was isolated from muscle specimens (weighing approximately 50 mg) using the SV Total RNA Isolation System (Promega Co., Madison, WI) following the manufacturer's instructions. The concentration of freshly extracted total RNA was quantitated (in nanograms per microliter) using a UV/visible spectrophotometer (UItrospec 4300 pro, Biochrom Ltd., Cambridge, UK). The integrity of the isolated RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) by loading samples onto a eukaryote total RNA nano-chip.

Table 1 Primers used for real-time fluorescent quantitative PCR

Gene symbol	Accession number	Strand	Nucleotide sequence	Position	Size of PCR products (bp)
β-Actin	NM_031144	Sense	5′-TATCGGCAATGAGCGGTTCC-3′	828–847	151
		Antisense	5′-AGCACTGTGTTGGCATAGAGG-3′	957–978	
GAPDH	NM_017008	Sense	5′-AAGTTCAACGGCACAGTCAAG-3′	233–253	119
		Antisense	5′-TACTCAGCACCAGCATCACC-3′	332–351	
RPL32	NM_013226	Sense	5′-CACAGCTGGCCATCAGAGTCA-3′	402–422	83
		Antisense	5′-AAACAGGCACACCAAGCCATCTATTC-3′	458–484	
PGK1	NM_05329	Sense	5′-AAGTCGGTTGTGCTTATGAG-3′	170–189	123
		Antisense	5′-TCCTTCAAGAACAGAACATCC-3′	277–297	
SDHA	NM_130428	Sense	5′-GGTCACTCGGGCTGGTTTAC-3′	854–874	132
		Antisense	5′-CCTTGGCTGTTGATGAGAATGC-3′	964–985	
RPL13	NM_173340	Sense	5′-AAGGTGGTGGTTGTACGCTGTG-3′	108–129	110
		Antisense	5′-CGAGACGGGTTGGTGTTCATCC-3′	196–217	
Hprt	NM_012583	Sense	5′-CCAGCGTCGTGATTAGTGATGATG-3′	83–106	135
		Antisense	5′-GAGCAAGTCTTTCAGTCCTGTCC-3′	195–217	
Tbp	NM_001004198	Sense	5′-CACCAATGACTCCTATGACC-3′	624–643	111
		Antisense	5′-GTTTACAGCCAAGATTCACG-3′	715–734	
Ywhaz	NM_013011	Sense	5′-GAAAATGAAGGGTGACTACTAC-3′	418–440	111
		Antisense	5′-CTGATTTCAAATGCTTCTTGG-3′	508–528	

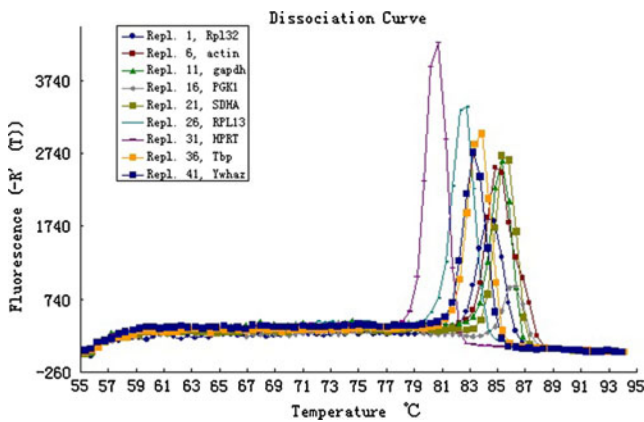

Fig. 1 The dissociation curves of candidate genes. Note: The dissociation curves of nine potential reference genes showed single cusps, and the dissociation temperatures were $Tm_{\beta\text{-actin}}$=84.8°C,Tm_{GAPDH}=85.3°C, Tm_{RPL32}=84.2°C,Tm_{PGK1}=86.3°C, Tm_{SDHA}=85.3°C,Tm_{RPL13}=82.8°C, Tm_{HPRT}=80.8°C,Tm_{Tbp}=83.8°C, and Tm_{Ywhaz}=83.2°C, respectively. The results could be explained by the fact that there were no other PCR productions besides our target genes and there was no primer dimmer

Reference genes primer design

Nine potential reference genes were chosen based on their common use as endogenous control genes in rat gene expression studies. Primers were designed based on sequences obtained from GenBank and imported into AlleleID 6 software (Premier Biosoft International), a program designed to generate primer pairs suitable for real-time PCR. The assays setting "SYBR Green Design" were chosen to limit primer sequences to regions with little secondary template structure. The designed primer sequences were validated on BLAST in order to ensure high efficiency. Primers were synthesized by Invitrogen Biotechnology. The sequences, length of products, and source sequences are listed in Table 1.

Real-time fluorescent quantitative PCR

The first-strand cDNA was synthesized using the Prime Script RT-PCR Kit (Takara Biotechnology Co. Ltd., Dalian, China) according to the standard protocol. For reverse transcription, 0.4 μg of total RNA was used in a reaction volume of 10 μL. Real-time PCR amplification was performed in a 25-μL reaction mixture that included 12.5 μL SYBR Premix Ex Taq, 9.5 μL dH_2O, 0.5 μL (10 μM) of each primer, and 2 μL cDNA as recommended in the manufacturer's instructions provided with SYBR Premix Ex Taq (Takara Biotechnology Co. Ltd., Dalian, China). Amplification was performed by one round of pre-denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing and extension at 60°C for 20 s. Fluorescence signals were detected at the end of every cycle. All reactions were performed using the Mx3005P Real-Time PCR System (Stratagene Inc., La Jolla, CA).

A standard curve was constructed to calculate the gene-specific PCR efficiency over a 5-fold dilution series (1, $1{:}10^1$, $1{:}10^2$, $1{:}10^3$, and $1{:}10^4$) of template cDNA for each

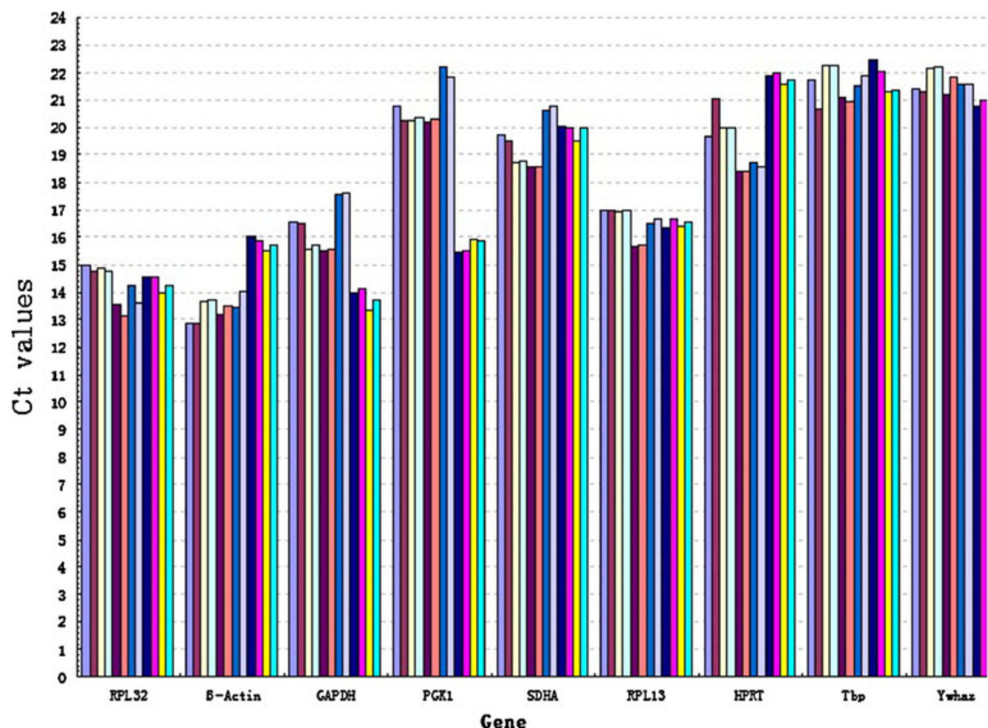

Fig. 2 Distribution of the expression (Ct) of the nine candidate reference genes. Note: A total of 12 Sprague Dawley male rats and 108 reactions were performed using the Mx3005P Real-Time PCR System. The Ct values of RPL13, Ywhaz, and RPL32 were more concentrated than the other candidate reference genes

primer pair. The efficiency values (Eff.) were obtained from the standard curves.

Data analysis

The PCR results were analyzed by Stratagene MxPro software. Baseline and threshold values were automatically set by the program. The number of PCR cycles required to reach the fluorescence threshold in each sample was defined as the Ct value, and each sample was analyzed in duplicate to obtain an average Ct for each sample. To confirm results, NormFinder [8], GeNorm [9], and BestKeeper [10] applications were also used. These bioinformatics packages calculate a stability value where a lower value indicates a higher stability (lower variability) in gene expression. The genes were ranked according to these gene stability values.

Results

Integrity of total RNA

All samples were performed on an Agilent 2100 Bioanalyzer using the RNA 6000 LabChips Kit. Approximately 0.1–0.4 μg of total RNA was extracted per milligram of muscle tissue using the SV Total RNA Isolation System. Only those RNA samples with a RNA Integrity Number above 8.0 and with clearly visible 28/18S peaks were used for real-time qPCR.

Calculation of the amplification efficiency of potential reference genes

The dissociation curves of the nine potential reference genes exhibited signal cusps, and the dissociation temperatures were all greater than 80°C (Fig. 1). The amplification efficiency of the nine genes were $Eff._{\beta\text{-actin}}=122.9\%$, $Eff._{GAPDH}=110.5\%$, $Eff._{RPL32}=115.7\%$, $Eff._{PGK1}=137.5\%$, $Eff._{SDHA}=113.9\%$, $Eff._{RPL13}=113.2\%$, $Eff._{HPRT}=114.2\%$, $Eff._{Tbp}=121.1\%$, and $Eff._{Ywhaz}=120.0\%$, respectively.

Validation of potential reference genes

Figure 2 presents the Ct values for each primer pair from the 12 samples (four controls, four at 6 h post-contusion, and four at 12 h post-contusion) analyzed by the Stratagene MxPro software.

Determination of the stability of reference genes by NormFinder

NormFinder analysis is a RT-qPCR data normalization tool that ranks the expression values of each of the

Table 2 Relative quantities of candidate reference genes

Sample	Genes								
	β-Actin	GAPDH	RPL32	PGK1	SDHA	RPL13	HPRT	Tbp	Ywhaz
Sample 1	1	0.091717851	0.426759802	0.017142896	0.403593791	0.374202354	0.380517389	0.438361128	0.165682366
Sample 2	0.976358723	0.093088977	0.397525931	0.025194809	0.480254878	0.357605129	0.132159797	1	0.083438897
Sample 3	0.511756658	0.188367766	0.364501205	0.025585867	0.866179449	0.382787638	0.302865898	0.276748016	1
Sample 4	0.499658077	0.167282881	0.385184281	0.02314841	0.827761264	0.365809624	0.296031525	0.276748016	0.091718993
Sample 5	0.774759746	0.199886807	0.746970771	0.025783667	1	1	0.977434326	0.728187284	0.201781784
Sample 6	0.609903542	0.188367766	0.888457377	0.024243113	1	0.96289963	1	0.800885007	0.122787927
Sample 7	0.614787014	0.043352324	0.594293569	0.005569058	0.212237349	0.514074366	0.777971083	0.517790899	0.067439514
Sample 8	0.384039973	0.041464767	1	0.007405077	0.181076502	0.473046891	0.885388336	0.386126201	0.150725095
Sample 9	0.077923864	0.621984628	0.461770062	1	0.319261683	0.589025745	0.06922256	0.241845399	0.122787927
Sample 10	0.087128432	0.560620469	0.461770062	0.96966529	0.326586469	0.455496676	0.064151421	0.340115565	0.232550908
Sample 11	0.121794632	1	0.758843226	0.690974785	0.469483568	0.567172672	0.09103253	0.597234685	0.125726941
Sample 12	0.102195199	0.754321816	0.608518395	0.734883505	0.32906522	0.495002017	0.077590709	0.583194278	0.249652593

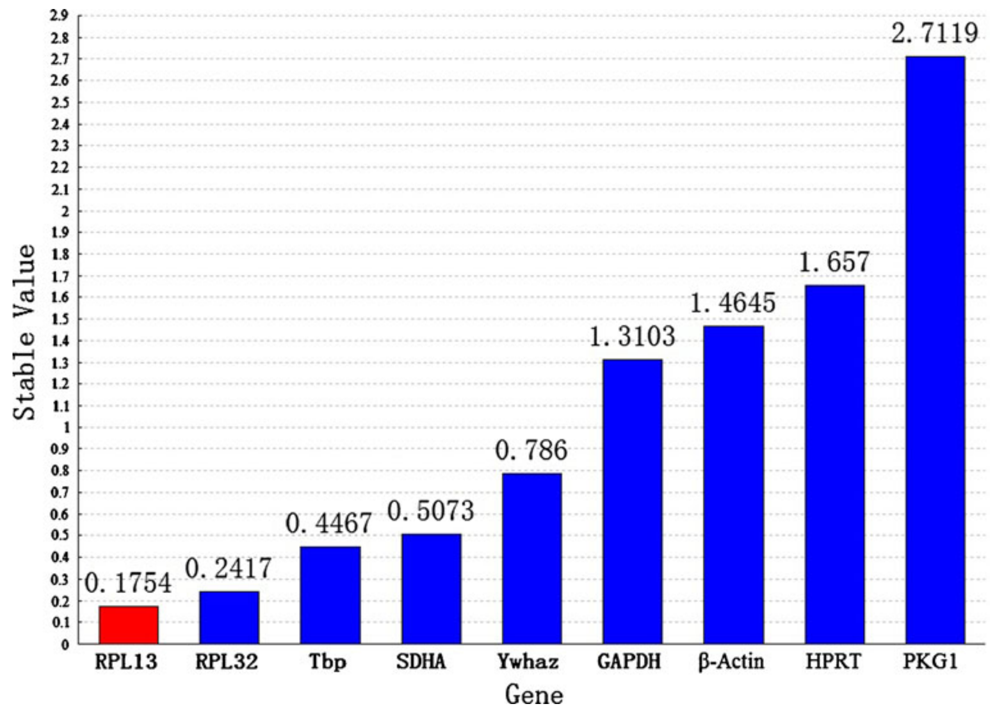

Fig. 3 Stability analysis of candidate reference genes by NormFinder. Note: The variability of candidate reference genes was calculated by NormFinder. The stability values of RPL13 and RPL32 were 0.1754 and 0.2417, significantly lower than the other candidates, including the ubiquitous control genes GAPDH (SD=1.3103) and β-actin (SD=1.4645), indicating that RPL13/32 were the most stably expressed genes in the contused skeletal muscle of rats

candidate reference genes. The program calculates a "stability value" that is inversely correlated with the stability of gene expression (so a higher stability value indicates lower stability). All raw Ct values were transformed to relative quantities using the ΔΔCt method (Table 2), and then, each data point was calculated using NormFinder. The results of the NormFinder analysis are presented in Figs. 3 and 4.

Determination of the stability of reference genes by geNorm

The geNorm program is a VBA applet for Microsoft Excel that provides a measure of gene expression stability (M values). An M value is the mean pair-wise variation between an individual gene and the other putative reference genes tested [9]. The geNorm program has a cutoff limit of variability suggesting that any gene with an M >1.5 should

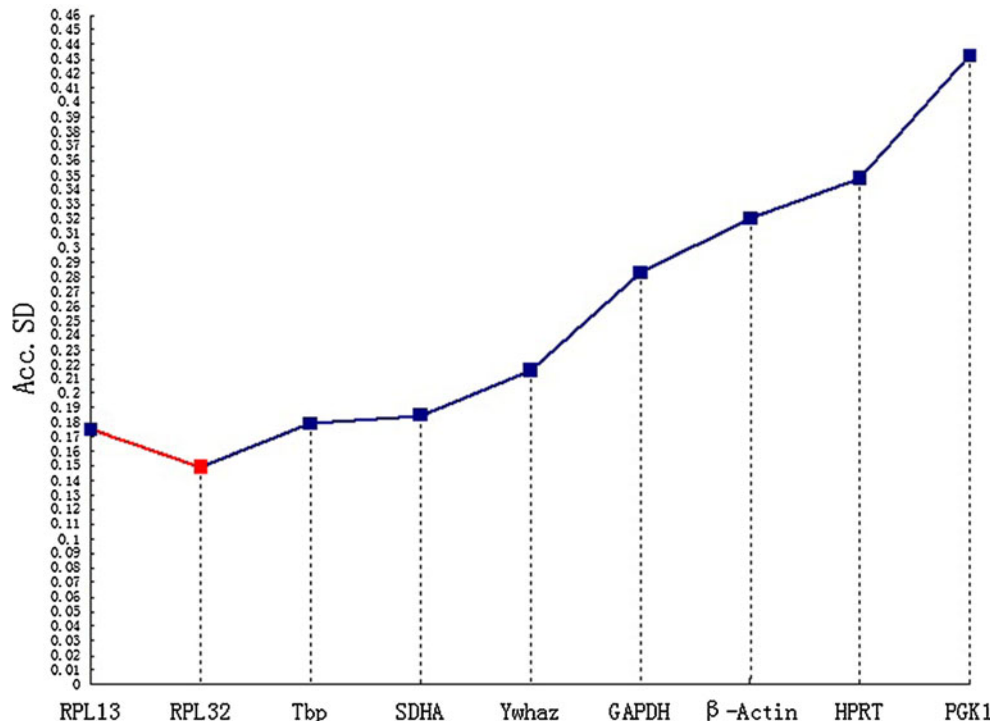

Fig. 4 Determination of the optimal number of reference genes for normalization based on the calculation of the Acc. SD. Note: The NormFinder algorithm also allowed for the determination of the optimal number of control genes to be used in the normalization processes through the calculation of the accumulated standard deviation (Acc. SD). The lowest value for the Acc. SD was achieved when using RPL13 and RPL32 (Acc. SD=0.1493)

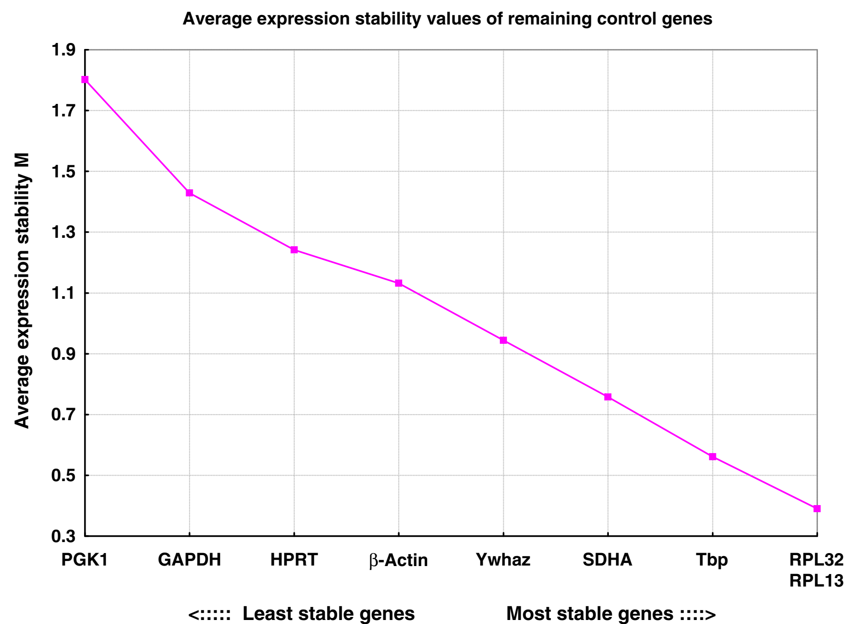

Fig. 5 Average expression stability values of remaining reference genes. Note: Average expression stability values (*M*) during stepwise exclusion of the least stable reference gene by geNorm application. The highest *M* values corresponded to least stable genes, PGK1 and GAPDH, while the lowest *M* values corresponded to the most stable genes, RPL13 and RPL32

be considered unreliable as a stable reference gene. For geNorm, all raw Ct values were transformed to relative quantities using the ΔΔCt method (Table 2), and then, each data point was calculated using geNorm. From the transformed data, geNorm produced a graph based on expression stability values (*M* values) (Fig. 5). The most stably expressed genes were RPL13 and RPL32 (M=0.374), followed by (from most to least stable) Tbp, SDHA, Ywhaz, β-actin, HPRT, GAPDH, and PGK1 (Table 3). From these results, we suggest that PGK1, GAPDH, HPRT, β-actin, and Ywhaz should not be considered as reliable internal reference genes for RT-qPCR analysis of contused muscle.

Determination of the stability of reference genes by BestKeeper

Raw Ct values are imported directly into the Microsoft Excel-based BestKeeper program. BestKeeper determines the "optimal" reference genes by employing a pair-wise correlational analysis of all pairs of candidate genes and calculates the geometric mean of the best choice [10]. The BestKeeper program produces a series of descriptive statistics, including a "BestKeeper index" and the standard deviation. It is recommended that any gene with a standard deviation of greater than 1 (SD >1) should be excluded from consideration as a stable

Table 3 The *M* values of each candidate reference gene

Sample	Genes									
	β-Actin	GAPDH	RPL32	PGK1	SDHA	RPL13	HPRT	Tbp	Ywhaz	Normalization factor
Sample 1	1.00E+00	9.17E−02	4.27E−01	1.71E−02	4.04E−01	3.74E−01	3.81E−01	4.38E−01	1.66E−01	0.8232
Sample 2	9.76E−01	9.31E−02	3.98E−01	2.52E−02	4.80E−01	3.58E−01	1.32E−01	1.00E+00	8.34E−02	1.0263
Sample 3	5.12E−01	1.88E−01	3.65E−01	2.56E−02	8.66E−01	3.83E−01	3.03E−01	2.77E−01	1.00E+00	0.8586
Sample 4	5.00E−01	1.67E−01	3.85E−01	2.31E−02	8.28E−01	3.66E−01	2.96E−01	2.77E−01	9.17E−02	0.8510
Sample 5	7.75E−01	2.00E−01	7.47E−01	2.58E−02	1.00E+00	1.00E+00	9.77E−01	7.28E−01	2.02E−01	1.7242
Sample 6	6.10E−01	1.88E−01	8.88E−01	2.42E−02	1.00E+00	9.63E−01	1.00E+00	8.01E−01	1.23E−01	1.8267
Sample 7	6.15E−01	4.34E−02	5.94E−01	5.57E−03	2.12E−01	5.14E−01	7.78E−01	5.18E−01	6.74E−02	0.8594
Sample 8	3.84E−01	4.15E−02	1.00E+00	7.41E−03	1.81E−01	4.73E−01	8.85E−01	3.86E−01	1.51E−01	0.8562
Sample 9	7.79E−02	6.22E−01	4.62E−01	1.00E+00	3.19E−01	5.89E−01	6.92E−02	2.42E−01	1.23E−01	0.7643
Sample 10	8.71E−02	5.61E−01	4.62E−01	9.70E−01	3.27E−01	4.55E−01	6.42E−02	3.40E−01	2.33E−01	0.7850
Sample 11	1.22E−01	1.00E+00	7.59E−01	6.91E−01	4.69E−01	5.67E−01	9.10E−02	5.97E−01	1.26E−01	1.1834
Sample 12	1.02E−01	7.54E−01	6.09E−01	7.35E−01	3.29E−01	4.95E−01	7.76E−02	5.83E−01	2.50E−01	0.9845
M<1.5	1.949	1.929	1.360	3.105	1.456	1.304	2.043	1.418	1.649	

Table 4 BestKeeper description statistics

	β-Actin	GAPDH	RPL32	PGK1	SDHA	RPL13	HPRT	Tbp	Ywhaz
n	12	12	12	12	12	12	12	12	12
Geo Mean (CP)	14.16	15.43	14.26	18.91	19.55	16.53	20.11	21.61	21.38
Ar Mean (CP)	14.20	15.49	14.27	19.08	19.56	16.53	20.16	21.62	21.39
Min (CP)	12.85	13.34	13.14	15.47	18.53	15.65	18.39	20.66	20.78
Max (CP)	16.05	17.63	14.97	22.21	20.79	17.01	22.00	22.45	22.18
SD (±CP)	1.06	1.13	0.49	2.25	0.63	0.34	1.24	0.48	0.39
CV (% CP)	7.44	7.32	3.41	11.81	3.22	2.05	6.15	2.23	1.84

Standard deviation was used to evaluate reference gene stability. A standard deviation >1 represented an unstable reference gene

reference gene. The descriptive statistics from our study are presented in Table 4. A range of standard deviations were observed, from 0.34 for RPL13 to 2.25 for PGK1. Those with a standard deviation greater than 1 were removed from analysis of the BestKeeper index (Table 5). Using the reference gene data sets from the remaining candidates for a new BestKeeper index calculation, RPL13 was shown to be the most correlated (r=0.775, p=0.003). Overall, BestKeeper ranked the stability of the reference genes from the most to the least stable as RPL13, RPL32, SDHA, Ywhaz, and Tbp.

Discussion

RT-qPCR has become a valuable tool for accurate gene expression profiling. A critical step for accurate analysis is to minimize the sample and experimental variation by normalizing the raw expression data to the expression of a reference gene. The "ideal" reference gene should be transcribed in all cell types and tissues under study, and transcription should be constant relative to global cellular transcription rates under different experimental conditions [15]. Expression of individual reference genes does differ among samples under different experimental conditions, however. Nevertheless, few studies have compared the stability of reference genes. Injured tissues like contused skeletal muscle exhibit large dynamic changes in mRNA expression mediated by endogenous repair mechanisms and cell death, making the choice of a stable reference all the more critical.

In the present study, among the nine candidate reference genes evaluated, RPL13 and RPL32 were the most stably expressed in contused skeletal muscle, whereas PGK1 was the least stable. Ribosomal proteins RPL13 and RPL32 are both components of the 60S ribosomal subunit. Although several ribosomal proteins are variably expressed under disease conditions (gastrointestinal cancer) [16], they have been used as normalization genes. In a study of inflammatory gene expression associated with ischemia–reperfusion brain injury, RPL32 was suggested as an optimal normalization gene [17]. Indeed, RPL13 and RPL32 have been confirmed as suitable normalization genes after elaborate control experiments investigating several potential candidates, while a number of other near-ideal housekeeping genes for internal reference have been proposed for work on humans, rodents, rhesus monkeys, dogs, and horses [18–23].

Historically, the most commonly used reference genes were β-actin and GAPDH [12], as they were used for many years in Northern blots, RNase protection assays, and later, in conventional RT-PCR. However, recent studies have indicated that these common reference genes are not stably expressed under all experimental conditions [24]. In our study, β-actin and GAPDH were found to have highly variable expression in contused muscle (Figs. 3 and 5, Tables 4 and 5) and were deemed unsuitable for normalization of RT-qPCR data. They were both rejected by the geNorm program and by the BestKeeper program due to high M values and high SDs (±Ct) compared to other candidates.

In recent years, the use of a single reference gene for data normalization has been discouraged in light of variations in reference gene expression and resultant misinterpretation of expression data [4, 25]. Some authors have proposed that at least three well-validated housekeeping genes should be used to normalize expression data. In

Table 5 BestKeeper index comparing genes which had a standard deviation <1

BestKeeper vs.	β-Actin	β-Actin GAPDH	RPL32	PGK1	SDHA	RPL13	HPRT	Tbp	Ywhaz
Coeff. of corr. (r)			0.558		0.489	0.775		0.430	0.475
p value			0.059		0.107	0.003		0.162	0.118

our study, we also estimated the optimal number of reference genes by calculating the accumulated standard deviation (Acc. SD) using the NormFinder algorithm. The lowest value for the Acc. SD was achieved using RPL13 and RPL32 (Acc. SD=0.1493); however, if three housekeeping genes were used to normalize the data (RPL13/RPL32/Tbp), the Acc. SD was slightly higher (0.1791). This result corresponded to predictions using geNorm and BestKeeper and indicated that normalization using these two genes yielded the best results in gene expression profiling of contusion skeletal muscle from rats.

In conclusion, although there is limitation of using an animal model for estimating wound age and the data obtained on animal model might not be fully valid on human material, the use of an animal model system seems justified in the present situation. This study analyzed a set of reference genes for normalization of gene expression profiles in contused rat skeletal muscle using RT-qPCR. Though we saw surprisingly high variations in expression, RPL13 and RPL32 emerged as the most appropriate reference genes following contusion of skeletal muscle, while the commonly used reference genes β-actin and GAPDH appeared to be too unstable under this experimental design. Our goal was not to suggest a "best" approach for all conditions, but rather to define the most stably expressed genes in rat skeletal muscle following contusion. Thus, the identified pair of stable reference genes may be used for future expression studies to help determine the age of early wounds in forensic medicine. This study further emphasizes the need to accurately validate candidate reference genes in the model under study before use in gene expression studies using RT-qPCR.

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