Stability of small non-coding RNA reference gene expression in the rat retina during exposure to cyclic hyperoxia

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Purpose: Oxygen-induced retinopathy (OIR) is a robust animal model of human retinopathy of prematurity that readily allows changes in retinal gene and microRNA (miRNA) expression in response to fluctuations in oxygen levels to be studied. We sought to identify small non-coding RNA (ncRNA) genes that showed stable expression upon exposure to varying levels of oxygen, with different developmental stages and in different rat strains, to act as reference genes for normalizing miRNA expression in a rat model of OIR.

Methods: Expression of five small ncRNAs (U6 snRNA, miR-16, U87, 4.5S RNA (H) “Variant 1”, and 5S ribosomal RNA [rRNA]) were tested on a standard RNA pool and representative retinal samples from P5, P6, P9, and P14 from room air– and cyclic hyperoxia–exposed rats using reverse transcription (RT)-qPCR, to assess the effect of developmental stage and exposure to fluctuations in oxygen levels, respectively. Two strains of inbred albino rats, Fischer 344 (F344, resistant to OIR) and Sprague-Dawley rats (SD, susceptible to OIR), were used to assess the effect of rat strain on the stability of the small ncRNAs.

Results: In this rat model of OIR, SS rRNA expression was variable with strain, fluctuations in oxygen levels, and developmental stage. U6 snRNA was stably expressed with changes in oxygen levels, and minimal variation was observed with strain and developmental stage. MiR-16 showed less stable expression with changes in oxygen levels and between strains compared to U6 snRNA. Some variation in expression in response to developmental stage was also observed. The PCR amplification efficiencies of the U6 snRNA and miR-16 TaqMan assays were 56% and 78%, respectively. U87 and 4.5S RNA (H) “Variant 1” expression varied with strain, exposure to cyclic hyperoxia, and in particular developmental stage, and was at low levels in the neonatal rat retina.

Conclusions: We conclude that U6 snRNA and miR-16 are the most suitable reference RNAs for normalizing miRNA expression, as they are relatively stable with strain, exposure to cyclic hyperoxia, and developmental stage in a rat model of OIR.

Normalization of reverse transcription (RT)-qPCR data using stably expressed reference genes is essential for accurately profiling gene and microRNA (miRNA) expression [1-4]. Genes that are constitutively expressed and are associated with housekeeping or structural functions are commonly used as reference genes, as they tend to be expressed across many cell and tissue types and show minimal variation among samples or experimental conditions [4-7]. However, evidence suggests that the expression of commonly used reference genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and beta-actin varies between cell and tissue types [8,9]. Identifying and characterizing stably expressed reference genes through a series of preliminary experiments is recommended, to accurately determine gene and miRNA expression levels and to avoid incorrect conclusions being drawn [3,6,10,11].

Retinopathy of prematurity (ROP) is a potentially blinding condition that affects premature infants who undergo supplemental inspired oxygen therapy. Oxygen-induced retinopathy (OIR) is a robust animal model of ROP that mimics the pathology seen in human disease [12,13]. The rat model of OIR described in this study utilizes exposure of neonatal rats to cyclic hyperoxia and relative hypoxia [14-17]. As changes in retinal gene expression in response to exposure of neonates to hyperoxia and relative hypoxia are involved in inducing OIR [18-21], identifying reference genes that are stable with variations in oxygen levels is vital. Previous studies from our laboratory identified two RNA reference genes encoding acidic ribosomal phosphoprotein and hypoxanthine guanine phosphoribosyl transferase as being stably expressed in response to cyclic hyperoxia and during early retinal development in several different rat strains [5], making these reference genes suitable for normalizing gene expression data.

MicroRNAs expressed in the eye are associated with physiologic and pathological processes and display tissue specificity and spatiotemporal expression [22,23]. In mouse
Total RNA was isolated from the retinas of neonatal rats as described previously [31], using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer’s method. Total RNA was also prepared from the rat liver, brain and lung, and mouse lens and liver using the same method. Mouse liver and lens tissue were kindly provided by Dr. Shiwani Sharma (Flinders University, Adelaide, South Australia). Quantification of total RNA was performed using the NanoDrop 8000 (Thermo Scientific, Wilmington, DE). An absorbance ratio of the RNA at 260 and 280 nm was used as a guide to determine RNA purity. Total RNA with a ratio of 1.8 or more was considered acceptable. Agarose gel electrophoresis was used to assess RNA integrity and residual DNA contamination. Total RNA with a 28S:18S ratio of approximately 2:1 and showing minimal DNA contamination was considered acceptable for use.

**RT-qPCR analysis of 5S RNA expression:** RT-qPCR was performed with reference to the Minimum Information for
Publication of Quantitative Real-Time PCR Experiments guidelines [32]. Each 10 μl reaction mixture contained 5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA), 1 μl each forward and reverse primers (0.5 μM final concentration), and 3 μl cDNA sample diluted 1:5 with Ultrapure water (Fisher Biotech, WA, Australia). Reactions were run on a StepOne Plus real-time PCR System (Applied Biosystems). The cycling conditions were initial denaturation (95 °C, 15 min) followed by 40 cycles of denaturation (95 °C, 15 s), annealing, and extension (60 °C, 30 s).

Primers specific for rat 5S rRNA were published previously [33]. The sequences for each primer were as follows: The forward primer was 5'-TCT CGT CTG ATC TCG GAA GC-3'; and the reverse primer was 5'-AGC CTA CAG CAC CCG GTA TT-3'. Primers were synthesized by Geneworks Ltd (Thebarton, South Australia, Australia).

**Assays for RT-qPCR analysis of miRNA expression**: TaqMan miRNA assays (Applied Biosystems) were used to determine miRNA expression levels in the retinal, liver, brain, lung, and lens samples. Eight commercially available small ncRNA controls, referred to as “endogenous controls” by Applied Biosystems, were chosen for analysis. In addition to these endogenous controls, four miRNAs considered stably expressed with strain and treatment were included. They were manually identified from data derived from Exiqon miRNA arrays (Exiqon A/S, Vedbaek, Denmark) performed at the Adelaide Microarray Centre (Adelaide, Australia) using Exiqon miRNA version 8.1 and version 11 all species array libraries. Briefly, microarray libraries were printed onto slides. Retinal-derived total RNA were labelled, hybridized and scanned according to the manufacturer’s protocol. Preliminary Exiqon microarray data analyses were performed at the Adelaide Microarray Centre using the software package LIMMA R (WEHI, Melbourne, VIC, Australia) to identify differentially expressed miRNAs. The full microarray processing protocol and statistical analyses has been described elsewhere [34]. The arrays were used to identify miRNAs that were differentially expressed as a result of rat strain and/or changes to oxygen levels. Thirteen ncRNAs (including 5S rRNA) were tested before RT-qPCR quantification of miRNA expression. The corresponding Applied Biosystems TaqMan miRNA assay reference numbers are shown in Table 1. Primer amplification efficiencies for each small ncRNA were determined using the standard RNA sample.

**RT-qPCR analysis of miRNA expression**: cDNA synthesis and qPCR reactions were performed using a StepOne Plus real-time PCR System (Applied Biosystems). Conversion of total RNA to single-stranded cDNA was performed in accordance with the manufacturer’s instructions for the TaqMan miRNA assays with slight modifications as detailed. Briefly, total RNA samples were diluted to a concentration of 4, 20, or 40 ng/μl depending on the optimized template concentration determined for each miRNA of interest. A total of 5–40 ng of RNA was used in each reverse transcription reaction.

The reverse transcription reaction was modified to 7.5 μl rather than 15 μl reactions and the volumes adjusted accordingly. A bulk reverse transcription master mix was prepared containing 100 mM deoxynucleotide triphosphates (with deoxythymidine triphosphate, 0.075 μl/reaction), Multi-scribe™ Reverse Transcriptase 50 U/μl (0.5 μl/reaction), 10 x Reverse Transcriptase Buffer (0.75 μl/reaction), RNase Inhibitor 20 U/μl (0.095 μl/reaction) and nuclelease-free water (2.08 μl/reactions). The reverse transcription master mix (3.5 μl/reaction) was then aliquoted into a 96 well reaction plate with 0.1 ml well volume (Applied Biosystems) and miRNA-specific RT primer (1.5 μl/reaction) added. The diluted total RNA (2.5 μl/reaction) was then added to bring the final volume to 7.5 μl.

The reactions were incubated for 30 min at 16 °C and then followed by 30 min of incubation at 42 °C. The reaction was then terminated at 85 °C for 5 min before being held at 4 °C until required for qPCR that was performed on the same day.

A PCR master mix was prepared for each reference small ncRNA gene. Each 10.3 μl reaction contained 0.5 μl 20X TaqMan miRNA assay mix, 5 μl TaqMan 2X Fast Universal PCR Master Mix No Amperase UNG, 3.8 μl of nuclelease-free water, and 1 μl of the reverse transcription product. Each reaction was performed in triplicate. Nuclelease-free water was used in place of the reverse transcription template as a negative control for non-specific amplification. PCR reactions were placed in the StepOne Plus cycler, and the AmpliTaq DNA polymerase was activated at 95 °C for 20 s, followed by 40 cycles of denaturing at 95 °C for 1 s, and then annealing and extension at 60 °C for 20 s. Again, a single representative room air- or cyclic hyperoxia–exposed rat was used at each time point for each strain of interest.

**Determination of PCR primer amplification efficiencies**: PCR primer amplification efficiencies were determined for the 5S rRNA primer pair using ten threefold serial dilutions of the standard cDNA sample derived from rat retinal tissue to generate a standard curve. Dilutions were made over five orders of magnitude; however, reliable data were generated only to four logs. For miRNA primer pairs, nine fourfold serial dilutions of the standard RNA sample from rat retinal tissue were used to generate the standard curve. Depending on the miRNA primer pair, reliable data were generated to a
minimum of two logs and a maximum of four logs. Although data from the standard curve would ideally extend to five orders of magnitude to determine PCR amplification efficiency accurately, the abundance of each mRNA or miRNA in the sample of interest was a limiting factor.

Standard curves for five small ncRNA primer pairs were also performed on total RNA derived from rat liver samples to determine if poor amplification efficiencies were due to the tissue-specificity of the ncRNA in question. In these instances, six twofold serial dilutions of the standard RNA sample were used to generate the standard curve over two orders of magnitude. A smaller dilution factor was used for the miRNA primer pairs to ensure that the majority of the threshold cycle values fell within the range of 20–30.

For all primer pairs, the mean threshold cycle value for each dilution was plotted against the log cDNA concentration, and the gradient of the regression line of the standard curve was used to calculate the PCR amplification efficiency. Amplification efficiencies were used in quantifying gene and miRNA expression in the context of OIR [35,36].

Statistics: All statistical analyses were performed using the software package PASW Statistics version 18.0 (SPSS Inc., Chicago, IL). Independent-samples Student *t* tests or Mann–Whitney U tests were performed to compare stability of the small ncRNAs between strains or cyclic hyperoxia exposure, and the significance level (alpha) was set at 0.05. Statistical analysis for small ncRNA expression in response to development was performed using one-way between-groups analysis of variance (ANOVA), with the significance (alpha) level adjusted for multiple comparisons (Tukey’s honestly significant difference test) to *p*<0.0125. Where appropriate, a Kruskal–Wallis test with the significance (alpha) level set at 0.05 was used instead.

RESULTS

Eight small ncRNA endogenous controls available from Applied Biosystems, four miRNAs manually identified from in-house Exiqon microarrays as being stably expressed with strain and oxygen exposure, and the significance level (alpha) was set at 0.05. Statistical analysis for small ncRNA expression in response to development was performed using one-way between-groups analysis of variance (ANOVA), with the significance (alpha) level adjusted for multiple comparisons (Tukey’s honestly significant difference test) to *p*<0.0125. Where appropriate, a Kruskal–Wallis test with the significance (alpha) level set at 0.05 was used instead.

**RESULTS**

Eight small ncRNA endogenous controls available from Applied Biosystems, four miRNAs manually identified from in-house Exiqon microarrays as being stably expressed with strain and exposure to cyclic hyperoxia, and the small ncRNA 5S rRNA were tested using RT-qPCR. Primer PCR amplification efficiencies from total RNA derived from rat retinal samples were determined using the standard RNA pool to a minimum of 2 orders of magnitude to generate a standard curve. The order of magnitude of the standard curve dilution series, the *R*² value for each standard curve and the mean Ct across all test samples, including the standard pool is shown. N/A=not available; ND=not able to be determined; *=small ncRNA assays referred to as endogenous controls by Applied Biosystems; ++=miRNA identified from in-house Exiqon microarrays as being stably expressed with strain and oxygen exposure.

| Small ncRNA                | Species reactivity | Assay ID  | Amplification efficiency in rat retina | Order of magnitude | R² value | Mean Ct |
|----------------------------|--------------------|-----------|----------------------------------------|-------------------|----------|---------|
| 4.5S RNA(H)* “Variant 1”  | Rat                | 001716    | 49.3%                                  | 2.4               | 1.00     | 24.1    |
| 4.5S RNA(H)* “Variant 5”  | Rat                | 001717    | ND – low abundance                     | ND                | ND       | ND      |
| snoRNA202*                 | Mouse              | 001232    | ND – low abundance                     | ND                | ND       | ND      |
| snoRNA*                    | Rat                | 001718    | ND – low abundance                     | ND                | ND       | ND      |
| U6 snRNA*                  | Human, mouse and rat | 001973 | 56.2%                                  | 4.2               | 1.00     | 20.9    |
| RNU6B*                     | Human              | 001093    | ND – low abundance                     | ND                | ND       | ND      |
| Y1*                        | Rat                | 001727    | ND – low abundance                     | ND                | ND       | ND      |
| U87*                       | Rat                | 001712    | 62.0%                                  | 2.4               | 0.98     | 23.8    |
| 5S rRNA                    | Rat                | N/A       | 92.5%                                  | 4.0               | 0.98     | 23.0    |
| hsa-miR-16+                | Human, mouse and rat | 000391 | 77.5%                                  | 4.2               | 0.98     | 24.2    |
| hsa-miR-379+               | Human, mouse and rat | 001138 | ND – low abundance                     | ND                | ND       | ND      |
| hsa-miR-191+               | Human, mouse and rat | 002299 | ND – low abundance                     | ND                | ND       | ND      |
| hsa-let-7d+                | Human, mouse and rat | 002283 | ND – low abundance                     | ND                | ND       | ND      |

Table 1. Small ncRNA Suitability as Reference Genes for Normalization of miRNA Expression in Rat Retinal Samples.
rat liver, 4.5S RNA (H) “Variant 1” was expressed, however 4.5S RNA (H) “Variant 5” was not highly expressed and the PCR amplification efficiency could not be determined. Interestingly, the same expression pattern was observed in the rat brain and lung (data not shown), suggesting that the 4.5S RNA (H) variants are not expressed to the same extent in different rat tissues. Y1 expression levels were low in the rat retina and liver. No cross-reactivity of the mouse-specific snoRNA202 assay was observed in the rat retina or liver; however, expression was confirmed in the mouse lens and liver (data not shown).

U6 snRNA, miR-16, U87, 4.5S RNA (H) “Variant 1”, and 5S rRNA were chosen for further analysis, as the expression levels of these small ncRNAs were adequate for determining primer PCR amplification efficiencies. The small ncRNAs’ stability with strain, treatment, and developmental stage was tested using representative samples from individual rats from P5, P6, P9, and P14, relative to the standard pool.

**Effect of strain:** The expression of each small ncRNA was separated based on the rat strain. The small ncRNA reference gene commonly used for normalizing miRNA expression in OIR, 5S rRNA, was the least stable of the five tested (Table 3). Analysis of the average expression level of each small ncRNA, relative to the standard pool, confirmed that little variation was present in U6 snRNA, miR-16, U87, and 4.5S RNA (H) “Variant 1” expression. However, the changes in expression among strains were not statistically significant for any gene.

**Effect of exposure to cyclic hyperoxia:** Expression of each small ncRNA separated based on room air or cyclic hyperoxia exposure showed 5S rRNA was the least stable reference transcript; however, this was not statistically significant (Table 4). Expression of U87, miR-16, and 4.5S RNA (H) “Variant 1” was somewhat variable with exposure to cyclic hyperoxia, although not to the same extent as for 5S rRNA. U6 snRNA showed the smallest change in average expression with fluctuations in oxygen levels. Overall, no changes in response to cyclic hyperoxia exposure were statistically significant.

**Effect of developmental stage:** Levels of U6 snRNA and miR-16 showed slight increases with increasing developmental age; however, these differences were not statistically significant (Figure 1). U87 levels were stable between P5, P6, and P9; however, by P14, expression had increased to a statistically significant level after correction for multiple comparisons. Levels of 4.5S RNA (H) “Variant 1” varied with developmental stages. A statistically significant decrease in expression levels occurred between P6 and P9, and a statistically significant increase in expression levels was observed

### Table 2. PCR primer amplification efficiencies of small ncRNA endogenous controls in rat liver.

| Small ncRNA                  | Amplification efficiency in rat liver | R² value | Ct at 1/2 dilution |
|------------------------------|--------------------------------------|----------|-------------------|
| 4.5S RNA(H) “Variant 1”      | 44.6%                                | 0.99     | 25.4              |
| 4.5S RNA(H) “Variant 5”      | ND – low abundance                   | ND       | 33.1              |
| snoRNA202                    | ND – low abundance                   | ND       | 33.0              |
| snoRNA                       | 50.4%                                | 1.00     | 26.9              |
| Y1                           | ND – low abundance                   | ND       | 34.5              |

Five small ncRNA endogenous controls were tested to determine the PCR primer amplification efficiencies using total RNA derived from rat liver samples. Six serial twofold dilutions of the total RNA were used to generate the standard curves for 4.5S RNA (H) “Variant 1” and snoRNA. Cts were unable to be generated for all six twofold serial dilutions for the remaining small ncRNAs; therefore amplification efficiencies for these assays could not be determined. The R² value for each standard curve is shown, as is the mean Ct at a 1/2 dilution. ND=not able to be determined.

### Table 3. Expression of five small ncRNAs and miRNAs relative to a standard pool tested for stability between rat strains.

| Assay               | Expression relative to the standard pool in F344 rats | Expression relative to the standard pool in SD rats | Difference in mean expression between F344 and SD rats | p value |
|---------------------|------------------------------------------------------|----------------------------------------------------|------------------------------------------------------|---------|
| U6 snRNA            | 0.48 (±0.13)                                         | 0.45 (±0.13)                                       | 0.03                                                 | 0.70    |
| miR-16              | 0.66 (±0.33)                                         | 0.56 (±0.23)                                       | 0.10                                                 | 0.50    |
| U87                 | 0.93 (±0.19)                                         | 0.90 (±0.21)                                       | 0.03                                                 | 0.56    |
| 4.5S RNA(H) “Variant 1” | 0.96 (±0.08)                                      | 0.90 (±0.21)                                       | 0.06                                                 | 0.47    |
| 5S rRNA             | 0.63 (±1.13)                                         | 0.47 (±0.39)                                       | 0.16                                                 | 0.53    |

Average expression relative to the standard pool and the standard deviation for each strain is shown. F344 n=8, SD n=8.
between P9 and P14. Levels of 5S rRNA, relative to the standard pool, increased with developmental age up to P9 and then decreased at P14.

**DISCUSSION**

The current convention for quantifying miRNA expression is to use a single small ncRNA reference gene for normalizing RT-qPCR data [37]. The choice of small ncRNA reference gene is important to avoid introducing bias or misleading conclusions. Gee et al. found that the expression of small ncRNA reference genes typically used for normalizing miRNA expression levels in cancer were themselves highly variable [10]. When these reference genes were used to normalize expression levels of miRNAs of interest from tumor samples, the potential prognostic value of these miRNAs was lost, as their normalized expression levels did not accurately reflect the associations between miRNA expression and tumor pathology or disease outcome [10]. These reference transcripts were later found to be derived from the introns of genes that themselves are dysregulated in cancer [10].

An ideal reference gene for use with rodent models of OIR is stable with strain, exposure to cyclic hyperoxia, and developmental stage. Previous studies of miRNA expression in mouse oxygen-induced retinopathy have used 5S rRNA as the reference RNA for normalizing miRNA expression [24-26], although the suitability of this reference gene for the purpose was hitherto unknown. Here, we showed that 5S rRNA levels varied with rat strain, changes in oxygen levels, and developmental stage, but this variability was not statistically significant. Some of the variation observed in 5S rRNA expression may be due to the different chemistries used to

### Table 4. Expression of five small ncRNAs and miRNAs relative to a standard pool tested for stability with exposure to cyclic hyperoxia.

| Assay           | Expression relative to the standard pool in room air-exposed rats | Expression relative to the standard pool in cyclic hyperoxia-exposed rats | Difference in expression between room air- and cyclic hyperoxia-exposed rats | p value |
|-----------------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|--------|
| U6 snRNA        | 0.49 (±0.16)                                                 | 0.47 (±0.11)                                                 | 0.03                                                          | 0.70   |
| miR-16          | 0.67 (±0.26)                                                 | 0.55 (±0.31)                                                 | 0.12                                                          | 0.41   |
| U87             | 0.85 (±0.15)                                                 | 0.98 (±0.22)                                                 | 0.13                                                          | 0.08   |
| 4.5S RNA(H)     | 0.96 (±0.08)                                                 | 0.90 (±0.21)                                                 | 0.06                                                          | 0.47   |
| “Variant 1”     | 0.96 (±0.08)                                                 | 0.90 (±0.21)                                                 | 0.06                                                          | 0.47   |
| 5S rRNA         | 0.28 (±0.25)                                                 | 0.82 (±1.10)                                                 | 0.54                                                          | 0.20   |

Mean expression relative to the standard pool and the standard deviation are shown for each treatment. Room air-exposed rats n=8, cyclic hyperoxia-exposed rats n=8.

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**Figure 1.** Average expression of five small ncRNAs and miRNAs compared at P5, P6, P9, and P14. Mean expression relative to the standard pool for each developmental stage. n=4 at each time point. Error bars represent ± standard deviation. Statistical analysis was performed using one-way ANOVA, with the significance level (alpha) adjusted for multiple comparisons (Tukey’s HSD test) at p<0.0125. For 5S rRNA, a Kruskal–Wallis test was used and the significance (alpha) level set at 0.05. * p<0.0125.
assay 5S rRNA expression levels (SYBR Green) compared with miRNA expression levels (TaqMan). However, expression of the 5S rRNA transcript was compared across samples within an assay method, rather than between methods, so that the variability is likely due to strain, oxygen exposure, or developmental stage. Previous studies of miRNA expression in mouse oxygen-induced retinopathy also used SYBR Green chemistry and TaqMan assays for analyzing 5S rRNA and miRNA expression levels, respectively [24,25]. Given that 5S rRNA expression levels varied between samples, we conclude that in at least this rat model of OIR, 5S rRNA is not a suitable reference RNA for normalizing miRNA expression levels.

U6 snRNA levels were stable with fluctuations in oxygen, strain, and developmental stage compared to the remaining four reference genes. However, the PCR amplification efficiency was only 56% for this particular assay. In comparison, miR-16 showed less stable average expression with changes in oxygen levels and between rat strains compared with U6 snRNA. Some variation in expression was observed with developmental stage, but less than that seen with 5S rRNA. The PCR amplification efficiency for the miR-16 TaqMan assay was better than for U6 snRNA, at 78%. The remaining two reference RNAs, 4.5S RNA (H) “Variant 1” and U87, showed poor PCR amplification efficiencies in neonatal rat retinas with efficiencies of 49% and 62%, respectively, and were variable with strain, exposure to cyclic hyperoxia, and developmental stage. Statistically significant changes in small ncRNA expression were observed in response to developmental stage for 4.5S RNA (H) “Variant 1” and U87.

5S rRNA has typically been used for normalizing miRNA expression levels in mouse models of oxygen-induced retinopathy, although the stability of this reference gene with changes in oxygen levels has not previously been examined. We have established that data derived from the use of 5S rRNA for normalization is statistically valid. However, the lower degree of variability observed in expression levels of U6 snRNA and miR-16 suggest they may be more appropriate for use in normalizing miRNA expression levels. Taken together, the data suggest that from the limited number of reactive small ncRNA control TaqMan assays available U6 snRNA and miR-16 are most suitable for normalizing miRNA expression in rat models of OIR, as these reference genes are relatively stable with strain, exposure to cyclic hyperoxia, and developmental stage.

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REFERENCES
1. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endocrinol 2002; 29:23-39. [PMID: 12200227].
2. Szabo A, Perou CM, Karaca M, Perreard L, Palais R, Quackenbush JF, Bernard PS. Statistical modeling for selecting housekeeper genes. Genome Biol 2004; 5:S9.-[PMID: 15287981].
3. Vandesonpele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002; 3:H0034.1-0034.11-[PMID: 12184808].
4. Galivi CR, Rozhdestvensky TS, Brosius J, Lehrach H, Kontthur Z. Application of housekeeping npcRNAs for quantitative expression analysis of human transcriptome by real-time PCR. RNA 2010; 16:450-61. [PMID: 20040593].
5. van Wijngaarden P, Breteron HM, Coster DJ, Williams KA. Stability of housekeeping gene expression in the rat retina during exposure to cyclic hyperoxia. Mol Vis 2007; 13:1508-15. [PMID: 17893650].
6. Suzuki T, Higgins PJ, Crawford DR. Control selection for RNA quantitation. Biotechniques 2000; 29:332-7. [PMID: 10948434].
7. Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 2004; 313:856-62. [PMID: 14706621].
8. Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 1999; 259:523-6. [PMID: 10364451].
9. Kouadjio KE, Nishida Y, Cadrin-Girard JF, Yoshioka M, St-Amand J. Housekeeping and tissue-specific genes in mouse tissues. BMC Genomics 2007; 8:127-[PMID: 17519037].
10. Gee HE, Buffa FM, Camps C, Ramachandran A, Leek R, Taylor M, Patil M, Sheldon H, Betts G, Homer J, West C, Ragoussis J, Harris AL. The small-nucleolar RNAs commonly used for microRNA normalisation correlate with tumour pathology and prognosis. Br J Cancer 2011; 104:1168-77. [PMID: 21407217].
11. Latham GJ. Normalization of microRNA quantitative RT-PCR data in reduced scale experimental designs. Methods Mol Biol 2010; 667:19-31. [PMID: 20827524].
12. Fleck BW, McIntosh N. Pathogenesis of retinopathy of prematurity and possible preventive strategies. Early Hum Dev 2008; 84:83-8. [PMID: 18234458].
13. Ricci B. Oxygen-induced retinopathy in the rat model. Doc Ophthalmol 1990; 74:171-7. [PMID: 2209374].
14. van Wijngaarden P, Coster DJ, Breteron HM, Gibbins IL, Williams KA. Strain-dependent differences in oxygen-induced retinopathy in the inbred rat. Invest Ophthalmol Vis Sci 2005; 46:1445-52. [PMID: 15790914].
15. Penn JS, Tolman BL, Henry MM. Oxygen-induced retinopathy in the rat: relationship of retinal nonperfusion to subsequent neovascularization. Invest Ophthalmol Vis Sci 1994; 35:3429-35. [PMID: 8056518].

16. Reynaud X, Dorey CK. Extranetal neovascularization induced by hypoxic episodes in the neonatal rat. Invest Ophthalmol Vis Sci 1994; 35:3169-77. [PMID: 8045712].

17. Penn JS, Tolman BL, Lowery LA. Variable oxygen exposure causes preretal neovascularization in the newborn rat. Invest Ophthalmol Vis Sci 1993; 34:576-85. [PMID: 8449677].

18. Gao G, Li Y, Zhang D, Gee S, Crosson C, Ma JX. Unbalanced expression of VEGF and PEDF in ischemia-induced retinal neovascularization. FEBS Lett 2001; 489:270-6. [PMID: 1165263].

19. van Wijngaarden P, Brereton HM, Gibbins IL, Coster DJ, Williams KA. Kinetics of strain-dependent differential gene expression in oxygen sensitivity in the retina. EXP Eye Res 2007; 85:508-17. [PMID: 17692314].

20. Gao G, Li Y, Fant J, Crosson CE, Becerra SP, Ma JX. Difference in ischemic regulation of vascular endothelial growth factor and pigment epithelium–derived factor in brown norway and sprague dawley rats contributing to different susceptibilities to retinal neovascularization. Diabetes 2002; 51:1218-25. [PMID: 11916948].

21. Chan CK, Pham LN, Zhou J, Spee C, Ryan SJ, Hinton DR. Differential expression of pro- and antiangiogenic factors in mouse strain-dependent hypoxia-induced retinal neovascularization. Lab Invest 2005; 85:721-33. [PMID: 15856049].

22. Karali M, Peluso I, Marigo V, Banfi S. Identification and characterization of microRNAs expressed in the mouse eye. Invest Ophthalmol Vis Sci 2007; 48:509-15. [PMID: 17251443].

23. Ryan DG, Oliveira-Fernandes M, Lavker RM. MicroRNAs of the mammalian eye display distinct and overlapping tissue specificity. Mol Vis 2006; 12:1175-84. [PMID: 17102797].

24. Shen J, Yang X, Xie B, Chen Y, Swaim M, Hackett SF, Campo-chiaro PA. MicroRNAs Regulate Ocular Neovascularization. Mol Ther 2008; 16:1208-16. [PMID: 18500251].

25. Bai Y, Bai X, Wang Z, Zhang X, Ruan C, Miao J. MicroRNA-126 inhibits ischemia-induced retinal neovascularization via regulating angiogenic growth factors. Exp Mol Pathol 2011; 91:471-7. [PMID: 21586283].

26. Peltier HJ, Latham GJ. Normalization of microRNA expression levels in quantitative RT-PCR assays: identification of suitable reference RNA targets in normal and cancerous human solid tissues. RNA 2008; 14:844-52. [PMID: 18375788].

27. Filipowicz W, Pogacic V. Biogenesis of small nucleolar ribonucleoproteins. Curr Opin Cell Biol 2002; 14:319-27. [PMID: 12067654].

28. Mestdagh P, Van Vlierbergh B, De Weer M, Weis D, Westermann F, Speleman F, Vandesompele J. A novel and universal method for microRNA RT-qPCR data normalization. Genome Biol 2009; 10:R64. [PMID: 19551210].

29. Ventresca MR, Gonder JR, Tanswell AK. Oxygen-induced proliferative retinopathy in the newborn rat. Can J Ophthalmol 1990; 25:186-9. [PMID: 23543949].

30. Penn JS, Henry MM, Wall PT, Tolman BL. The range of PaO2 variation determines the severity of oxygen-induced retinopathy in newborn rats. Invest Ophthalmol Vis Sci 1995; 36:2063-70. [PMID: 7675745].

31. Tea M, Fogarty R, Brereton HM, Michael MZ, Van der Hoek MB, Tsykin A, Coster DJ, Williams KA. Gene expression microarray analysis of early oxygen-induced retinopathy in the rat. J Ocul Biol Dis Infor. 2009; 2:190-201. [PMID: 20157446].

32. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem 2009; 55:611-22. [PMID: 19246619].

33. Uchida S, Haru K, Kobayashi A, Funato H, Hohara T, Otsuki K, Yamagata H, McEwen BS, Watanabe Y. Early life stress enhances behavioral vulnerability to stress through the activation of REST4-mediated gene transcription in the medial prefrontal cortex of rodents. J Neurosci 2010; 30:15007-18. [PMID: 21068306].

34. Neal CS, Michael MZ, Rawlings LH, Van der Hoek MB, Gleadle JM. The VHL-dependent regulation of microRNAs in renal cancer. BMC Med 2010; 8:64- [PMID: 20964835].

35. Meijerink J, Mandigers C, van de Locht L, Tonnissen E, Goodsaid F, Raemaekers J. A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. J Mol Diagn 2001; 3:55-61. [PMID: 11333000].

36. Simon P. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 2003; 19:1439-40. [PMID: 12874059].

37. Roa W, Brum B, Giao L, Amanie J, Fairchild A, Gabos Z, Nijjar T, Scrimger R, Yee D, Xing J. Identification of a new microRNA expression profile as a potential cancer screening tool. Clin Invest Med 2010; 33:E124. [PMID: 20370992].