Diabetes, Metabolic Syndrome and Obesity

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ORIGINAL RESEARCH

Assessment of Reference Genes Stability in Cortical Bone of Obese and Diabetic Mice

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Introduction: Bone, a pivotal structural organ, is susceptible to disorders with profound health implications. The investigation of gene expression in bone tissue is imperative, particularly within the context of metabolic diseases such as obesity and diabetes that augment the susceptibility to bone fractures. The objective of this study is to identify a set of internal control genes for the analysis of gene expression.

Methods: This study employs reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR) to assess gene expression in bone tissue. We selected fourteen housekeeping genes and assessed their stability in the cortical bone of mouse models for obesity and diabetes using four well-established algorithms (GeNorm, BestKeeper, NormFinder, and the comparative Delta Ct method).

Results and Conclusion: We identified *Rpl13a* as the mostly stably expressed reference gene in cortical bone tissue from mouse models of obesity and diabetes (db/db), while *Gapdh* was found to be the most stable reference gene in another diabetes model, KKAy mice. Additionally, *Ef1a, Ppia, Rplp0*, and *Rpl22* were identified as alternative genes suitable for normalizing gene expression in cortical bone from obesity and diabetes mouse models. These findings enhance RT-qPCR accuracy and reliability, offering a strategic guide to select reference gene for studying bone tissue gene expression in metabolic disorders.

Keywords: reference genes, *Rpl13a*, *Gapdh*, cortical bone, metabolic disorders

Introduction

Gene expression serves as the pivotal regulator in orchestration of the intricate processes involved in bone morphogenesis, homeostasis, and repair; variations in gene expression led to different bone diseases.[1,2] Within the continually remodeling bone tissue, a harmonious interplay of distinct cellular cohorts, notably osteoblasts, osteoclasts, and osteocytes, mediates the synthesis and secretion of an array of extracellular matrix proteins, growth factors, and cytokines.[3] This complex process of bone homeostasis is meticulously governed at the transcriptional level, guided by a multifaceted interplay, involving transcription factors, epigenetic modulators, and elaborate signaling pathways. RNA, synthesized from DNA via transcription, is key in gene expression and comes in two forms: protein-coding and noncoding. Protein-coding genes produce messenger RNAs (mRNAs) which are translated into proteins, crucial for bone modeling and remodeling.[4,5] The expression of non-coding RNAs, including small, long, and circular types, is also involved in bone development and the pathogenesis of bone-related disorders.[6–8] Circular RNAs affect bone formation by regulating specific gene expression.[6] MicroRNAs (like miR-30, miR-124, miR-204) and long non-coding RNAs also play significant roles in bone growth and stability.[7,8] Dysregulations that disrupt this genetic equilibrium within the skeletal framework can engender a spectrum of skeletal disorders, encompassing conditions such as osteoporosis, osteoarthritis, and osteogenic

Received: 6 December 2023
Accepted: 15 February 2024
Published: 2 March 2024

neoplasms.[9] Hence, delving into the nuanced complexities of gene regulation in bone is crucial for the pursuit of pioneering therapeutic modalities for these incapacitating disorders. In recent years, an array of sophisticated gene expression analysis methodologies,[10] including microarray profiling, RNA sequencing, droplet digital PCR[11] and reverse transcription-quantitative real-time PCR (RT-qPCR), have substantially enriched our comprehension of the molecular substratum governing the dynamics of bone development, preservation, and pathological changes, offering the promise of groundbreaking strides in this field.

When the expression levels of genes are low, PCR becomes essential for their detection. Housekeeping genes such as *RN18S1, GAPDH*, and *ACTB* (β-actin) are commonly used as reference genes for gene expression analysis in published studies;[12] however, their use as reference genes is not always appropriate, as their expression can vary across species, tissue types, cell lines, developmental stages, and in response to experimental treatments.[13–16] Moreover, the expression of housekeeping genes can vary among different diseases.

The prevalence of obesity and diabetes, which are considered significant metabolic diseases, is increasing worldwide.[17] Metabolic bone diseases encompass a diverse range of disorders characterized by disrupted skeletal homeostasis; these conditions often exhibit varying patterns of genetic inheritance, resulting in different genetic profiles converging to produce a similar phenotype.[4] RT-qPCR is frequently performed to explore the role of alternative genes in these metabolic diseases, and normalization is a crucial factor in obtaining reliable qPCR results.[18] Obesity and diabetes are also linked to an increased risk of bone fractures, highlighting the importance of studying gene expression in bone tissue related to these conditions; however, selecting suitable reference genes can be challenging and time-consuming, and use of a panel of reference genes is often necessary to increase the reliability of results. As rodents, such as mice, serve as models for humans in many experiments, evaluating the stability of reference genes in murine models is crucial. High-fat diet-induced obese mice, along with KKAy and db/db strains, are key models for diabetes research. These models are essential for investigating bone health and skeletal disorders, as both show notable bone quality impairments. It is necessary to evaluate the stability of reference genes in the cortical bone of three murine models of metabolic disorders.[19] A previous study evaluated the stability of reference genes in human trabecular bone and provided the optimum choice for human bone disease;[20] however, similar studies in cortical bone from mice fed with standard diet, or from mouse models of metabolic disorders, have not been reported. As different conclusions are obtained using different reference genes,[21,22] it is necessary to screen for a set of stable internal control genes in mice for gene expression analysis.

In our study, we aimed to evaluate the stabilities of 14 selected housekeeping genes in three mouse models of general metabolic conditions and to identify a set of internal control genes for the analysis of gene expression. Our findings will provide a reference for research using these models and will save researchers time and expense.

Materials and Methods

Sample Preparation

Seven-week-old KK-Ay and wild-type mice (n = 8 per group) were purchased from Beijing HFK Bioscience Co., Ltd, and eight-week-old db/db mice and littermate controls were obtained from GemPharmatech Co., Ltd. Mice were housed in a specific pathogen-free room, and provided with standard diet and free access to water. Rooms were maintained at a suitable temperature and humidity, with a 12 h/12 h light/dark cycle. KKAy and db/db mice and their age and gender matched controls were kept for 18 weeks and 12 weeks, respectively; both of these mouse strains have a typical diabetic phenotype.[23]

To observe the influence of obesity in bone tissue, a control group and obese mice, fed with a high-fat diet for 9 weeks (referred to as HFD mice), were kept under the same environment described above for KKAy and db/db mice. After euthanasia, humerus bones were collected and soft tissue removed. All animal experiments were approved by the Ethics Committee of the Chongqing Medical University (IACUC-CQMU-2023-0036, approved on 16 March 2023), adhered to the Guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T 35892–2018.

cDNA Synthesis

Cortical bones of humeri without bone marrow were acquired by centrifugation,[24] after cutting off the epiphyseal plates from both ends, and homogenized in a tube containing 1 mL of TRIzol reagent (Thermo Fischer Scientific, Waltham, MA, USA). Total RNA extraction was then performed according to the standard protocol.[25] The RNA was extracted by adding 200 µL of chloroform to each sample, mixing it, and incubating for 3 minutes. After centrifugation at 12,000 rpm for 15 minutes at 4°C, the supernatant was mixed with isopropanol and left to rest for 10 minutes before being centrifuged again. The resulting precipitate was washed three times with 75% ethanol and resuspended in DEPC-treated water.

RNA was quantified using a Nanophotometer (IMPLEN). The RNA quality control was followed the previous method.[26] A260: A280 ratios were between 1.8 and 2.0. After extraction, 1.5 µg aliquots of total RNA were used for cDNA synthesis, following the standard protocol for the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) in a final volume of 20 µL, by incubation for 5 min at 25°C, 60 min at 42°C, and 5 min at 70°C, followed by 5 min at 65°C.

RT-qPCR

RT-qPCR was performed using QuantStudio5 (Applied Biosystems, Thermo Fischer Scientific) with SYBR Green. Reaction mixtures contained PowerUp SYBR Green Master Mix (Thermo Fischer Scientific), 0.5 µM of forward and reverse primers (Table 1), and 0.25 µL of cDNA, in a final volume of 10 µL. Reactions were carried out in MicroAmp1 Optical 384-well plates (Thermo Fischer Scientific), using the following program: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15s at 95°C and 1 min at 60°C, and dissociation curves were plotted. From the fourteen candidate reference genes, *Rpl22, Hprt, Atpf1, Ubc, Ppia* primer pairs were obtained from the literature.[27] The rest of primers were designed using Primerbank and verified using the BLAST tool, or acquired from previous publications.[28,29]

Statistical Analysis

For the physiological analyses of mice, unpaired student's *t*-test was used for comparisons between two different groups. The confidence interval was 95%. Four algorithms previously described in the literature were used to select the most suitable reference genes for analysis of bone tissues from HFD, KK-Ay and db/db mice: GeNorm,[29] BestKeeper,[30] NormFinder,[31] and the comparative Delta Ct method.[32] All analyses were conducted using the web-based RefFinder tool[33] (http://blooge.cn/RefFinder).

Results

To obtain suitable housekeeping genes for analysis of the expression of target genes in cortical bone tissue using RT-qPCR, we established three frequently used mouse models of metabolism: KKAy, db/db, and HFD. Primers to amplify candidate genes were chosen from published studies after a review of investigations that included reference gene selection.

Stable Reference Gene Selection in Cortical Bone from Wild-Type Mice

Mice are frequently used as animal models in medical research.[19] We first analyzed the stability of candidate genes in 20–25-week-old mice fed with normal diet (Table 2), by determining the expression levels of each candidate gene in bone samples (Figure 1A). The CT value of the gray line was 15, while that of the red line was 33; values outside of these lines were considered to indicate unreliable low and high expression of candidate genes in mouse cortical bone tissue, respectively. As shown in Figure 1B, stability of housekeeping gene expression was comprehensively analyzed in wild type mice. Based on comparative delta Ct, NormFinder, and Bestkeeper data, *Rplp0* was the most stably expressed gene. Analysis using the GeNorm algorithm selected *Ppia* and *Gapdh* as the best combined group. Further, *Gapdh* was selected as the best among three commonly used internal control genes (*Gapdh, Rn18s*, and *ActB*) by the four algorithms. *ActB* and *B2m* emerged as the least stable among the evaluated candidate genes.

Table 1 Primer Sequences of Candidate Genes in Cortical Bone Tissue of Humeri in Mice

Gene	Gene Name	Sequence (5' to 3')		Function
		Forward	Reverse	
Rpl22[a]	Ribosomal protein L22	ACCCTGGACTGCACTCACCCTG	CCGCCGAGGTTGCCAGCTTT	Translation of mRNA in protein
Hprt [a]	Hypoxanthine phospho-ribosyltransferase 1	CCCTGGTTAAGCAGTACAGCCCC	AGTCTGGCCTGTATCCAACACTTCG	Purine metabolism
Atpf1[a]	ATP synthase mitochondrial F1 complex assembly factor 1	TGGCGACAGGCTGGACTCAG	GCTGCCCGAAGTCTTCTCAGCG	Energy metabolism
Ubc[a]	Ubiquitin C	AGCCCAGTGTTACCACCAAGAAGG	TCACACCCAAGAACAAGCACAAGGA	Protein degradation
Tbp	TATA-box-binding protein	CCTTGTACCCTTCACCAATGAC	ACAGCCAAGATTCACGGTAGA	General transcription factor
Rplp0	Ribosomal protein lateral stalk subunit P0	GGGCATCACCACGAAAATCTC	CTGCCGTTGTCAAACACCT	Translation of mRNA in protein
Ppia[a]	Peptidylprolyl Isomerase A or Cyclophilin A	CAGACGCCACTGTCGCTTT	TGTCTTTGGAACTTTGTCTGCAA	ER cyclosporine-binding protein
Rpl13a	Ribosomal protein L13a	CTGTGAAGGCATCAACATTTCTG	GACCACCATCCGCTTTTTCTT	Structural component of the large 60S ribosomal subunit
Ef1a	Elongation factor 1 alpha	ATAACCCCAGGAAGTACCTTCG	GTCTGCTGCGTATTTACTGCC	Protein synthesis
Sdha	Succinate dehydrogenase complex, subunit A	GGAACACTCCAAAAACAGACCT	CCACCACTGGGTATTGAGTAGAA	Citric acid cycle
Rn18s	18S ribosomal RNA	CGCCGCTAGAGGTGAAATTCT	CATTCTTGGCAAATGCTTTCG	Ribosome RNA, translation of mRNA in protein
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	Carbohydrate metabolism
B2m	Beta-2-microglobulin	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC	MHC class I protein
Actb	Actin beta	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC	Cytoskeletal protein

Notes: [a]These primer pairs were obtained from the literature.[27]

Stable Selection of Reference Genes in Cortical Bone of HFD Mice

Increasing numbers of people are living with obesity, which is disruptive to their quality of life. Here, we assessed the suitability of housekeeping genes for analysis of control and HFD group mice, with an obese phenotype; excess energy leads to obesity, and often occurs in mice fed a high fat diet. Expression levels of the candidate genes are presented in Figure 1C. For comparisons between control and obese animals, *Rpl13a* was identified as having the most stable expression by RefFinder, comparative delta Ct, and GeNorm; however, NormFinder selected *Ef1a* as the most stable reference gene, while *B2m* was the most stable gene according to BestKeeper (Table 3). In general, *Rpl13a* was stably expressed, while *Sdha* was the most unstable reference gene in comparisons between control and obese animals (Figure 1D).

Stable Reference Gene Selection in Cortical Bone of db/db Mice

Diabetes is severe threat to public health.[34] We analyzed gene expression in cortical bone tissue of control group and diabetic model db/db mice (Table 4). *Rpl13a, Ef1a, Ppia*, and *Atpf1* were identified as the more stable reference genes

Table 2 Stability of Expression of Candidate Genes in Cortical Bone Tissue of C57BL/6J Mice (20–25 Weeks Old)

Gene	RefFinder		Delta CT Method		BestKeeper		NormFinder		Genorm	
	Stability Value	Rank	Average of STDEV	Rank	Std Dev [± CP]	Rank	Stability Value	Rank	M value	Rank
Rplp0	1.5	1	0.93	1	0.61	1	0.279	1	0.616	5
Rpl13a	3.31	2	0.95	2	0.74	5	0.401	3	0.583	4
Rpl22	3.72	3	0.99	4	0.71	4	0.528	4	0.53	3
Ppia	4.26	4	1.25	10	0.68	3	1.041	10	0.356	1
Ef1a	4.41	5	0.96	3	0.88	9	0.293	2	0.73	7
Gapdh	4.82	6	1.23	9	0.77	6	1.006	9	0.356	1
Rn18s	5.83	7	1.21	8	0.66	2	0.854	8	0.816	9
Hprt	6.47	8	1.08	5	0.84	7	0.592	5	0.869	10
Atpf1	7.33	9	1.14	6	0.92	10	0.766	6	0.763	8
Sdha	8.77	10	1.19	7	1.18	11	0.782	7	0.92	11
Ubc	9.3	11	1.31	11	0.86	8	1.111	12	0.676	6
Actb	12.74	12	1.38	12	1.35	12	1.102	11	1.048	12
B2m	14.24	13	1.64	13	1.78	14	1.463	13	1.138	13
Tbp	14.74	14	1.73	14	1.55	13	1.547	14	1.217	14

using the four algorithms. GeNorm identified *Atpf1* and *Rpl13a* as the best stable gene pair. *B2m* was expressed more stable than *Actb, Gapdh*, and *Rn18s* in control and db/db mice. Comprehensive analysis of the expression stability of internal control genes is presented in Figure 1E and F.

Stable Reference Gene Selection in Cortical Bone from KKAy Mice

We next analyzed the stability of selected housekeeping genes in the control group and another diabetic mouse model, KKAy (Table 5). Several genes were excluded from the gene expression analysis, due to the difference between the two groups, and further analysis was conducted using the remaining selected genes. *Gapdh* was identified as the best reference gene using comparative delta Ct and RefFinder. *Rpl22* was identified as the most stable gene by BestKeeper, while *Ubc* was chosen as the best reference gene using NormFinder. *Rpl22* and *Ppia* were the best gene pair for gene expression analysis, according to the GeNorm algorithm. Gene expression data in control and KKAy mice are presented in Figure 1G, and comprehensive analysis of gene stability is presented in Figure 1H.

Overall, our findings show that the most frequently used reference genes (ie, *Gapdh, Actb*, and *Rn18s*) are not consistently stable in the mouse models analyzed. We found that *Gapdh* was more stable in mice fed with a standard or high-fat diet and in KKAy mice. The best reference gene was not the same in the two diabetic models; *Rpl13a* was identified as better in db/db mice, while *Gapdh* was selected in KKAy mice. We found that *Rpl22, Rplp0, Ppia*, and *Ef1a* were expressed relatively stable and may be better choices as candidate reference genes in analysis of mouse cortical bone tissue.

Discussion

To date, there remains no standard criterion for selection of reference genes, which are generally used generally for qPCR analyses.[28] The qPCR technique is constantly being improved[35] and the selection of reference genes in various samples is an important factor to consider.[36] Different reference genes may have variable influence on the analysis of target genes, unlike immunohistochemical or other gold standard analysis techniques.[13,15] A review reported that none of the popular reference genes, *RN18S1, ACTB, B2M, GAPDH*, and *HPRT*, were stable in more than 50% of analyses.[13] Few studies have validated the stability of housekeeping genes, making the precision of results questionable. Bone tissue is comprised of bone cells (predominantly osteocytes) and abundant bone matrix, and it is difficult to obtain high-quality RNA for identification of stable reference genes.[37] Some investigations have validated the stability of reference genes in

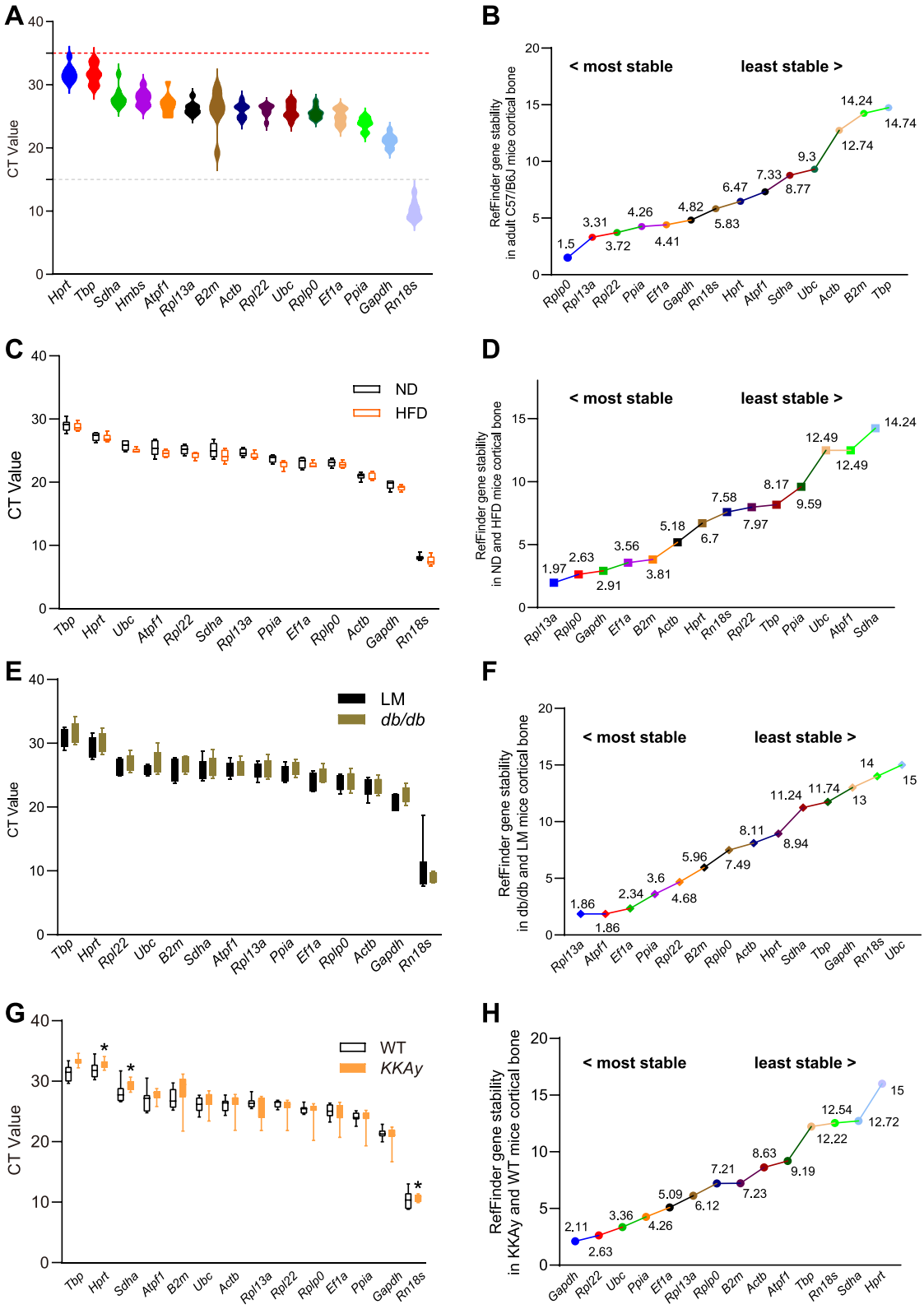

Figure 1 CT Values and Gene Stability Analysis Using RefFinder algorithm in Different Mouse Groups. (**A**) CT value of wild type (C57BL/6J) mice. (**B**) Gene stability in wild-type mice (RefFinder). (**C**) CT values in normal vs high-fat diet mice. (**D**) Gene stability in diet-treated mice (RefFinder). (**E**) CT values in *db*/*db* mice vs littermates. (**F**) Gene stability in *db*/*db* mice (RefFinder). (**G**) CT values in KKAy mice vs C57BL/6J mice. (**H**) Gene stability in KKAy vs C57BL/6J mice (RefFinder).

Table 3 Stability of Expression of Candidate Genes in Cortical Bone Tissue of Control and HFD Mice

Gene	Student's t-test	RefFinder		Delta CT Method		BestKeeper		NormFinder		Genorm	
		Stability Value	Rank	Average of STDEV	Rank	Std Dev [± CP]	Rank	Stability Value	Rank	M value	Rank
Rpl13a	0.313	1.97	1	0.49	1	0.52	5	0.235	3	0.143	1
Rplp0	0.616	2.63	2	0.5	3	0.48	4	0.277	4	0.143	1
Gapdh	0.310	2.91	3	0.49	2	0.54	6	0.189	2	0.25	3
Ef1a	0.603	3.56	4	0.5	4	0.62	10	0.165	1	0.283	4
B2m	0.762	3.81	5	0.58	5	0.43	1	0.401	7	0.339	6
Actb	0.836	5.18	6	0.6	8	0.44	2	0.437	9	0.321	5
Hprt	0.775	6.7	7	0.58	6	0.58	8	0.396	6	0.356	7
Rn18S	0.278	7.58	8	0.68	11	0.47	3	0.539	10	0.443	10
Rpl22	0.100	7.97	9	0.59	7	0.6	9	0.417	8	0.386	8
Tbp	0.690	8.17	10	0.6	9	0.63	11	0.383	5	0.417	9
Ppia	0.088	9.59	11	0.68	10	0.57	7	0.551	11	0.468	11
Ubc	0.106	12.49	12	0.73	13	0.64	12	0.566	13	0.508	12
Atpf1	0.296	12.49	13	0.7	12	0.86	13	0.559	12	0.547	13
Sdha	0.338	14.24	14	0.82	14	0.97	14	0.718	14	0.591	14

Table 4 Stability of Expression of Candidate Genes in Cortical Bone Tissue of Control and db/db Mice

Gene	Student's t-test	RefFinder		Delta CT Method		BestKeeper		NormFinder		Genorm	
		Stability Value	Rank	Average of STDEV	Rank	Std Dev [± CP]	Rank	Stability Value	Rank	M value	Rank
Rpl13a	0.461	1.86	1	1.99	1	2.12	3	0.236	4	0.471	1
Atpf1	0.519	1.86	2	2.04	4	1.91	1	0.236	3	0.471	1
Ef1a	0.116	2.34	3	1.99	2	2.19	4	0.227	1	0.531	3
Ppia	0.138	3.6	4	2.04	3	2.3	6	0.227	2	0.589	4
Rpl22	0.260	4.68	5	2.13	6	1.94	2	0.3	5	1	8
B2m	0.173	5.96	6	2.13	5	2.21	5	0.303	6	0.931	7
Rplp0	0.480	7.49	7	2.26	7	2.62	9	1.167	8	0.77	5
Actb	0.429	8.11	8	2.29	8	2.57	8	1.196	9	0.83	6
Hprt	0.269	8.94	9	2.49	9	2.42	7	1.125	7	1.199	9
Sdha	0.446	11.24	10	2.6	10	3.17	11	1.812	10	1.317	10
Tbp	0.252	11.74	11	2.94	11	2.76	10	1.849	11	1.454	11
Gapdh	0.098	13	12	3.43	12	4.22	12	3.048	12	1.678	12
Rn18s	0.289	14	13	5.01	13	6.21	13	4.921	13	2.068	13
Ubc	0.312	15	14	8.81	14	20.52	14	8.792	14	2.967	14

osteogenic differentiation during various treatments,[38–41] but only a few have focused on bone tissue,[20,42] particularly in the context of metabolic disorders.

Several advancements in methods for studying metabolic disorders have been made recently. Chen et al[43] employed metabolomics and machine learning to identify 26 plasma markers for early and personalized detection of metabolic syndrome. Mass spectrometry and spectroscopy, enhanced by machine learning, are advancing metabolic in vitro analysis for faster and more accurate results.[44] Cao et al utilized a novel single-cell metabolomics method to explore diverse metabolic states in stem cells.[45] Metabolomics, which involves the analysis of cellular metabolites, provides insights into the final products of cellular processes; however, it lacks the direct gene expression information offered by transcriptomics. Transcriptomics is gaining importance in bone research for its ability to detect real-time gene changes, aiding in

Table 5 Stability of Expression of Candidate Genes in Cortical Bone Tissue of C57BL/6J and KKAy Mice

Gene	Student's t-test	RefFinder		Delta CT Method		BestKeeper		NormFinder		Genorm	
		Stability Value	Rank	Average of STDEV	Rank	Std Dev [± CP]	Rank	Stability Value	Rank	M value	Rank
Gapdh	0.788	2.11	1	1.05	1	0.92	2	0.591	2	0.484	4
Rpl22	0.826	2.63	2	1.17	5	0.91	1	0.894	7	0.374	1
Ubc	0.132	3.36	3	1.07	2	1.18	7	0.485	1	0.626	7
Ppia	0.957	4.26	4	1.32	9	0.93	3	1.126	10	0.374	1
Ef1a	0.681	5.09	5	1.12	3	1.05	6	0.787	5	0.449	3
Rpl13a	0.438	6.12	6	1.17	4	1.24	8	0.698	4	0.577	6
Rplp0	0.878	7.21	7	1.24	8	0.98	4	0.994	9	0.514	5
B2m	0.052	7.23	8	1.21	6	1.46	11	0.695	3	0.804	9
Actb	0.189	8.63	9	1.23	7	1.32	9	0.812	6	0.691	8
Atpf1	0.076	9.19	10	1.36	10	1.02	5	0.993	8	1.025	11
Tbp	0.466	12.22	11	1.51	11	1.4	10	1.153	11	0.929	10
Rn18s[a]	0.023	12.54	12	-	-	-	-	-	-	-	-
Sdha[a]	0.013	12.72	13	-	-	-	-	-	-	-	-
Hprt[a]	0.001	15	14	-	-	-	-	-	-	-	-

Notes: [a]These genes were excluded from further analysis due to differential expression.

early bone disease detection and management.[46] Despite this, RT-qPCR remains widely used in life sciences for its cost-effectiveness and simplicity.

In this study, we aimed to validate the most stable reference genes for gene expression analysis of cortical bone tissue samples from mice fed a standard diet, as well as mouse models of obesity and diabetes.[47,48] The expression stability of 14 genes was evaluated using four different algorithms. Both the comparative delta Ct and GeNorm methods rely on pairwise comparisons of gene expression. The BestKeeper algorithm estimates gene stability by considering the standard deviation of Cq values. While three of the aforementioned algorithms are based on intragroup variation, the NormFinder algorithm incorporates both intragroup and intergroup variation to estimate gene stability. The final results achieved with each algorithm often differed from one another, due to various methods of approaching the problem of calculating the expression stability used by the algorithms. The RefFinder algorithm uses the geometric mean of the results of the other algorithms to conduct a general analysis. Although this approach may not be biologically relevant in some cases, the result of RefFinder is consistently corroborated by those of the majority of other algorithms,[27] indicating that the reference genes chosen using this approach are reliable.

In this study, the RefFinder algorithm validated *Rpl13a* as the most stable gene in the db/db and HFD models, but not in KKAy mice, possibly because ribosomal proteins are crucial components involved in basic physiological cell processes, which are key to maintaining cell development and tissue homeostasis.[49,50] Ribosomal proteins are established to have essential roles in ribosome assembly and protein translation, and are also reported to have ribosome-independent functions. RPLP0 can be recruited by irradiation-induced paraspeckles, interact with NONO in the nucleus, and is involved in DNA repair.[51] Eukaryotic translation elongation factor 1A (EF1A) is required for recruitment of aminoacyl tRNAs to the A-site of the ribosome during translation elongation and binding to GTP.[52] *GAPDH* was chosen as a reference gene in most published studies, possibly because its sequence is relatively conserved and due to its established reliability as a reference gene;[53] however, GAPDH is involved in various physical process in cells, and can be significantly influenced by other factors.[12] In our study, *Gapdh* was considered the most stable housekeeping gene in KKAy mice, but found to be unstable in db/db mice. These genes are relatively stable in the three models studied. The gene that encodes the 18S rRNA subunit, *Rn18s*, exhibited highly variable expression in the mouse models analyzed, despite its high expression levels in mouse cortical bone tissue. The theoretical assumptions applicable to reference genes are generally unlike those for target genes, because reference gene transcripts are often present at much greater abundance than that of the studied gene.[12] Moreover, while high level expression may lead to greater errors, dilution

of samples before RT-qPCR may prevent detection of target genes. Previous reports concluded that *Rn18s* in inadequate for use in normalization of RT-qPCR analysis of osteogenic and chondrogenic differentiation[54] and in mouse osteoblasts and osteoclasts.[55] The *Hprt1* gene encodes hypoxanthine-guanine phosphoribosyl transferase (HPRT) may regulate bone metabolism,[56,57] and was also variably expressed in the models analyzed in this study. In addition, differences in levels of modification-dependent protein catabolic processes and protein ubiquitination in db/db mice may be due to variation in *Ubc* expression.

There are several limitations in our study. Firstly, the sample size is relatively small. Additionally, each sample in our study required the analysis of 14 genes, accompanied by a duplicate test, surpassing the capacity of a single 384-well reaction plate. Consequently, we were compelled to employ multiple plates. This experimental setup may introduce potential inconsistencies across different plates due to the limited capacity for accommodating all samples and their replicates on a single plate. Moreover, we have not yet verified the selected stable reference genes through normalization of target genes. Future work will be dedicated to validating the most suitable set of these stable genes for effective normalization of target genes.

Conclusion

Cautious selection of reference genes can contribute to the accuracy of gene expression analysis. It is recommended that RT-qPCR data are normalized using at least two validated reference genes. We identified *Rpl13a* as the mostly stably expressed reference gene in cortical bone tissue from mouse models of obesity and diabetes (db/db), while *Gapdh* was the most stable reference gene in another diabetes model, KKAy mice. *Ef1a, Ppia, Rplp0*, and *Rpl22* are alternative genes that could be used for the normalization of gene expression in cortical bone tissue from mouse models of obesity and diabetes. In conclusion, we selected a set of housekeeping genes for use in analysis of bone from three mouse models of metabolic conditions, which will provide a convenient reference for researchers.

Acknowledgment

This research was funded by Natural Science Foundation of Chongqing (China), CSTB2022NSCQ-MSX0110, cstc2021jcyj-msxmX0320; the Future Medical Youth Innovation Program of Chongqing Medical University, W0046. The APC was funded by Chongqing Medical University.

Disclosure

The authors report no conflicts of interest in this work.

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