Identification of appropriate reference genes for gene expression studies in mice left ventricles from different developmental stages

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Research Article

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Abstract

Aims: Real-time quantitative polymerase chain reaction (RT-qPCR) is the standard assay used for revealing the gene expression characteristics. However, the RT-qPCR studies all need reference genes for normalization to make the results comparable, which should hold a high expression stability during all experimental datasets. So far, there was no optimal set of reference genes identified in mice left ventricles (LV) across embryonic and postnatal stages. The objective of our research was to identify the appropriate reference genes in mice LV from different developmental stages.

Methods and Results: we investigated the gene expressions of common 21 candidate housekeeping genes in mice LV from 7 different developmental stages, almost throughout the whole period of the mouse lifespan. The expression of some candidate reference genes, such as *18S* and *Actb*, apparently fluctuated. The stability of potential reference genes was evaluated by a number of methods, such as GeNorm, NormFinder, BestKeeper, Delta-Ct and RefFinder method. we identified a set of optimal reference genes that can be reliably used for normalization of RT-qPCR experiments in different developmental stages of mice LV. Our results showed that following genes should not be used as reference genes in mice LV development studies: *18S*, *Hmbs*, *Ubc*, *Psmc4*, *Tfrc* and *Actb*. And the *Rplp0* appeared to represent a good choice.

Conclusions: Our study provides the expression stability of the commonly used reference genes in process of LV development and maturation. We also identified a set of optimal reference genes under different conditions. Our findings may be helpful in future studies to investigate the gene expression patterns and mechanism of mammalian heart development.

Introduction

The heart is the first organ to develop in the embryo, which held significantly different physiological function and gene expression profiling among different developmental stages[1]. Cardiac malformation is the most common form of congenital anomalies associated with a serious societal impact because of the significant morbidity and mortality[2]. Meanwhile, multiple studies have found that the distinct gene expression pattern during cardiac maturation[3,4]. The main function of the heart, pumping blood to make a constant supply of oxygen and nutrients for body, is mainly determined by the left ventricular (LV) status[5,6]. And the LV remodeling is the major manifestations of many cardiac disorders[7,8]. However, the specific molecular mechanism involved in LV development/maturation remained not fully understanding[9].

Mice have been used frequently as a model in the area of cardiac development/maturation, because of the similar anatomical, genetical and physiological characteristics[10]. Transcriptome analysis have been confirmed to providing a broad insight into the molecular regulatory networks that underlie stage-specific differences in mice hearts[11,12]. Until today real-time quantitative polymerase chain reaction (RT-qPCR) is the standard assay used for gene expression quantification analysis with high sensitivity and accuracy[13]. However, the RT-qPCR studies all need reference genes for normalization to make the results comparable among different groups[14]. The unstable reference genes would lead to erroneous results, which makes the selection of the internal reference genes an important factor in the design of a RT-qPCR experiment[15]. The reference genes should hold a high expression stability during all experimental datasets[16,17]. So far, there was no ideal reference gene that is appropriate for all kinds of conditions[18]. Thus, it is critical to identify the reference genes that have relatively stable expression in the specific context of study.

The common reference genes used in RT-qPCR for heart samples were Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*), 18S ribosomal RNA (*18S*), and actin beta (*Actb*)[19]. And the other genes also have been used as reference genes in several studies[17]. But not all are suitable for all experimental conditions or different developmental samples[20]. Some housekeeping genes were demonstrated significantly altered in heart development or maturation process[21]. A recent study identified the appropriate reference genes for normalizing gene expression data of heart with different developmental stages in mouse[22]. So far, there was little optimal set of reference genes had been identified in LV tissues across embryonic and postnatal stages in mice.

Our work systematically assessed the expression stability of the commonly used reference genes (n=21) in mice LV across heart development and maturation. Synthesizing the results of a number of methods, such as GeNorm[21], NormFinder[23], BestKeeper[24], Delta-Ct[25] and RefFinder[26] method, we identified a set of optimal reference genes that can be reliably used for normalization of RT-qPCR experiments in different specific conditions.

Methods

Sample collection

The C57BL/6 mice were maintained in plastic cages at 23 to 25 °C with a 12/12-hour light/dark cycle. The protocol for the experiment was approved by the Animal Care and Use Committee of Fuwai hospital. The animals were mated overnight and pregnancy was detected at 0.5 gestation day by the observation of the mating plug. The mice of various ages were euthanized by CO₂ inhalation to collect LV specimens (**Figure 1**). The mouse embryonic hearts were collected when the pregnant female mice were sacrificed on exact gestation day. LV in embryonic hearts were identified and collected by careful microdissection under stereomicroscope (Leica) using sharpened microdissection scissors. Tissue from 10 to 15 embryos were pooled in one tube to ensure a sufficient final RNA per sample, so did in mice within 7 days of birth.

RNA isolation and cDNA synthesis

Each RNA isolation was performed from 30 mg of the frozen LV tissue. MagNA Lyser Green Beads (Roche, Switzerland) containing TRIzol (Invitrogen, USA) and the MagNA lyser instrument (Roche, Switzerland) were used for tissue homogenate. Then the standard instructions of Trizol manufacturer were carried out for total RNA extraction. The concentration and quality were evaluated by NanoDrop2000 (NanoDrop Technology, USA). For each sample, First Strand cDNA was synthesized from 500ng total RNA by using the Takara Reverse Transcription Kit (Takara, Japan). All samples within this experiment were processed simultaneously to avoid interexperimental variations.

Selection of candidate reference genes and Primer design

In this study, the 21 housekeeping genes (**Table 1**), which were commonly used as reference genes in previous cardiac studies, were analyzed to provide a better reference guide for identification of molecular mechanisms underlying cardiac development and maturation. Those candidate genes included the *Actb* (Actin beta), *Gapdh* (Glyceraldehyde-3-phosphate dehydrogenase), *Reep5* (Receptor accessory protein5), (Ribosomal protein L5), *Psmb4* (Proteasome subunit beta 4), *Vcp* (Valosin containing protein), *B2m* (Beta-2-microglobulin), *Gusb* (Glucuronidase beta), *Hmbs* (Hydroxymethylbilane synthase), *Hprt1* (Hypoxanthine phosphoribosyltransferase 1), *Ipo8* (Importin 8), *Pgk1* (Phosphoglycerate kinase 1), *Polr2a* (RNA polymerase II subunit A), *Ppia* (Peptidylprolyl isomerase A), *Rplp0* (Ribosomal protein lateral stalk subunit P0), *Tbp* (TATA box binding protein), *Tfrc* (Transferrin receptor), *Ubc* (Ubiquitin C), *Ywhaz* (14-3-3 protein zeta), *18S* (eukaryotic 18S ribosomal RNA) and *Sdha* (succinate dehydrogenase complex flavoprotein subunit A). According to the published mouse sequences, the NCBI primer designing tool was used to generate the RT-qPCR primers sequences (**Table 1**).

RT-qPCR

RT-qPCR was performed in 384 well plates using the Viia7 Real Time PCR System (Applied Biosystems). Each 10 µl reaction mixture contained the 5 µl 2× SYBR Green Real-Time PCR Master Mix reaction mixture, 0.4 µL of each primer (10 µM), 2µl cDNA (2.5 ng/µl) and 2.2 µL double-distilled water. The thermal cycling program: 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was performed in three technical replicates and the resulting Ct values averaged.

Evaluation of Expression Stability

The gene expression stability was evaluated by analyzing the raw Ct values in the four independent statistical applications: GeNorm, Normfinder, BestKeeper and Delta-Ct method. And we also performed a consensual analysis by RefFinder to make a comprehensive variability score for each reference gene.

Statistical analysis

Continuous variables are expressed as the mean $\pm$ the standard deviation (SD) without special instructions. A one-way analysis of variance (ANOVA) was performed to calculate whether there is statistically different among groups. Differences with a P-value <0.05 were considered statistically significant. All statistical analyses were performed using SPSS Statistics, version 23.0 (IBM Corp, Armonk, NY), and graphs were generated using GraphPad Prism 7 (GraphPad Software Inc., CA).

Results

Expression characteristics of the reference genes

We showed the full gene names and corresponding GeneBank accession numbers of the 21 candidate housekeeping genes in **Table 1**. The **table 2** demonstrated the RNA concentrations at each stage of study, which ranged from 61.08 - 83.38 ng/ μ l. No significant between-group differences were apparent in RNA content. The extracted RNA quality is shown as 260 nm /280 nm ratio and 260 nm /230 nm ratio assessed by Nanodrop 2000 Spectrophotometer. Both ratios were close to 2.0, indicating the high quality of the RNA samples.

For the primer sequences and the amplified product length also see **Table 1**. Analysis of the melting curve assays, which showed the single peak, indicated the RT-qPCR amplification had good specificity (**Figure 2A**). The Ct (cycle threshold) -values obtained by RT-qPCR were used to quantify the gene expression levels, with a low Ct value indicating a high gene expression level and vice versa. **Figure 2B** illustrated the expression in each candidate reference gene abundance across the prenatal to postnatal periods of heart development. The candidate reference genes studied presented different expression levels with the mean Ct value ranged from 16.16 (*Gapdh*) to 25.19 (*Gusb*). The expression of the most frequently used internal reference gene in RT-qPCR experiments, *Actb*, revealed the unexpectedly significant variations at 7 different developmental stages of mice hearts. We found that some genes hold a more stable expression (such as *Ubc*, *B2m*, *Vcp*), whereas others were discrete, e.g., *Ppia*, *Tfrc*, *Actb*. These data illustrated the need for selection of appropriate reference genes for gene expression studies in mice hearts from different developmental stages.

For more efficient analysis of the expression pattern of those candidate reference genes at different development or maturation phases, we used the hierarchical cluster analysis (HCA) and orthogonal projections to latent structures - discriminant analysis (OPLS-DA) to globally visualize the expression classifications. Three phases can be distinguished in the gene expression features, that are (A) embryo stage, (B) first 7 days after birth, (C) 1 to 9 months after birth (**Figure 3**).

Stability of candidate reference genes

To assess the stability of gene expression, four different tools were used for each dataset tested in the two developmental stages addressed: GeNorm, NormFinder, Delta-Ct and BestKeeper. **Table 3** demonstrated the results of the different statistical methods for analyzing the temporality expression variabilities of 21 housekeeping genes not only at earlier stages (embryonic stages). Although the scores and rank orders are not exactly the same as those reported in each of the calculation methods, the ranges of the dynamics are roughly similar in GeNorm, NormFinder, and Delta-Ct (**Table 3**).

GeNorm, NormFinder, and Delta-Ct, working on similar principles, held different computational approaches with BestKeeper. The stability values derived from BestKeeper based on the CV and SD values, while the former methods by comparing the average variation of a gene to all other. So, a consensual statistical analysis of the variabilities of the housekeeping genes was needed. Based on previous studies, we employed the RefFinder analysis to obtain a comprehensive evaluation of candidate reference genes by integrating all four analysis programs of the above-mentioned. The overall rank order of the most stable reference genes is shown in **Table 3** for different comparisons across different developmental stages.

We summarized these results of the overall gene stability in each dataset with different condition using the heatmap or dot plot graph to illustrate the most suitable 5 reference genes for calculating the gene expression (**Figure 4**). Concerning the embryo development of LV, the *Ppia*, *Rplp0*, *B2m*, *Vcp*, and *Gapdh* were selected as the optimal reference genes when comparing the embryonic LV at 14-16 (E14-16) with LV at E17-20. In contrast, the *Ppia* and *Gapdh* were no more reliable choices for comparison of embryonic LV VS. hearts within 7 days of birth (E14-20 VS. D1-7). And the best selections of reference genes in this condition were the *Vcp*, *Rplp0*, *Ywhaz*, *B2m*, and *Hprt1* (**Table 3, Figure 4**). Regarding the gene expression stabilities in LV on embryonic (E14-20) and postnatal maturation stages (M1-9), the results indicated the *Rplp0*, *Gapdh*, *Tbp*, *Vcp*, *Gusb* should be the appropriate reference genes for RT-qPCR experiments. To make the results of RT-qPCR as accurate as possible related to the early postnatal (D1-7) and postnatal maturation stages (M1-9), *Reep5*, *Rplp0*, *Polr2a*, *Pgk1*, *Rpl5* represent the best set of reference genes (**Table 3, Figure 4**). Finally, among the 21 reference genes evaluated for their expression variabilities in comparing all LV samples under different phases of developmental maturity, the optimal set of reference genes should be *Rplp0*, *Tbp*, *Vcp*, *Gusb*, and *Rpl5*. On different conditions, the appropriate set of reference genes were not the same across groups. Our results showed that following genes should not be used for normalization in RT-qPCR experiments in mice hearts: *18S*, *Hmbs*, *Ubc*, *Psm4*, *Tfrc* and *Actb*. And the *Rplp0* appeared to represent a good choice as reference gene when performing all the comparisons in the mice LV samples.

Gene expression levels normalized by different reference gene

We further verified the results by using the different internal reference genes to normalize the RT-qPCR data sets and to calculate the expression differences among groups. The most and least stable reference gene (*Rplp0* and *18S*) were included for RT-qPCR data analysis with the the same samples. As the results showed in **Figure 5**, the selection of proper internal reference gene induced very profound effects on gene expression levels. There was a large and significant difference in *Vcp* gene expression profile among the groups when *Rplp0* as reference gene for normalization (**Figure 5A**), however, no significance when *18S* used as reference gene (**Figure 5B**). As for the expression of *Pgk1* in mice LVs with different stages, it is evident from **Figure 5** that the statistical significance was obviously increased by the selecting of reference gene as *Rplp0* rather than *18S*.

Discussions

The previous literatures pointed out that there is no perfect internal reference gene appropriate to all situations for the quantification of target gene expression levels in RT-qPCR experiments[18]. We presented a detailed analysis of the selection of reference genes in mice LV tissues from different developmental stages in this study. And a set of the most suitable internal reference genes were proposed when normalizing the RT-qPCR data obtained from different developmental stages of mice LV tissues.

The RT-qPCR has become the most common and useful method for revealing the gene expression characteristics. Selecting an appropriate reference gene for normalization in RT-qPCR experiments was important to reduce the effect of sample heterogeneity and provide more accurate results[13,20]. The mechanism of heart development, which was often studied by transcriptomics, was not completely elucidated. Thus, it is crucial to identify the stable reference genes during those periods. The previous reference genes studies primarily based on myocardial tissues from adult mice or whole hearts[27]. The number of reference genes studies during heart development and maturation was relatively small. It is the limitations of previous researches that only a limited number of candidate reference genes and a small number of samples with different developmental stages were included[22,27,28]. In this study, we investigated the gene expressions of common 21 candidate housekeeping genes in 7 different developmental stages, almost throughout the whole period of the mouse lifespan. We therefore believed the results from our systematical study is likely to be more applicable and robust.

Previous investigators demonstrated that the expression of some candidate reference genes can significantly vary in different condition[22]. For example, gene *18S*, *Actb* and *Gapdh* held considerably alterations of expression level depending upon developmental stages and experimental conditions[29]. It is worth mentioning that the expression of *Gapdh* and *Actb* apparently fluctuated even in normal myocardial tissues of mice. In agreement, our data also demonstrated there were altered expressions of above reference genes in developing hearts. *18S*, *Actb* and *Gapdh* were the most frequently used internal reference genes in cardiac-related researches. But in our study, it is indicated the *18S* and *Actb* did not have a sufficiently stable expression. Thus, it

cannot be used as ideal reference gene in RT-qPCR experiments on LV development or maturation. The Gapdh may be redeemed as a better candidate reference gene only under some specific conditions (Regarding the gene expressions in LV at embryonic stages or comparison between embryonic (E14-20) and postnatal maturation stages (M1-9)). There is a congruence between this data with our transcriptomic data of human heart samples in different developmental stages (in-house data), suggesting the *Actb* and *18S* unsuitable as reference control in heart development studies. The results listed in **Table 3** indicated the *Rplp0* have a greater gene expression stability across the sample sets. The protein, *Rplp0* encoding, is a component of the 60S subunit and responsible for the synthesis of proteins in the cell[30]. It was the only one reference gene that could apply to all subgroup analysis. Based on the results, we considered the *Rplp0* is an optimal reference gene to be used to normalize data in mice LV development or maturation.

Moreover, selecting the different reference gene as control, yielded a completely different results of the expression levels of target genes (**Figure 5**), which could lead to biased results and, in the worst case, wrong conclusions. This also emphasizes the importance of selecting the optimal reference gene when studying the transcriptomic signatures in heart development or maturation process.

Conclusions

Our study provides for the first time the expression stability of the commonly used reference genes in process of LV development and maturation. Through four analysis approaches (GeNorm, Normfinder, BestKeeper and the Delta-Ct) and a consensual analysis (RefFinder), we identified a set of optimal reference genes under different conditions. Our findings may be helpful in future studies to investigate the gene expression patterns and mechanism of mammalian LV development.

Declarations

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Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The study protocol was reviewed and approved by the Animal Care and Use Committee of Fuwai hospital.

Conflict of interest: None declared

Authors' contribution: JR and NNZ participated in the design of the study, performed the measurements and the statistical analysis. XJL helped in data collection and the interpretation of data. JR, XGS and JPS wrote the manuscript. All authors read and approved the manuscript.

Data availability: Data sharing are applicable to this article without restriction.

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Tables

Table 1. Candidate reference genes and primer sequences

Gene symbol	Gene Name	GenBank Accession	5'-Primer Sequences (Forward/Reverse)-3'	Product size
Actb	Actin beta	NM_007393	GGCTGTATTCCCCTCCATCG / CCAGTTGGTAACAATGCCATGT	154 bp
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	NM_001289726	AGGTCGGTGTGAACGGATTTG / TGTAGACCATGTAGTTGAGGTCA	123 bp
Reep5	Receptor accessory protein 5	NM_007874	GGTTCCTGCACGAGAAGAACT / GAGAGAGGCTCCATAACCGAA	140 bp
Rpl5	Ribosomal protein L5	NM_016980	TTGGTGATCCAGGACAAGAATAA / GCACAGACGATCATATCCCC	125 bp
Psmb4	Proteasome subunit beta 4	NM_008945	ATGGAAGCGTTTTGGGAGTCA / GTTCTGGGTCCGAGTGATGG	144 bp
Vcp	Valosin containing protein	NM_009503	GCTTGTAAGTGGCCATTCTG / GATCTCAGGCACTGGATCGT	114 bp
B2m	Beta-2-microglobulin	NM_009735	TTCTGGTGCTTGTCTCACTGA / CAGTATGTTTCGGCTTCCCATTCT	104 bp
Gusb	Glucuronidase beta	NM_010368	GGCTGGTGACCTACTGGATTT / GGCCTGGGAACCTGAAGT	131 bp
Hmbs	Hydroxymethylbilane synthase	NM_001110251	AAGGGCTTTTCTGAGGCACC / AGTTGCCCATCTTTCATCACTG	78 bp
Hprt1	Hypoxanthine phosphoribosyltransferase 1	NM_013556	GGTTAAGCAGTACAGCCCCA / GGCCTGTATCCAACACTTCG	81 bp
Ipo8	Importin 8	NM_001081113	ACGTGACAGTAGATACCAACGC / GCATAGCACTCGGCATCTTCT	115 bp
Pgk1	Phosphoglycerate kinase 1	NM_008828	ATGTCGCTTTCCAACAAGCTG / GCTCCATTGTCCAAGCAGAAT	164 bp
Polr2a	RNA polymerase II subunit A	NM_001291068	AAATACCCAGAAACAACGGAGG / CCAGTCCGCTCAATCACCC	83 bp
Ppia	Peptidylprolyl isomerase A	NM_008907	GAGCTGTTTTGCAGACAAAGTTC / CCCTGGCACATGAATCCTGG	125 bp
Rplp0	Ribosomal protein lateral stalk subunit P0	NM_007475	AGATTCGGGATATGCTGTTGGC / TCGGGTCTAGACCAGTGTTCT	109 bp
Tbp	TATA box binding protein	NM_013684	GTGGGGAGCTGTGATGTGA / TCCAGGAAATAATTCTGGCTCA	96 bp
Tfrc	Transferrin receptor	NM_011638	GTTTCTGCCAGCCCTTATTAT / GCAAGGAAAGGATATGCAGCA	152 bp
Ubc	Ubiquitin C	NM_019639	GAGGTGGCATGCAGATCTTT / CCCTCCTTGCTCTGGATCTT	112 bp
Ywhaz	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	NM_011740	GAAAAGTTCTTGATCCCCAATGC / TGTGACTGGTCCACAATTCCTT	134 bp
18S	eukaryotic 18S ribosomal RNA	NR_003278	CTCAACACGGGAAACCTCAC / CGCTCCACCAACTAAGAACG	110 bp
Sdha	succinate dehydrogenase complex flavoprotein subunit A	NM_023281	GGAACACTCCAAAAACAGACCT / CCACCACTGGGTATTGAGTAGAA	106 bp

Table 2. The quality of RNA samples isolated from left ventricles

	N	RNA content (ng/ul)	A260 (Abs)	260nm/280 nm ratio		260nm/230 nm ratio	
				mean	SD	mean	SD
E 14~16	4	67.65±2.92	1.70±0.07	1.97	0.03	2.13	0.09
E 17-20	5	66.98±2.40	1.67±0.06	1.99	0.03	2.08	0.13
D 1~3	6	61.08±4.56	1.53±0.11	2.02	0.01	2.09	0.07
D 4~7	5	69.12±9.59	1.73±0.24	2.03	0.01	2.05	0.14
M 1~2	5	83.38±22.58	2.08±0.56	2.05	0.02	2.21	0.05
M 3~5	7	65.57±4.78	1.64±0.12	2.08	0.02	1.97	0.17
M 6~9	6	66.17±3.67	1.65±0.09	2.06	0.02	2.16	0.08

E= embryonic day; D= postnatal day; M= postnatal month; N= sample size; SD= standard deviation

Table 3. Reference gene expression variability and rankings

GeNorm		NormFinder		BestKeeper		Delta CT		RefFinder	
Gene name	Stability value	Gene name	Stability value	Gene name	std dev	Gene name	STDEV	Gene name	Geomean of ranking values
E 14~16/E 17~20(n=4/5)									
Ppia	0.064	Ppia	0.032	Hprt1	0.24	Ppia	0.46	Ppia	1.57
Rplp0	0.064	B2m	0.039	Reep5	0.26	B2m	0.46	Rplp0	2.82
Vcp	0.081	Rplp0	0.099	Gapdh	0.27	Rplp0	0.47	B2m	3.36
B2m	0.093	Vcp	0.13	Vcp	0.35	Vcp	0.47	Vcp	3.94
Tbp	0.109	Ywhaz	0.138	Ubc	0.35	Tbp	0.5	Gapdh	6.42
Rpl5	0.12	Tfrc	0.161	Ppia	0.4	Ywhaz	0.5	Hprt1	6.57
Gapdh	0.14	Rpl5	0.185	Rplp0	0.41	Rpl5	0.5	Tbp	6.69
Ywhaz	0.174	Tbp	0.186	B2m	0.44	Tfrc	0.51	Rpl5	7.17
Tfrc	0.206	Gapdh	0.258	Rpl5	0.44	Gapdh	0.52	Ywhaz	7.33
Ubc	0.233	Ubc	0.263	Tbp	0.45	Ubc	0.55	Ubc	7.95
Hprt1	0.254	Gusb	0.317	Tfrc	0.47	Gusb	0.57	Tfrc	8.3
Gusb	0.284	Actb	0.334	Ywhaz	0.57	Actb	0.59	Reep5	9.36
Actb	0.303	Hprt1	0.394	Sdha	0.62	Hprt1	0.61	Gusb	12.35
Ipo8	0.341	Ipo8	0.487	Ipo8	0.67	Ipo8	0.68	Actb	13.55
Reep5	0.378	Sdha	0.573	Hmbs	0.68	Sdha	0.75	Ipo8	14
Sdha	0.409	Reep5	0.593	Gusb	0.7	Reep5	0.76	Sdha	14.71
Hmbs	0.462	Hmbs	0.806	Pgk1	0.7	Hmbs	0.94	Hmbs	16.48
Psm4	0.51	Psm4	0.808	Actb	0.74	Psm4	0.95	Psm4	18.24
Polr2a	0.552	Polr2a	0.857	Psm4	0.85	Polr2a	0.95	Polr2a	19.25
18S	0.605	18S	1.063	Polr2a	1.01	18S	1.14	Pgk1	19.92
Pgk1	0.68	Pgk1	1.351	18S	1.19	Pgk1	1.4	18S	20.25
E 14~20/D1~7(n=9/11)									
Vcp	0.103	Vcp	0.075	Hprt1	0.23	Vcp	0.46	Vcp	1.19
Rplp0	0.103	Rplp0	0.097	Vcp	0.25	Rplp0	0.47	Rplp0	2.11
Ywhaz	0.175	Ywhaz	0.162	B2m	0.28	Ywhaz	0.48	Ywhaz	3.71
B2m	0.21	B2m	0.194	Tbp	0.28	Gusb	0.49	B2m	4.23
Tbp	0.225	Gusb	0.2	Rplp0	0.31	B2m	0.49	Hprt1	5.2
Gusb	0.243	Ppia	0.218	Ppia	0.36	Ppia	0.51	Tbp	5.57
Actb	0.255	Actb	0.242	Ywhaz	0.37	Actb	0.52	Gusb	6.03
Ppia	0.277	Tbp	0.256	Reep5	0.39	Tbp	0.52	Ppia	6.45
Hprt1	0.292	Hprt1	0.261	Ipo8	0.39	Hprt1	0.54	Actb	8.47

Rpl5	0.306	Tfrfc	0.285	Tfrfc	0.39	Tfrfc	0.55	Tfrfc	10.24
Tfrfc	0.326	Rpl5	0.32	Gusb	0.4	Ipo8	0.56	Ipo8	10.93
Ipo8	0.341	Ipo8	0.321	Sdha	0.43	Rpl5	0.56	Rpl5	11.45
Polr2a	0.38	Polr2a	0.576	Ubc	0.44	Polr2a	0.71	Sdha	13.71
Sdha	0.419	Gapdh	0.604	Actb	0.44	Sdha	0.75	Polr2a	13.9
Ubc	0.45	Sdha	0.62	Rpl5	0.44	Gapdh	0.75	Reep5	14.07
Reep5	0.477	Ubc	0.643	Hmbs	0.53	Ubc	0.77	Ubc	15.23
Gapdh	0.507	Psm4	0.655	Polr2a	0.55	Reep5	0.79	Gapdh	15.92
Psm4	0.535	Reep5	0.656	Psm4	0.65	Psm4	0.8	Psm4	17.99
Hmbs	0.561	Hmbs	0.67	Gapdh	0.65	Hmbs	0.82	Hmbs	18.2
18S	0.586	18S	0.733	18S	0.73	18S	0.85	18S	20
Pgk1	0.65	Pgk1	1.197	Pgk1	0.88	Pgk1	1.26	Pgk1	21
E 14~20/ M1~9 (n=9/18)									
Rplp0	0.236	Rplp0	0.068	Reep5	0.35	Rplp0	0.64	Rplp0	1.78
Gapdh	0.236	Tbp	0.07	Ubc	0.35	Tbp	0.65	Gapdh	3.22
Rpl5	0.268	Gapdh	0.248	B2m	0.39	Gapdh	0.69	Tbp	3.44
Hprt1	0.278	Vcp	0.276	Vcp	0.43	Gusb	0.69	Vcp	5.26
Tbp	0.295	Gusb	0.281	Sdha	0.49	Rpl5	0.71	Gusb	6.03
Gusb	0.319	Rpl5	0.371	Ipo8	0.51	Vcp	0.71	Rpl5	6.06
Ywhaz	0.35	Hprt1	0.409	Tbp	0.64	Hprt1	0.73	Hprt1	7.24
Vcp	0.393	Ipo8	0.438	Hmbs	0.69	Ipo8	0.78	Ipo8	8.06
Hmbs	0.433	Hmbs	0.455	Polr2a	0.71	Ywhaz	0.79	Reep5	8.44
Psm4	0.47	Polr2a	0.514	Rplp0	0.76	Hmbs	0.8	Hmbs	8.97
Ipo8	0.506	Psm4	0.531	Gusb	0.81	Polr2a	0.83	Ubc	9.46
Polr2a	0.533	Ywhaz	0.545	Gapdh	0.84	Psm4	0.84	Polr2a	10.44
Actb	0.572	Reep5	0.669	Psm4	0.86	Reep5	0.91	Ywhaz	10.65
Ppia	0.609	18S	0.77	Hprt1	0.95	Actb	0.98	Psm4	11.45
Reep5	0.649	Actb	0.835	Rpl5	1	18S	1.01	B2m	11.65
18S	0.683	Ppia	0.936	18S	1.05	Ppia	1.06	Sdha	14.67
Pgk1	0.723	Tfrfc	1.008	Ywhaz	1.07	B2m	1.15	Actb	15.09
Tfrfc	0.762	Pgk1	1.014	Pgk1	1.21	Pgk1	1.16	18S	15.23
B2m	0.812	B2m	1.03	Actb	1.29	Tfrfc	1.18	Ppia	16.36
Ubc	0.855	Ubc	1.101	Ppia	1.42	Ubc	1.2	Pgk1	17.74
Sdha	0.893	Sdha	1.155	Tfrfc	1.51	Sdha	1.26	Tfrfc	18.69
D 1~7/ M1~9 (n=11/18)									

Reep5	0.174	Rplp0	0.075	Ubc	0.18	Rplp0	0.48	Reep5	2.38
Polr2a	0.174	Reep5	0.085	B2m	0.32	Reep5	0.48	Rplp0	2.58
Pgk1	0.195	Polr2a	0.106	Sdha	0.32	Polr2a	0.48	Polr2a	2.82
Rplp0	0.211	Rpl5	0.126	Ipo8	0.33	Rpl5	0.5	Pgk1	5.23
Rpl5	0.223	Pgk1	0.165	Vcp	0.34	Pgk1	0.51	Rpl5	5.89
Tbp	0.248	Tbp	0.212	Gapdh	0.5	Tbp	0.52	Vcp	6.88
Gusb	0.263	Gusb	0.249	Polr2a	0.51	Vcp	0.53	Tbp	7.14
Vcp	0.284	Vcp	0.283	Reep5	0.52	Gusb	0.54	Ipo8	7.35
Ipo8	0.299	Ipo8	0.32	Hmbs	0.52	Ipo8	0.55	Ubc	8
Hprt1	0.321	Hprt1	0.33	Pgk1	0.61	Hprt1	0.58	Gusb	8.9
Hmbs	0.345	Hmbs	0.395	Rplp0	0.62	Hmbs	0.62	Sdha	9.65
Psmb4	0.37	Psmb4	0.41	Tbp	0.64	Gapdh	0.64	Hmbs	10.46
Gapdh	0.39	Gapdh	0.455	18S	0.66	Psmb4	0.64	Gapdh	10.5
18S	0.422	Ywhaz	0.55	Psmb4	0.67	Ywhaz	0.7	Hprt1	11.42
Sdha	0.452	18S	0.551	Rpl5	0.71	18S	0.73	B2m	11.82
Ubc	0.475	Ubc	0.643	Gusb	0.73	Ubc	0.76	Psmb4	12.72
Ywhaz	0.5	Sdha	0.665	Hprt1	0.84	Sdha	0.77	18S	14.23
B2m	0.536	Ppia	0.755	Ywhaz	0.98	Ppia	0.85	Ywhaz	15.65
Ppia	0.572	B2m	0.888	Ppia	1.18	B2m	0.95	Ppia	18.49
Actb	0.615	Actb	0.929	Actb	1.32	Actb	0.99	Actb	20
Tfrc	0.664	Tfrc	1.051	Tfrc	1.47	Tfrc	1.14	Tfrc	21
D 1~3/ D 4~7 (n=6/5)									
Vcp	0.056	Gusb	0.072	Ipo8	0.13	Vcp	0.23	Vcp	1.78
Rplp0	0.056	Vcp	0.08	B2m	0.14	Gusb	0.23	Rplp0	2.45
Gusb	0.074	Rpl5	0.088	Rplp0	0.14	Rplp0	0.23	Gusb	2.45
B2m	0.095	Rplp0	0.09	Tbp	0.14	Rpl5	0.24	Ipo8	4.6
Polr2a	0.112	Ppia	0.094	Gusb	0.15	Ppia	0.24	Rpl5	4.9
Rpl5	0.125	Sdha	0.099	Vcp	0.15	Sdha	0.24	B2m	6.45
Sdha	0.133	Ipo8	0.102	Hprt1	0.17	Hprt1	0.25	Sdha	7.09
Ipo8	0.141	Hprt1	0.107	Polr2a	0.17	Ipo8	0.25	Ppia	7.24
Hprt1	0.149	Actb	0.124	Sdha	0.17	B2m	0.25	Hprt1	7.71
Ppia	0.153	Reep5	0.125	Rpl5	0.17	Polr2a	0.26	Polr2a	8.39
Reep5	0.157	Polr2a	0.128	Ppia	0.18	Reep5	0.26	Tbp	9.06
Actb	0.161	B2m	0.133	Ywhaz	0.18	Actb	0.26	Reep5	11.2
Ywhaz	0.169	Ywhaz	0.206	Reep5	0.19	Ywhaz	0.29	Actb	11.81
Pgk1	0.182	Pgk1	0.217	Ubc	0.19	Pgk1	0.31	Ywhaz	12.74

Tbp	0.196	Tbp	0.269	Actb	0.21	Tbp	0.34	Pgk1	14.48
Gapdh	0.21	Gapdh	0.289	Pgk1	0.26	Gapdh	0.36	Ubc	16.66
Ubc	0.225	18S	0.313	18S	0.26	18S	0.38	Gapdh	16.7
18S	0.237	Ubc	0.325	Tfric	0.33	Ubc	0.38	18S	17.24
Tfric	0.253	Tfric	0.345	Gapdh	0.35	Tfric	0.41	Tfric	18.74
Psm4	0.283	Psm4	0.529	Hmb	0.36	Psm4	0.57	Psm4	20.25
Hmb	0.314	Hmb	0.575	Psm4	0.4	Hmb	0.61	Hmb	20.75
M1~9 (n=18)									
Pol2a	0.102	Reep5	0.039	Ubc	0.17	Reep5	0.28	Reep5	2.45
Sdha	0.102	Sdha	0.047	Vcp	0.24	Sdha	0.28	Sdha	2.69
Reep5	0.118	Ppia	0.073	B2m	0.25	Ppia	0.29	Pol2a	3.76
Ywhaz	0.13	Rplp0	0.079	lpo8	0.31	Rplp0	0.29	Ppia	5.3
Gusb	0.137	Pol2a	0.108	Gusb	0.33	Pol2a	0.29	Rplp0	6.16
lpo8	0.14	Ywhaz	0.115	Tbp	0.33	Tbp	0.29	Ywhaz	6.24
Tbp	0.143	Hmb	0.119	Hmb	0.35	Ywhaz	0.3	Gusb	6.51
Ppia	0.149	Tbp	0.123	Pol2a	0.36	Gusb	0.3	Tbp	6.7
Rplp0	0.152	Gusb	0.137	Ywhaz	0.37	Hmb	0.3	lpo8	7.33
Hmb	0.155	Hprt1	0.144	Rplp0	0.38	lpo8	0.31	Hmb	8.15
Hprt1	0.164	Pgk1	0.152	Reep5	0.39	Hprt1	0.32	Vcp	8.9
Pgk1	0.174	lpo8	0.164	Ppia	0.39	Pgk1	0.34	Ubc	9.1
Rpl5	0.183	Rpl5	0.166	Sdha	0.41	Rpl5	0.34	Hprt1	11.41
Vcp	0.192	Actb	0.236	Hprt1	0.43	Vcp	0.35	B2m	11.5
Actb	0.203	Gapdh	0.242	Actb	0.43	Actb	0.38	Pgk1	12.81
Gapdh	0.215	Vcp	0.245	Rpl5	0.48	Gapdh	0.39	Rpl5	13.69
Psm4	0.237	Psm4	0.356	Pgk1	0.49	Psm4	0.47	Actb	14.74
B2m	0.261	B2m	0.471	Psm4	0.52	B2m	0.52	Gapdh	16.44
Ubc	0.281	Ubc	0.473	Gapdh	0.54	Ubc	0.53	Psm4	17.24
18S	0.321	18S	0.625	18S	0.72	18S	0.7	18S	20
Tfric	0.402	Tfric	1.15	Tfric	1.25	Tfric	1.17	Tfric	21
Total (n=38)									
Rplp0	0.221	Rplp0	0.044	Ubc	0.33	Rplp0	0.61	Rplp0	1.78
Tbp	0.221	Tbp	0.188	B2m	0.37	Tbp	0.64	Tbp	2.45
Gusb	0.268	Vcp	0.267	Vcp	0.39	Gusb	0.66	Vcp	3.83
Rpl5	0.299	Gusb	0.277	Sdha	0.4	Vcp	0.67	Gusb	4.65
Hprt1	0.321	Rpl5	0.312	lpo8	0.43	Rpl5	0.67	Rpl5	6.12
Vcp	0.373	Hprt1	0.389	Reep5	0.46	Hprt1	0.71	lpo8	6.44

Ipo8	0.429	Ipo8	0.405	Hmbs	0.64	Ipo8	0.73	Hprt1	7.33
Polr2a	0.457	Polr2a	0.432	Polr2a	0.65	Polr2a	0.75	Polr2a	8
Gapdh	0.486	Gapdh	0.476	Tbp	0.66	Gapdh	0.78	Ubc	8.98
Hmbs	0.51	Hmbs	0.502	Rplp0	0.68	Ywhaz	0.79	Hmbs	9.37
Ywhaz	0.533	Psm4	0.52	Gapdh	0.69	Hmbs	0.8	Gapdh	9.46
Psm4	0.552	Ywhaz	0.568	Psm4	0.77	Psm4	0.81	Reep5	10.72
Reep5	0.58	Reep5	0.57	Gusb	0.81	Reep5	0.83	B2m	11.1
18S	0.61	18S	0.683	Rpl5	0.82	18S	0.92	Psm4	11.74
Ppia	0.651	Ppia	0.882	18S	0.85	Ppia	1.01	Ywhaz	12.42
Actb	0.682	Pgk1	0.902	Hprt1	0.91	Actb	1.04	Sdha	13.87
Pgk1	0.716	Actb	0.918	Pgk1	0.93	Pgk1	1.07	18S	14.24
Tfrc	0.748	Tfrc	0.932	Ywhaz	1.11	Ubc	1.07	Ppia	16.12
Ubc	0.79	Ubc	0.946	Tfrc	1.34	B2m	1.09	Pgk1	16.74
B2m	0.823	B2m	0.973	Ppia	1.4	Tfrc	1.1	Actb	17.39
Sdha	0.851	Sdha	1.004	Actb	1.42	Sdha	1.12	Tfrc	18.73

Figures

Figure 1

Flow chart of the study design. Illustration of the developmental stages of the mice left ventricle tissues sampled in this study and the short description of the core experiment manipulations. The statistical applications for evaluating the expression stability of reference genes were also shown. E= embryonic day; D= postnatal day; M= postnatal month

A **B** **Figure 2**

Overall expression characteristics of the reference genes during development of mouse heart. (A) The melting curve assays of the 21 candidate reference genes across all samples showed the single peak, indicated the RT-qPCR amplification had good specificity; (B) The expression of cycle threshold (Ct) -values in each candidate reference gene. Bar graph representative of mean values of the Ct value.

Figure 3

Clustering analysis of the candidate reference genes expression. (A) Heat map of hierarchical cluster analysis of samples performed on the profiles of 21 candidate reference genes; (B) Supervised OPLS-DA score plot. Three phases can be distinguished in the gene expression features, that are (1) embryo stage, (2) first 7 days after birth, (3) 1 to 9 months after birth.

Figure 4

Stability of candidate reference genes. (A) Heatmap to illustrate the most suitable 5 reference genes with high stability in each dataset with different condition using. The color scale indicates the stability: Green color indicate higher stability, while red color represents a decreased stability. (B) Dot plot graph to show the optimal reference genes in each condition.

Figure 5

Relative expression of two target genes. (A) Vcp expression detection normalized by reference gene Rplp0 or 18S; (B) Pgk1 expression detection normalized by reference gene Rplp0 or 18S. to illustrate the variability in Vcp and Pgk1 expression in different heart developmental periods depending on the selection of the reference gene.