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Differential expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β actin and hypoxanthine phosphoribosyltransferase (HPRT) in postnatal rabbit sclera

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Abstract

Purpose. GAPDH, β-actin, HPRT and 18S rRNA are constitutively expressed in all mammalian cells. In accordance with the nature of invariant control, these genes have been used to standardize genes of interest in expression studies. Recent studies have suggested that GAPDH, β-actin and HPRT in special situations may come under temporary regulatory control, but that 18S rRNA may be more likely to remain constitutive. However, little is known about the quantitative expression of these genes in fibroblasts and in particular during early postnatal development, a time of rapid changes in cell metabolism. In this study we have examined the differential expression of these genes in association with scleral development from an early postnatal age up to young adult status.

Methods. GAPDH, β-actin, HPRT, and 18S rRNA gene expression were analyzed in the rabbit sclera from 1 day to 8 weeks postnatally by real-time, comparative PCR.

Results. Real-time PCR analysis showed that the expression levels of GAPDH, β-actin, and HPRT were higher in the first postnatal week and then declined. However, from 2 to 8 weeks, the mRNA levels of these three genes underwent significant variations ($P < 0.01$) in their levels of expression. In contrast, the expression level of 18S rRNA showed no significant variation ($P \geq 0.5$) over this time period.

Conclusions. The present study shows that GAPDH, β actin and HPRT gene were differentially expressed in early postnatal scleral development. It also suggests that these gene products could be implicated in the developmental process and have a crucial role in the early postnatal period. This study demonstrates that 18S rRNA may be preferable to normalize genes of interest in studies of early development.

Keywords: GAPDH; β actin; HPRT; 18S rRNA; differential expression; sclera; postnatal development

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β actin and hypoxanthine phosphoribosyltransferase (HPRT) are constitutively expressed housekeeping genes present in most mammalian tissues. Several studies have reported the genetic organization, expression and regulation of these genes. Moreover, recent studies demonstrated that GAPDH, β actin and HPRT display a number of diverse activities. In consideration of these genes as being expressed in an invariant fashion, they are commonly used to standardize the expression of genes of interest. However, there may be circumstances that temporarily affect constitutive control.

GAPDH, is a glycolytic protein, crucial for energy production. Recent studies have shown that GAPDH exhibits a number of activities including phosphotransferase activity,[1,2] nuclear RNA export,[3] DNA replication and DNA repair.[4] Moreover, GAPDH was detected as an essential part of gene expression observed in apoptosis,[5] as part of the cellular phenotype of age-related neuronal disorders like Alzheimer's disease[6] and CAG triplet repeat disorders.[7] The multifunctional nature of GAPDH suggests versatility in the mechanisms regulating its expression and a few reports have found

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changes in the number of GAPDH transcripts in specific situations.[8,9]

Actin is the most abundant protein in eukaryotic cells and the role of actin in cell function is ubiquitous. A large number of cellular process, including cytokinesis, endocytosis, chemotaxis, or neurite outgrowth is mediated by polymerization of actin filaments. A number of signal transduction pathways have been identified recently that regulate actin polymerization and contractility. GTP-binding proteins, protein kinases, phosphoinositide kinases, and protein phosphatases were reported to play a role in determining the location and extent of actin polymerization.[10,11] Although there were a number of reports on the functional analysis of the actin gene, little is known about the changes in number of transcripts, in particular, during the developmental period.

HPRT is a housekeeping gene characterized by high constitutive expression in the mammalian brain. The enzyme it encodes is key to purine salvage in humans and involved in recovery of preformed bases for use in cellular metabolism. Partial deficiency of this enzyme can result in the overproduction of uric acid leading to a severe form of gout, whilst a virtual absence of HPRT activity causes the Lesch-Nyhan syndrome.[12] Although, functional roles, structural significance and differential regulatory mechanisms of GAPDH, β actin and HPRT in different cell types have been elucidated, there was no report on the differential expression of these genes in scleral fibroblasts and in particular during postnatal development.

Another gene that has been used frequently for comparison in gene expression studies, 18S rRNA, is also constitutively expressed in most tissues.[13,14] Schmittgen and Zkrajsek, recently reported that commonly used house keeping genes, β actin, β-2 microglobulin and GAPDH, were differentially expressed in NIH 3T3 fibroblasts transfected with an inducible chimeric gene and 15% serum treatment. Under these conditions, 18S rRNA showed constant expression suggesting the importance of validation of internal control genes.[15]

Our preliminary studies of scleral development examining the expression of structural proteins suggested that GAPDH expression was elevated in early development compared to later times.[16,17] This observation prompted us to examine a number of genes that are commonly used as analysis standards.

Material and methods

Total RNA extraction and cDNA synthesis

New Zealand White rabbits of different ages were obtained from the Laboratory Animal Center of the National University of Singapore. The animals were used in accordance with the ARVO Statement for the Use of Animals in Research. Two randomly chosen rabbits from each of two litters of 1 day, 1, 2, 4, 6, and 8-weeks old were sacrificed. The posterior sclera was dissected from each eye and isolated from the adjacent and adherent tissues. The sclera of each group of each litter was immediately frozen in liquid nitrogen. Two sclera were pooled together and used for RNA isolation.

Total RNA was isolated using TRIzol Reagent (Life Technologies) in accordance with the manufacture's instructions. Total RNA was quantified by spectrophotometer (Spectronic, Genesys 5) and the integrity of the samples was analyzed by 260/280 nm absorbance ratios and by 1.2% agarose gel electrophoresis. Total RNA was treated with DNase and reverse transcription was carried out with 1 μg of total RNA from each sample by SUPERSCRIPT II (Life Technologies) using 0.5 μg of random primer in accordance with the manufacture's instructions.

Real-time comparative PCR

Real-time comparative PCR (SYBR Green) was performed in a 96-well microtiter plate format on a ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) equipped with a Sequence Detection System (SDS) software version 1.6.3. PCR was performed using SYBR Green PCR master mix following the manufacture's protocol with slight modification. The primers for the GAPDH and β actin were obtained from previously published primer sequences.[18,19] The primer for HPRT was constructed with the Primer Express software version 1.0 (PE Applied Biosystems) using a rabbit specific sequence (Table 1). Quantum RNA Classic II 18S Internal Standard (Ambion) was used as endogenous control. The 18S primer-competimer ratio of 1 : 4 was used in all experiments. To standardize and evaluate scleral gene expression, aliquots of the same cDNA preparation were used as templates in all PCR reactions. The Hot Start technique was employed using AmpliTaq Gold DNA polymerase (PE Applied Biosystems). The conditions for the PCR were

Table 1. Primer sequences and sizes of PCR products.

Gene	Primers	Sizes
GAPDH	5′-TCACCATCTTCCAGGAGCGA-3′ (Fw)	
	5′-CACAATGCCGAAGTGGTCGT-3′ (Rev)	293 bp
β Actin	5′-ACGTTCAACACGCCGGCCAT-3′ (Fw)	
	5′-GGATGTCCACGTCGCACTTC-3′ (Rev)	493 bp
HPRT	5′-ACTGAACGGCTTGCTCGAGAT-3′ (Fw)	
	5′-AGCAGGTCAGCAAAGAACTTATAGC-3′ (Rev)	101 bp

as follows: 55°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 2 min. The comparative quantification values were obtained from the threshold cycle (C_T) number at which the increase in signal associated with an exponential growth of PCR products began to be detected using SDS software. The PCRs were performed (n = 5) for each sample-primer set, and the mean of the experiments was used as the relative quantification value. At the end of the PCR cycles, amplification specificity was confirmed by dissociation curve analysis and the reaction products were separated on 3% agarose gel and stained with ethidium bromide for visual confirmation of PCR products.

Data analysis by comparative C_T ($\Delta\Delta C_T$) method

The C_T value represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value (optimal ΔRn) was first detected. The calculation for comparative C_T ($\Delta\Delta C_T$) method was previously described.[20] Briefly, the ΔC_T value was determined by subtracting the corresponding average 18S C_T value from the average GAPDH, β actin, and HPRT C_T value. The standard deviation of the difference was calculated from the standard deviations of the gene of interest and the corresponding 18S values. The $\Delta\Delta C_T$ value was obtained by subtraction of the ΔC_T calibrator value. This is the subtraction of an arbitrary constant, so the standard deviation of the $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. The value given for the gene of interest relative to their corresponding 1-day value was determined by evaluating the expression by $2^{-\Delta\Delta C}{}_T$.

Results

Comparison of gene expression assay C_T value

Utilization of gene expression detection assay was initially determined by comparing the mean C_T value of all genes. The C_T value of 18S rRNA gene expression assay was relatively constant throughout the observation period while the C_T value of the other three genes was variable over the same time (Fig. 1).

Validation of amplification efficiency

To determine the relationship of PCR efficiency of GAPDH compared to 18S internal standard PCR, PCR reactions were performed using several cDNA dilutions from the 1 day RNA sample. Efficiency of target and reference were approximately equal at cDNA dilutions from 1 µl to 0.0625 µl (Fig. 2). The diagram shows a linear relationship between threshold cycles (C_T) of target (GAPDH) and internal standard (18S rRNA) control plotted against the relative amount of loaded cDNA in serial dilutions from 1 : 1 through 1 : 32. The correlation coefficient (r) of 18S C_T value was 0.954 (P = 0.05) and GAPDH was 0.968 (P = 0.05).

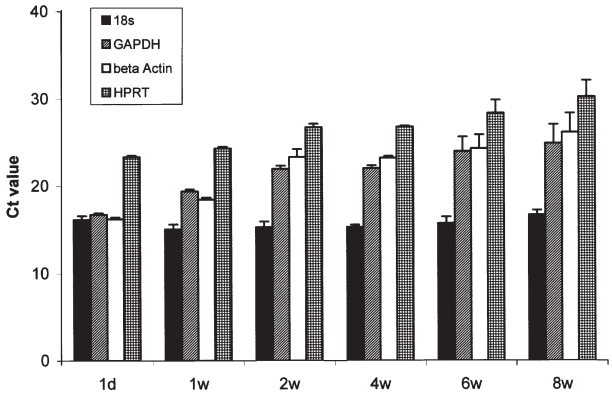

Figure 1. Threshold amplification levels of the genes. Significant PCR-signals were analyzed as C_T values. One µg of total RNA was analyzed and the means of 18S, GAPDH, β actin and HPRT (n = 5) means and standard deviations are shown. The C_T appears higher for lower levels of RNA abundance. Thus, at 1 day HPRT message is present in the lowest amount.

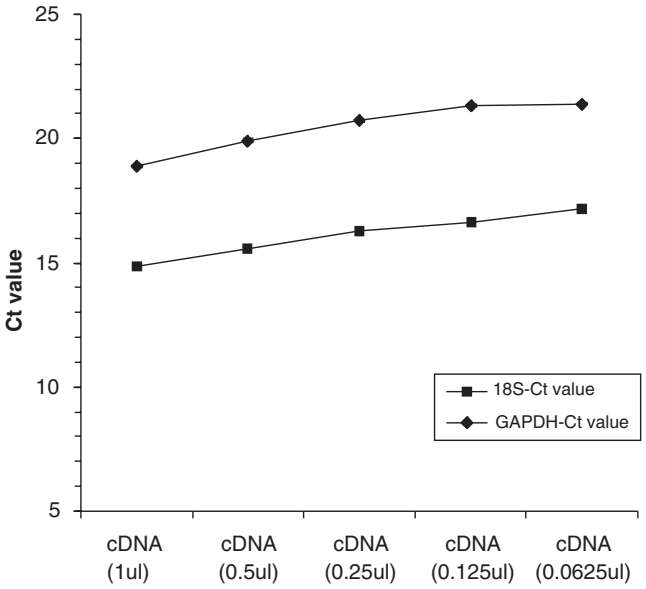

Figure 2. Validation of amplification efficiency for 18S rRNA and GAPDH. The diagram shows the linear relationship between threshold cycles (C_T value; mean and standard deviation) of target and endogenous control plotted against the relative amount of cDNA in serial dilutions from 1 µl to 0.625 µl.

Specific amplification

Dissociation curve analysis, performed with ABI Prism 7700 Dissociation Curve Software demonstrated that each of the primer pairs amplified a single product with a distinct dissociation temperature (Tm). Once the predicted size of each product had been confirmed by agarose gel electrophoresis, the Tm was used to identify specific products in subsequent analyses. Figure 3 shows the Tm of 18S rRNA PCR products

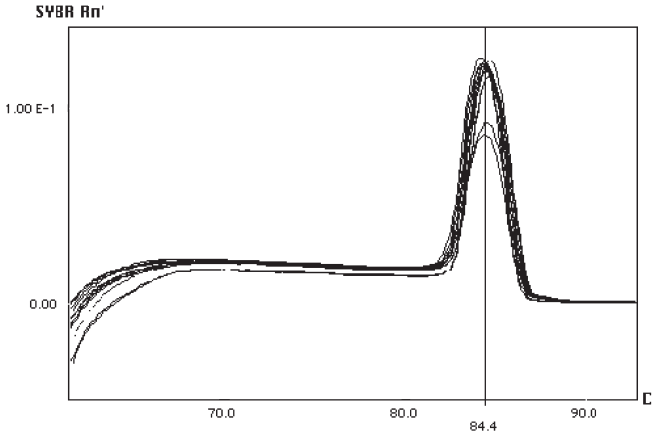

Figure 3. Dissociation curve analysis of 18S rRNA. The dissociation curve analysis was performed at the end of PCR reaction and Tm of the specific PCR product is seen as a single peak in a first derivative plot. The diagram shows the dissociation curve of 18S rRNA from the triplicates of each cDNA sample (1 day, 1, 2, 4, 6 and 8 weeks) and Tm of 18S rRNA is 84.4°C.

at 84.4°C. Tm of GAPDH, β actin and HPRT were 85.6, 89.7 and 81.0°C respectively (data not shown).

Comparative analysis by real time PCR

Table 2 shows the average C_T value used to determine the number of copies of GAPDH, β actin and HPRT, ΔC_T and $\Delta\Delta C_T$, and the relative number of copies for each gene of interest compared to the 1 day mRNA of that gene. The range was obtained from $\Delta\Delta C_T$ and the standard deviations of $\Delta\Delta C_T$. GAPDH, β actin, and HPRT mRNA levels showed a similar pattern of expression over the observation period of 1 day to 8 weeks (Fig. 4A,B,C). Message levels were highest for all genes at day 1. GAPDH mRNA level decreased after 1 day, reaching at two weeks, 0.014-fold lower than that of 1 day and appeared under constitutive control until 6 weeks (0.005-fold of 1 day mRNA) (Fig. 4A). A similar finding was also observed for β-actin gene expression. The mRNA level decreased after 1 day of the postnatal period and reached the lowest level at 6 and 8 weeks (0.002 times of 1 day level)

Table 2A. Comparative quantification of GAPDH using comparative C_T method.

Time	GAPDH Average C_T	18S Average C_T	ΔC_T GAPDH-18S	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T\ 1\text{-day}}$	GAPDH (Relation to 1-day with range)
1 day	16.74+/–0.18	16.15+/–0.42	0.58+/–0.45	0+/–0.45	1 (1.374–0.727)
1 week	19.36+/–0.22	15.06+/–0.56	4.3+/–0.61	3.71+/–0.61	0.076 (0.116–0.049)
2 week	21.96+/–0.36	15.31+/–0.63	6.65+/–0.73	6.07+/–0.73	0.014 (0.024–0.008)
4 week	22.04+/–0.32	15.32+/–0.24	6.72+/–0.41	6.13+/–0.41	0.014 (0.018–0.01)
6 week	23.96+/–1.66	15.76+/–0.73	8.20+/–1.81	7.62+/–1.81	0.005 (0.017–0.001)
8 week	24.88+/–2.15	16.71+/–0.54	8.17+/–2.22	7.58+/–2.22	0.005 (0.024–0.001)

Table 2B. Comparative quantification of β actin using comparative C_T method.

Time	β actin Average C_T	18S Average C_T	ΔC_T β actin-18S	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T\ 1\text{-day}}$	β actin (Relation to 1-day with range)
1 day	16.23+/–0.19	16.15+/–0.42	0.07+/–0.46	0+/–0.46	1 (1.3–0.7)
1 week	18.44+/–0.23	15.06+/–0.56	3.38+/–0.61	3.30+/–0.61	0.1 (0.154–0.065)
2 week	23.32+/–0.90	15.31+/–0.63	8.00+/–1.10	7.93+/–1.10	0.004 (0.008–0.001)
4 week	23.23+/–0.19	15.32+/–0.24	7.90+/–0.31	7.83+/–0.31	0.004 (0.005–0.003)
6 week	24.30+/–1.57	15.76+/–0.73	8.54+/–1.74	8.47+/–1.74	0.002 (0.009–0.0008)
8 week	26.14+/–2.19	16.71+/–0.54	9.43+/–2.25	9.36+/–2.25	0.002 (0.007–0.0003)

Table 2C. Comparative quantification of HPRT using comparative C_T method.

Time	HPRT Average C_T	18S Average C_T	ΔC_T HPRT-18S	$\Delta\Delta C_T$ $\Delta C_T - \Delta C_{T\ 1\text{-day}}$	HPRT (Relation to 1-day with range)
1 day	23.31+/–0.18	16.15+/–0.42	7.15+/–0.46	0+/–0.46	1 (1.37–0.72)
1 week	24.3+/–0.18	15.06+/–0.56	9.23+/–0.59	2.08+/–0.59	0.236 (0.358–0.156)
2 week	26.71+/–0.42	15.31+/–0.63	11.4+/–0.76	4.24+/–0.76	0.052 (0.089–0.031)
4 week	26.77+/–0.12	15.32+/–0.24	11.45+/–0.27	4.29+/–0.27	0.05 (0.061–0.042)
6 week	28.35+/–1.52	15.76+/–0.73	12.58+/–1.69	5.43+/–1.69	0.023 (0.075–0.007)
8 week	30.24+/–1.86	16.71+/–0.54	13.53+/–1.94	6.37+/–1.94	0.012 (0.046–0.003)

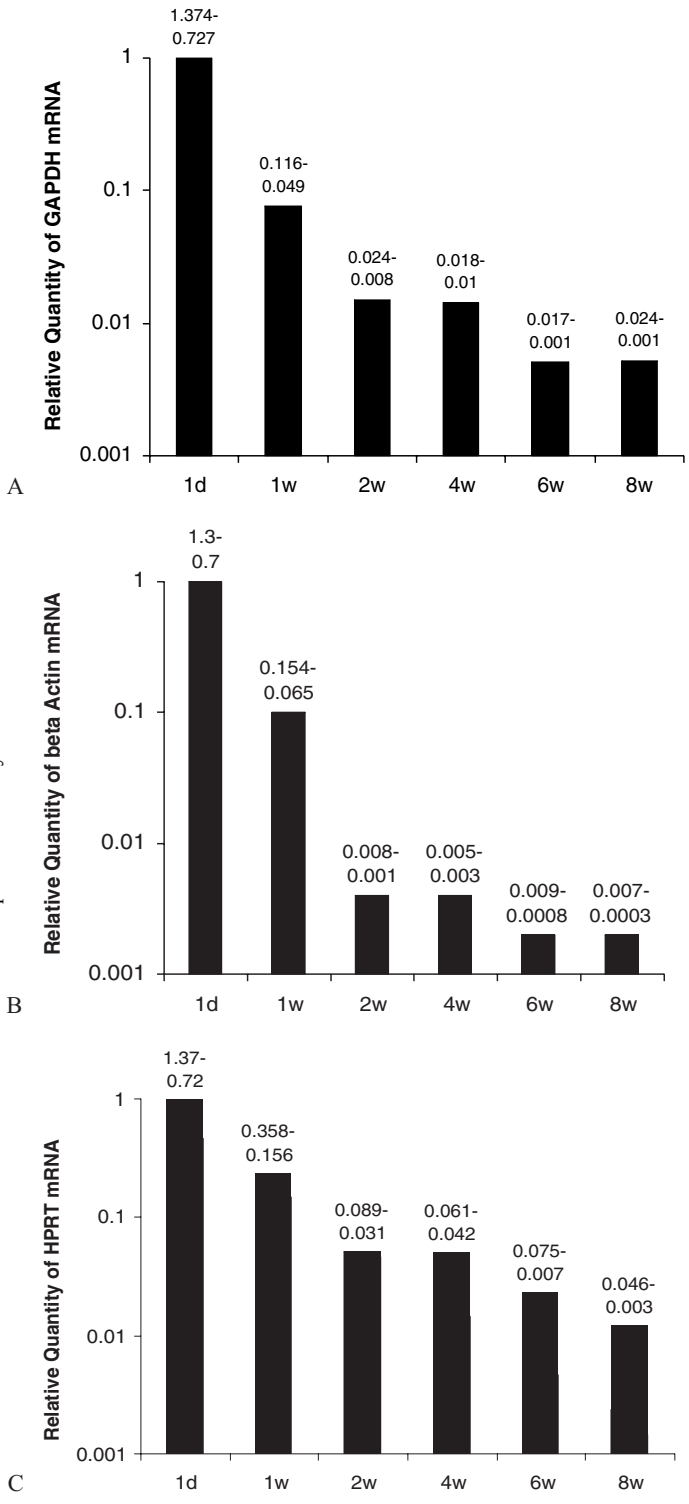

Figure 4. Bar graphs show the relative gene expression of GAPDH (A), β actin (B), and HPRT (C) to the corresponding 1 day mRNA level, with range, after normalization with 18S rRNA internal standard. The mRNA levels of these genes at 1 day are higher and decreased after 1 week of the postnatal period. The mRNA levels from 2 to 8 weeks were found relatively constant in all genes.

(Fig. 4B). The mRNA level of HPRT showed a similar expression trend as did GAPDH and β-actin. The highest level was found at 1 day and decreased after 1 week. Then HPRT showed relatively constant expression from 2–8 weeks (Fig. 4C).

Discussion

Several studies have reported that GAPDH, β actin and HPRT are constitutively expressed in mammalian tissues. Their complex genetic organization and functional diversity have been investigated in recent years. A few studies have demonstrated the mRNA and protein level of these genes in diverse organs.[21,22] However, there have been few studies on differential expression of these genes in a particular tissue during early development. The present study showed that GAPDH, β-actin and HPRT were differentially expressed in postnatal rabbit sclera during the observation period of 1 day to 8 weeks. The data also showed that 18S rRNA expression was quite constant over the same time period.

GAPDH has been reported as a classical glycolytic protein and used as a model protein for analysis of protein structure and enzyme mechanisms. Because of a consistent expression of GAPDH in different tissue, this gene is often used as a non-varying control in many gene expression studies. Our study does show that expression after the early postnatal period does attain more consistent expression. Recent studies of the regulation of messages representing genes of the TGF-β family in cells of cornea, limbus and conjuntiva, expression levels were compared to those of GAPDH.[23,24] However, an independent study noted that the expression level of GAPDH mRNA was cell cycle specific and regulated with a characteristic temporal sequence of expression in relation to DNA synthesis.[25] The present study showed that GAPDH expression is the highest in the first postnatal week, followed by a decline becoming more constant during the rest of the observation period. Our finding could suggest the need for high level of energy production as the growth rate of axial elongation of the eye was found to be greatest in the first week after birth.[16,17]

This finding was corroborated by the fact that the β-actin gene exhibited the highest expression at 1 day of the postnatal period in this study. Although actin polymerization is controlled by a number of proteins at different levels, our study suggests that the increase in the actin gene product early in the postnatal period may be used for the more rapid fibroblast cell division and secretion as well as in interactions with matrix. Increased actin could also provide for intracellular transport in fibroblasts as well as various signal transduction pathways. Although proteins that regulate actin polymerization during scleral development remains to be determined, our findings suggest an important role of β-actin in the postnatal scleral development.

Purine phosphoribosyltransferase (PRTs) are the enzymes that catalyze the recovery of preformed bases for use in cellular metabolism. The mammalian HPRT is a purine salvage

enzyme that catalyzes the conversion of hypoxanthine and guanine to their respective mononucleotides.[26] HPRT gene has been found at low level in all tissues and at higher levels in the brain although the significance and mechanism of this differential expression still remains unknown.[27] The other independent study reported that HPRT activity increased during the first 7 days of life and reached a mature level in the developing mouse brain and suggested that HPRT might play an important role in developing hippocampus.[28] Our study shows that the HPRT gene is expressed at a higher level in the first week of postnatal period in the sclera, suggesting that HPRT gene product is crucial during this active growth period.

In the present study, 18S rRNA was found expressed at consistent levels throughout the observation period and therefore was very useful for normalizing genes of interest. Although rRNA is expressed abundantly in the tissues, the use of primer-competimer technique at a constant concentration of 2 : 8 made the PCR amplification comparable to the gene of interest. 18S RNA could be amplified in the same PCR reaction in 96 well microtiter plate with the genes of interest. Our present study suggested that GAPDH, β actin, and HPRT are differentially regulated in postnatal scleral development and 18S rRNA could be preferable for the normalization of expression levels of other genes during this period of postnatal development.

Acknowledgements

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