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U6 snRNA is a Suitable Endogenous Control for microRNA-124 and -134 in Cultured Rat Hippocampal Neurons

(U6 snRNA adalah Kawalan Endogen yang Sesuai untuk microRNA-124 dan -134 dalam Neuron Hipokampus Tikus Berkultur)

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ABSTRACT

As researchers seek to determine the cellular mechanisms underlying biological processes, they have turned to analyze the functional role of microRNAs to understand this process in details. Here, we investigated the expression pattern of two microRNAs, miR-124 and -134 in maturing neurons and found that the choice of endogenous controls influenced the observed expression levels of these microRNAs. We have cultured rat hippocampal neurons and performed quantitative PCR on the microRNAs using Taqman gene expression assays. The expression of miRNAs was normalised with selected endogenous controls. Using BestKeeper and NormFinder software, we found that 18S rRNA and 5S rRNA to be unsuitable as endogenous controls in this system, while normalising to U6 snRNA produced more consistent results. Our study would like to highlight the importance of empirically testing proposed endogenous controls in any model system before data interpretation is carried out.

Keywords: Endogenous controls; hippocampal neuron culture; microRNAs; miR-124; miR-134

ABSTRAK

Dalam usaha untuk menentukan mekanisme sel dalam proses biologi, para penyelidik telah beralih untuk menganalisis peranan fungsi mikroRNA untuk memahami proses ini dengan lebih mendalam. Kami telah menentukan tahap ekspresi untuk dua jenis mikroRNA, miR-124 dan -134 dalam sel-sel neuron yang semakin matang dan mendapati bahawa pilihan untuk gen endogen boleh mempengaruhi tahap ekspresi yang diperhatikan untuk mikroRNA tersebut. Kami telah mengkultur sel neuron daripada hipokampus tikus dan menggunakan teknik PCR kuantitatif dengan asai ekspresi gen Taqman. Ekspresi miRNA gen telah dipenormal dengan gen endogen. Dengan menggunakan perisian BestKeeper dan NormFinder, kami mendapati bahawa rRNA 18S dan rRNA 5S merupakan gen endogen yang tidak sesuai dalam sistem ini, manakala keputusan lebih konsisten apabila snRNA U6 digunakan. Hasil penyelidikan kami menunjukkan kepentingan untuk menggunakan pendekatan secara empirik semasa menimbangkan pilihan gen endogen yang ingin digunakan.

Kata kunci: Kawalan endogen; kultur neuron hipokampus; mikroRNAs; miR-124; miR-134

INTRODUCTION

Since the discovery of microRNAs (miRNAs), there has been an explosion of research looking at how these key players regulate gene expression. For researchers interested in the central nervous system, there is a wealth of miRNAs that are specifically expressed in the brain and spinal cord. Some miRNAs show distinct expression patterns during development and have differential temporal and spatial expression in brain regions (Bak et al. 2008; Krichevsky et al. 2003). For example, miR-103 is highly expressed throughout embryonic brain development and into adulthood (Bak et al. 2008; Krichevsky et al. 2003), while others like miR-9, are highly expressed during embryonic development, but then decrease significantly in adulthood (Krichevsky et al. 2003). Some miRNAs, like miR-7 are enriched in the pituitary gland compared to miR-479 which is more enriched in the olfactory bulb (Bak et al. 2008).

To obtain the data for expression studies, many technical variables need to be taken into consideration. The method of isolation and all 'upstream' processing of the RNA samples must be standardised to ensure that the final comparison will be an accurate representation of the expression in the cell. From tissue collection to quantification, there can be as many as five steps involved, all of which carry the potential for error. In a recent study by Wang et al. (2008), they showed that even the method of RNA isolation can result in variable results in a microarray study.

Endogenous controls are genes that are assumed to be expressed at a constant level within the cell throughout a developmental stage or during an experimental intervention. We were interested in determining the expression pattern of two miRNAs, miR-134 and -124 during synapse formation in hippocampal neurons. Previous studies had looked at the expression of these

miRNAs using different endogenous controls (Table 1). We wondered whether the choice of endogenous control would affect the pattern of expression seen with the miRNAs

METHODS

PRIMARY HIPPOCAMPAL CELLS (HPC) CULTURE

All animal procedures were accepted by the University of Malaya Animal Ethic Committee and conformed to international standards. The protocol for culturing hippocampal cells was followed as described by Dotti et al. (1988). Briefly, hippocampi from Sprague-Dawley (SD) rats embryonic day 18.5 (E18.5) were isolated and trypsinised with 200 µL of 2.5% trypsin (Gibco) together with 50 µL of DNase I (Thermo Scientific). The neurons were plated on the poly-L-lysine-coated in 96-well culture plates at 1.6×10^4 cells per well. MEM-10 medium was used as the neuronal plating medium which containing 10% of heat-inactivated horse serum (Sigma Aldrich), 200 µL of penicillin/streptomycin (Gibco), 14.5 mg of L-glutamine (Gibco), 5.5 mg of sodium pyruvate (Sigma Aldrich) and topped up to 50 mL with Dulbecco's Modified Eagle Medium (D-MEM) (Gibco). The MEM-10 medium was replaced with Neurobasal medium when the neurons are attached on the culture plate. The hippocampal neurons were maintained in Neurobasal Medium (Gibco) supplemented with B-27 serum-free supplement (Gibco) and GlutaMAX-I (Gibco) together with 660 µL of glucose (Sigma Aldrich) and 500 µL of penicillin/streptomycin. Cell lysates were harvested at 2, 4, 6, 10 and 18 days *in vitro* (DIV) and stored in RNAlater solution (Ambion) to avoid RNA degradation. For brain-derived neurotrophic factor (BDNF) stimulation, hippocampal neurons at 4, 6 and 18 DIV were treated with 100 pg μL^{-1} BDNF (Origene) for 3 h at 37°C.

TOTAL RNA ISOLATION

Total RNA from the cell lysates was isolated using the mirVana miRNA isolation kit (Ambion). The quantity and purity of the total RNA were determined using Nanodrop (Thermo Scientific) and the RNA 6000 Pico kit (Agilent) was used in conjunction with the Agilent 2100 Bioanalyzer to determine the integrity of RNA. Any total RNA which has integrity number (RIN) lower than 5 was excluded from the study.

MIRNAS EXPRESSION IN CULTURED HIPPOCAMPAL NEURONS

cDNA was synthesised from 500 ng total RNA using Taqman MiRNA Reverse Transcription kit (Applied Biosystems) together with 5× Taqman MiRNA assay stem-loop RT primers (Applied Biosystems) for miR-124 (ID: 001182) and miR-134 (ID: 001186), accordingly. For endogenous control, U6 small nuclear RNA (snRNA; ID: 001973) and 500 ng μL^{-1} of universal oligo$(dT)_{18}$ primer (Thermo Scientific) for 18S and 5S ribosomal RNA (rRNA) were used for cDNA synthesis. The amount of reverse transcription reagents were added according to the manufacturer's instructions. Once the RNA samples were added into the reverse transcription reagents, the cDNA synthesis steps were performed in a thermocycler (Applied Biosystems) according to the manufacturer's instructions. cDNA products were then kept at -20°C.

Quantitative PCR (qPCR) was carried out in an Applied Biosystems 7500 Fast Real-Time PCR System with 5 µL of undiluted cDNA product, 2× Maxima Probe/ ROX qPCR Master Mix (Thermo Scientific) without AmpErase UNG and 20× Taqman MiRNA assay probes (miR-124 or miR-134) or 20× Taqman U6 snRNA assay probe or 20× Taqman primers and MGB probes for 5S rRNA (forward primer: 5'-ATCTCGGAAGCTAAGCA-3'; revers primer: 5'-GGTCTCCCATCCAAGTACT-3'; MGB probe: 5'-FAM-TCGGGCCTGGT-NFQ-MGB-3') or 18S rRNA (Applied Biosystems). The amount of qPCR master mix and assay probes were added according to the manufacturer's instructions. The 96-well PCR plate was sealed with transparent adhesive film. All the Taqman assays were validated according to the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) guidelines (Bustin et al. 2009).

The BestKeeper software (Pfaffl et al. 2004), version 1 (http://www.gene-quantification.de/bestkeeper.html) and NormFinder software (Andersen et al. 2004) (http://moma.dk/normfinder-software), were used for validation of the endogenous controls. The BestKeeper software is an Excel-based software which utilises the raw cycle threshold (Ct) values to evaluate the expression. For Normfinder, the ΔCt of the endogenous control was determined by using the following equation:

$$\Delta Ct = Ct \text{ of Time X} - Ct \text{ of Time 0}, \quad (1)$$

where Time X represents any time point (i.e. 4, 6, 10 or 18 DIV) and Time 0 represents the control time point used which was 2 DIV (Livak & Schmittgen 2001).

Studies have shown that BDNF treatment in neurons will induce gene transcription (Kang & Schuman 1996; Schratt et al. 2004). Hence, to ensure the expression of the endogenous control was stable even after experimental manipulation, the hippocampal neurons at the 4, 6 and 18 DIV time points were stimulated with 100 pg μL^{-1} BDNF for 3 h at 37°C. The hippocampal neurons were harvested immediately after and the cell lysates were processed as mentioned earlier. Statistical analysis was done by unpaired student's T-test with *P*-value set at 0.05.

The relative expression of miRNAs were obtained by using $2^{-\Delta\Delta Ct}$. The value of ΔΔCt was calculated by following the equation:

$$\Delta\Delta Ct = (Ct_{,target} - Ct_{,U6\ snRNA}) \text{ of Time X} - (Ct_{,target} - Ct_{,U6\ snRNA}) \text{ of Time 0}, \quad (2)$$

TABLE 1. Review of studies on miR-124 and- 134 in terms of the endogenous controls used

Types of miRNA	Endogenous control	Expression	Notes	Organism, brain region	Age of tissue/ neuronal culture	Author
miR-124	5.8S rRNA	Constant	Northern blot	E18 rat, cortex	1.5, 7, 14 DIV	(Kim et al. 2004)
miR-124	5S rRNA	13-fold increase from E12- 21, then constant	Northern blot	Rat, forebrain	E12, 13, 21, P5, adult	(Krichevsky et al. 2003)
miR-124	5S rRNA	Detection of expression	qRT-PCR	Mice	8 week old	(Mishima et al. 2010)
miR-124	5S rRNA	High expression in neuroblasts	qRT-PCR	Mice, Neuroblasts	N/A	(Cheng et al. 2009)
miR-124	U6 snRNA	Upregulated (in *EfnB1*-deficient NPCs)	qRT-PCR	E14.5 *EfnB1* mutant mice, cortex- neural progenitor cells	0, 3, 6 DIV	(Arvanitis et al. 2010)
miR-124	5S rRNA, U6 snRNA	Highest expression during E14, E17 and postnatally	Northern blot	Mouse, cortex & cerebellum	E 12-17, P0 P2, 5, 8, 14	(Smirnova et al. 2005)
miR-124	snoRNA-55 snoRNA-135	Expression of miR-124 in microglia was similar to cultured cortical cells	qRT-PCR	C57BL/6 mice, Microglia, cortical neurons	5 DIV	(Ponomarev et al. 2011)
miR-134	miR-124a, U6 snRNA	Upregulated	Northern blot	Rat, hippocampus	P1, 7, 13, 19 4, 7, 10, 14 and 18 DIV	(Schratt et al. 2006)
miR-134	β-3 tubulin	Depolarization induced expression in soma and dendritic compartments	qRT-PCR	E18 Rat, hippocampus & cortex	5 DIV cortical 7 DIV hippocampal	(Fiore et al. 2009)

where Time X represents any time point (i.e. 4, 6, 10 or 18 DIV) and Time 0 represents the control time point (Livak & Schmittgen 2001).

In our study, we chose 2 DIV as our control time point in order to measure the increase in expression as the neurons mature in culture. All experiments were done at least in triplicate. Standard curves with serial dilutions for the target gene and endogenous control (18S or 5S rRNAs or U6 snRNA) were used to ensure the percentage of amplification efficiency is within 90 to 110%. All slopes of the standard curves fell within ±10% of-3.3 and R^2 value was more than 0.98.

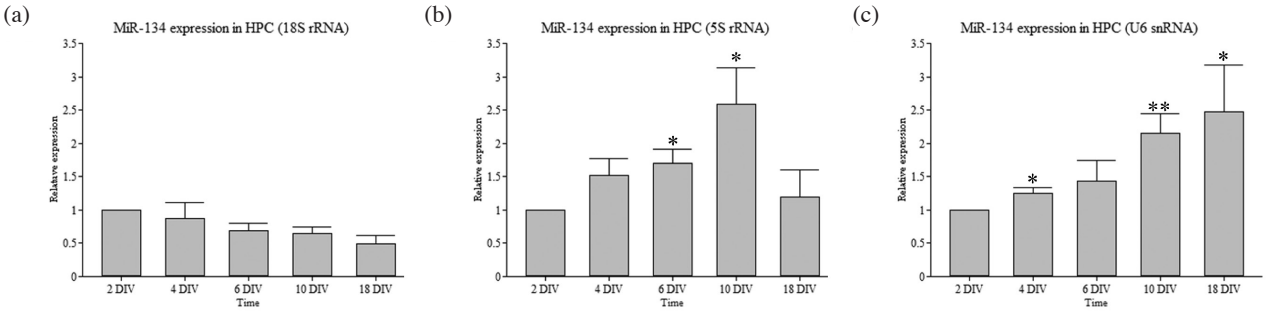
STATISTICAL TECHNIQUES

Statistical analysis for real time RT-PCR data was performed by applying the independent student's T-test and one way analysis of variants (ANOVA) using the BioStat 2008 Professional analysis software.

RESULTS AND DISCUSSION

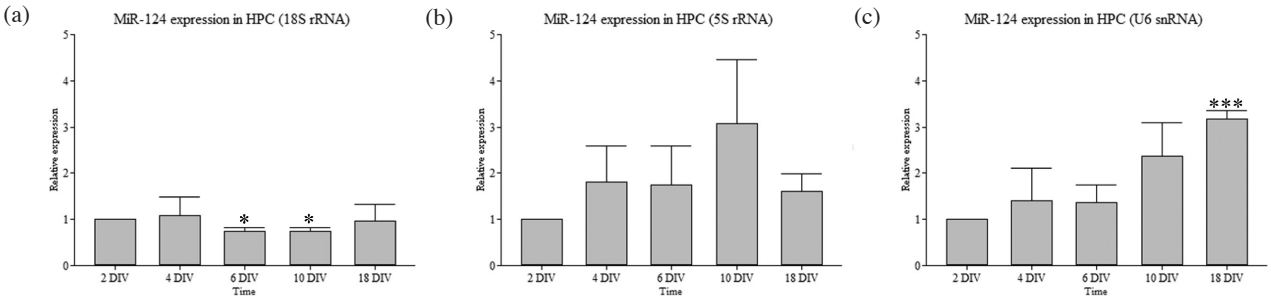
First, we performed a screen of three commonly used endogenous controls – 18S rRNA, U6 snRNA and 5S rRNA - to determine which was the most appropriate for use in a hippocampal neuron system as the neurons developed from immature neurons at 2 - 18 DIV. 5S and 18S rRNAs are one of the components of 60S (Allison et al. 1993) and 40S ribosomal subunits, respectively (Rabl et al. 2011), while U6 snRNA is part of the spliceosome (Madhani et al. 1990). These genes are present in all cell types, hence, they are the common endogenous controls used in qPCR studies (Godlewski et al. 2008; Krichevsky et al. 2003; Schratt et al. 2006). We observed a differential expression pattern of miR-134 (Figure 1) depending on which endogenous control was used. As shown in Figure 1(a), the expression of miR-134 showed constant expression from 2 to 18 DIV when normalised with 18S rRNA. However with 5S rRNA (Figure 1(b)), miR-134 expression significantly increased from 2 to 10 DIV (*P*-value: 0.011) and then decreased by 18 DIV, although this decrease was not significant (*P*-value >0.05). Meanwhile, when normalised with U6 snRNA, the expression of miR-134 increased significantly from 2 to 18 DIV (*P*-value: 0.036).

When 18S rRNA was the endogenous control for miR-124, we detected a significant downregulation of its expression at 6 DIV (*P*-value: 0.039) and at 10 DIV (*P*-value: 0.040) compared to 2 DIV (Figure 2(a)). However, this expression pattern was different when 5S rRNA was used as the endogenous control (Figure 2(b)). Here, miR-124 was seen to increase from 2 DIV to 10 DIV, although it was not



The relative expression was calculated using $2^{-\Delta\Delta Ct}$. The error bars of real-time RT-PCR data represent standard error of the mean of three independent experiments. (**P*-value <0.05, ***P*-value <0.01)

FIGURE 1. miR-134 expression in hippocampal neurons from 2 to 18 DIV. The expression was normalised with three different endogenous controls a) 18S rRNA; b) 5S rRNA and c) U6 snRNA



The relative expression was calculated using $2^{-\Delta\Delta Ct}$. The error bars of real-time RT-PCR data represent standard error of the mean of three independent experiments. (**P*-value <0.05, *** *P*-value <0.001)

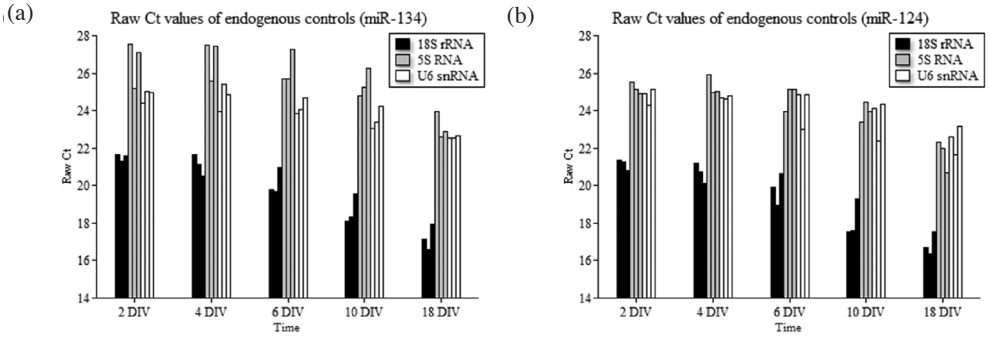
FIGURE 2. Expression of miR-124 in hippocampal neurons from 2 to 18 DIV. The expression was normalised with three different endogenous controls a) 18S rRNA; b) 5S rRNA and c) U6 snRNA

significant, and then showed a decrease at 18 DIV (*P*-value >0.05). In the case of U6 snRNA, miR-124 showed a trend of increasing expression from 2 to 18 DIV with a *P*-value of 0.0003 (Figure 2(c)).

The best endogenous control is thought to be the one that shows constant expression in the cells regardless of age (in culture) or experimental manipulation (e.g. addition of growth factors). However, even in the best of hands, there will be some variability in each reaction. Therefore, the general rule is that the cycle threshold (Ct) values of the endogenous gene of interest should not be more than 10% of the mean of the Ct. Based on this, we examined the raw Ct value of each endogenous control for miR-134 (Figure 3(a)) and miR-124 (Figure 3(b)). The percentage of outliers in 18S rRNA assay was as high as 20%, compared to 5S rRNA (13.33%) and U6 snRNA had no outliers (Table 2). Statistical analysis indicated that the 18S and U6 results were reproducible (*p*-value>0.05), while there was some indication that the 5S expression was unstable (*p*-value<0.05).

In order to confirm this finding, we validated all three endogenous controls using the BestKeeper software. Based on the evaluation by the software, any endogenous control with a standard deviation (SD) larger than 1.0 is considered to be unstable. We observed that 18S rRNA and 5S rRNA did not meet this criterion for either miR-124 or -134, while U6 snRNA did (Table 3). Additionally, the selection of the most stable endogenous control by BestKeeper software is also based on the lowest coefficient of variance (CV) and here, the U6 snRNA showed the lowest CV value (Table 3). As a result, U6 snRNA was selected by the BestKeeper software as the most stable endogenous control compared to the other two genes.

The NormFinder analysis tool was also used to rank the endogenous controls based on their stability values (Andersen et al. 2004). We transformed the raw Ct values into linear scale expression quantities using the ΔCt method and performed NormFinder analysis. The U6 snRNA was selected as the gene with the most constant



N=3, independent experiments for each endogenous control. The input RNA was standardised at 500 ng for each reaction

FIGURE 3. Raw Ct values of 18S rRNA, 5S rRNA and U6 snRNA endogenous controls during a) Taqman miR-134 and b) Taqman miR-124 assays

TABLE 2. Mean Ct values of three independent experiments across all DIVs were determined

	18S rRNA		5S rRNA		U6 snRNA	
	miR-134	miR-124	miR-134	miR-124	miR-134	miR-124
Mean	19.7310	19.3381	25.6511	24.1668	23.9736	23.9609
+10% of mean	21.7041	21.2719	28.2162	26.5835	26.3709	26.3570
-10% of mean	17.7579	17.4043	23.0860	21.7502	21.5762	21.5648
% of Outlier (n=15)	13.33%	20%	13.33%	6.67%	0%	0%
P-value	0.543		0.0137		0.9375	

TABLE 3. Comparison of the performance of endogenous controls as evaluated by the BestKeeper software

	18S rRNA		5S rRNA		U6 snRNA	
	miR-134	miR-124	miR-134	miR-124	miR-134	miR-124
N	15	15	15	15	15	15
SD (± Ct)	1.44	1.52	1.24	1.16	0.78	0.93
CV (% Ct)	7.29	7.84	4.83	4.80	3.24	3.88

'N': numbers of Ct value input (five time-points of three independent experiments), SD: standard deviation; CV: coefficient of variance.

expression with the lowest stability value followed by 5S rRNA and 18S rRNA (Table 4).

TABLE 4. Stability values of endogenous control as evaluated by the NormFinder software

Endogenous control	Stability value
U6 snRNA	0.333
5S rRNA	0.384
18S rRNA	0.586

The above analyses suggested that U6 snRNA was the most suitable endogenous control for cultured hippocampal neurons amongst the three genes tested. We then tested whether the expression of U6 snRNA could be affected by BDNF treatment, as studies have suggested that some endogenous controls are sensitive to different culture conditions (Livak & Schmittgen 2001). Upon treatment with BDNF for 3 h, we found that there was no significant change in U6 snRNA expression compared to the untreated controls at 4, 6 and 18 DIV (P-value >0.05, Table 5). Therefore, this suggests that U6 snRNA is expressed in a stable manner in hippocampal neurons.

We note that a previous study has suggested that 5S rRNA and U6 snRNA are not suitable as housekeeping genes (Lim et al. 2011). However, this study was performed in cell lines which were induced into neuronal differentiation by retinoic acid, whilst our cells are primary hippocampal neurons which were not treated with any inducible factors. We found that the U6 snRNA was stable under BDNF treatment, so perhaps the issue is that U6 snRNA is affected by the retinoic acid treatment performed by Lim et al. (2011) rather than their argument that it is inconsistently expressed. Furthermore, the miRNAs that they propose as endogenous controls are not suitable in a system looking at maturing neurons undergoing synaptogenesis as the expression of these miRNAs have been reported to change in developing neurons - for miR-103 (Paschou & Doxakis 2012) and in response to BDNF for miR-26b (Caputo et al. 2011). Meanwhile contrary to Lim et al. (2011), a study has reported that miR-106b expression changes during neuronal differentiation and it is involved in neurogenesis (Brett et al. 2011), therefore this miRNA is also not a good candidate as an endogenous control. In keeping with this, whilst we acknowledge the difference in size and abundance between miRNAs and U6 snRNA, the use of miRNAs as endogenous controls may not be without its problems as the proposed candidate miRNAs themselves may play a role in the process under investigation (in this case, synapse formation).

We propose that it is very difficult to ascertain a panel of genes that would act as consensus endogenous control genes in every cell type and condition, and this study is limited to rat hippocampal neurons. Different cell types and their response to the experimental set-up may affect the expression of these genes. Indeed when we analysed the expression of U6 snRNA using the same NormFinder analysis tool as Lim et al. (2011), we found no significant differences in our primary hippocampal neurons (with and without BDNF treatment) which contrasted with their findings that U6 snRNA was unstable in the cell lines. This difference is likely to be due to the different cell types used and the different experimental conditions. This may also explain the contrasting report by Genovesi et al. (2012) on U6 snRNA in stem cells. Most of these studies have looked at endogenous controls in neuronal differentiation in cell lines whilst our study is focused on maturing neurons involved in synapse formation.

These results have important implications in analysing the expression of the miRNAs tested. We found that when 5S RNA and U6 snRNA were used, the expression of miR-134 was seen to be up-regulated during neuronal development which closely matches the expression seen by Schratt et al. (2006).

In the case of miR-124, we found that miR-124 expression appears to be more sensitive to different endogenous controls. MiR-124 has been shown to be involved in a range of neuronal roles including neurogenesis (Cheng et al. 2009) and neuronal differentiation (Makeyev et al. 2007). We found that when 18S rRNA was used as a control, the expression of miR-124 appeared to be downregulated at certain time points. In contrast, when 5S rRNA was used, the expression of miR-124 had a more inconsistent pattern. Meanwhile with U6 snRNA, there was a significant increase in expression. In a previous study, Schratt et al. (2006) found that miR-124 levels were highest at 4 DIV and 7 DIV and then appeared to decrease and remain constant at lower levels from 10 - 14 DIV,

TABLE 5. Statistical analysis of real-time RT-PCR data of U6 snRNA expression in hippocampal neurons culture

Time	BDNF treatment	Mean	Variance	P-value
4 DIV	No	4.5345	0.0015	0.6380
	Yes	4.5557	0.0014	
6 DIV	No	4.4822	0.0014	0.4529
	Yes	4.5298	0.0039	
18 DIV	No	4.5844	0.0016	0.3031
	Yes	4.522	0.0006	

The mean values were obtained from the mean of log transformed Ct number, and sample size equals to 3. The P-value is set at 0.05, any P-value which is larger than 0.05 is considered as not significant. The P-values were determined by independent T-test

although there is some indication that the level increases at 18 DIV again. If we were to base our argument on U6 snRNA being the more stable endogenous control, then it appears that the expression of miR-124 is up-regulated in cultured hippocampal neurons, suggesting a significant role during maturing and matured neurons, beyond neuronal differentiation. A recent study has suggested that miR-124 has a role to play in long term plasticity of mature synapses by modulating the transcriptional factor CREB (Rajasethupathy et al. 2009), which would fit in with a high expression of miR-124 in mature neurons.

CONCLUSION

We acknowledge that our study is limited as a comprehensive screen of many endogenous controls was not performed. However, this study does show that amongst the tested endogenous controls, the U6 snRNA was the most suitable for miR-124 and -134, based on two independent softwares for endogenous control selection (BestKeeper and NormFinder) - at least in a maturing neuronal system. There may be other endogenous controls that would also be suitable for these miRNAs, providing careful evaluation is performed. The authors declare no competing interests exists in the interpretation of this study.

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