Selection and evaluation of stable housekeeping genes for gene expression normalization in carbon nanoparticle-induced mice acute pulmonary inflammation

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Abstract

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) is a highly specific and sensitive technique for the quantification of gene expression on the mRNA levels. But use of unconfirmed housekeeping genes (HKGs) could lead to misinterpretation of the expression of genes of interest (GOI). In this study, The stability and suitability of eleven frequently used housekeeping genes, namely 18S rRNA, ACTB, B2M, CYPA, GADPH, GUSB, HMBS, HPRT1, RPL13A, SDHA and TBP in 36 lung tissues isolated from either wild type (WT) mice or p50 knock out (p50-/-) mice or p105 knock out (p105-/-) mice which were treated with either CNP or H2O or non-treated, have been validated by geNorm, NormFinder and BestKeeper programs. The expression levels of ACTB, GUSB and RPL13A were the most constant in lung tissues across three genotypes and three kinds of treatments. A set of three most stable genes is found sufficient used as housekeeping genes for lung tissues in studies of similar design.

Keywords: Housekeeping genes, acute lung inflammation, NF-kB, quantitative RT-PCR

Introduction

Inhalation of carbon nanoparticles (CNP), a main constituent of urban air pollution, is believed to trigger pulmonary or even systemic inflammation via the generation of oxidative stress [1, 2]. However, the redox-sensitive transcription factor NF-κB, which controls a majority of inflammatory genes, is thought to play an important role in onset of pulmonary inflammation [3, 4]. In mammalian cells, the NF-kB family is composed of five members, NF-kB1 (p50, precursor p105), NF-kB2 (P52, precursor p100), RelA, RelB, and c-Rel, which function as various hetero- and homo-dimmers [5]. It has been reported that NFkB1 (p50 and p105) plays import roles in NFkB functions, however, whether subunit p50 and p105 of NF-kB could be control acute pulmonary inflammation and injury after 24 hours upon CNP treatment is not clear.

One approach to understanding p50 and p105 roles in CNP-induced acute pulmonary inflammation is to study gene expression in animal models using qRT-PCR. The data obtained by qRT-PCR is typically normalized with an internal control, often referred to as a housekeeping gene. However, the use of unconfirmed HKGs may lead to misinterpretation of the expression of GOI. Up to now, several mathematical methods, such as geNorm [6], NormFinder [7] and BestKeeper [8], have been developed to analyze the variability of the expression of candidate HKG. The ideal HKG for qRT-PCR would be one whose mRNA is consistently expressed at the same level in all samples under investigation, regardless of tissue type, disease state, medication or experimental conditions, and could have expression levels comparable to that of the target [9-11].

However, the systematic study of the suitability of HKGs for qRT-PCR normalization in the field of CNP-induced acute pulmonary inflammation has thus far been lacking. Therefore, the

aim of the present study is to identify candidate genes in the CNP-induced acute pulmonary inflammation models that could be used in qRT-PCR experiments as housekeeping genes to normalize the expression of GOI.

Methods

Animal treatment and lung tissue processing

Animal treatment and lung tissue processing as described in our previous study [36]. Briefly, all mice were female, 10-12 weeks of age with body weights between 17.39 and 20.5 g during the study. Each of 3 genetically modified mice consisted of three groups (each group consisted of between 6 and 8 animals), and one group was instillation with 20µg CNP (primary particle size: 10nm, OC<5%), the other two served as control and sham exposed groups. After 24h, mice were anesthetized by intraperitoneal injection of a mixture of xylazine (4.1mg/kg body weight) and ketamine (188.3 mg/kg body weight) and killed by exsanguination. The lung tissue after bronchoalveolar lavage (BAL) either stored at -80℃ or performed further study. Four completely lung tissues of each group were chose for gene expression levels analysis. We treated animals humanely and with regard for alleviation of suffering; experimental protocols were reviewed and approved by the Bavarian Animal Research Authority (approval no. 211-2531-108/99).

Total RNA extraction and first strand cDNA synthesis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with an additional peqGOLD TriFast (Peqlab, Erlangen, Germany) extraction to improve protein exclusion. RNA concentration and purity was determined by A260 and A280 measurements using a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The mean ratio value of A260/A280 for all RNA samples was 2.05±0.4, reflecting high purity and protein absence. RNA integrity was evaluated by the ratio of 28S/18S ribosomal RNA bands after

115 eletrophotesis in denaturing 1% agarose gel. To guarantee of the quality necessary for
116 expression analysis all samples used in this study presented a 28S/18S rRNA ratio ≥1.7.
117 One microgram total RNA was reverse-transcribed using the superscript TM II Reverse
118 Transciptase kit (Invitrogen, Karlsruhe, Germany) for first strand cDNA synthesis with 5μM
119 Random Nonamer (N9; MWG Biotech, AG, Ebersberg, Germany) primer according to the
120 manufacture's recommendations. In brief, RNA and primers were mixed and incubated at 70
121 ℃ for 5 min followed by cooling on ice for 5 min and room temperature for 5 to 10 min
122 before transcription. The first strand cDNA synthesis was started after adding transcription
123 mixture at 42℃ lasting 1 hour for reverse transcription reaction. Finally, the reaction was
124 inactivated by heating 70℃ for 15 min. All cDNA samples were diluted 1:5 with DNase- and
125 RNase- free H2O and stored at -20℃.

126 **Real-Time quantitative PCR with SYBR green**

127 qRT-PCR was conducted using the ABI PRISM® 7000 detection system (Applied Biosystems,
128 Foster city, CA, USA), based on ABsolute™ QPCR SYBR® Green ROX Mix (Thermo
129 Scientific, Wilmington, USA). The PCR reaction mixture contained 1μl cDNA (10ng/μl), 1μl
130 (5μM) of each primer, 12.5μl ROX mix and PCR-grade H_2O up to a total volume of 25μl.
131 After initial enzyme activation (one cycle at 95℃ for 15min), 40 cycles amplification (95℃
132 for 15 s, 60℃ for 30s and 72℃ 30s) were performed in 96-well optical reaction plates
133 (Applied Biosystems, Foster city,CA, USA). To verify that the used primer pair produced
134 only a single product, a dissociation protocol was added after thermocycling, determining
135 dissociation of the PCR products from 60℃ to 95℃ by increasing 0.5℃ per cycle. In all
136 negative control samples no amplification of the fluorescent signal was detected, proving that

the extraction procedure, including the DNase treatment, effectively removed genomic DNA from all RNA samples.

Statistical data analysis

The Ct is defined as the number of cycles needed for fluorescence to reach a specific threshold level of detection and is inversely correlated with the amount of RNA or DNA template present in the reaction [36]. The stability of HKGs expression was analysed with geNrom, NormFinder and BestKeeper software packages. Relative expression of GOI applies ΔΔCt method was used where ΔΔCt = (Ct target gene, test sample – Ct endogenous control, test sample) - (Ct target gene, calibrator sample - Ct endogenous control, calibrator sample) [37]. Relative quantities were corrected for efficiency of amplification and fold change in gene expression between groups was calculated as $E^{-\Delta\Delta Ct}$ ± SEM. Where more than one endogenous control are used, fold change estimates were calculated using the geometric mean of EC quantities relative to the calibrator sample which could be the minimum, maximum or a named sample or an average.

Results

Selection of housekeeping genes and identification of primers

For the selection and evaluation of stable housekeeping genes for gene expression normalization in mice acute pulmonary inflammation induced by CNP, we selected 11 commonly used HKGs (18S rRNA, ACTB, B2M, CYPA, GAPDH, GUSB, HMBS, HPRT1, RPL13A, SDHA, and TBP) of varying functional classes (for full gene information see Table 1). Particular attention was paid to selecting HKGs that belong to different functional classes, which significantly reduce the chance that genes might be co-regulated [12, 13]. Primers were then designed and tested (Table 2). The specificity of the amplifications was confirmed by the presence of a single band of expected size for each primer pairs in agarose gels following electrophoresis and by the single peak dissociation curves of the amplicon. Efficiency of PCR

reactions ranged between 94.97% for TBP and 112.19% for GUSB, and correlation coefficients varied from 0.9887 to 1 for HMBS and ACTB, respectively (Table 2).

Transcriptional profiles of housekeeping genes

For comparison of HKGs transcriptional profiles, the cycle threshold (Ct) values were plotted directly and indicated in figure 1. The median expression range of the 11 tested HKGs was calculated from raw Ct values and spanned 16.43 cycles for ACTB to 24.76 cycles for HMBS. As presented in figure1, expression levels of GUSB, HMBS, HPRT1 and TBP were low, with median Ct values between 22 and 25 cycles. GADPH, RPL13A and SHDA displayed intermediate expression levels with median Ct values between 20 and 21.74 cycles. In contrast, high expression of 18S rRNA, ACTB, B2M and CYPA was detected, with Ct values between 16.5 and 19 cycles. Among the 11 HGKs, the maximum and minimum expression range was 2.63 cycles for HPRT1 and 1.13 cycles for CYPA, respectively.

Expression stabilities of candidate housekeeping genes

Our main objective was to identify HKGs with minimal variability among our set of samples. In order to determine the least variable HKGs, we evaluated expression stabilities of the 11 candidate HKGs using the three most commonly used Excel-based tools: geNorm, NormFinder and BestKeeper.

geNorm analysis

For ranking the various candidate HKGs, geNorm is a useful program using the principle that the expression ratio of two ideal HKGs is identical in all tested samples [6]. The 11 candidate HKGs for normalization were ranked according to their expression stability M values using the geNorm program. The M value is defined as the average pair-wise variation of a certain gene with all other tested HKGs. Consequently, genes with low M value have a low variation and a stable expression, while genes with high M value have a high variation and a less stable expression. The average expression M values of the eleven HKGs were plotted in Figure 2. As shown in the upper line of Figure 2, M value of RPL13A and ACTB were the lowest

(0.218), and that of 18S rRNA was the highest (0.466), indicating that RPL13A and ACTB had the most stable expression and that 18S rRNA was expressed most variably.

NormFinder

NormFinder, another VBA applet, is a model-based program calculating HKGs expression stability (more stable gene expression is indicated by lower average expression stability values) based on the intra-group variance, and includes the inter-group variance if applicable [7]. In this sense, Using this program, we identified the same HKGs as having the greatest stability: GUSB, ACTB and RPL13A (stability values 0.005, 0.008 and 0.009, respectively, Figure 2 downer line), although here GUSB was more stable than ACTB and RPL13A. The three least HKGs were 18S rRNA, HPRT1 and B2M (stability values 0.037, 0.022 and 0.017, respectively).

BestKeeper

The Excel-based program BestKeeper, determining the optimal HKGs employing the pair-wise correlation analysis of all pairs of candidate genes (up to ten HKGs) and calculating the geometric mean of the best suited ones by raw Ct values of each gene. More important, all genes may be include in the calculation of the BestKeeper index, which can be used to rank the best HKGs because of stable HKGs showing a strong correlation with the BestKeeper index [8]. The ten HKGs studied in our analysis compared with BestKeeper index, also correlated gene one with another, except for 18S rRNA (the least gene determined by geNorm and NormFinder). BestKeeper analysis showed that the four stable genes were CYPA, GUSB, ACTB and RPL13A (BestKeeper index 0.949, 0.945, 0.928 and 0.900, respectively), while the three variable genes were GADPH, HPRT1 and TBP (BestKeeper index 0.744, 0.756 and 0.831, respectively).

The optimal number of HKGs for normalization

To evaluate the optimal number of HKGs for accurate normalization, pair-wise variations V_n/V_{n+1} between two sequential normalization factors (NF) are calculated to determine the

effect of adding the next HKG in normalization [6]. A large variation implies that the added gene has a significant effect and should preferably be included for calculation of a reliable NF. As shown in Figure 3, the threshold of 0.15 is not exceeded at any point, indicating that two HKGs would be sufficient under this condition. However, normalization using three HKGs, instead of two, is generally considered as a more robust manner to generate a much more accurate and reliable estimate of the actual transcript level of GOI [14, 6]. So the three most stable HKGs (ACTB, RPL13A and GUSB) we selected using geNorm, NormFinder and BestKeeper would be sufficient for accurate normalization of GOI.

Evaluation of selected candidate HKGs and normalization approach

In order to assess the value of the validation of housekeeping genes, the relative expression of CXCL1 which is known to be involved in acute pulmonary inflammation [18, 19], was normalized using the following approach: i) the three best HKGs combination (NF) selected by geNorm, NormFinder and BestKeeper ACTB, RPL13A and GUSB; ii) the frequently cited endogenous gene 18S rRNA [15-17]; iii) ACTB, RPL13A and GUSB were used individually. CXCL1 protein concentration was measured by ELISA in lung BAL fluids collected 24 hours after treated with 20μg CNP. Results indicated that concentration of Cxcl1 was 40.97 fold induced in p50-/- mice (130.29± 29.70 pg/ml), 12.3 fold induced in p105-/- mice (39.13±0.79 pg/ml) and 9.02 fold induced in wt mice (28.67±7.43 pg/ml) upon CNP exposure, as compared with wt control mice (3.18±1.07 pg/ml).

Consequently, Figure 4 showed a significant increase in the CXCL1 expression in group of CNP exposure was normalization to both the HKGs selected in this study and the commonly cited housekeeping gene 18S rRNA, as compared with wt control group. When normalized to the top three stable HKGs (ACTB, GUSB and RPL13A), CXCL1 was up-regulated (in comparison to the wt control group) in wt mice by 6.03 fold, p50-/- mice by 15.71 fold and p105-/- mice by 10.25 fold, respectively, upon CNP exposure. However, normalization to the commonly cited 18s rRNA, CXCL1 was up-regulated (also in comparison to the wt control

group) in wt mice by 3.95 fold, p50-/- mice by 9.57 fold and p105-/- mice by 7.21 fold, respectively, in response to CNP exposure. But there is a decrease of approximately 1.53 fold in wt mice, 1.64 fold in p50-/- mice and 1.42 fold in p105-/- mice was seen in the same treatment group when normalizing against 18S rRNA, compared with normalized to top three stable HKGs combination. When normalization to ACTB and RPL13A, the relative expression of CXCL1 less than 1 fold compared with normalizing against the top three HKGs combination, while normalizing to GUSB up-regulated 1.14 to 1.27 fold compared with the top three stable HKGs combination.

Therefore, these results demonstrate how the explanation of GOI expression levels can be affected by the choice of the HKGs in real-time quantitative RT-PCR analysis.

Discussion

In this study we have selected and evaluated the stable housekeeping genes for using as qRT-PCR normalizing factors in CNP-induced acute pulmonary inflammation. Based on our results, we conclude that use of a single normalization housekeeping gene is potentially hazardous, and suggest a panel of housekeeping genes for more accurate transcript quantification.

qRT-PCR is a sensitive and accurate technique for measuring gene expression [20], and constitutes a powerful tool for increasing our understanding of the subunit p50 and p105 of NF-kB roles in CNP-induced acute pulmonary inflammation. However, in CNP-induced acute pulmonary inflammation system, little is known about the ideal genes to use for normalization and many previous studies have only utilized a single housekeeping gene in normalizing gene expression data [21-23]. Normalization of Real-time RT-PCR data using a single, non-validated housekeeping gene may lead to inaccurate biological conclusions, and previous studies have highlighted the need to validate housekeeping genes for each experimental condition [24-29].

The geNorm [6], NormFinder [7] and BestKeeper [8] algorithms are now widely used to determine the most stable housekeeping genes from a set of candidate genes with invariable expression [30-34]. Among 11 candidate housekeeping genes in this study, both geNorm and NormFinder identified ACTB, GUSB and RPL13A as the most stable combination of housekeeping gens for the CNP-induced acute pulmonary inflammation. And BestKeeper identified CYPA, GUSB, ACTB and RPL13A as the top four stable housekeeping according to the BestKeeper index (shown in Table 3). Considering the both results, ACTB, GUSB and RPL13A could be enough as a validation combination of housekeeping genes for normalization of real-time RT-PCR data in our study system.

In order to check the value of the validation of endogenous controls, we have used different housekeeping genes selected in this study to normalize the expression of CXCL1, gene which is known to be involved in acute pulmonary inflammation in response to CNP exposure. We have observed differences in the results obtained when suitable and unsuitable housekeeping genes are used.

Conclusion

Our current results showed that ACTB, GUSB and RPL13A were the most stably expressed genes in lung tissues from CNP-induced acute lung inflammation mice, regardless of genotype and treatment. Thus, these are good housekeeping genes for quantitative real-time PCR studies. Since the current study also observed fluctuations in expression in frequently used housekeeping genes, including 18S rRNA, B2M, CYPA, GAPDH, HMBS, HPRT1, SDHA, and TBP, it is recommend that ACTB, GUSB and RPL13A be used as housekeeping genes for lung tissues in studies of similar design and that the stability of housekeeping genes be validated prior to expression studies.

Competing interests

The authors declare no competing interests. Non-financial competing interests exist.

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Fig. 1. The transcriptional profiles of eleven candidate housekeeping genes in mice lung tissue from carbon nanoparticle induced acute pulmonary inflammation. Raw Ct values are represented for gene by a box-plot. The central box represents the interquartile interval (25%-75%), the line inside the box is the median value (50%), and whiskers (error bars) above and below the box indicate the 90^{th} and 10^{th} percentiles. Ct: (real-time PCR cycle threshold number)

Fig. 2. Gene expression stability and ranking of the eleven candidate housekeeping genes were calculated using the software packages geNorm and NormFinder, respectively. The average expression stability M values (M) and the best combination of two genes for11 HKGs were calculated by geNorm program (upper line with solid circle) in lung tissue. The cut-off for an unstable gene is $M \geq 1.5$ and the lower the M, the more stable the gene among the candidate HKGs; lower line with open circle from NormFinder, also calculating a stable value which is inversely proportional to the stability of the candidate gene. With both approaches, the most stable genes (lowest stability value) are identified as ACTB, RPL13A and GUSB, whereas 18S rRNA and HPRT1 are two least stable HKGs.

Fig. 3. The optimal number of HKGs for normalization was determined by pair-wise using geNorm. Pair-wise variation ($V_n/<V_{n+1}$) analysis between the normalization factor NF_n and NF_{n+1} to determine the number of HKGs required for accurate normalization. Each bar represents the variation between the means of n most stable genes versus the group of n+1 most stable genes (e.g., column 1 represents the variation between the mean of the two most stable genes, that is, ACTB, RPL13A and three most stable genes, that is ACTB, RPL13A, and GUSB).

Fig. 4. Relative expression of CXCL1 was normalization by different HKGs combination. The relative expression of CXCL1 in lung homogenates after 24 hours of instillation with CNP mRNA level were calculated using $E^{-\Delta\Delta Ct}$ method and normalized to NF (the most stable

three HKGs, ACTB, GUSB and RPL13A, were determined by geNorm, NormFinder and BestKeeper), or frequently cited 18s rRNA or individual of the most three stable HKGs, respectively. Each bar represents the mean of twice measurements from 4 animals, ± SEM.

Table1

Name, function and accession number of candidate housekeeping genes considered in this work

Table 2

Primer sequences and amplicon characteristics of housekeeping genes and genes of interest

Table 3

Inter-gene relations and correlation between the housekeeping genes and the bestkeeper index

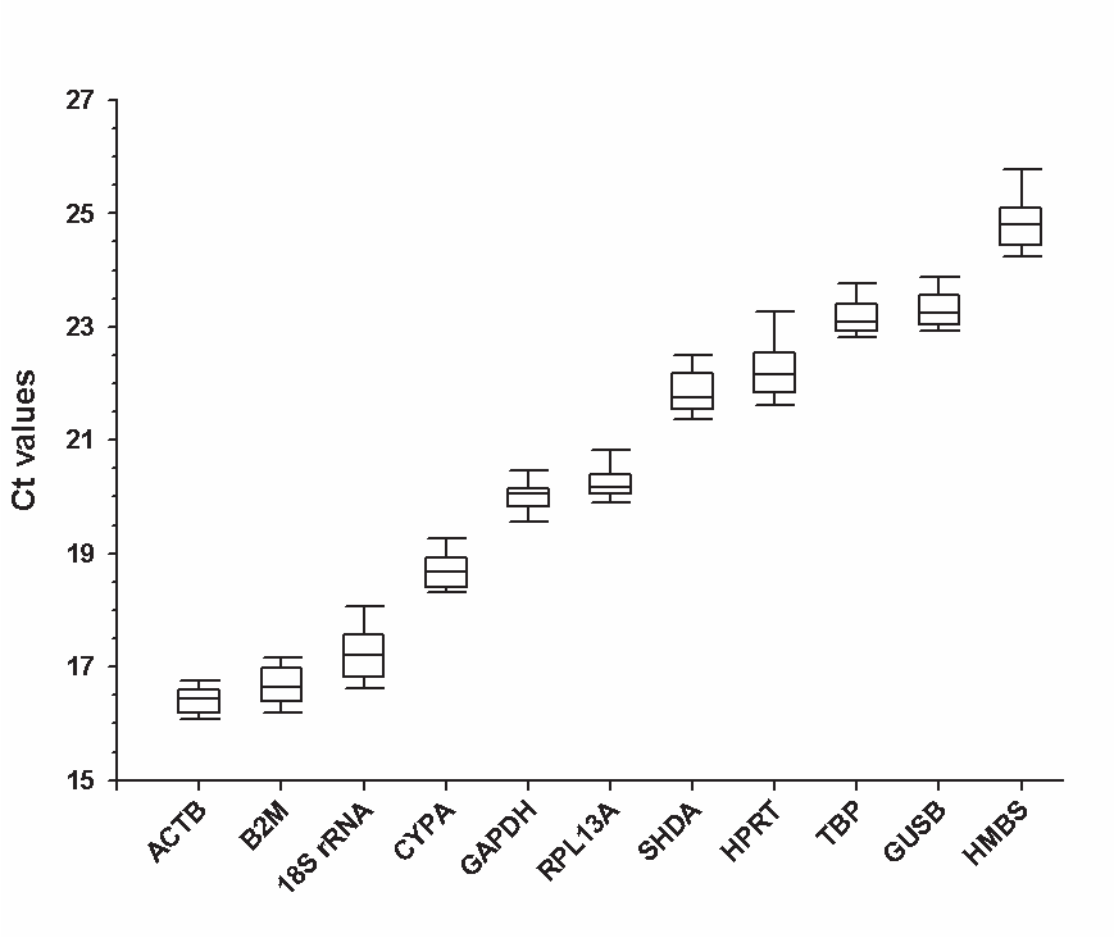

Figure 1

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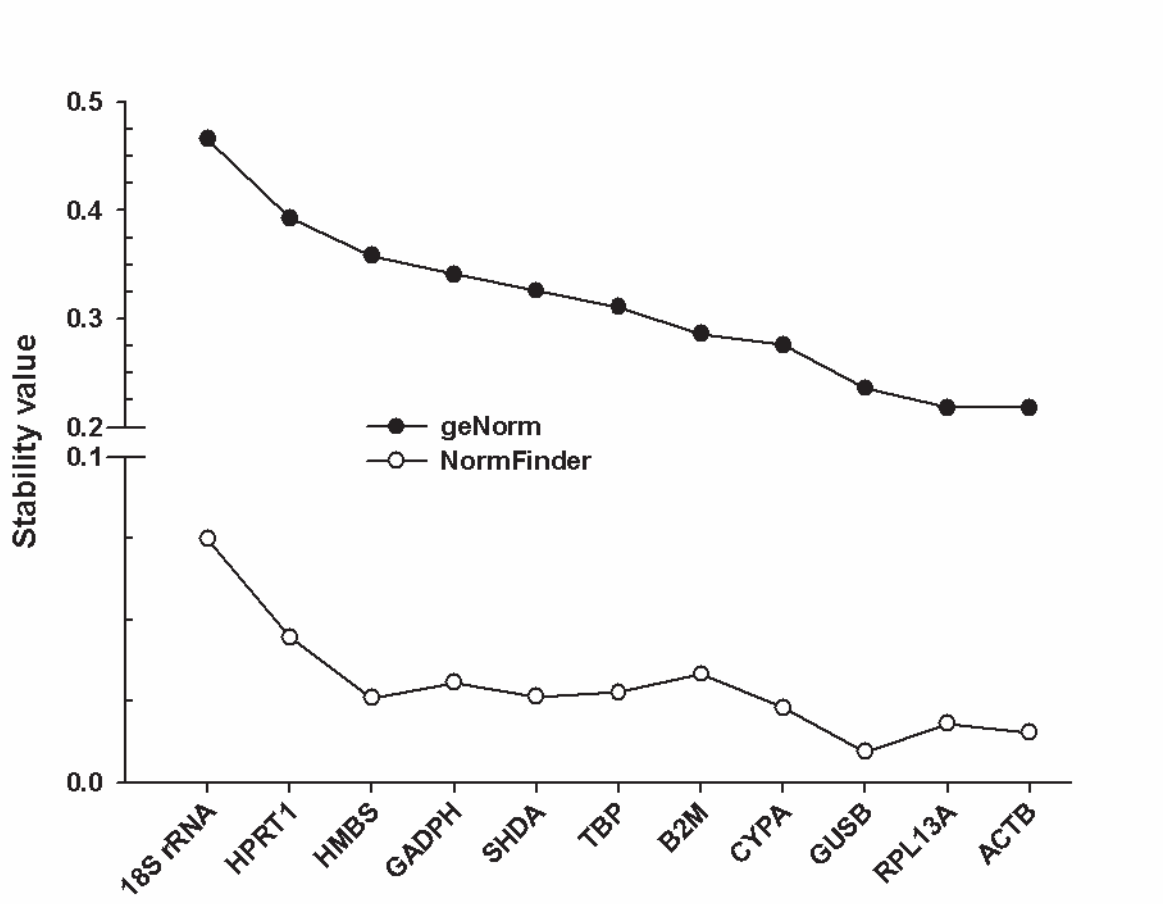


Figure 2

Gene expression stability and ranking of the eleven candidate housekeeping genes were calculated using the software packages geNorm and NormFinder, respectively. The average expression stability M values (M) and the best combination of two genes for11 HKGs were calculated by geNorm program (upper line with solid circle) in lung tissue. The cut-off for an unstable gene is M≥1.5 and the lower the M, the more stable the gene among the candidate HKGs; lower line with open circle from NormFinder, also calculating a stable value which is inversely proportional to the stability of the candidate gene. With both approaches, the most stable genes (lowest stability value) are identified as ACTB, RPL13A and GUSB, whereas 18S rRNA and HPRT1 are two least stable HKGs.

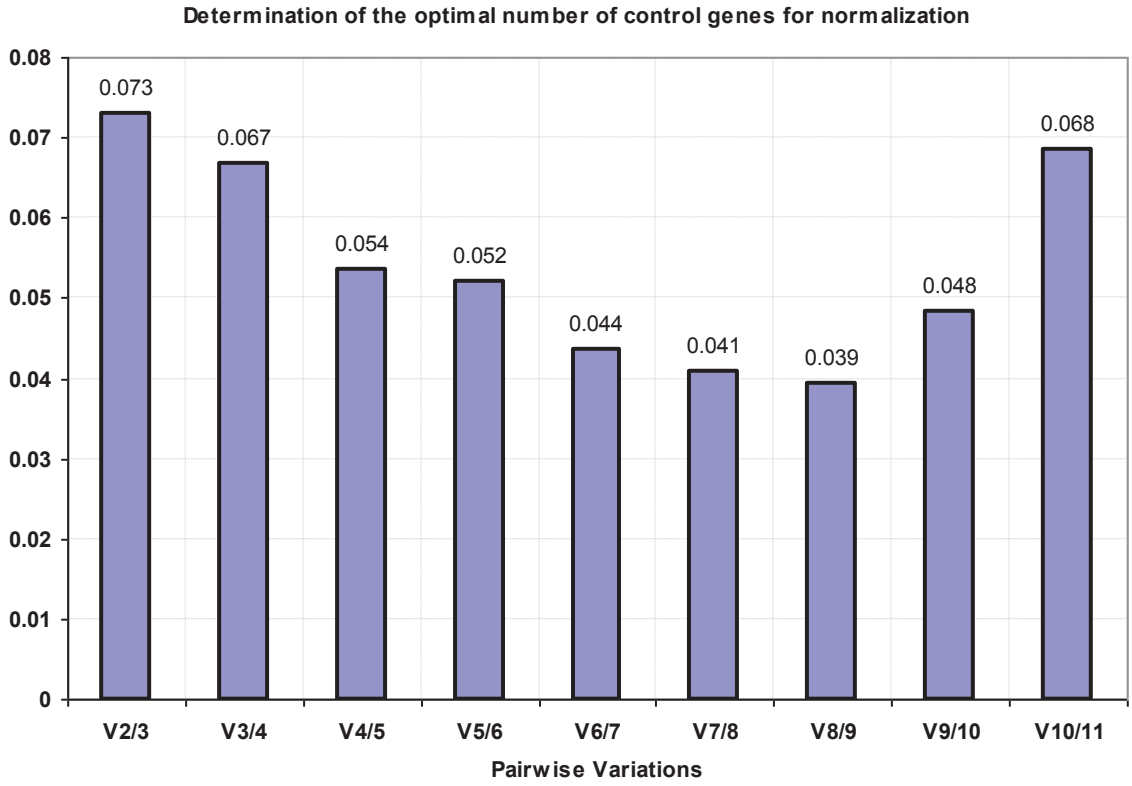
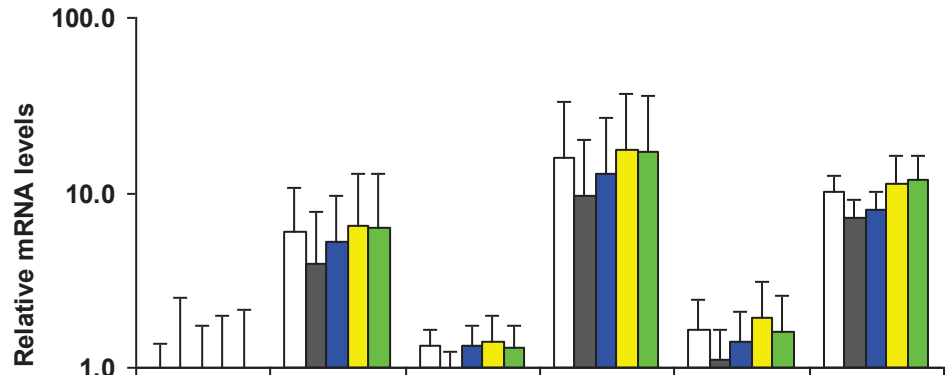


Figure 3

The optimal number of HKGs for normalization was determined by pair-wise using geNorm. Pair-wise variation ($V_n/<V_{n+1}$) analysis between the normalization factor NF_n and NF_{n+1} to determine the number of HKGs required for accurate normalization. Each bar represents the variation between the means of n most stable genes versus the group of n+1 most stable genes (e.g., column 1 represents the variation between the mean of the two most stable genes, that is, ACTB, RPL13A and three most stable genes, that is ACTB, RPL13A, and GUSB).



	WT		p50-/-		p105-/-	
	Control	CNP	Control	CNP	Control	CNP
NF	1.00	6.03	1.35	15.71	1.64	10.25
18S rRNA	1.00	3.95	0.97	9.57	1.10	7.21
GUSB	1.00	5.30	1.33	12.84	1.40	8.05
ACTB	1.00	6.53	1.41	17.69	1.95	11.38
RPL13A	1.00	6.34	1.31	17.08	1.62	11.76

Figure 4

Relative expression of CXCL1 was normalization by different HKGs combination. The relative expression of CXCL1 in lung homogenates after 24 hours of instillation with CNP mRNA level were calculated using $E^{-\Delta\Delta Ct}$ method and normalized to NF (the most stable three HKGs, ACTB, GUSB and RPL13A, were determined by geNorm, NormFinder and BestKeeper), or frequently cited 18s rRNA or individual of the most three stable HKGs, respectively. Each bar represents the mean of twice measurements from 4 animals, ± SEM.

Table1: Name, function and accession number of candidate housekeeping genes considered in this work

Symbol	Gene name	Function	Accesion Number
18S rRNA	18S ribosomal RNA	Cytosolic small ribosome subunit, translation	NR_003278
ACTB	Actin, beta	Cytoskeletal structural protein	NM_007393
B2M	Beta-2 microglobulin	Beta-chain of major histocompatibility complex class I molecules	NM_009735
CYPA (Ppia)	Cyclophilin A (peptidyprolyl isomerase A)	Catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides, accelerating folding	NM_008907
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Catalysis of conversion of D-glyceraldehyde-3-phosphate to 3-phospho-D-glyceroyl phosphate	NM_008084
GUSB	Beta-glucuronidase	Exoglycosidase in lysosomes	NM_010368
HMBS (PBGD)	Hydroxymethylbilane synthase	Third enzyme of the heme biosynthetic pathway and catalyzes the head to tail condensation of four porphobilinogen molecules into the linear hydroxymethylbilane	NM_013551
HPRT1	Hypoxanthine guanine phosphoribosyl transferase	Purine synthesis in salvage pathway	NM_013556
RPL13A	ribosomal protein L13A	Structural component of the large 60S ribosomal subunit	NM_009438
SDHA	Succinat dehydrogenase complex, subunit A, flavoprotein (Fp)	Succinate dehydrogenase/fumarate reductase, flavoprotein subunit involved in energy production and conversion	NM_023281
TBP	TATA box binding protein	General RNA polymerase II transcription factor	NM_013684

Table 2: Primer sequences and amplicon characteristics of housekeeping genes and genes of interest

Name	Sequence (5'→3')	Amplicon (bp)	TM (°C)*	E (%)[#]	R^2
18S rRNA	F: GAC TGT CTC GCC GGT GTC R: GGA GAG CCG GAA CGT CGA	98	88.86±0.03	96.8	0.9983
ACTB	F: TCC ATC ATG AAG TGT GAC GT R: GAG CAA TGA TCT TGA TCT TCA T	154	83.02±0.03	99.3	1.0000
B2M	F:CTG ACC GGC CTG TAT GCT A R:CAG TCT CAG TGG GGG TGA AT	244	82.95±0.04	98.33	0.9998
CYPA	F:TTT GCA GAC GCC ACT GTC R:CAG TGC TCA GAG CTC GAA AG	165	87.09±0.05	107.5	0.9988
GAPDH	F: TGC ACC ACC AAC TGC TTA GC R: GGC ATG GAC TGT GGT CAT GAG	101	83.6±0.04	102.8	0.9981
GUSB	F:CAG GGT CAA CTT CAG GTT CC R:GCT CTT TGT GAC AGC CAC TG	165	84.16±0.04	112.19	0.9948
HMBS	F:GGT CCC TGT TCA GCA AGA AG R:AAG CCA GAA GTA GGC AGT GG	242	86.8±0.00	109.8	0.9887
HPRT1	F:GTT GGA TAC AGG CCA GAC TTT GT R: CAC AGG ACT AGA ACA CCT GC	224	81.56±0.03	97.6	0.9985
RPL13A	F:CCC TCC ACC CTA TGA CAA GA R:CTG CCT GTT TCC GTA ACC TC	221	85.45±0.06	105.93	0.997
SDHA	F:CAG TTC CAC CCC ACA GGT AT R:GAT CTT TCT CAG GGC CAC AG	208	84.8±0.06	102.7	0.9978
TBP	F:GCC TTC CAC CTT ATG CTC AG R:GCT ACT GCC TGC TGT TGT TG	202	84.22±0.03	94.97	0.991
KC	F:CCG AAG TCA TAG CCA CAC R:GTG CCA TCA GAG CAG TCT		83.14±0.07	110	0.99

* The dissociation temperature of amplicon was calculated by ABI PRISM® 7000 Sequence Detection System. [#]Amplification efficiency calculation was performed from the slopes of the dissociation curve according to the equation $E=10^{(-1/slope)}$.

Table 3: Inter-gene relations and correlation between the housekeeping genes and the bestkeeper index

	RPL13A	SHDA	HPRT1	ACTB	TBP	CYPA	HMBS	B2M	GUSB	GADPH
SHDA	0.710	-	-	-	-	-	-	-	-	-
HPRT1	0.600	0.840	-	-	-	-	-	-	-	-
ACTB	0.894	0.721	0.566	-	-	-	-	-	-	-
TBP	0.687	0.883	0.787	0.656	-	-	-	-	-	-
CYPA	0.821	0.836	0.698	0.878	0.727	-	-	-	-	-
HMBS	0.737	0.664	0.444	0.830	0.555	0.829	-	-	-	-
B2M	0.802	0.722	0.601	0.780	0.727	0.789	0.583	-	-	-
GUSB	0.876	0.780	0.567	0.917	0.719	0.892	0.873	0.815	-	-
GADPH	0.668	0.522	0.328	0.824	0.458	0.687	0.869	0.468	0.767	-
BestKeeper	0.900	0.892	0.756	0.928	0.831	0.949	0.845	0.851	0.945	0.744
p-value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001