Reference genes for the developing mouse lung under consideration of biological, technical and experimental confounders

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For gene expression analysis, the raw data obtained from RT-qPCR are preferably normalized to reference genes, which should be constantly expressed regardless of experimental conditions. Selection of reference genes is particularly challenging for the developing lung because of the complex transcriptional and epigenetic regulation of genes during organ maturation and injury repair. To date, there are only limited experimental data addressing reliable reference genes for this biological circumstance. In this study, we evaluated reference genes for the lung in neonatal C57BL/6 mice under consideration of biological, technical and experimental conditions. For that, we thoroughly selected candidates from commonly used reference genes side-by-side with novel ones by analyzing publicly available microarray datasets. We performed RT-qPCR of the selected candidate genes and analyzed their expression variability using GeNorm and Normfinder. Cell-specific expression of the candidate genes was analyzed using our own single-cell RNA-sequencing data from the developing mouse lung. Depending on the investigated conditions, i.e., developmental stages, sex, RNA quality, experimental condition (hyperoxia) and cell types, distinct candidate genes demonstrated stable expression confirming their eligibility as reliable reference genes. Our results provide valuable information for the selection of proper reference genes in studies investigating the neonatal mouse lung.

Real-time quantitative PCR (RT-qPCR) is a commonly used method for the quantification of gene expression levels due to its numerous advantages, including high sensitivity and specificity; reliable reproducibility and significant accuracy even in low amount samples. With the increasing application of RT-qPCR in different research areas, the role of so-called reference genes becomes more important to allow for the accurate interpretation of experimental data and the comparability across replicates. Relative quantification, i.e., normalization of the expression level of a gene of interest to a reference gene used as an endogenous controls compensates gene expression variability induced by experimental conditions such as differences in sample volume, RNA integrity or cDNA synthesis.

In order to ensure robust performance, reference genes need to meet critical quality criteria including (i) consistent expression levels in the investigated tissue, (ii) resilience toward tissue processing conditions and (iii) robustness to experimental challenges imposed on the tissues investigated. These prerequisites are especially

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challenged in the developing organ undergoing rapid and significant functional and structural changes. After birth, the mouse lung undergoes alveolarization while being exposed to the environment and its different conditions. This not only renders the lung as a prime target to study repair and regeneration processes under clinically relevant conditions, but marks a challenging condition for the selection of reference genes at the same time due to the ever-changing pathways and cellular crosstalk during postnatal development.

Despite this need, little is known about reliable reference gene candidates. Previous studies demonstrated that commonly used reference genes are significantly affected by tissue origin, developmental stages and experimental conditions.

To address this challenge, we have used several complimentary tools to perform a comprehensive evaluation of pre-selected reference gene candidates in neonatal lung tissue targeting their expression variability across critical biological, technical and experimental conditions.

**Results**

**Identification of potential reference gene candidates by public and commercial databases.** Reference gene candidates were pre-selected using the four data sources a–d as described in the methods and summarized in one comprehensive candidate list (n = 122 genes before considering overlaps, Supplementary Table S1):

(a) PubMed search revealed seven reference genes from six RT-qPCR studies performed in lung tissue of C57BL/6 mice.

(b) Four studies including one meta-analysis evaluating reference genes in murine lung tissue and other organs identified 30 reference gene candidates.

(c) Genevestigator analysis revealed 58 reference gene candidates considering three different expression levels (range of gene expression variability: SD 0.16–0.96).

(d) The commercial reference gene catalogue (Roche RealTime Ready panel) included 27 reference genes.

In a subsequent step, 27 genes were excluded according to one or more of the following criteria: overlap between selection steps (n = 14) or presence of pseudogenes (n = 12; www.informatics.jax.org). Due to conflicting findings regarding the eligibility of ribosomal genes as reference genes, n = 10 genes coding for ribosomal proteins were excluded leaving one representative candidate for evaluation (Rpl4). Hbb-b1, coding for a subunit of the oxygen carrier was intentionally excluded as regulation under hyperoxia conditions has to be expected. This resulted in a final list of n = 95 reference gene candidates.

**Selection of final reference gene candidates and validation of expression variability by RT-qPCR.** Pulmonary expression of 46 out of 95 reference gene candidates was confirmed in our own transcriptome data from neonatal mice. Of these, 20 reference gene candidates yielding the lowest overall SD (0.118–0.242) in our own transcriptome data and in the Genevestigator dataset were selected for further analysis. As the majority of these candidates (18 of 20 genes) derives from Genevestigator, four additional candidates were randomly selected from the candidate genes ranking 21–46 according to the overall SD in order to broaden the spectrum of data sources considered (Table 1).

In the next step, expression variability of all 24 reference gene candidates was validated by RT-qPCR. Expression of Fkbp1a was not detectable in 6 of 60 samples (no measurable Cq values) and was therefore excluded from further analysis to guarantee reliable expression of the selected reference genes. This finding was potentially related to the low RNA quality (RIN < 5) in these samples. It resulted in a total of 23 final reference gene candidates (exclusion of Fkbp1a from the 24 initially selected genes). Furthermore, Psm4 showed low expression levels in 13 out of 86 samples, so that the suitability of this gene as a reference gene likely depends on the particular experimental setting, which needs to be considered with caution.

**Expression variability of the reference gene candidates under physiologic condition with relation to developmental stage.** In order to address expression variability in the neonatal mouse lung, we first characterized expression variability using all lung samples obtained under physiologic conditions (n = 17, PND2.5–28.5, FiO2 = 0.21, RIN > 5). Here, GeNorm identified 7 out of the 23 reference gene candidates with acceptable gene stability values (M-value < 0.5)\(^{50}\). Among them, Nupl2 and Rpl4 showed the lowest expression variability (M-value 0.280 for both) followed by Csnk1a1, Maca, Eif3a, Elac2 and Dolpp1 with increasing M-values. Likewise, Normfinder identified 8 out of the 23 reference gene candidates with acceptable gene stability values (SV < 0.5)\(^{51}\) and among them, Maca demonstrated the lowest expression variability (SV = 0.205) followed by Csnk1a1, Nupl2, Rpl4, Zc3h11a, Eif3a, Pcsk7 and Dolpp1 with increasing SV values (Tables 2, 3).

In a second step, we characterized pulmonary gene expression variability for all 23 candidates in each developmental stage, i.e., PND2.5, 5.5 and 14.5–28.5 (FiO2 = 0.21; RIN > 5). Here, 9 (GeNorm) and 5 (Normfinder) genes out of the 23 reference gene candidates were identified demonstrating low variability (M- or SV-value < 0.5) in all three groups. Among these, Elac2 (GeNorm) and Maca, Nupl2 (Normfinder) showed the highest mean stability ranking in all developmental stages (Tables 2, 3). Intra- and intergroup variation analysis (Normfinder) identified Maca (re-calculated SD 0.2207) as the reference gene with lowest variation and Maca together with Nupl2 as the best combination for reference gene performance (re-calculated SD 0.1492).

**Effect of biological, experimental and technical variables on reference gene expression variability.** Sex-related effects were investigated in samples spanning all developmental stages while only includ-
Table 1. The 24 final reference gene candidates. After the exclusion of 27 genes according to the criteria outlined in the text and subsequent confirmation of gene expression in our own transcriptomic data, 46 out of 122 reference gene candidates, shown in the table, remained for further analysis. Next, these 46 genes were ranked according to the overall SD covering both microarray data from our own and Genevestigator. The top 20 candidates with lowest overall SD and four additional genes chosen randomly from the rank 21–46 were selected for validation in neonatal murine lungs by RT-qPCR. Finally, it resulted in the 24 final reference gene candidates (highlighted in bold letters): four genes from reference gene studies, 18 genes from Genevestigator and two genes from the Roche commercial panel. *Fkbp1a was later excluded from further raw data analysis due to its undetectable low expression levels in several RNA samples. Gene expression levels are represented as log2-scaled signal intensity according to Genevestigator analysis: > 13.00 for high level, 10.00–13.00 for medium level, < 10.00 for low level. SDref stands for the SD from RefGenes analysis of Genevestigator and SDarray for the SD from our own microarray data. The overall SD was calculated by averaging SDref and SDarray. The source a for commonly used reference gene, b for reference gene study, c for Genevestigator and d for Roche reference gene panel.

| Gene symbol | Entrez ID | Source | Expression level | SDref | SDarray | SDoverall | Rank |
|-------------|-----------|--------|------------------|-------|---------|-----------|------|
| Dolpp1      | 57170     | c      | Medium           | 0.16  | 0.05    | 0.18      | 1    |
| Mprr1L1     | 93734     | c      | Medium           | 0.18  | 0.03    | 0.12      | 2    |
| Eluc2       | 68626     | c      | Medium           | 0.19  | 0.05    | 0.13      | 3    |
| Psk7        | 18554     | c      | Medium           | 0.20  | 0.03    | 0.14      | 4    |
| Rdmc3b      | 277230    | c      | Medium           | 0.19  | 0.07    | 0.14      | 5    |
| Maca        | 59803     | c      | Medium           | 0.21  | 0.05    | 0.15      | 6    |
| Fkbp1a*     | 14225     | c      | High             | 0.24  | 0.06    | 0.17      | 7    |
| Wars2       | 70560     | c      | Medium           | 0.24  | 0.08    | 0.19      | 8    |
| Auh         | 11392     | c      | Low              | 0.24  | 0.08    | 0.18      | 9    |
| Efhand2     | 27984     | c      | High             | 0.27  | 0.02    | 0.19      | 10   |
| Zc3h11a     | 70579     | c      | High             | 0.28  | 0.02    | 0.19      | 11   |
| Rexo5       | 434234    | c      | Low              | 0.28  | 0.03    | 0.19      | 12   |
| Napl2       | 231042    | c      | Low              | 0.27  | 0.10    | 0.20      | 13   |
| Tmed2       | 56334     | c      | High             | 0.31  | 0.02    | 0.22      | 14   |
| Grn         | 14824     | c      | High             | 0.33  | 0.02    | 0.23      | 15   |
| Eif3a       | 13669     | b      | Low              | 0.32  | 0.09    | 0.23      | 16   |
| Tbp         | 23134     | d      | Medium           | 0.33  | 0.04    | 0.23      | 17   |
| Cokk1a1     | 93687     | c      | High             | 0.33  | 0.08    | 0.24      | 18   |
| Eif4g2      | 13690     | c      | High             | 0.34  | 0.03    | 0.24      | 19   |
| Tnks2       | 74493     | c      | High             | 0.34  | 0.04    | 0.24      | 20   |
| F2r         | 14462     | c      | High             | 0.32  | 0.13    | 0.24      | 21   |
| Rab23       | 19335     | c      | Medium           | 0.34  | 0.10    | 0.25      | 22   |
| Mau1cmn     | 50918     | c      | High             | 0.33  | 0.15    | 0.26      | 23   |
| Rpg1b       | 72462     | c      | Medium           | 0.37  | 0.07    | 0.26      | 24   |
| Hprt1       | 13452     | d      | High             | 0.37  | 0.07    | 0.27      | 25   |
| Canx        | 12330     | c      | High             | 0.35  | 0.15    | 0.28      | 26   |
| Eif4b       | 22384     | c      | Medium           | 0.39  | 0.04    | 0.27      | 27   |
| Nc335a5     | 74102     | c      | High             | 0.41  | 0.03    | 0.29      | 28   |
| Atp6v1k     | 11658     | c      | High             | 0.45  | 0.06    | 0.32      | 29   |
| Tnha        | 22631     | d      | High             | 0.45  | 0.06    | 0.32      | 30   |
| Mx2         | 17858     | d      | Low              | 0.43  | 0.17    | 0.32      | 31   |
| Cldn1p2     | 15193     | c      | Medium           | 0.52  | 0.00    | 0.36      | 32   |
| Tufu60      | 67595     | b      | High             | 0.54  | 0.01    | 0.38      | 33   |
| Tfe         | 22042     | d      | High             | 0.51  | 0.21    | 0.39      | 34   |
| Zimh20      | 67592     | c      | High             | 0.56  | 0.04    | 0.39      | 35   |
| Pumad4      | 19185     | b      | High             | 0.62  | 0.00    | 0.43      | 36   |
| Sdha        | 66495     | d      | High             | 0.69  | 0.06    | 0.49      | 37   |
| G6pdc       | 14381     | d      | High             | 0.71  | 0.04    | 0.50      | 38   |
| Rhd1        | 59040     | c      | High             | 0.53  | 0.48    | 0.50      | 39   |
| Ing3        | 71777     | c      | Medium           | 0.78  | 0.05    | 0.53      | 40   |
| Guab        | 110006    | a, b, d| Medium           | 0.79  | 0.01    | 0.55      | 41   |
| Mya2g2a     | 18780     | d      | Low              | 0.80  | 0.03    | 0.56      | 42   |
| Actb        | 11461     | a, b, d| High             | 0.83  | 0.04    | 0.58      | 43   |
| Rpl4        | 67891     | b      | High             | 0.85  | 0.02    | 0.60      | 44   |
| Gapdh       | 14433     | a, b, d| High             | 0.88  | 0.05    | 0.63      | 45   |
| Hpgi0b4d    | 15516     | d      | High             | 0.92  | 0.47    | 0.73      | 46   |
ing RNA quality of RIN > 5 (n = 21 male, n = 20 female). Out of the 23 reference gene candidates, 16 (GeNorm) and 14 (Normfinder) genes revealed low expression variability (M- or SV-value < 0.5) in both sexes. Among these, GeNorm identified Tbp, Tmed2 and Normfinder Eif4g2 with the highest mean stability ranking in both sexes (Tables 2, 3). Intra- and intergroup variation analysis by Normfinder identified Eif4g2 (re-calculated SD 0.0476) as the reference gene with the lowest variation and Tbp together with Eif4g2 as the best combination for reference gene performance (re-calculated SD 0.0466).

With regard to the effect of RNA quality on expression variability of the reference gene candidates, a comparative analysis between low (n = 33; PND2.5–28.5; RIN < 5) and high (n = 17; PND2.5–28.5; RIN > 5) RNA quality samples identified no gene with an acceptable M-value of < 0.5 in both RNA quality groups (GeNorm). However, Normfinder revealed 3 out of the 23 candidate genes with low expression variability (SV < 0.5) in both RNA quality conditions. Of them, Maea demonstrated the highest mean stability ranking in both RNA quality conditions (Tables 2, 3).

Intra- and intergroup variation analysis by Normfinder identified Pcsk7 as the reference gene with lowest expression variability (re-calculated SD 0.1382) and Rpl4 and Zc3h11a as the best combination for reliable reference gene performance (re-calculated SD 0.1460).

In lung tissues acquired from hyperoxia-exposed mice (n = 36; PND2.5–28.5; FiO2 = 0.8, RIN > 5), GeNorm identified 8 out of all 23 reference gene candidates with low expression variability (M-value < 0.5) with Auh and Zc3h11a demonstrating the lowest expression variability (M-value 0.299), followed by Rpl4, Dolpp1, Csnk1a1, Maea, Pcsk7 and Elac2. Likewise, Normfinder revealed 8 out of all 23 candidate genes with low expression variability (SV < 0.5) with Rpl4 demonstrating the lowest expression variability (SV = 0.235) followed by Zc3h11a, Auh, Maea, Dolpp1, Csnk1a1, Nupl2 and Eif3a (Tables 2, 3).

When comparing expression variability of all 23 candidates under physiologic (n = 17; PND2.5–28.5; FiO2 = 0.21, RIN > 5) and hyperoxia conditions (n = 36; PND2.5–28.5; FiO2 = 0.8, RIN > 5), GeNorm and 7 (Normfinder) genes were identified with low expression variability (M- or SV-value < 0.5) in both experimental conditions. Among them, GeNorm recognized Rpl4 and Normfinder Maea, Rpl4 with the highest mean stability ranking in both groups, i.e., physiologic and hyperoxia conditions (Tables 2, 3).

Intra- and intergroup variation analysis by Normfinder demonstrated Rpl4 (re-calculated SD 0.1004) as the best reference gene and Maea together with Zc3h11a as the best combination for reliable reference gene performance (re-calculated SD 0.0711).

Table 2. GeNorm result represented with M-values and corresponding stability ranking for different analytic approaches compared with the pre-selection.
| Reference gene candidates | Pre-selection | Physiologic condition (FiO2 = 0.21, PND2.5–28.5, RIN > 5; n = 17) | Developmental stages (FiO2 = 0.21, RIN > 5) | Sex (FiO2 = 0.21, PND2.5–28.5, RIN > 5) | Low RNA quality (FiO2 = 0.21, PND2.5–28.5, RIN < 5; n = 33) | Hyperoxia exposure (FiO2 = 0.8, PND2.5–28.5, RIN > 5; n = 36) |
|---------------------------|--------------|-------------------------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| *Auh*                     | 0.151        | 8                                               | 0.553 10                        | 0.201 7                         | 0.755 16                         | 0.541 17                        |
| *Csnk1a1*                 | 0.199        | 19                                              | 0.278 2                         | 0.217 9                         | 0.275 3                         | 0.314 8                         |
| *Dolpp1*                  | 0.113        | 1                                               | 0.496 8                         | 0.403 18                        | 0.366 4                         | 0.808 22                        |
| *Ephd2*                   | 0.157        | 9                                               | 0.810 15                        | 0.586 20                        | 0.717 15                        | 0.665 19                        |
| *Eif3a*                   | 0.197        | 16                                              | 0.409 6                         | 0.271 12                        | 0.505 9                         | 0.418 12                        |
| *Eif4g2*                  | 0.198        | 17                                              | 0.993 19                        | 0.508 14                        | 0.439 12                        | 0.970 8                         |
| *Elac2*                   | 0.119        | 4                                               | 0.527 9                         | 0.376 16                        | 0.596 14                        | 0.125 2                         |
| *Grm*                     | 0.191        | 14                                              | 1.452 22                        | 0.336 13                        | 1.363 21                        | 0.270 6                         |
| *Kdm3b*                   | 0.121        | 5                                               | 0.608 11                        | 0.257 10                        | 0.519 10                        | 0.578 18                        |
| *Maea*                    | 0.137        | 6                                               | 0.205 1                         | 0.366 15                        | 0.106 2                         | 0.086 1                         |
| *Mpv17l*                  | 0.113        | 2                                               | 2.444 23                        | 1.815 23                        | 1.902 23                        | 2.871 23                        |
| *Nupl2*                   | 0.178        | 12                                              | 0.311 3                         | 0.216 8                         | 0.395 6                         | 0.254 4                         |
| *Peak7*                   | 0.118        | 3                                               | 0.476 7                         | 0.390 17                        | 0.492 8                         | 0.704 20                        |
| *Pmd4*                    | 0.358        | 22                                              | 0.616 12                        | 0.509 21                        | 0.475 7                         | 0.258 5                         |
| *Psg89*                   | 0.312        | 20                                              | 1.341 21                        | 0.811 22                        | 1.537 22                        | 0.730 21                        |
| *Rcos5*                   | 0.166        | 11                                              | 0.868 17                        | 0.259 11                        | 0.381 5                         | 0.336 9                         |
| *Rpl4*                    | 0.491        | 23                                              | 0.329 4                         | 0.104 5                         | 0.522 11                        | 0.278 7                         |
| *Tbp*                     | 0.194        | 15                                              | 0.729 13                        | 0.090 4                         | 0.567 13                        | 0.515 16                        |
| *Tfrc*                    | 0.333        | 21                                              | 0.897 18                        | 0.168 6                         | 1.104 20                        | 0.387 11                        |
| *Tmed2*                   | 0.185        | 13                                              | 1.003 20                        | 0.053 2                         | 1.084 19                        | 0.426 13                        |
| *Trnk2*                   | 0.198        | 18                                              | 0.850 16                        | 0.412 19                        | 0.762 17                        | 0.474 15                        |
| *Wars2*                   | 0.150        | 7                                               | 0.774 14                        | 0.089 3                         | 0.545 12                        | 0.462 14                        |
| *Zc3h11a*                 | 0.166        | 10                                              | 0.368 5                         | 0.355 14                        | 0.106 1                         | 0.365 10                        |

Table 3. Normfinder result represented with SV-values and corresponding stability ranking for different analytic approaches compared with the pre-selection.

By RT-qPCR validation, we observed differences in expression stability rankings for selected candidates, e.g., *Mpv17l*, presenting the lowest expression variability in the pre-selection in contrast to highest expression variability in RT-qPCR analysis (Tables 2, 3).

Expression of the 23 candidates in diverse cell types in the course of neonatal development PND0–PND14. Gene expression profiles in three major cell types (epithelial, endothelial, mesenchymal) during the course of neonatal lung development (PND0–PND14) from a recently published single-cell atlas of the developing mouse lung demonstrated high expression level in the majority of the investigated cells, while the former three genes showed slightly lower expression levels in the epithelium (Figs. 1, 2). The variance of each candidate gene in the scRNA-Seq dataset was calculated during SCTransform normalization and ranked from lowest to highest. The five genes with the lowest variance were: *Rexo5*, *Mpv17l*, *Wars2*, *Nupl2* and *Elac2*.

Discussion

Accurate interpretation of experimental data obtained by RT-qPCR, i.e., presented as raw Cq values, is of critical importance for result interpretation. A widely applied method is the so-called relative quantification using reference genes as endogenous control. This normalization strategy is easy to apply and allows for compensation of non-specific gene expression variability resulting from technical and experimental conditions. These include variation due to different amount of starting material, variance in RNA integrity and inconsistent efficiency of cDNA synthesis. For optimal performance, these reference genes need to fulfill critical criteria such as low expression variability with regard to different variables that might act as ‘hidden confounders’ when considering reference gene regulation.

We therefore thoroughly assessed important reference gene quality criteria in a clinically and experimentally relevant, challenging context presented in the developing lung. Here, organ development progresses rapidly, sample numbers are often limited and different biological, technical and experimental variables can exhibit significant effects on gene expression that need to be eliminated or taken into account when a reference gene is used. Through the application of GeNorm and Normfinder algorithms, broadly used to evaluate gene expression variability, in combination with group comparisons, we successfully characterized reference genes in the developing lungs of neonatal C57BL/6 mice. The main challenge while selecting a reference gene for studies...
in a developing organ, was that pre-selection of the reference gene candidates only relied on limited data from neonatal tissue. Their characterization during postnatal lung development while considering the sex and RNA quality indicated the significant impact of these conditions on reference gene expression. However, it revealed promising candidates with lower expression variability. Studies addressing the broadly used experimental condition ‘hyperoxia’ to induce lung injury further emphasized this pattern.

Although the analyses did not reveal one single gene with universally low expression variability in all conditions investigated, the results allowed the selection of individual genes under consideration of the different biological, technical and experimental conditions studied (summarized results Table 4, Fig. 3).

Specific considerations regarding reference gene selection reflect both, the insight generated by the study as well as the limitations of interpretability. Differences in expression stability rankings of the candidates obtained in the pre-selection process and by RT-qPCR measurements (e.g., Mpv17l) might reflect effects of age or analysis method and should be considered for the individual experimental design. The low expression variability, i.e., high expression stability of Rpl4 observed in different experimental conditions has to be interpreted in light of the controversial discussion around genes encoding ribosomal proteins. The discussion is reflected by the publications of de Jonge et al.11, supporting reference gene qualities for this group of genes due to low expression variability, in contrast to Thorrez et al.15, discouraging their use as reference genes due to the significant differences between specific tissues. In contrast to the exclusion of an entire group of genes, analysis results further required single case decisions: The lack of Fkbp1a expression in 10% of the neonatal lung samples resulted in the exclusion of this gene for further analysis to ensure stable reference gene expression in neonatal lung tissue. Potential effects of development on Fkbp1a expression were likely aggravated by low RNA quality.

Cell specific expression levels of the reference gene candidates obtained by single-cell RNA sequencing confirmed promising candidates (Rexo5, Mpv17l, Wars2, Nupl2, Elac2) with low expression variability in the course of lung development, i.e., PND0-PND14. These findings should be considered when addressing specific cell types or lung cellular compartments by gene expression analysis in total lung homogenates despite the overall low expression levels of these genes (Figs. 1, 2).

In summary, 16 out of 23 reference gene candidates can be suggested as ‘reliable’ based on their expression stability in at least one of the experimental settings investigated (Fig. 4). For the majority of genes such as Elac2, Csnk1a1, Eif3a, Eif4g2, Tmed2 and Mpv17l, a role in the pathogenesis of lung diseases was indicated by previous studies22–27, although their functions in the neonatal lung remain unexplored. The data resource provided by the study not only gives insight into an unexplored field of significant relevance for studies in lung development but could serve as a guide for reference gene selection, specifically considering commonly relevant biological, technical and experimental conditions.

![Figure 1. Profile of reference gene expression time in the developing lung by single-cell RNA sequencing.](image-url)
Methods

After pre-selection of reference gene candidates by reviewing literature and publicly available databases including Genevestigator, we confirmed expression variability of the reference gene candidates using our own transcriptome data obtained from murine lungs during alveolarization [postnatal day (PND) 5–7] as well as microarray data available through Genevestigator. We validated the gene expression profiles of the final reference gene candidates by RT-qPCR in lung tissue of neonatal C57BL/6 considering different biological, technical and experimental conditions. These analyses were performed using GeNorm and Normfinder algorithms for the characterization of reference gene expression variability. Cell-specific expression patterns of the reference gene candidates were assessed using single-cell data obtained during postnatal lung development (PND0–PND14).

In silico selection of reference gene candidates. Reference gene candidates were selected from four independent public and commercial databases (see a–d) taking into account studies that used both adult and neonatal mice as well as different experimental conditions in order to allow for broad coverage at the initial step. The search was limited by (i) underrepresentation of studies in neonatal mice in public and commercial databases, (ii) unavailability of sex-specific information and (iii) insufficient data on RNA quality for the majority of studies. The selection process combined reference gene candidates from the following four sources: (a) PubMed
Figure 3. Reference genes with low expression variability (RT-qPCR). Black, bold: Genes with high expression stability identified by both GeNorm and Normfinder. Colored: Genes selected for low expression variability by GeNorm (green) or Normfinder (red). Pcsk7 (blue) was identified by GeNorm in the hyperoxia condition and by Normfinder in the physiologic condition.

Figure 4. The reliable reference genes with regard to the analytical approaches. 16 out of 23 reference gene candidates that were identified in at least one analytical approach (colored bars) as a reliable reference gene are presented. Maea was most commonly identified as stably expressed gene among the different analytical approaches. For detailed information about experimental conditions of the analytical approaches see the text.
search using the terms ‘reference gene’, ‘murine lung’, ‘C57BL/6’, ‘neonatal’, ‘lung development’, ‘RT-qPCR’ and ‘housekeeping gene’ (single and combined search). Here, reference gene candidates were selected from the studies performed in lung tissue from C57BL/6 mice with the age from PND3 to 26 months, one study investigated neonatal mice between PND3 and PND21; (b) publications that specifically evaluated the expression variability of commonly-used murine reference genes including studies in C57BL/6, p50 and p105 transgenic mice aged 8–15 weeks\(^{11–14}\). Here, one meta-analysis of murine microarray data covered different murine strains, experimental conditions and age groups\(^{11}\); (c) the subtool ‘RefGenes’ of Genevestigator (https://genevestigator.com/gv) using the selection criteria ‘lung tissue’ and ‘C57BL/6’ resulting in 10 gene expression profiling studies from 2 to 22 week-old C57BL/6 mice (n = 122 wild-type; n = 17 transgenic mice; total n = 139 samples) with one study in 2 week old C57BL/6 mice\(^{24}\). These studies included different experimental conditions such as simvastatin treatment, cigarette smoke exposure and ovalbumin sensitization\(^{15–30}\). All genes identified with RefGenes at three different expression levels (log2-scaled signal intensity: > 13.00 high level, 10.00–13.00 medium level, < 10.00 low level; n = 20 each) were selected excluding doublet candidates; (d) a commercial reference gene catalogue (Roche Applied Science, n = 27 reference genes).

**Validation of reference expression variability using our own lung transcriptome data and Genevestigator.** As the majority of gene candidates were derived from data obtained in adult mice, we next assessed their expression in neonatal murine lung tissues (PND 5–7) using our own transcriptome data to then rank expression variability of all reference gene candidates according to the overall SD (SD\(_{\text{array}}\)). Overall SD, sensitively detecting variability, was calculated as the square root of the averaged variances of the mean expression values from our own neonatal mouse microarray data (SD\(_{\text{array}}\)) and Genevestigator RefGene analysis (SD\(_{\text{ref}}\)), predominantly derived from adult mice:

$$SD_{\text{overall}} = \sqrt{\frac{SD_{\text{ref}}^2 + SD_{\text{array}}^2}{2}}.$$  

**Investigation of pulmonary expression variability under consideration of biological, technical and experimental variables.** RT-qPCR for selected reference gene candidates in lung tissue included C57BL/6 mice (n = 17) during lung development under physiologic conditions (FiO\(_2\) = 0.21) and good RNA quality [RNA Integrity Number (RIN) > 5] with alveolarization [PND2.5 (n = 6), PND5.5 (n = 7)] and post-alveolarization [PND14.5–PND28.5 (n = 4)] stages and subsequently addressed the impact of biological and technical conditions, i.e., sex and RNA quality\(^{37}\). Sex: Expression variability of the reference gene candidates was compared in male (PND2.5–28.5, RIN > 5; n = 21) and female (PND2.5–28.5, RIN > 5; n = 20) mice. RNA quality: Expression variability was analyzed under consideration of RNA quality comparing results in low (RIN < 5, PND2.5–28.5; n = 33) and high (RIN > 5, PND2.5–28.5; n = 17) RNA quality samples\(^{37}\) under physiologic conditions (FiO\(_2\) = 0.21). In a subsequent comparison, expression variability was evaluated in the experimental setting of hyperoxia (FiO\(_2\) = 0.8, PND2.5–28.5, RIN > 5; n = 36) and compared to results obtained under physiologic conditions (FiO\(_2\) = 0.21, PND2.5–28.5, RIN > 5; n = 17).

**Assessment of expression variability.** Analyses of raw Cq data were performed using GeNorm and Normfinder integrated in GenEx 7.0 software (MultiD Analyses AB; https://multid.se/genex/). For each analysis group, GeNorm identified the two reference genes with similar gene expression by pairwise comparison, whereas Normfinder identified the reference gene based on intra- and intergroup gene expression variation as introduced by different conditions, e.g., biological or technical conditions\(^{19,38}\).

For identification of the candidates with lowest expression variabilities, GeNorm and Normfinder, were applied as follows: (i) When considering a biological variable or experimental condition, all reference gene candidates with M- or SV-values below 0.5 were regarded as reliable reference genes according to a previous study\(^{16,17}\) and ranked according to the lowest M- or SV-value. (ii) When comparing two or more biological, technical variables or experimental conditions, expression variability of the reference gene candidates was ranked according to M- or SV-values in each group and subsequently, the mean expression variability rank for each gene was calculated by averaging the ranks from each group for genes with M- or SV-values below 0.5 in all groups. (iii) For comparison of two groups by means of Normfinder, intra- and intergroup variation analysis with sufficient sample numbers was performed.

**In vivo experiments and mRNA analysis in neonatal murine lungs.** Microarray experiments were performed in lung tissue obtained from 5 to 7-day-old C57BL/6 mice (FiO\(_2\) = 0.21). Lungs were excised and immediately snap frozen in liquid nitrogen before total RNA was extracted from homogenized tissues using the miRNeasy Mini Kit (Qiagen). Extracted total RNA was subjected to cRNA synthesis, cRNA fragmentation and finally hybridization on Mouse CodeLink 10 K arrays using the CodeLink Expression Assay Kit (GE Healthcare) according to the manufacturer’s instructions. The spot signals of obtained microarray images were quantified using the CodeLink System Software 5.0.0.31312 which generated local background corrected raw as well as median centered intra-slide normalized data. For further analysis only the intra-slide normalized data consisting of 10,181 probe sets and 6 samples were used.

The genes represented by the probe sets were annotated using the biocLite package (BioConductor) with the library “mwgcod.db” for CodeLink Mouse Whole Genome arrays. The expression data were processed using an automated quality control workflow which includes omission of control genes, removal of genes with poor quality flags and removal of probe sets with high proportion (≥ 50%) of missing values. A total of 3651 probe
RT-qPCR experiments and data analysis. Wild-type male and female mice (C57BL/6J) were randomly assigned to the experimental groups to spontaneously breath room air (FIo2=0.21) or undergo hyperoxia exposure while spontaneously breathing (FIo2=0.8). Total RNA was extracted from homogenized whole lungs using the PeqGOLD total RNA kit (PeqLab). Samples were stored at – 80 °C until further analysis. Real time qPCR: RNA quality was assessed using the RNA 6000 Nano Kit (Agilent Technologies) and RT-qPCR was then performed according to the manufacturer’s instructions. Briefly summarized, equivalents of 1 µg total RNA were transcribed into cDNA (Transcriptor First Strand cDNA synthesis kit, Roche) and subsequently diluted 1:10 using PCR-grade water. All 24 reference gene candidates were assessed in the lung samples by RT-qPCR using FastStart essential DNA probes master (Roche) and RealTime Ready Assays (Roche) providing gene specific primers. For data analysis, inter-run differences were adjusted for by calibration using a fixed cDNA mix generated from 31 cDNA samples with the best RNA quality from each age group (RIN = 5.4–8.1). The cDNA mix was applied to every plate with the expression level of the gene Tbp (Entrez ID 21374) serving as an inter-run calibrator. Differences between plates were compensated through the inter-run calibrated Cq value using the formula demonstrated in the study of Ståhlberg et al.49.

Sex was confirmed using Y chromosome identification by qPCR (60 samples of 86 samples in total). Age groups were identified as neonatal, i.e., during alveolarization (PND2.5 and PND5.5) and adolescent/young adult, i.e., post-alveolarization (PND14.5–PND28.5).

Single-cell RNA-sequencing analysis. Data from single-cell RNA sequencing in lung tissue samples were analyzed for marker gene expression in different lung cell types as previously reported67. This dataset was generated from a mouse lung cellular suspension that was depleted of Ter119 + blood cells and CD45 + immune cells and sequenced using the 10x Genomics Chromium platform. Data from P0, P3, P5, P7 and P14 mice were analyzed. Variability of marker genes across individual cells in the lung was calculated by determining the standard deviation of each gene across all cells in the presented dataset. Genes with the lowest standard deviation are considered the least variable.

Approval for animal experiments. The study was conducted in accordance with the German animal welfare law (TierSchG and TierSchVersV) and the European legislation for the protection of animals used for scientific purposes (2010/63/EU). All animal experiments were approved by the Ethic-Commission of the Medical Faculty of the Justus-Liebig-University Giessen (Approval No. TVA B2/277) and the Institution for Animal Care, Ludwig-Maximilians-University Munich (Approval No. TVA 117-10). The study complies with the ARRIVE guidelines.

Data availability The microarray data generated during this study have been deposited in NCBI’s Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE189505. The raw data obtained from RT-qPCR validation were provided as supplemental data.

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Author contributions

H.S. performed RT-qPCR experiment with data analysis and wrote the manuscript draft. R.E.M. performed the hyperoxia experiments in mice of different age groups, provided the samples and advised for interpretation of the results. J.M.S. and N.M.N. performed the single cell RNA sequencing including data interpretation and presentation. M.M. and H.H. performed the microarray experiment with data analysis and presentation. A.H., S.K.-E. and S.D. conceived the study, supervised the experiments, interpreted the results and substantially revised the manuscript.

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Competing interests

The authors declare no competing interests.
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