



Selection of best reference genes for qRT-PCR analysis of human neural stem cells preconditioned with hypoxia or baicalein-enriched fraction extracted from *Oroxylum indicum* medicinal plant



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ABSTRACT

Whilst the potential of neural stem cell (NSC)-based treatment is recognized worldwide and seems to offer a promising therapeutic option for stroke treatments, there is currently no full understanding regarding the effects of hypoxic and baicalein-enriched fraction (BEF) preconditioning approaches on the therapeutic potential of these cells for stroke. The potential of preconditioned NSC can be determined based on the expression of several key neuroprotective genes using qRT-PCR technique. However, prior to that, it is imperative and extremely important to carefully select reference gene(s) for accurate qRT-PCR data normalization to avoid error in data interpretation. This study aimed to evaluate the stability of ten candidate reference genes via comprehensive analysis using three algorithms software: geNorm, NormFinder and BestKeeper. Our results revealed that *HPRT1* and *RPL13A* were the most reliable reference genes for BEF-preconditioned NSCs, but ironically, *HPRT1* was ranked as the least stable reference gene for hypoxic-preconditioned NSCs. On the other hand, *RPLP1* and *RPL13A* were selected as the most stably expressed pair of reference genes for hypoxic-preconditioned NSCs. In conclusion, this study has pointed out the importance of identifying valid reference genes and has presented the first significant validation on best reference genes recommended for qRT-PCR study involves NSC preconditioned with hypoxia or with BEF extracted from *Oroxylum indicum* medicinal plant.

1. Introduction

Ischemic stroke is caused by physical blockage of blood flow to brain, typically by blood clot or cholesterol plaque, resulting in an area of ischemia where the brain tissue becomes irreversibly damaged due to lack of oxygen and nutrients. Neuronal cell apoptosis that occurs following ischemic stroke can be reduced if the occlusion is removed quickly and tissue reperfusion is restored early (Tachibana et al., 2017). Although current pharmacological drugs such as tissue plasminogen activator, thrombolytic agents and statin allow rapid restoration of blood supply (Carandang, 2016; Xian et al., 2016), these treatments are unable to trigger regeneration of new viable neuronal cells to replace the damaged brain tissue and thus cannot completely restore the brain function. As a consequence, poor prognosis and high rate of recurrent events are observed among the stroke patients (Cheah et al., 2016). This is indicated by previous report which showed that about 13% of stroke

patients who had suffered a first stroke developed a second stroke within a year and the observed mortality rate was 7-fold higher among the patients who experienced a recurrent stroke (Aarnio et al., 2014). The constraint of these therapies has led to the demand for an alternative therapy. Recently, cell grafting has emerged as a potential therapy for the stroke (Locatelli et al., 2009).

Neural stem cells (NSCs) were first identified within the central nervous system (CNS) in 1992 (Reynolds and Weiss, 1992). These cells are multipotent progenitors that are able to grow and self-renew in culture for extensive periods, yet retaining a stable differentiation capacity to give rise to matured, functional brain cells (Shi et al., 2008). In animal studies, NSC transplantation successfully improved brain function by regenerating the damaged brain tissue (Stroemer et al., 2009; Zhao et al., 2008). However, the major limitation of stem cell-based therapy is its inability to retain most of the donor stem cells after grafting into hypoxic sites with excessive free radicals, glutamate excitotoxicity and

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inflammation (Fischer et al., 2009; Roybon et al., 2006). A study showed that as little as 0.09% retention rate of grafted stem cells was observed in mice with transient middle cerebral artery occlusion (MCAO) (Bacigaluppi et al., 2009). Low cell survival rate limits the capacity of stem cell to repair the injured area and this poses a much more difficult challenge to the stem cell-based therapy for stroke.

Several strategies have been applied to circumvent this limitation. In our lab, hypoxic and baicalein-enriched fraction (BEF) preconditioning of NSCs were employed to enhance the stem cell-based therapy. Hypoxic preconditioning involves brief periods of hypoxia that triggers various protective signaling pathways and enhances resilience to ischemia (Sharp et al., 2004; Wei et al., 2017). Transcriptional responses to hypoxia are mediated by the hypoxia-inducible factor (HIF) (Sharp et al., 2004). Hypoxic preconditioning has been shown to promote NSC survival after transplantation into ischemic rat brain (Wakai et al., 2015). On the other hand, BEF preconditioning involves brief periods of exposure to baicalein compounds extracted from *Oroxylum indicum* - a medicinal herb belongs to family of Bignoniaceae that is commonly found in many regions of South-east Asia (Harminder et al., 2011). In Malaysia, *O. indicum* is commonly known as “Beko” and the young leaves as well as the fruits are consumed raw as salad by the local community. The baicalein active compound is postulated to trigger intrinsic neuroprotective mechanisms and enhance resilience to subsequent lethal insults (Harminder et al., 2011). It also has been documented as HIF stabilizer under normoxic conditions (Cho et al., 2008). Hence, BEF preconditioning could exert neuroprotective effects mimicking the hypoxic preconditioning under ambient conditions. We are determined to investigate the fundamental gene pathway involved in both preconditioning strategies in enhancing NSC survival rate within an ischemic environment using qRT-PCR based analysis.

qRT-PCR is the most well-known technique for absolute and relative quantification of mRNA transcription level. This technique offers greater sensitivity and specificity than Northern blot and semi-quantitative PCR (Rumlow et al., 2016). The expression of target gene is often normalized to reference gene to eliminate sample-to-sample biases. Reference genes are genes that are expressed constantly in all cells to maintain essential cellular functions (Scrideli et al., 2008). However, there is no universal reference gene for qRT-PCR as the expression of reference gene could vary under different experimental conditions, tissue types or species. As such, normalization using non-validated reference genes may introduce errors in gene studies (Gutierrez et al., 2008). Since the validity and accuracy of qRT-PCR are highly dependent on the reference gene, hence, it is imperative to identify the ideal candidate reference genes prior to qRT-PCR in this study.

Here, we evaluated the suitability of ten reference genes primarily used for gene expression study in human cells, namely β -actin (*ACTB*), eukaryotic initiation factor 4A (*eIF4A*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), importin 8 (*IPO8*), peptidyl-prolyl isomerase A (*PPIA*), ribosomal protein L13A (*RPL13A*), 60S ribosomal protein large P1 (*RPLP1*), TATA box binding protein (*TBP*) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (*YWHAZ*) for qRT-PCR normalization of hypoxic and BEF-preconditioned human NSCs, respectively. These candidate genes were carefully selected based on literature review of relevant studies which examined over 7,000 human genes in major tissue types to identify and designate house-keeping genes (Hsiao et al., 2001; Lee et al., 2007). The information of the candidate reference genes are summarized in Table 1. In this study, three algorithms software: geNorm, Normfinder and BestKeeper were used to analyse the stability of these candidate reference genes. This is because a single statistical approach could not possibly cover all variables associated with gene expression studies. Therefore, interpretation based on only one calculation algorithms can be associated with false positive results and misleading conclusions.

Table 1

Main physiological function and accession number of candidate reference genes used in this study.

Gene	Main physiological function	Accession no.
<i>ACTB</i>	Formation of cellular cytoskeleton	NM_0011101.3
<i>eIF4A</i>	Translation initiation	NM_014740.3
<i>GAPDH</i>	Carbohydrate metabolism	NM_001289745.1
<i>HPRT1</i>	Nucleotide synthesis	NM_000194.2
<i>IPO8</i>	Nuclear protein import	NM_006390.3
<i>PPIA</i>	Protein biosynthesis	NM_021130.4
<i>RPLP1</i>	Protein synthesis	NM_001003.2
<i>RPL13A</i>	Protein biosynthesis	NM_012423.3
<i>TBP</i>	Transcription factor	NM_003194.4
<i>YWHAZ</i>	Signal transduction	NM_001135699.1

2. Materials and methods

2.1. Neural stem cell culture

Human neural stem cell line derived from the National Institutes of Health (NIH) approved H9 human embryonic stem cells (H9-hNSCs) were purchased from GIBCO, USA. The H9-hNSCs were seeded at a density of 1.0×10^5 cells/cm² in a CELLstart™ CTS™ (GIBCO, USA) coated T25 flask added with 3 mL of complete StemPro® NSC SFM culture medium comprised of 1X Knockout™ D-MEM/F-12 (GIBCO, USA), 2 mM GlutaMAX™-I Supplement (GIBCO, USA), 20 ng/mL basic Fibroblast Growth Factor (GIBCO, USA), 20 ng/mL Epidermal Growth Factor (GIBCO, USA) and 2% StemPro® Neural Supplement (GIBCO, USA). The cells were allowed to adhere for 24 h in a CO₂ incubator at 37 °C in the presence of 5% CO₂. The medium was replaced with equal volume of pre-warmed complete StemPro® NSC SFM every two days and cultured until confluence prior to subsequent preconditioning treatments.

2.2. Study design

In present study, H9-hNSCs were cultured and subjected to two groups. Group 1: H9-hNSCs preconditioned under hypoxic cell culture condition (hypoxic-preconditioned H9-hNSCs) vs. H9-hNSCs without hypoxic preconditioning (non-hypoxic H9-hNSCs; control); and Group 2: H9-hNSCs preconditioned with BEF treatment (BEF-preconditioned H9-hNSCs) vs. H9-hNSCs without the BEF treatment (non-BEF H9-hNSCs; control). Reference genes were carefully selected from each of these groups, respectively.

2.3. Hypoxic preconditioning of H9-hNSCs

Confluent H9-hNSCs were subjected to hypoxic cell culture condition set at 2% O₂ for 24 h at 37 °C using a tri-gas hypoxic incubator flushed with 5% CO₂, 2% O₂ and 93% N₂. On the other hand, the non-hypoxic H9-hNSCs (control) were subjected to normoxic cell culture condition maintained at 21% O₂ and 5% CO₂ for 24 h using a standard CO₂ incubator.

2.4. BEF preconditioning of H9-hNSCs

Fresh leaves of *O. indicum* were collected from Kampung Pasir Parit, Pasir Mas (LGPS coordinate: latitude 5.905471, longitude 102.1884469). The plant material was verified by Dr. Rahmad Zakaria and voucher specimens (USM. Herbarium 11751) were deposited in the Universiti Sains Malaysia herbarium by Mr. V. Shunmugam (Fig. 1). The leaves were oven-dried at 50 °C and then crumbled into powder. 25 g of the *O. indicum* powder was transferred into a thimble added with 300 mL of petroleum ether solvent. The temperature of the extraction was set at the boiling point of petroleum ether (42–62 °C) and the extraction was carried out until the color of solvent turned clear. Then, the solvent was discarded and replaced with 500 mL of methanol. The temperature of the



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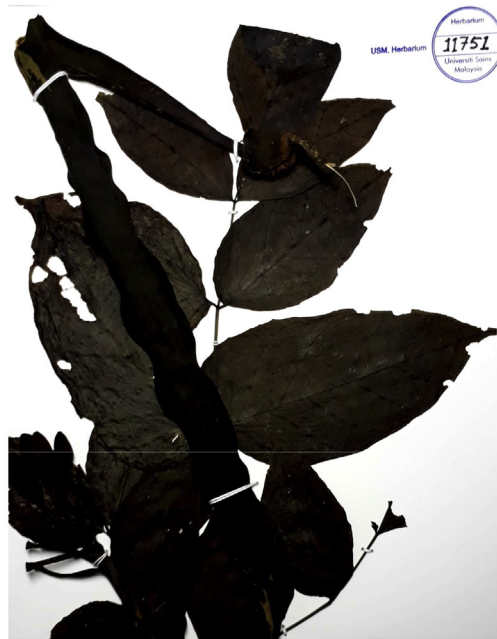


SOARING
UPWARDS
MALAYSIAN HIGHER EDUCATION

Bilik Herbarium
Kompleks Rujukan Biodiveristi (D32)
Pusat Pengajian Sains Kajihayat

Herbarium No.: 11751

Picture of Herbarium



Picture of Label

USM Herbarium
Pusat Pengajian Sains Kajihayat
Universiti Sains Malaysia

Family: Bignoniaceae

Genus/Species: *Oroxylum indicum* (L.) Benth. Ex Kurz

Date: 29/01/2018

Locality: Pasir Mas, Kelantan.

Habitat: Small tree up to 12m tall, mostly sighted along the river bank or slopes of the hill and often cultivated as an ornamental plant.

Notes: A evergreen tree or partly deciduous tree up to 15m high sparingly branched with gray-brown bark. Somewhat fissured glabrous throughout twigs pale grey, massive with big scars pimply from lenticels. Leaves 1m-3m long wide, very large, spreading, the main stalk and side stalk arched, swollen and jointed at the attachment of the side stalk and leaflets $\frac{3}{4}$ m – $1\frac{1}{4}$ m x $\frac{1}{2}$ m elliptic, shortly tipped, stalk thin with wavy edge, light green and dark along the vein, base narrowed or heart shaped, general asymmetric. Flowers with foxy stink, inflorescences up to $1\frac{1}{2}$ m-3m long, flowering upper part, fruiting below calyx, fleshy, lurid reddish purple to liver brown, outside greenish white and yellow on the inside. Pods $\frac{3}{4}$ m- 1m, curved at the base with fine edge on each side.

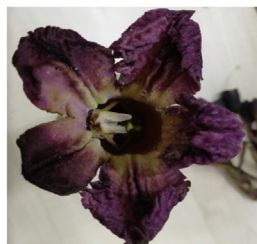
Collector: Kang In Nee

Identified by: Dr. Rahmad Zakaria

Picture of Plant



Stem and leaves



Flower



Fruit

Fig. 1. Voucher specimen of *Oroxylum indicum* plant (USM Herbarium 11751) verified by Dr. Rahmad Zakaria and deposited in the Universiti Sains Malaysia (USM) herbarium by Mr. V. Shunmugam. The leaves of *O. indicum* plant were used to extract baicalein-enriched fraction (BEF) for H9-hNSCs preconditioning treatment in this study.

extraction was the adjusted to the boiling point of methanol (62–65°C) and the extraction was carried out again until the color of solvent turned clear. The clear solvent was collected and dried using rotary evaporator at 40 °C for 12 h. The dried crude extract was subjected to column chromatography packed with 70 g of Diaion HP20 resin and flushed with mixtures of distilled water and methanol at gradient concentrations to obtain baicalein-enriched fraction (BEF). Confluent H9-hNSCs were subjected to BEF preconditioning by culturing the cells with complete StemPro® NSC SFM medium added with 1.56 µg/mL of BEF for 24 h at 37 °C in a standard humidified chamber with 21% O₂ and 5% CO₂. The concentration was selected based on a series of optimization procedure

done in our laboratory (data not shown). On the other hand, the non-BEF H9-hNSCs were cultured with complete StemPro® NSC SFM medium added with 0.1% DMSO for 24 h at 37 °C in a standard humidified chamber with 21% O₂ and 5% CO₂.

2.5. Primer design and optimization

Primers were designed using Primer3Plus program (Table 2). Each primer pair flanking the exon-exon boundary were designed to avoid genomic DNA amplification that possibly present in the sample. The designated primer sequences were BLAST against human genome

Table 2

List of primer sequences used in this study.

Gene	Forward (5' to 3')	Reverse (5' to 3')	Product size
ACTB	ATTGGCAATGAGCGGTTTC	TGAAGGTAGTTTCGTGGATGC	91
eIF4A	GAAGCTGGATTACGGACAGC	GCACGTGCTCTTAGGCTTCT	87
GAPDH	CCAAGGTCATCCATGACAAC	ACAGTCTTCTGGGTGGCAGT	82
HPRT1	TGAGGATTGGAAAGGGTGT	CCTCCCATCTCCTTCATCAC	90
IPO8	AGCTGGCTTGGCAGTTTATT	GCTGAATACGAGGCAGGAAT	118
PPIA	AGGGTTCCTGCTTTCACAGA	CATAGATGGACTTGCACCA	93
RPLP1	AGCCGGTGTAATGTTGAGC	CAGATGAGGCTCCCAATGTT	81
RPL13A	GGATGAACACCAACCCCTCC	AACACCTTGAGACGGTCCAG	133
TBP	TTCGAGAGTTCTGGGATTG	GGATTATATTCGGCGTTTCG	117
YWHAZ	CCGTACTTGCGCTGAGGTTG	TGCAATTTCCTTTTGCTGA	106

database to avoid any possibility of homologous sequences.

2.6. Quantitative real-time PCR (qRT-PCR)

Briefly, total RNA was isolated from H9-hNSCs using Trizol[®] reagent (Thermo Scientific, USA). Isolated RNA samples underwent DNase treatment using Ambion[®] TURBO DNA-free[™] DNase Treatment and Removal Reagents (Thermo Scientific, USA). cDNA were synthesized from 1 µg of total RNA samples RevertAid[™] H minus first strand cDNA Synthesis kit (Thermo Scientific, USA). The qRT-PCR was carried out in 20 µL total volume reactions contained 1X Power SYBR[®] Green Master Mix (Thermo Scientific, USA), 2 µL of 10X diluted cDNA template and 0.1 µM of forward and reverse primers using Applied Biosystems[®] 7500 Real-Time PCR system. The thermal cycling program was set up with an initial heat activation step at 95 °C for 10 min followed by 40 cycles of denaturation step at 95 °C for 15 s, an annealing step at 57 °C for 30 s and an extension step at 72 °C for 45 s. Fluorescence was measured at the end of extension step (72 °C for 45 s). At the end of the PCR reactions, dissociation or melt curves were run by heating PCR products at 95 °C for 15 s, cooling to 60 °C for 1 min and gradually re-heating to 95 °C for 15 s. Dissociation curve analysis was performed to confirm specificity of amplification. Moreover, in order to confirm the primer specificity and accuracy, agarose gel electrophoresis analysis was done to verify the PCR product size. PCR efficiency (E) was calculated based on the data obtained from the exponential phase of each individual amplification plot via equation $E = [10^{(-1/\text{slope})} - 1] \times 100\%$.

2.7. Data analysis

The stability and ranking of all candidate reference genes were evaluated using geNorm, NormFinder and BestKeeper. To compare the transcription level of the reference genes, the average cycle threshold (C_T) value of duplicate reactions was used as input for subsequent analysis using the geNorm, NormFinder and BestKeeper programs.

2.8. geNorm analysis

geNorm software was downloaded from <http://allserv.urgent.be/~jvdesomp/genorm/index.html>. The C_T value was transformed into linear scale expression quantity, Q using the standard formula $Q = E^{\Delta C_T}$ where where E is the exponential amplification and ΔC_T is the difference between the lowest C_T value and sample C_T value (Vandesompele et al., 2002). Reference gene with the highest expression has the highest Q, which was set to 1. Q values were then used as the input of Microsoft Excel for geNorm analysis. The software generated an average expression stability measure, M as the mean of the stability values of all other reference genes. The most stably expressed reference gene deemed the lowest M value. geNorm also was used to determine the gene expression stability by calculating the average pairwise variation, $V(n/n+1)$ of a pair particular reference genes with all other candidate genes of the study. The cutoff value for pairwise variation was set at 0.15 (Vandesompele et al., 2002).

2.9. NormFinder analysis

NormFinder was obtained from <http://www.mdl.dk/publicationsnormfinder.html>. Similar to geNorm analysis, the C_T value was converted to linear scale expression quantity (Q) by standard formula $Q = E^{\Delta C_T}$ (Andersen et al., 2004). The highest relative quantity for each reference gene was set to 1. The transformed Q values were then used as input data for NormFinder program. NormFinder estimated the overall expression variation of all reference genes and the variation between sample subgroups. Stability value (M) for each gene which represented expression variability within samples and between groups were calculated. Lower M indicated lower intra- and inter-group variations, hence implying a higher stability (Andersen et al., 2004).

2.10. BestKeeper analysis

The BestKeeper application was obtained from <http://www.genequantification.com/bestkeeper.html>. The average raw C_T values were used directly as the input data. The geometric mean of the C_T values of all reference genes were grouped together to obtain the BestKeeper Index (Pfaffl et al., 2004). BestKeeper index was determined based on three criteria: The pairwise correlation coefficient (r) between each reference gene, the standard deviation (SD) of the C_T values between the entire data set and the coefficient of variance (CV) of the C_T values between the entire data set. Stable reference gene had r value closer to 1, while lower SD and CV value indicated lower variability and high stability of genes across samples. Besides, BestKeeper also excluded any reference gene with SD value more than 1 which corresponding to inconsistent input amount. In this study, the stability of all reference genes were ranked according to the three criteria (r, SD and CV) individually and subsequently calculated the mean of the rankings to generate the final rank of each reference genes (Olias et al., 2014).

2.11. Comprehensive analysis

The three software applied in this study utilized different algorithms in ranking the stability of reference genes. Therefore a geometric mean of the stability values obtained from all three software was calculated as comprehensive ranking to ensure a comprehensive analysis of 10 references genes based on the stability at different aspects to select the most reliable reference gene for qRT-PCR analysis in this study (Ma et al., 2016; Xie et al., 2012).

3. Results

3.1. PCR amplification specificity and efficiency for candidate reference genes

Amplification specificity for each primer pair was successfully confirmed by a single peak in the melting curve analysis (Fig. 2). The amplification specificity was further validated by a 3% agarose gel with a specific PCR product of expected size yielded for each primer pair

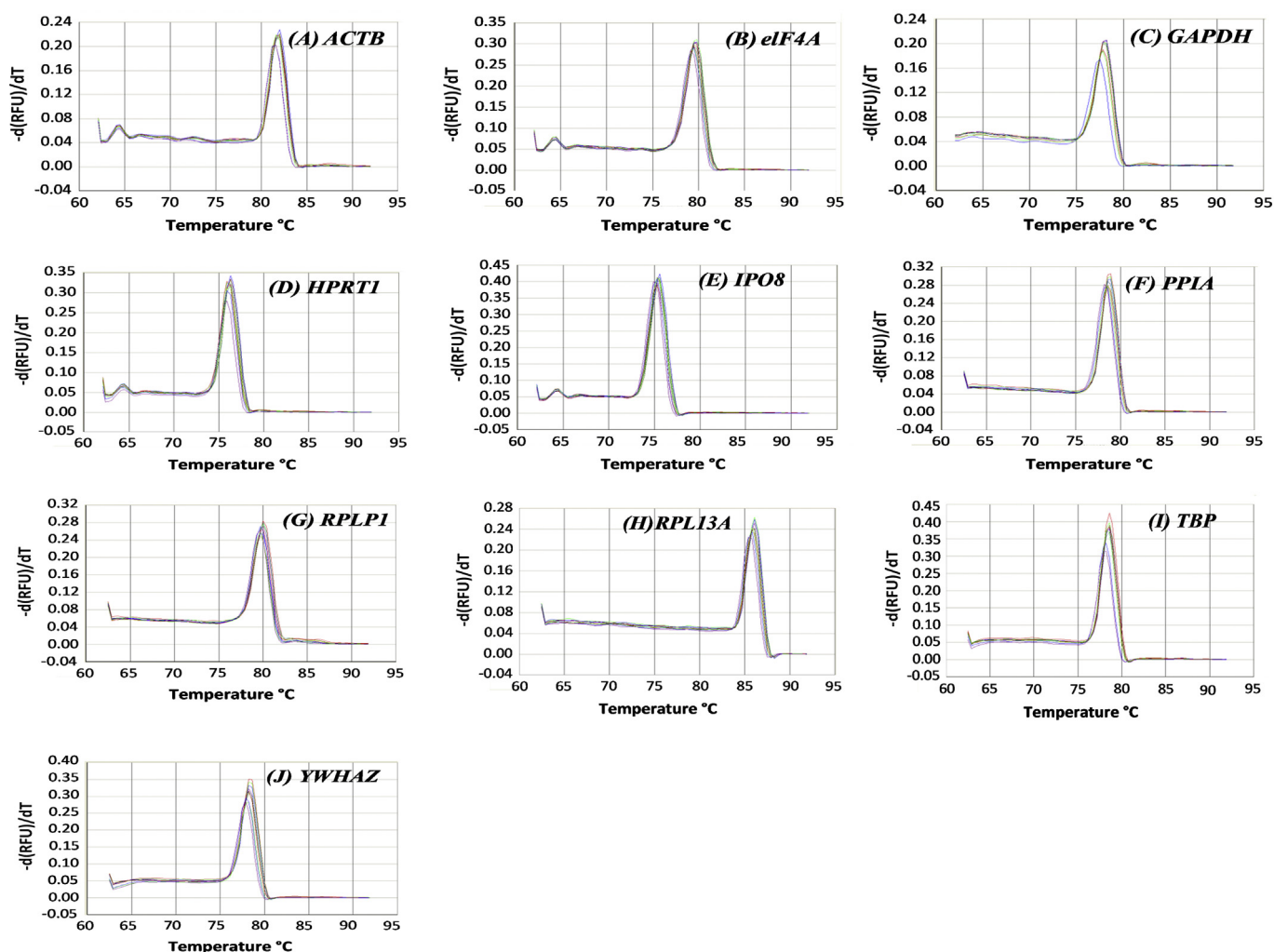


Fig. 2. Determination of qRT-PCR primer amplification specificity for (A) *ACTB*, (B) *eIF4A*, (C) *GAPDH*, (D) *HPRT1*, (E) *IPO8*, (F) *PPIA*, (G) *RPLP1*, (H) *RPL13A*, (I) *TBP* and (J) *YWHAZ* based on melt curve analysis. Single melt curve indicated that only the desired product was specifically amplified in each reaction. $d(RFU)/dT$ = Relative fluorescence units (RFU) with time (T).

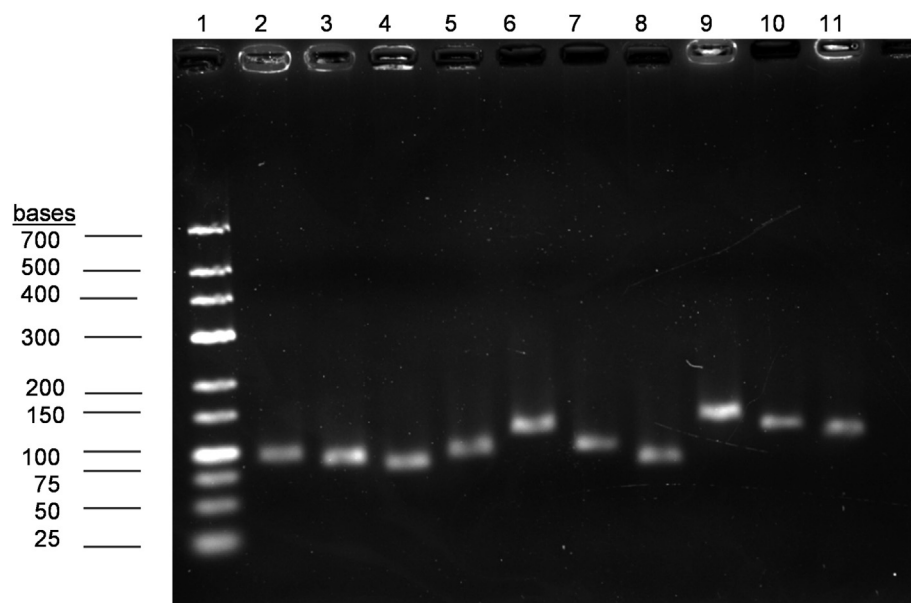


Fig. 3. Representative image of agarose gel (3%) electrophoresis showing amplification of a specific qRT-PCR product of the expected size for each reference gene ($n = 3$). No primer dimers and other unspecific amplification were found in the gel. Lane 1: GeneRuler™ low range DNA ladder; Lane 2: qRT-PCR product amplifying *ACTB* (91 bp); Lane 3: *eIF4A* (87 bp); Lane 4: *GAPDH* (82 bp); Lane 5: *HPRT1* (90 bp); Lane 6: *IPO8* (118 bp); Lane 7: *PPIA* (93 bp); Lane 8: *RPLP1* (81 bp); Lane 9: *RPL13A* (133 bp); Lane 10: *TBP* (117 bp) and Lane 11: *YWHAZ* (106 bp).

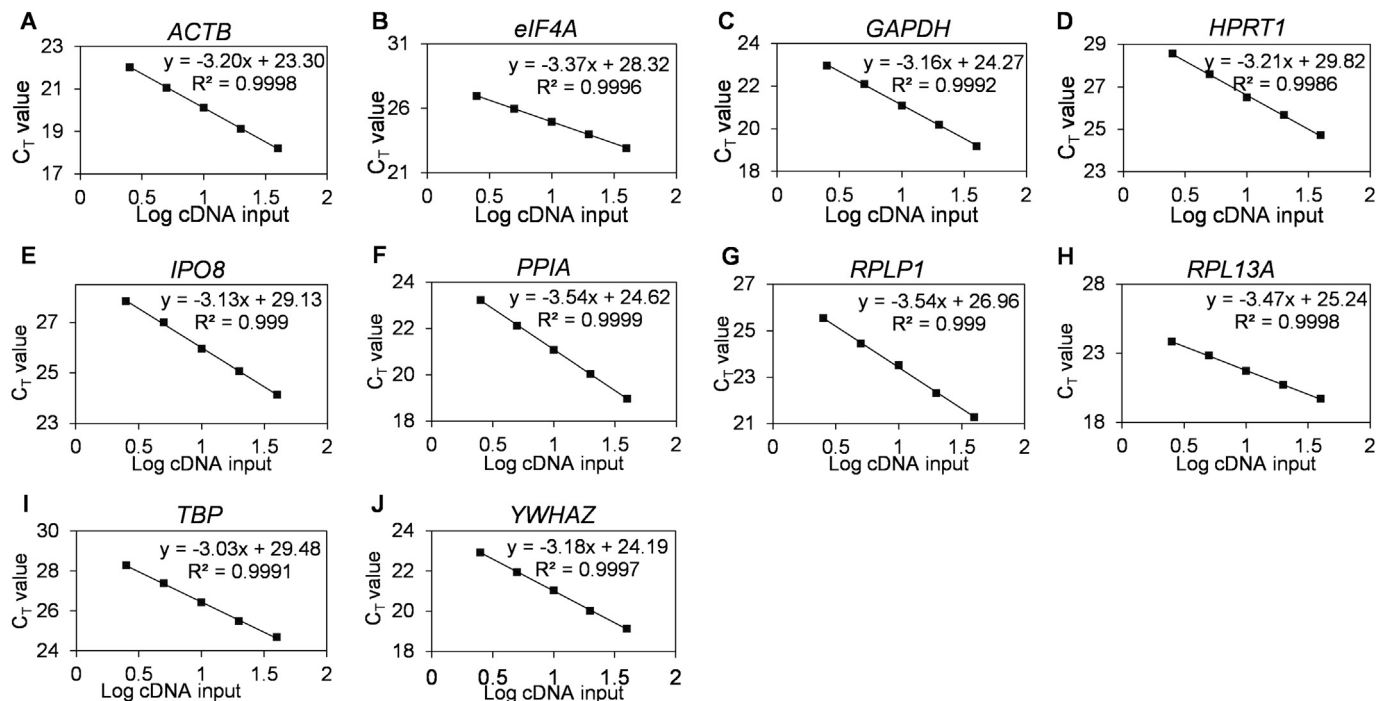


Fig. 4. Determination of qRT-PCR primer amplification efficiency for (A) *ACTB*, (B) *eIF4A*, (C) *GAPDH*, (D) *HPRT1*, (E) *IPO8*, (F) *PPIA*, (G) *RPLP1*, (H) *RPL13A*, (I) *TBP* and (J) *YWHAZ* based on standard curves calculation. The standard curve was generated by plotting the mean C_T values against the 2-fold serial dilutions of cDNA.

Table 3

The amplification efficiency and R² obtained from standard curves for each candidate reference gene used in the study.

Gene	Efficiency ($E = [10(-1/\text{slope}) - 1] \times 100\%$)	Correlation coefficient (R ²)
<i>ACTB</i>	105.6	0.9998
<i>eIF4A</i>	98.0	0.9996
<i>GAPDH</i>	107.5	0.9992
<i>HPRT1</i>	104.9	0.9986
<i>IPO8</i>	108.9	0.9990
<i>PPIA</i>	91.7	0.9999
<i>RPLP1</i>	91.6	0.9990
<i>RPL13A</i>	94.4	0.9998
<i>TBP</i>	113.9	0.9991
<i>YWHAZ</i>	106.1	0.9997

(Fig. 3). Amplification efficiency of each primer pair was also successfully determined using serial dilution curves (Fig. 4). The amplification efficiency of all the reference genes ranged from 91.6% to 113.9% with a positive correlation coefficient (R²) ranged from 0.9986 to 0.9990 (Table 3). Efficiency between 90% and 110% can be considered as good. The amplification efficiency test showed that all primer pairs consistently performed throughout the amplification.

3.2. Expression level of candidate reference genes

The expression levels of all these 10 candidate reference genes were relatively similar across hypoxic-preconditioned and non-hypoxic H9-hNSCs except *eIF4A* and *HPRT1* which showed significant down-regulation under hypoxic condition (Fig. 5A). On the other hand, the expression levels of candidate reference genes across BEF-preconditioned and non-BEF H9-hNSCs were relatively similar except *TBP* which showed significant increase after BEF preconditioning (Fig. 5B). Due to their unstable transcription expressions across samples, *eIF4A*, *HPRT1* and *TBP* would not be the reliable reference genes for normalization in their respective group. However, selection of suitable reference genes based on

solely the raw C_T value may mask genomic context, hence, more appropriate measures using statistical approach were carried out in subsequent steps to determine the reliability of each reference gene for qRT-PCR normalization.

3.3. Expression stability of candidate reference genes by geNorm

geNorm ranked the candidate reference genes differently across hypoxic- and BEF-preconditioned H9-hNSCs (Table 4). *RPL13A* and *RPLP1* were ranked the most stable pair of reference genes whilst *HPRT1* ranked the least stable reference gene across hypoxic- and non-hypoxic H9-hNSCs. To our surprise, the least stable reference gene (*HPRT1*) across hypoxic- and non-hypoxic H9-hNSCs was ranked the most stable reference genes across BEF- and non-BEF H9-hNSCs together with *RPL13A*. On the other hand, *TBP* was ranked the least stable in the BEF group. The V2/3 score which was lower than the cutoff value of 0.15 supported the use of two reference genes for qRT-PCR normalization of both hypoxic- and BEF-preconditioned H9-hNSCs (Fig. 6).

3.4. Expression stability of candidate reference genes by NormFinder

The reference genes for hypoxic- and BEF-preconditioned groups ranked by NormFinder was found consistent to geNorm analysis. *RPLP1* was selected as the most stably expressed gene whilst *HPRT1* deemed the least stable for hypoxic-preconditioned H9-hNSCs. On the other hand, *HPRT1* was the most reliable gene whilst *TBP* ranked the least reliable for BEF-preconditioned H9-hNSCs (Table 5).

3.5. Expression stability of candidate reference genes by BestKeeper

The ranking of reference gene based on BestKeeper SD, CV and r values exhibited differences across hypoxic- and BEF-preconditioned group (Table 6). Based on the geometric mean, *RPLP1* was the most stably expressed reference gene whilst *HPRT1* ranked the least reliable for qRT-PCR normalization of hypoxic-preconditioned and non-hypoxic

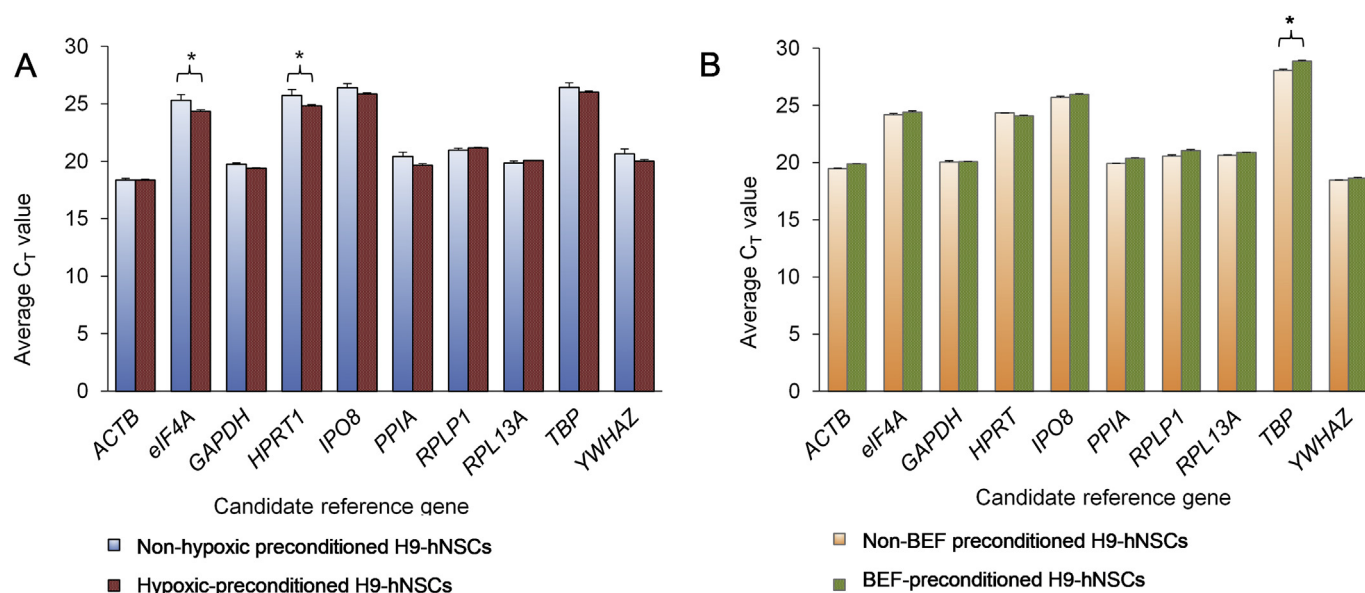


Fig. 5. The expression levels (C_T value) of candidate reference genes in (A) hypoxic-preconditioned vs. non-hypoxic H9-hNSCs and (B) BEF-preconditioned vs. non-BEF H9-hNSCs. The average C_T values were relatively similar across both cells in two respective groups, except the *eIF4A*, *HPRT1* and *TBP*. The values were expressed as mean $C_T \pm$ SEM from three independent experiments. Statistical analysis was performed using the two-tailed Student's t-test (* $p < 0.05$).

Table 4

geNorm analysis of the candidate reference genes across hypoxic- and BEF-preconditioned H9-hNSCs. The most stably expressed reference genes had lowest M value.

Ranking	Hypoxic		BEF	
	M	Gene	M	Gene
1	0.0817	<i>RPL13A/RPLP1</i>	0.0513	<i>RPL13A/HPRT1</i>
3	0.1313	<i>ACTB</i>	0.0942	<i>PPIA</i>
4	0.1889	<i>TBP</i>	0.0985	<i>ACTB</i>
5	0.2063	<i>GAPDH</i>	0.1211	<i>RPLP1</i>
6	0.2343	<i>PPIA</i>	0.1390	<i>YWHAZ</i>
7	0.2957	<i>IPO8</i>	0.1520	<i>IPO8</i>
8	0.3213	<i>YWHAZ</i>	0.1609	<i>eIF4A</i>
9	0.3663	<i>eIF4A</i>	0.1787	<i>GAPDH</i>
10	0.4237	<i>HPRT1</i>	0.2157	<i>TBP</i>

Table 5

NormFinder analysis for best candidate reference genes between hypoxic- and BEF-preconditioned H9-hNSCs. The most stably expressed reference genes had lowest M value.

Ranking	Hypoxic		BEF	
	M	Gene	M	Gene
1	0.094	<i>RPLP1</i>	0.053	<i>HPRT1</i>
2	0.14	<i>GAPDH</i>	0.056	<i>PPIA</i>
3	0.159	<i>TBP</i>	0.064	<i>RPL13A</i>
4	0.172	<i>IPO8</i>	0.076	<i>ACTB</i>
5	0.176	<i>RPL13A</i>	0.090	<i>RPLP1</i>
6	0.177	<i>YWHAZ</i>	0.093	<i>IPO8</i>
7	0.216	<i>PPIA</i>	0.099	<i>YWHAZ</i>
8	0.288	<i>eIF4A</i>	0.118	<i>eIF4A</i>
9	0.365	<i>ACTB</i>	0.156	<i>GAPDH</i>
10	0.388	<i>HPRT1</i>	0.218	<i>TBP</i>

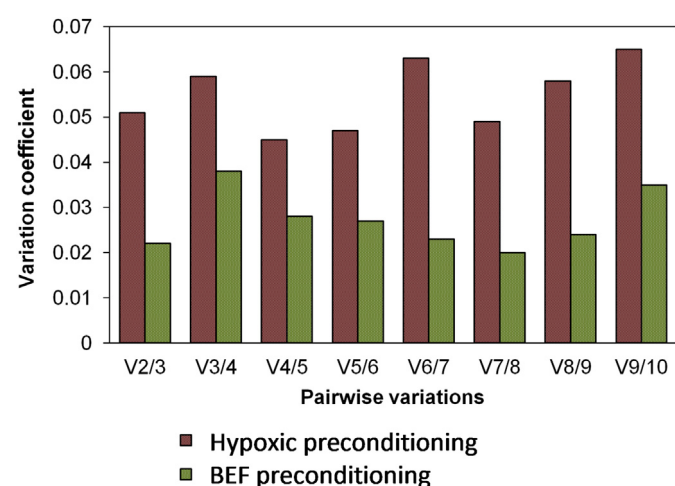


Fig. 6. Determination of the optimal number of reference genes based on geNorm analysis for hypoxic- and BEF-preconditioned H9-hNSCs. Cutoff value of 0.15 indicates the minimum number of reference genes for valid normalization. This result suggested a minimum two reference genes are necessary for qRT-PCR normalization.

H9-hNSCs. On the other hand, geometric mean revealed that *GAPDH* was the most stably expressed reference gene, while *RPLP1* was the least reliable reference gene across BEF-preconditioned and non-BEF H9-hNSCs (Table 6).

3.6. Overall expression stability of candidate reference genes

The order of stability ranking obtained from all three programs was similar but not identical. This discrepancy in ranking might attribute to distinct underlying theoretical algorithms of each program. Overall, *RPLP1* was ranked as the most reliable reference gene for qRT-PCR normalization of hypoxic preconditioning group (Table 7). *HPRT1* was overall ranked as the most unstable reference gene for hypoxic-preconditioned and non-hypoxic H9-hNSCs (Table 7). To summarize, the comprehensive ranking from the most to the least stable reference gene for hypoxic preconditioning group was *RPLP1*, *RPL13A*, *ACTB*, *TBP*, *GAPDH*, *IPO8*, *YWHAZ*, *eIF4A*, *PPIA* and *HPRT1*. To be congruent with recommendation by pairwise variation analysis, the two best performing reference genes, *RPLP1* and *RPL13A* were selected for qRT-PCR normalization of hypoxic preconditioning group.

On the other hand, the comprehensive ranking for BEF preconditioning group in descending stability was *HPRT1*, *RPL13A*, *PPIA*, *GAPDH*, *YWHAZ*, *ACTB*, *IPO8*, *RPLP1*, *eIF4A* and *TBP*. Overall, *HPRT1*

Table 6

Ranking of candidate reference genes across hypoxic- and BEF-preconditioned H9-hNSC based on BestKeeper analysis. Geometric mean was calculated for the final ranking.

Hypoxic					Final rank	BEF					Final rank
Gene	Ranking based on			Geometric mean		Gene	Ranking based on			Geometric mean	
	SD	CV	r				SD	CV	r		
<i>RPLP1</i>	1	1	9	2.08	1	<i>GAPDH</i>	1	1	10	2.15	1
<i>ACTB</i>	2	2	10	3.42	2	<i>HPRT1</i>	4	3	3	3.3	2
<i>RPL13A</i>	3	3	5	3.56	3	<i>YWHAZ</i>	2	4	5	3.42	3
<i>IPO8</i>	7	7	1	3.66	4	<i>RPL13A</i>	3	5	4	3.91	4
<i>TBP</i>	4	4	6	4.58	5	<i>IPO8</i>	4	2	8	4.00	5
<i>YWHAZ</i>	8	8	2	5.04	6	<i>TBP</i>	10	10	1	4.64	6
<i>GAPDH</i>	5	5	7	5.59	7	<i>PPIA</i>	8	9	2	5.24	7
<i>eIF4A</i>	9	9	3	6.24	8	<i>ACTB</i>	7	7	5	6.25	8
<i>PPIA</i>	6	6	8	6.6	9	<i>eIF4A</i>	6	6	9	6.87	9
<i>HPRT1</i>	10	10	3	6.69	10	<i>RPLP1</i>	8	8	7	7.65	10

Table 7

Comprehensive ranking of the 10 candidate reference genes based on geometric mean of geNorm, NormFinder and BestKeeper ranking across hypoxic- and BEF-preconditioned H9-hNSCs.

Hypoxic					BEF				
No.	geNorm	Norm-Finder	Best-Keeper	Overall ranking	No.	geNorm	Norm-Finder	Best-Keeper	Overall ranking
1	<i>RPL13A/RPLP1</i>	<i>RPLP1</i>	<i>RPLP1</i>	<i>RPLP1</i>	1	<i>RPL13A/HPRT1</i>	<i>HPRT1</i>	<i>GAPDH</i>	<i>HPRT1</i>
2		<i>GAPDH</i>	<i>ACTB</i>	<i>RPL13A</i>	2		<i>PPIA</i>	<i>HPRT1</i>	<i>RPL13A</i>
3	<i>ACTB</i>	<i>TBP</i>	<i>RPL13A</i>	<i>ACTB</i>	3	<i>PPIA</i>	<i>RPL13A</i>	<i>YWHAZ</i>	<i>PPIA</i>
4	<i>TBP</i>	<i>IPO8</i>	<i>IPO8</i>	<i>TBP</i>	4	<i>ACTB</i>	<i>ACTB</i>	<i>RPL13A</i>	<i>GAPDH</i>
5	<i>GAPDH</i>	<i>RPL13A</i>	<i>TBP</i>	<i>GAPDH</i>	5	<i>RPLP1</i>	<i>RPLP1</i>	<i>IPO8</i>	<i>YWHAZ</i>
6	<i>PPIA</i>	<i>YWHAZ</i>	<i>YWHAZ</i>	<i>IPO8</i>	6	<i>YWHAZ</i>	<i>IPO8</i>	<i>TBP</i>	<i>ACTB</i>
7	<i>IPO8</i>	<i>PPIA</i>	<i>GAPDH</i>	<i>YWHAZ</i>	7	<i>IPO8</i>	<i>YWHAZ</i>	<i>PPIA</i>	<i>IPO8</i>
8	<i>YWHAZ</i>	<i>eIF4A</i>	<i>eIF4A</i>	<i>eIF4A</i>	8	<i>eIF4A</i>	<i>eIF4A</i>	<i>ACTB</i>	<i>RPLP1</i>
9	<i>eIF4A</i>	<i>ACTB</i>	<i>PPIA</i>	<i>PPIA</i>	9	<i>GAPDH</i>	<i>GAPDH</i>	<i>eIF4A</i>	<i>eIF4A</i>
10	<i>HPRT1</i>	<i>HPRT1</i>	<i>HPRT1</i>	<i>HPRT1</i>	10	<i>TBP</i>	<i>TBP</i>	<i>RPLP1</i>	<i>TBP</i>

was ranked as the most reliable reference gene for qRT-PCR normalization of BEF-preconditioned and non-BEF H9-hNSCs. *TBP* was overall ranked as the most unstable reference gene. The two most reliable reference genes, *HPRT1* and *RPL13A* were selected to normalize gene expression data of BEF preconditioning group (Table 7).

4. Discussion

This study provided the first validation on reliability of ten frequently used candidate reference genes for hypoxic-preconditioned and BEF-preconditioned H9-hNSCs, respectively. We found that both experimental conditions required different reference genes even they are from the same cell type, demonstrating that reference genes can be regulated depending on experimental conditions.

The cell types under investigation in this study were H9-hNSCs preconditioned with either hypoxia or BEF. The expression levels of all ten candidate reference genes were similarly regulated in both groups, except the *eIF4A*, *HPRT1* and *TBP*. Downregulation of *eIF4A* in H9-hNSCs after hypoxic preconditioning was concomitant with previous studies which also reported significant decrease of *eIF4A* in human cells cultured under low oxygen condition (Ho et al., 2016). *eIF4A* encodes for protein functions as a subunit of the eukaryotic initiation factor (eIF) 4F (eIF4F) complex comprised on several initiation factors including the RNA helicase eIF4A, the cap-binding eIF4B and the scaffold eIF4G which altogether mediates the binding of mRNA to the ribosome (Jackson et al., 2010). In normoxia, the eIF4F complex typically initiates protein synthesis routinely. However, under hypoxia, low O₂ concentration inhibits binding of eIF4E to eIF4G, thereby inhibiting eIF4F activity, as well as its counterpart, the eIF4A (Connolly et al., 2006). This explained our finding which showed significant decreased *eIF4A* expression level in hypoxic-preconditioned H9-hNSCs compared to the non-hypoxic cells. Further, this also support our finding that *eIF4A* was among the least stable reference genes for hypoxia group. On the other hand, *HPRT1* has

a central role in the generation of purine nucleotides through the purine salvage pathway. *HPRT1* encodes hypoxanthine phosphoribosyl-transferase which catalyzes conversion of hypoxanthine to inosine monophosphate and guanine to guanosine monophosphate via transfer of the 5-phosphoribosyl group from 5-phosphoribosyl 1-pyrophosphate, and this biochemistry process consumes energy (Keebaugh et al., 2007). Under hypoxia, as adenosine triphosphate (ATP) generation by oxidative phosphorylation begins to fall off due to O₂ limitation, the cellular ATP demands of most mammalian cells and tissues tend to remain constant, trying to avoid unnecessary consumption of ATP. This could be the reason of the significant reduction of *HPRT1* expression in hypoxic-preconditioned cells. Thus, we are not surprised when all three algorithms software: geNorm, NormFinder and BestKeeper revealed that *HPRT1* as the least stable reference genes for hypoxic preconditioning group. Nonetheless, the least stable reference gene for BEF preconditioning was not *eIF4A* or *HPRT1*. For this group of cells, *TBP* scored the lowest rank in stability as compared to other nine candidate reference genes. Furthermore, *TBP* also was the only reference gene that showed significant different in expression level among the BEF-preconditioned vs. non BEF-preconditioned H9-hNSCs. Hence, it was not recommended for qRT-PCR normalization of BEF-preconditioned H9-hNSCs.

Out of the 10 reference genes evaluated, *RPLP1* was consistently ranked by geNorm, NormFinder and BestKeeper as the most suitable reference gene across hypoxic preconditioning group. The *RPLP1* gene was documented as crucial ribosomal protein for progenitor cell proliferation, embryonic and brain development (Perucho et al., 2014). However, to our surprise, the least stable reference gene (*HPRT1*) for hypoxic preconditioning group was found to be the most stable reference gene in BEF preconditioning group. In a previous study done by Amable et al. (2013), *HPRT1* was identified as one of the best reference gene in human bone marrow-derived mesenchymal stromal cells and dermal fibroblasts (Amable et al., 2013). Therefore, we suggest that the discrepancies of *HPRT1* expression between cells indicated that it was regulated

by experimental condition. This contradictory finding, again, highlighted the importance of identifying valid reference genes for each experimental set-up prior to gene expression analysis for accurate normalization of qRT-PCR data.

Normalization with multiple reference genes is becoming more prevalent. It was reported in previous studies that normalization based on multiple candidate genes provided the advantage of more accurate normalization calculation than a single gene (Tan et al., 2011; Tricarico et al., 2002). Based on geNorm analysis, we found that an optimum of two housekeeping genes were needed to perform a valid normalization for both hypoxic and BEF preconditioning group. In this analysis, *RPLP1* and *RPL13A* were selected as the best combination for normalization of gene expression between hypoxic and non-hypoxic H9-hNSCs while *HPRT1* and *RPL13A* were selected as the best two genes for normalization of gene expression between BEF- and non-BEF H9-hNSCs.

5. Conclusions

Ideally, reference genes should exhibit constant expression level across all tissue types and experimental settings. However, this study has pointed out that such reference genes do not exist. Not only that, we also found that the best reference gene for one experimental condition could be the worst for another experimental condition even using the same cell types. Therefore, this novel study clearly elucidated the importance of identifying valid reference genes for each experimental set-up prior to gene expression analysis. In conclusion, comprehensive analysis using geNorm, NormFinder and BestKeeper analysis revealed that *RPLP1* and *RPL13A* were the best reference genes for hypoxic-preconditioned human NSCs whilst *HPRT1* and *RPL13A* were the most reliable for BEF-preconditioned human NSCs.

Declarations

Author contribution statement

In Nee Kang: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Chong Yew Lee: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Suat Cheng Tan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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