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Selection of reference genes for quantitative real-time PCR evaluation of chronic erythropoietin treatment effect on the SH-SY5Y and PC12 cells

Research Article

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Abstract: The quantitative real-time polymerase chain reaction (qPCR) is a sensitive technique for examining the influence of erythropoietin (Epo) on gene expression. A critical and fundamental step for data analysis is the selection of and normalization to the optimal reference gene(s). We identified appropriate reference gene(s) among 32 genes during chronic recombinant human Epo (rHuEpo) treatment of SH-SY5Y cells using TaqMan human Express Endogenous Control Plate. Expression stability of the selected reference gene (*RPLP*) was retested with qPCR, together with two commonly used reference genes (*GAPDH*, *ACTB*) and six genes of interest (*EPOR, EPO, STAT5B, STAT5A, JUN, AKT*). In PC12 cells, three commonly used reference genes (*Gapdh*, *CycA* and *Ywhaz*) and seven genes of interest (*EpoR, Epo, Stat5b, Stat5a, Jun, Akt, Fos*) were evaluated. For the evaluation of expression stability, geNorm, NormFinder and BestKeeper software were used. All three gave similar results. We demonstrated that among the housekeeping genes, *RPLP* in SH-SY5Y and *CycA* and *Ywhaz* in PC12 are the most stable genes. Additionally, we showed that normalization with *GAPDH* gave misleading results compared to normalization with geNorm. In conclusion, selection of the appropriate normalization gene(s) is crucial for correct interpretation of rHuEpo treatment results.

Keywords: *Erythropoietin (Epo) • Chronic treatment • Quantitative real-time PCR • Reference gene selection • Normalization*

1. Introduction

Erythropoietin (Epo) is a highly glycosylated hormone with a molecular mass of approximately 30 kDa. It is the principal regulator of erythropoiesis with its main site of synthesis in the kidneys [1]. Epo stimulates the survival, proliferation and differentiation of erythroid progenitors and precursors in the bone marrow. It is a member of the cytokine family of growth factors which includes prolactin, interleukin 2 to 7, G-CSF, GM-CSF and others [2,3]. Epo binds to its cognate receptor (EpoR) on the cell surface activating several signaling pathways including Jak-STAT, Ras-MAPK and PI3K [4].

Epo appears to act on several non-haematopoietic tissues, exhibiting an anti-apoptotic effect [5]. Epo and its receptor were found to be expressed in brain [6], reproductive tract [7], lung, heart [8] and other tissues. EpoR is expressed in brain capillaries, enabling Epo to cross the blood-brain barrier [9]. Studies have shown that Epo can reduce experimental brain tissue injury and that

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it acts as a protective agent in cerebral ischemia models [10]. Additionally, it has been shown that the expression of Epo and EpoR is increased when brain injury occurs [11]. Epo exhibited a neuroprotective effect in studies of stroke and spinal cord injury in animal models [12]. For a recent review of this field see [13].

The human neuroblastoma cell line, SH-SY5Y, and a rat pheochromocytoma cell line, PC12, have become popular cell models for studies of neuroprotection, neurotoxicity and neurodegenerative diseases [14,15]. However, the molecular basis of Epo's neuroprotection is not well understood. Quantitative real-time polymerase chain reaction (qPCR) is a technique that enables the identification of the genes involved in Epo-induced neuroprotection on neural cell lines SH-SY5Y and PC12.

qPCR is a valuable method for the quantification of gene expression due to its high sensitivity, broad dynamic range, accuracy and speed. The method is based on measurement of PCR products after DNA amplification [16,17]. Since the expression of genes varies among organisms, tissues and cells and also among experimental groups of the same origin, there is a need for a good, reliable normalization gene or genes [18]. However, no gold standard is available, and different normalization approaches have been used. In the case of cell lines, normalization has been based on cell number, RNA quantity or cDNA quantity. But because of the significant variability of these parameters (amount of starting material, enzymatic efficiency, transcriptional activity, presence of inhibitors), "house keeping genes" (HKG) have become routinely used as normalization factors [19,20].

The ideal endogenous control should be expressed constantly, in all tissues, and under different conditions. The choice of reference genes depends on the cells studied and has to be validated for each experiment; if not, the differences in expression levels of genes of interest (GOI) may not be appreciated or may be incorrect [17]. Commonly known HKGs are glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), beta actin (*ACTB*) and 18s ribosomal RNA (*18s rRNA*) [21-25]. But in recent years, many studies have shown that the expression levels of these genes may vary under specific conditions [26,27].

Therefore, the first step in our qPCR experiment was the identification of genes being stably expressed in cell lines SH-SY5Y and PC12 under chronic recombinant human erythropoietin (rHuEpo) treatment, which could be used as reference genes. In the present study, we have evaluated the expression of 32 HKGs in SH-SY5Y cells under chronic rHuEpo treatment using TaqMan expressed human plates. Gene expression in cell lines SH-SY5Y and PC12 during chronic rHuEpo treatment was also validated with real-time qPCR with the aim to (re)test the stability of the most commonly used reference genes and determine the gene expression of several genes of interest involved in Epo signaling. The geNorm [28], NormFinder [29] and BestKeeper [30] software were used for evaluation of the expression stability of individual genes and the outcomes were very similar. Our results suggest that selection of the appropriate normalization gene(s) is crucial for correct interpretation of rHuEpo treatment results.

2. Experimental procedure

2.1. Cell lines

SH-SY5Y (human) and PC12 (rat) cell lines were from American Type Culture Collection, USA. SH-SY5Y cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient F12 Ham (Sigma Aldrich, St. Louis, Missouri) with 10% fetal bovine serum (Sigma Aldrich, St. Louis, Missouri) at 37°C, 5% CO_2. PC12 cells were cultured in DMEM (Sigma Aldrich, St. Louis, Missouri) with 2,5% fetal bovine serum (Sigma Aldrich, St. Louis, Missouri) and 15% horse serum (Sigma Aldrich, St. Louis, Missouri). Cells were untreated or treated with recombinant human erythropoietin (rHuEpo) (Neorecormon, Roche, Basel, Switzerland) at final concentrations of 1, 2, 5 IU mL^{-1} for 6 weeks. Isolation of RNA was performed during treatment every 3 to 5 days. 36 samples were collected from SH-SY5Y and 44 from PC12 cells (Table 1). Samples crossed out in Table 1 were collected but not tested due to poor RNA yield. At the time of RNA isolation, the cells were ~80% confluent.

Table 1. List of SH-SY5Y and PC12 cell sample collections during chronic rHuEpo treatment.

SH-SY5Y	Epo (IU mL^{-1})				PC12	Epo (IU mL^{-1})			
Exposure day	0	1	2	5	Exposure day	0	1	2	5
7					7				
10					10				
12					12				
17					16				
24					19				
27					22		X		
30					26				
33	I		II	III	29		X		
36	I	X	II	III	32				
					35		X		
					38				

2.2. RNA isolation and characterization

Total RNA from the cells was isolated using a FujiFilm QuickGene 810 machine with QuickGene RNA cultured cell HC kit S (FujiFilm LifeScience, FujiFilm, Tokio, Japan). The amount and quality of isolated RNA were measured on NanoDrop (A260/A280 and A260/A230) (Thermo Scientific, Massachusetts, USA) and the quality (RIN number) was checked using Agilent RNA chips (Agilent, Colorado, USA).

2.3. cDNA synthesis

All RNA samples were treated with DNAse I (Roche Applied Bioscience, Basel, Switzerland) according to the manufacturer's instructions (1 µg of cell RNA mixed with 2 µL of 10x DNAse buffer and 1 µL of DNAse I). RNA was reversed-transcribed using Superscript III reverse transcriptase (Invitrogen, California, USA). 1 µg of cell RNA was mixed with 10 µl of reverse transcriptase master mix (5 µL of 5x first strand buffer, 1.25 µL of 100mM DTT, 1.25 µL of 10mM dNTP mix, 0.65 µL of random primers (Promega, Wisconsin, USA), 0.5 µL SuperScript III (200 U μL^{-1}), 0.5 µl of RNAse OUT (Invitrogen)) and bdH_2O to a final volume of 30 µL. This was followed by incubation at 25ºC for 5 minutes, 50ºC for 60 minutes and 70ºC for 15 minutes. cDNA synthesis was carried out in a 96 well plate to ensure equal conditions for all samples containing the RNA isolated from SH-SY5Y and PC12 cells.

2.4. TaqMan Express Plate

TaqMan Express 96 well 32 format human reaction plates (TaqMan, Express Endogenous Control Plate, Gene Expression Assays, Applied Biosystems, California, USA) were used on three SH-SY5Y pooled cDNA samples. Three sample pools marked with I-III were prepared by mixing the equal amount of RNA from SH-SY5Y samples (Table 1) with the highest RNA yield and analyzed in one technical replicate. Pre-designed gene specific primers and hydrolysis probe sets enabled us to perform quantitative gene expression studies on cDNA. The reaction mix consisted of 10 µL of cDNA (final concentration of cDNA was 10 ng per 20 µL reaction) and of 10 µL TaqMan Gene Expression Master mixes. The run was performed on an Applied Biosystems 7500 Real Time PCR System. qPCR reaction steps were AmpErase UNG Activation at 50ºC for 2 minutes, AmpliTaq DNA Polymerase Activation at 95ºC for 10 minutes, 45 cycles of denaturation at 95ºC for 15 seconds and annealing at 60ºC for 1 minutes.

2.5. Real time qPCR

Real time quantitative polymerase chain reactions (qPCR) were performed using LighCycler 480I (Roche Applied Science, Basel, Switzerland) on a 384 well plate format with LightCycler SYBR Green I Master (Roche Applied Science). The PCR master mix consisted of 2.5 µL of SYBR Green I Master, 1.15 µL of RNAse free water, 0.6 µL of 300 nM primer mix and 0.75 µL of 4.16 time diluted cDNA in a total volume of 5 µL. All the samples were analyzed in three technical replicas. qPCR reaction steps were 10 minutes at 95ºC, 40 cycles for 10 seconds at 95ºC, 20 seconds at 60ºC and 20 seconds at 72ºC. Melting curve analysis was performed in a range of 65ºC to 95ºC.

Primers were designed using the Roche Universal ProbeLibrary (Tables 2 and 3). They were designed to be intron-spanning, when possible, to minimize inaccuracies due to genomic DNA. All primers were validated on a series of cDNA dilutions to verify the presence of a gene specific peak and the absence of primer dimers.

2.6. Data analysis

Statistical analysis of data values was done using GraphPad Prism 5.0 (GraphPad software) and Windows Excel (Windows). For stability comparison of candidate reference genes, geNorm (http://medgen.ugent.be/~jvdesomp/geNorm/), NormFinder software (http://www.mdl.dk/publicationsnormfinder.htm) and BestKeeper (http://gene-quantification.com/bestkeeper.html) and EqPCR Wizard were used.

3. Results and discussion

3.1. Material for qPCR

Cell lines SH-SY5Y and PC12 were untreated or treated with rHuEpo (1, 2, 5 IU mL^{-1}) for up to 6 weeks. During the time course of treatment, 36 samples from SH-SY5Y and 44 from PC12 cells were collected (Table 1) followed by isolation of total RNA, cDNA synthesis and TaqMan or SYBR Green real-time qPCR analysis.

3.2. Expression analysis of the candidate reference genes

Expression analysis of 32 reference/HKGs was performed on TaqMan Express Human plate in order to find the most stable genes during chronic rHuEpo treatment of SH-SY5Y cells. Analysis was performed on three pooled samples of RNA isolated from SH-SY5Y cells marked with I-III in Table 1. Average quantification cycle (Cq) and standard deviation (SD) were calculated and are shown in Table 4. Genes that show low SD are assumed to be more stably expressed in rHuEpo treated conditions compared to genes with higher SD.

Table 2. Housekeeping genes (HKG) and genes of interest (GOI) evaluated in SH-SY5Y human cell line.

	HUMAN Gene symbol	Accession no.	Gene name		Primer sequence	Primer efficiency	Amplicon length
HKG	*GAPDH*	NM_002046.3	Glyceraldehyde-3-phosphate dehydrogenase	F R	caacggatttggtcgtattgg gcaacaatatccactttaccagagttaa	1.88	72
HKG	*ACTB*	NM_001101.3	Actin beta	F R	ccaaccgcgagaagatga ccagaggcgtacagggatag	1.94	97
HKG	*RPLP*	NM_053275.3	Ribosomal protein large P0	F R	gcatcagtaccccattctatca aaggtgtaatccgtctccacaga	1.99	74
GOI	*EPOR*	NM_000121.2	erythropoietin receptor	F R	ttggaggacttggtgtgtttc agcttccatggctcatcct	1.81	101
GOI	*EPO*	NM_000799.2	erythropoietin	F R	tcccagacaccaaagttaatttcta ccctgccagacttctacgg	1.98	76
GOI	*STAT5B*	NM_012448.3	signal transducer and activator of transcription 5B	F R	gtccgagaagccaacaatg gaggtgtttctgggacatgg	1.89	69
GOI	*STAT5A*	NM_003152.3	signal transducer and activator of transcription 5A	F R	gtccctccctggacttttct ggaggagggaaaagttggac	1.97	90
GOI	*JUN*	NM_002229.2	jun B proto-oncogene	F R	atacacagctacgggatacgg gctcggtttcaggagtttgt	1.98	73
GOI	*AKT*	NM_005163.2	v-akt murine thymoma viral oncogene homolog 1	F R	gcagcacgtgtacgagaaga ggtgtcagtctccgacgtg	1.98	67

Table 3. Housekeeping genes (HKG) and genes of interest (GOI) evaluated in PC12 rat cell line.

	RAT Gene symbol	Accession no.	Gene name		Primer sequence	Primer efficiency	Amplicon length
HKG	*Gapdh*	NM_017008	Glyceraldehyde-3-phosphate dehydrogenase	F R	gcaagagaggaggccctcag tgtgagggagatgctcagtg	1.97	74
HKG	*CycA*	XM_345810.3	Cyclophilin A	F R	agcactggggagaaaggatt tgtgacgttaccaccctgac	1.98	87
HKG	*Ywhaz*	NM_013011	Tyrosin 3-monooxygenase/ tryptophan, 5-monooxygenase activation protein, zeta	F R	gctacttggctgaggttgct tgctgtgactggtccacaat	1.96	61
GOI	*EpoR*	NM_017002.2	erythropoietin receptor	F R	tgagtgtgtcctgagcaacc ccagcacagtcagcaacagt	1.95	200
GOI	*Epo*	NM_017001	erythropoietin	F R	agtcgcgttctggagaggta ccttctgcacagcccatt	1.97	71
GOI	*Stat5b*	NM_022380.1	signal transducer and activator of transcription 5B	F R	ggagagcctacggatccaa agggacacttgcttctgctg	1.81	102
GOI	*Stat5a*	NM_017064.1	signal transducer and activator of transcription 5A	F R	tcgctgtatccgtcacattc acaccagcaggggagcta	1.98	78
GOI	*Jun*	NM_021836.2	jun B proto-oncogene	F R	gggactgggagctcatacc aaagggtggtgcatgtgg	1.90	60
GOI	*Akt*	NM_033230.1	v-akt murine thymoma viral oncogene homolog 1	F R	aacgacgtagccattgtgaa ccatcattcttgaggaggaagt	1.84	95
GOI	*Fos*	NM_022197.2	murine osteosarcoma viral oncogene homolog	F R	gggacagcctttcctactacc gatctgcgcaaaagtcctgt	1.94	87

The predicted most stably expressed genes were *UBC*, *ELF1* and *RPLP*; the stability of *RPLP* was assessed further.

3.3. Expression analysis of the selected reference genes and genes of interest

The expression of one of the most stably expressed genes on TaqMan plates (*RPLP*), two most common used reference genes according to the literature (*GAPDH* and *ACTB*) [20,31,32] and six GOI (genes of interest) involved in Epo signal transduction (Table 2) were (re)evaluated with qPCR on 35 SH-SY5Y samples (Table 1). In PC12, the expression of three most common used reference genes according to the literature (*Gapdh*, *CycA* and *Ywhaz*) [20,31,32] and seven GOI involved in Epo signal transduction (Table 3) were evaluated with qPCR on 41 samples (Table 1). Low variability of quantification cycle (Cq) of tested GOI on each cell line

Table 4. TaqMan Endogenous Control Express Plate analysis of SH-SY5Y cells.

Gene Symbol	Gene Name	Avg Cq	SD
UBC	Ubiquitin C	21.93	0.018
ELF1	E74-like factor 1	28.53	0.039
RPLP	Ribosomal protein large P0	20.27	0.043
CDKN1A	Cyclin-dependent kinase inhibitor 1A	25.72	0.072
CASC3	Cancer susceptibility candidate 3	24.45	0.097
PUM1	Pumilio homolog 1	24.56	0.112
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	20.28	0.124
TBP	TATA box binding protein	28.23	0.142
B2M	Beta-2-microglobulin	24.99	0.167
YWHAZ	Tyrosin 3-monooxygenase/tryptophan, 5-monooxygenase activation protein, zeta	26.67	0.168
18S	RNA, 18S ribosomal	9.72	0.191
GUSB	Glucuronidase, beta	26.06	0.193
POP4	Processing of precursor 4, ribonuclease P/MRP subunit	25.97	0.203
IPO8	Importin 8	27.49	0.222
EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 alpha	26.80	0.227
HMBS	Hydroxymethylbilane synthase	25.51	0.231
RPL30	Ribosomal protein L30	24.07	0.232
HPRT1	Hypoxanthine phosphoribosyltransferase 1	26.29	0.236
ABL1	c-abl oncogene 1, non-receptor tyrosine kinase	25.91	0.247
CDKN1B	Cyclin-dependent kinase inhibitor 1B	24.97	0.270
PGK1	Phosphoglycerate kinase 1	23.80	0.271
TFRC	Transferrin receptor	26.40	0.292
PPIA	Peptidylprolyl isomerase A	20.57	0.310
PSMC4	Proteasome (prosome, macropain) 26S subunit, ATPase, 4	24.78	0.327
MT-ATP6	Mitochondrially encoded ATP synthase 6	19.27	0.336
POLR2A	Polymerase (RNA) II (DNA directed) polypeptide A	26.31	0.371
MRPL19	Mitochondrial ribosomal protein L19	27.69	0.420
RPL37A	Ribosomal protein L37a	20.62	0.442
GADD45A	Growth arrest and DNA-damage-inducible, alpha	31.50	0.570
ACTB	Actin beta	25.84	0.575
PES1	Pescadillo homolog 1, containing BRCT domain	27.05	0.783

shows high stability of our qPCR experiment (Fig. 1). The expression of *EPO* and *STAT5A* in SH-SY5Y cells and *Epo* in PC12 cells was too low for further analysis.

3.4. Evaluation of expression stability

Evaluation of expression stability of four GOI in SH-SY5Y cells, six GOI in PC12 cells and three HKG in both cell lines was performed with three independent software programs. The geNorm uses a pair-wise comparison; co-regulated genes belonging to the same system with a similar expression profile get a high score. It ranks potential reference genes according to their expression stability. The software calculates the normalization factor (NF), which can be directly used for normalization of qPCR data. NormFinder ranks the genes on a model based approach and calculates a stability value

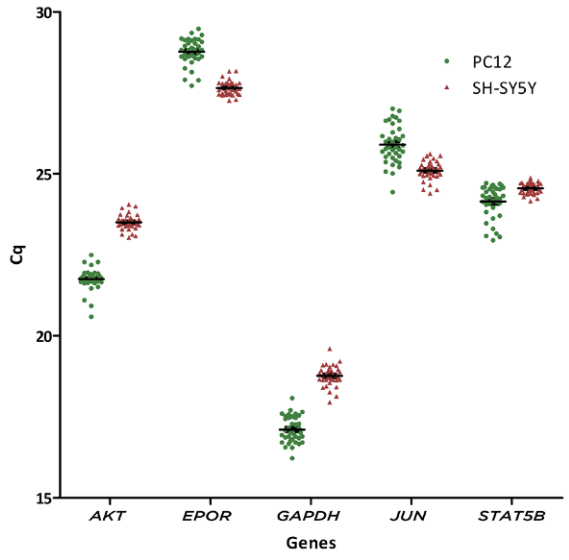

Figure 1. Gene expression level.

combining estimates of intra- and inter-group values. BestKeeper does pair wise correlation analysis; it uses a geometric mean of each gene's crossing point (CP) value.

According to geNorm, the 3 most stable genes with high average expression stability in SH-SY5Y samples are *RPLP*, *STAT5B* and *EPOR*, while in PC12 samples they are *Akt1*, *Stat5a* and *CycA* (Figs. 2A, 2E). Among these genes, we can find HKG as well as GOI. GeNorm also evaluates the number of reference genes that should be used for normalization. The value V (pairwise variations) in the SH-SY5Y samples is the lowest in V2/3 and rises in V3/4 when an additional fourth gene is selected (Fig. 2B), indicating that not more than 3 genes should be used for normalization. However, based on the low difference in average expression stability (M) between selected 3 genes (Fig. 2A), the normalization factor could be equally calculated from the 2 (*RPLP*, *STAT5B*) or 3 most stable genes (*RPLP*, *STAT5B*, *EPOR*). Pairwise variations in PC12 samples dramatically drop between V2/3 and V3/4 indicating that 4 genes would be the appropriate number (*Akt1*, *Stat5a*, *CycA and Ywhaz)* (Fig. 2F). However based on the lower difference in average expression stability (M) between the 3rd and 4th genes (Fig. 2E), the normalization factor could be equally calculated from the 3 most stable genes (*Akt1*, *Stat5a and CycA).*

The average expression stability was also performed with geNorm separately on untreated and Epo treated (5 IU mL^{-1}) samples with the aim of determining if Epo treatment has any influence on the selection of the most stably expressed genes. Results show that there is no major difference in the selection of the most stable genes; the only difference is in the order of the proposed genes (data not shown). In SH-SY5Y untreated samples, *EPOR*, *RPLP* and *STAT5B* were proposed, while in Epo treated samples, *EPOR*, *STAT5B* and *RPLP* were proposed. In PC12 untreated samples, *Akt*, *Stat5a* and *Ywhaz* were selected, while in Epo treated samples *Akt*, *Ywhaz* and *Stat5a* were selected.

Ranking of genes by NormFinder is made on the stability value calculated from the combined estimate of intra- and inter-group values. Most stable are genes with a low stability value. In SH-SY5Y cells, these are *STAT5B*, *RPLP* and *EPOR,* while in PC12 cells, they are *Akt1*, *Stat5a* and *Ywhaz* (Fig. 2C, 2G).

The last software or program used for reference gene determination was BestKeeper. This software uses average CP values for the calculation of the most stable genes used for normalization. Low SD of the CP values means that the gene could be used as a reference gene. In SH-SY5Y cells, this is *RPLP* followed by *STAT5B* and *EPOR* (Fig. 2D). While in PC12 cells, *CycA*, *Ywhaz*, *Stat5a* and *Akt* have almost the same SD of CP (Fig. 2H). Therefore, all three genes in SH-SY5Y and four genes in PC12 are suitable for normalization.

3.5. Reference gene selection

Real-time quantitative PCR is an accurate and reproducible method enabling evaluation of erythropoietin treatment on the level of gene expression. However, correct biological interpretation of qPCR data requires an appropriate normalization procedure, including selection of stable reference genes. Since Epo acts through several signaling pathways and modulates expression of several genes [34], we consider it important to note that some housekeeping genes (HKGs) may also be altered under Epo treatment. Previous results on Epo treated human breast cancer cell lines suggested that common HKGs are not stably expressed under these conditions (personal communications S. Berne). Therefore identification of HKGs under Epo treatment was performed with the aim of defining genes appropriate for normalization.

In this study, we analyzed the expression stability of several HKGs in Epo treated human SH-SY5Y cells with the aim of finding suitable reference genes for qPCR data analysis. Additionally, we analyzed the expression stability of the most commonly used HKGs and selected genes of interest (GOI), involved in Epo signal transduction, in human SH-SY5Y cells and rat PC12 cells during 6 weeks of rHuEpo treatment.

In order to find the most appropriate HKGs, experiments with TaqMan human Express Endogenous Control Plate were done on SH-SY5Y pooled samples. The experiments predict that among 32 genes, *UBC*,

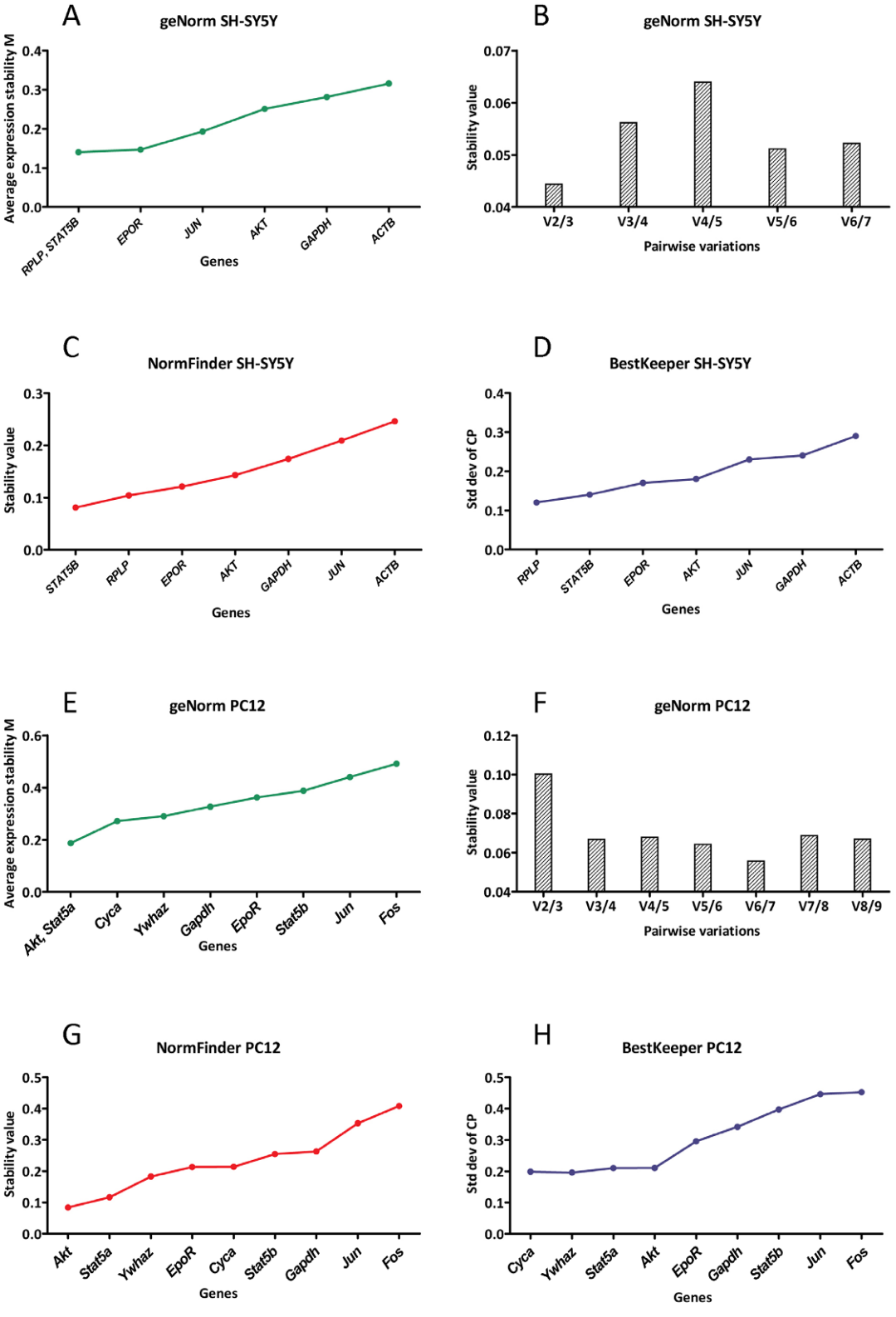

Figure 2. GeNorm, NormFinder and BestKeeper analysis of expression stability.

ELF1 and *RPLP* are the most stably expressed genes in Epo treated samples (Table 4). According to the literature, *RPLP*, *GAPDH* and *ACTB* are among the most commonly used endogenous controls in SH-SY5Y, all three also being present on TaqMan human Express Endogenous Control Plate [20,31,32]. While *RPLP* was confirmed to be an adequate gene for data normalization, *GAPDH* and *ACTB* expression fluctuates and, therefore, should not be used as reference genes under this treatment condition.

To confirm the results obtained on the pooled samples on TaqMan plates, the expression of *RPLP, GAPDH* and *ACTB* was re-evaluated with qPCR on all SH-SY5Y samples together with six genes involved in Epo signal transduction (Table 2). In PC12 cells, the expression of the three most commonly used genes, according to the literature (*Gapdh*, *CycA* and *Ywhaz*) [20,31,32], and seven genes involved in Epo signal transduction (Table 3) were evaluated. When comparing the data obtained with GeNorm, NormFinder and BestKeeper, we conclude that all three programs gave very similar results. We suggest the use of geNorm software, as it provides information on the stability of genes as well as information on the number of appropriate genes, to be used for normalization.

In the case of SH-SY5Y cells, *RPLP*, *STAT5B* and *EPOR* genes were always selected as the most suitable for normalization. According to geNorm the normalization to 2 most stable genes (*RPLP and STAT5B)* is also sufficient. Interestingly, only *RPLP* is a HKG, while *STAT5B* and *EPOR* are GOI. The selection of *RPLP* confirms the data obtained by TaqMan. This result also indicates that chronic rHuEpo treatment does not affect the expression level of two genes involved in Epo signal transduction, *STAT5B* and *EPOR*.

Similar results were obtained for PC12 cells. However, we selected the HKGs using data from the available literature (*CycA*, *Ywhaz* and *Gapdh)* [20,31,32]. All three programs suggested *Stat5a* and *Ywhaz* as a second and/or third choice. The difference in the results lay in the calculation of the most stable gene. In the case of geNorm and NormFinder, *Akt1* was selected as the most stable, but in the case of BestKeeper it was *CycA*. According to geNorm, 3 most stable genes (*Akt1*, *Stat5a* and *CycA)* would be the appropriate number for normalization.

3.6. Normalization with geNorm and GAPDH factor

In order to show the effect of the normalization procedure on data interpretation, we analyzed two genes (*JUN* in SH-SY5Y, *EpoR* in PC12) with two separate normalization factors (Fig. 3). Firstly, normalization was performed with a normalization factor calculated by geNorm from the three most stable genes (SH-SY5Y: *RPLP*, *STAT5B*, *EPOR;* PC12: *Akt1*, *Stat5a, CycA*). The results show stable relative gene expression of *JUN* under chronic Epo treatment (Fig. 3A), while the expression of *EpoR* appears to be increased during the first two weeks of Epo treatment (Fig. 3C). The second normalization was performed with *GAPDH*, one of the most often selected normalization factors. Normalization to *GAPDH* results in a very unstable relative gene expression of both, *JUN* and *EpoR* during the entire treatment period (Figs. 3B, 3D).

3.7. Influence of normalization factor on data interpretation

Normalization is the crucial step in qPCR data analysis; this is shown with two distinct approaches to the *JUN* and *EpoR* expression profile interpretation (Fig. 3). If an inappropriate normalization approach is selected, results and biological interpretation can be misleading. When normalization is performed with the three genes, determined by geNorm software to be the most stably expressed genes, the expression level of *JUN* in SH-SY5Y cells is not influenced by Epo treatment, while the expression of *EpoR* in PC-12 cells seems to be affected only at the beginning of treatment.

However, when normalization is performed with only *GAPDH*, the expression level varies during the whole period of treatment. In addition, compared to other genes, the expression level of *GAPDH* in both cell lines is very high (Cq under 20, Fig. 1), which may additionally lower the stability of results. Zhou and colleagues have also shown that G*apdh* is not recommended as a normalization factor when PC12 cells are treated with various pharmacological agents [33]. If the normalization of *JUN* and *EpoR* is performed only on *GAPDH* (Figs. 3B, 3D), the results lead to an incorrect interpretation that these two genes are differentially expressed under rHuEpo treatment. It was previously shown that the expression of several genes varies during rHuEpo treatment [34-37]. However, the interpretation of these data may need to be re-evaluated as quantification was performed with a single HKG (*GAPDH*, *ACTB*). Choosing the appropriate number of HKGs and the suitable software for evaluation and normalization are crucial steps adequate qPCR data interpretation.

4. Conclusions

We determined that the most commonly used reference gene, *GAPDH,* is not the most suitable gene for qPCR normalization of chronic rHuEpo treated SH-SY5Y and

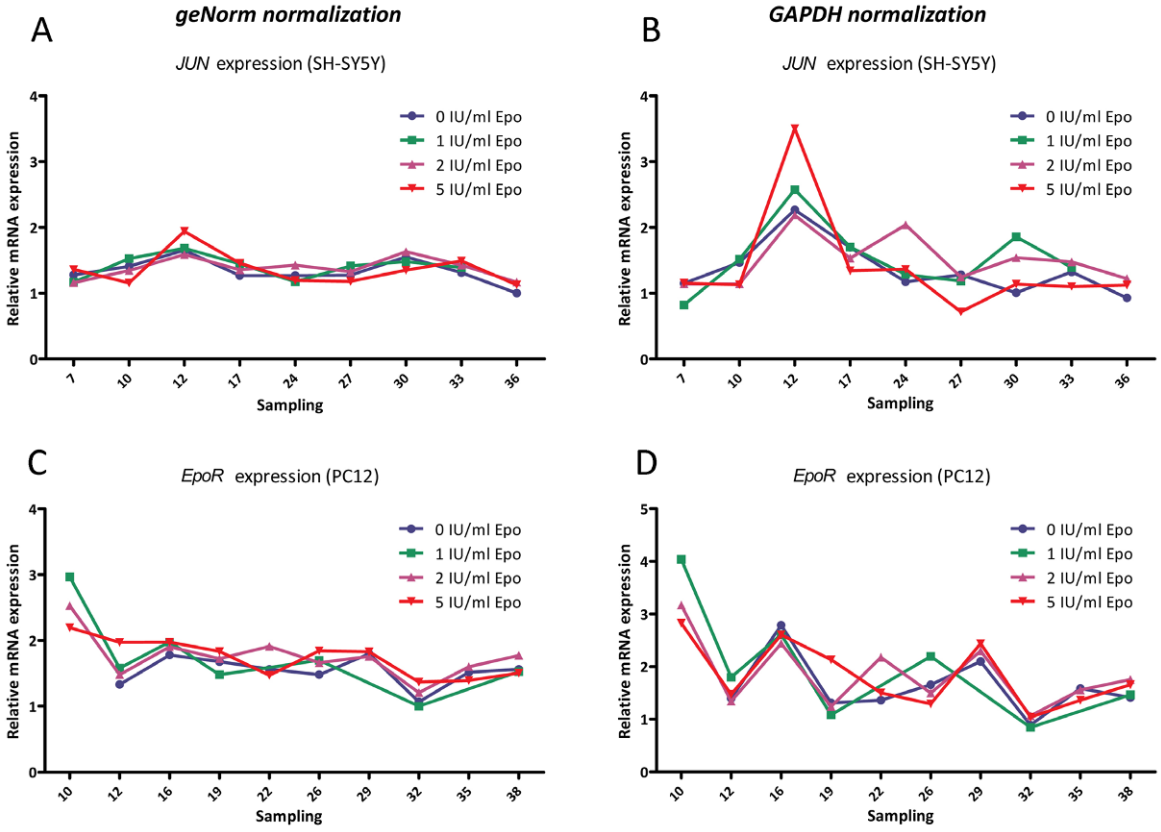

Figure 3. Normalization with geNorm and *GAPDH* factor.

PC12 cells. This was demonstrated using TaqMan human Express Endogenous Control Plate and qPCR analysis of selected genes. Epo alters the expression of several genes, including HKGs. It is very important that a careful selection of adequate reference genes is performed before qPCR normalization analysis. We propose *RPLP, UBC* and *ELF1* in SH-SY5Y cells and *CycA* and *Ywhaz* in PC12 cells as reference genes suitable for normalization during chronic rHuEpo treatment.

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