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Validation of suitable housekeeping genes for 3T3-L1 derived adipocytes cultured in obesity mimicking conditions and RAW 264.7 macrophage cells lines in hypoxic and normoxic conditions

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A B S T R A C T

Reverse transcription quantitative-polymerase chain reaction (RT-qPCR) is the most commonly used method for gene expression analysis, and reliable results depend on proper normalization by stable reference genes. Adipose tissue hypoxia is the new concept in understanding the major disorders associated with obesity and may facilitate cellular mechanisms associated with obesity like chronic inflammation, macrophage infiltration, adiponectin reduction, leptinelevation, adipocyte death, ER stress and mitochondrial dysfunction. The hypoxic conditions in the obese adipose tissue lead to differential expression of genes in the resident cells of adipose tissues including macrophages andhence reliable normalization of RT-qPCR data becomes crucial. In this study, the expression stability of 4 most commonly used candidate reference genes, ACTB, B2M, HMBS and 18SrRNA were evaluated in murine adipocytesand macrophage cell lines, in presence of high free fatty acids, under hypoxia and normoxia conditions. The results were analyzed by manually calculating fold difference using $2-\Delta\Delta Ct$ and validated by using two algorithms, NormFinder, and Bestkeeper. Our results showed HMBS and B2M as the most stably expressed genes in both cell lines under obesity mimicking conditions (in presence of high amount of FFAs and hypoxia). By contrast, ACTB and 18S rRNA were the least stable genes under the tested conditions. Furthermore, the expression levels of FABP4 andPPAR-γ (for adipocytes) and MCP-1 and IL-6 (for macrophages) were profiled in these cells to validate the selected reference genes. Our data provides a basis for gene expression analysis in future studies related to inflammation in hypoxia related obesity pathogenesis.

1. Introduction

Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) has become the most prevalent and powerful technique to analyze gene expression and quantification to understand the complex cell signaling networks. There are two methods of analyzing quantitative gene expression - absolute and relative quantification methods (Chen et al., 2005; Schmittgen and Livak, 2008). Often, it is more important to understand the changes in the expression level of a gene than the actual copy number. However, relative quantification of genes, requires robust endogenous control genes as references (Bustin et al., 2013; Huggett et al., 2005; Wong and Medrano, 2005). The ideal reference genes should be expressed at constant levels regardless of experimental conditions, cell types, tissues, developmental stages or stress treatments (Wong and Medrano, 2005). Nevertheless, increasing evidence suggests that the expression of reference genes often varies considerably under different experimental conditions (Suzuki et al., 2000). Thus, identification of reliable reference genes is a pre-requisite for qRT-PCR experiments.

Obesity, an excess of adipose tissue, is attributed to hypertrophy and hyperplasia of adipocytes (Brook et al., 1972; Helmlinger et al., 1997). Due to hypertrophy, adipocytes endure less than adequate oxygen supply (hypoxia) as the diffusion limit of O_2 is crossed. Reports suggest that while healthy adipocyte expansion may occur with appropriate angiogenesis, pathological expansion results in adipose tissue hypoxia (Sun et al., 2011). Hypertrophy of adipocytes and influx of immune cells like monocytes and macrophages in adipose tissues during obesity promotes the development of chronic low-grade inflammation eventually leading to insulin resistance and type 2 diabetes (Shoelson et al., 2007). In the presence of hypoxia, cells respond by coordinated expression of a wide variety of genes that regulate glycolysis, angiogenesis, erythropoiesis, and inflammation among others. This ensures adaptation to their altered hypoxic milieu. To understand the pathogenesis of hypoxic obese adipose tissue, it's necessary and important to analyze the gene expression changes at the transcriptional level. It has been reported that in adipose tissue some of the commonly used reference genes like glyceraldehyde-3-phosphate dehydrogenase (GAPDH), can be regulated in presence of insulin and in obesity conditions (Alexander-Bridges et al., 1992; Dugail et al., 1988; Matthae et al., 2013). Recent (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) MIQE guidelines make it mandatory to validate the gene expression analysis data with more than one

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stable endogenous controls. Hence this study was done to identify endogenous controls with stable gene expression levels in hypoxic and normoxic conditions. In this study, we analyzed four most commonly used reference genes (Beta Actin - ACTB, Beta 2 Microglobulin - B2M, Hydroxymethylbilane Synthase - HMBS and 18S ribosomal RNA - 18S rRNA) for their suitability as endogenous controls in 3T3-L1 (murine pre-adipocytes) and RAW 264.7 (murine macrophages) cell lines. The study was conducted in obesity mimicking conditions of high free fatty acids concentration coupled with hypoxia. The results were validated using software NormFinder, and BestKeeper (Andersen et al., 2004; Pfaffl et al., 2004; Sridharan et al., 2018; Vandesompele et al., 2002). This work provides a basis for the selection of reference genes for similar future studies associated with the inflammation in hypoxia linked obesity.

2. Materials and methods

2.1. Materials used

Dulbecco's Modified Eagle's Medium (DMEM), Free Fatty Acids (FFAs) such as, Palmitic acid (PA)(P5585), Oleic acid (OA) (O1383), Stearic acid (SA) (S4751) and Myristric acid (MA) (M3128), TRI reagent, ethanol, isopropanol, Endotoxin Free Bovine Serum Albumin (BSA), Dexamethasone, isobutyl-1-methylxanthine (IBMX), Insulin and Primers used were synthesized Sigma, USA. Fetal Bovine Serum (FBS) Gibco, USA. Penicillin/ streptomycin (Penstrep) Invitrogen, USA.

2.2. Free fatty acids (FFAs) conjugation with BSA

2.2.1. Stock preparation

PA, OA, SA, and MA were reconstituted in absolute ethanol to make the stock concentration at 200 mM, filtered and stored in dark at −20 °C till further use.

2.2.2. Working dilutions

The FFA stock solutions were diluted with 5 N NaOH and 150 mM NaCl as per protocol (L'homme et al., 2013) by heating at 60 °C in shaking water bath for 2 h. This was followed by addition of ice-cold 10% endotoxin-free BSA solution to the FFA solution and further incubation at 50 °C in shaking water bath till it dissolved completely (approx. 2 h). The pH was adjusted to 7.0 and the solutions were stored at −20 °C till further use.

2.3. Cell culture

3T3-L1 and RAW 264.7 cell lines were cultured in complete DMEM containing 10% FBS and 50I/U Penstrep at 37 °C in a humidified atmosphere containing 5% CO_2 and 21% O_2 (CO_2 incubator- ESCO, USA). Once the cells have reached 80% confluency, the cells were trypsinized with 0.25% trypsin-EDTA and centrifuged at 2000 rpm for 10 min. The cells from pellet were used for the further experiments mentioned below.

2.4. Differentiation and maturation of 3T3-L1 pre-adipocytes to adipocytes

3T3-L1 cells were cultured under the normoxic condition as mentioned in Section 2.3. Once the culture flasks were ~80% confluent, (day 0) cells were given differentiation medium (complete DMEM supplemented + 10 µg/mL insulin + 1 µM dexamethasone + 0.5 mM IBMX) for 2 days. On day 3, cells were replenished with maturation medium (complete DMEM medium + 10 µg/mL insulin) and incubated for the next 48 h (day 5) for complete maturation. On day 6, maturation media + 500 µM of different FFAs were added to each plate and incubated for 24 and 48 h (matured adipocytes).

For experiments in hypoxia, adipocytes were grown under normoxia till day 6. Cells were replenished with maturation media + 500 µM of different FFAs and then transferred to hypoxia (1.5% O_2) enabled CO_2 incubator at 37 °C in a humidified atmosphere containing 5% CO_2 (Heracell Vios 160i CO_2 incubator - Thermo Scientific, USA) for 24 and 48 h. Early exposure of adipocytes to hypoxic conditions inhibited their differentiation and maturation.

2.5. Culture of RAW 264.7 cells under a differential oxygen tension

RAW 264.7 cells were cultured under 37 °C in a humidified atmosphere containing 5% CO_2 and 21% O_2 (Normoxia). Once the cells have reached 80% confluency, the cells were washed twice using (Phosphate Buffered Saline) PBS. Further, RAW 264.7 cells were scraped and centrifuged at 900 rpm for 5 min. Cells were then cultured at 1.5% oxygen tension in the hypoxia enabled incubator.

2.6. RNA isolation and cDNA synthesis

Post incubation under hypoxic and normoxic conditions, total RNA was isolated from differentiated and matured adipocytes as well as RAW264.7 cells by standard protocol using TRI reagent and the total RNA was quantified using Nanodrop (NanoDrop™ 2000/2000c Spectrophotometers Thermo Scientific, USA). 1 µg of total RNA was converted to cDNA using a cDNA conversion kit. (Verso cDNA kit Thermo Scientific, USA).

2.7. Endogenous controls

For this study, four most commonly used endogenous genes were evaluated for stable expression under normoxic and hypoxic conditions. Each gene has their independent role in the cellular maintenance and the regulation of their expression is presumed not to be directly related. The genes and the primer pairs used for this study are listed in (Table 1).

2.8. Quantitative Real-Time PCR

The qPCR was performed on Real-Time system (CFX 96 Real-time PCR machine and C1000 PCR machine Bio-Rad, USA) using SYBR Green master mix (SYBR® Premix Ex Taq™ II (Tli RNase H Plus) TAKARA, Japan), gene-specific primers (10 pmol, Tables 1, 2) and 10 ng of cDNA template. Amplification was carried out in 20 µL reaction volume under following reaction conditions: Initial denaturation step at 95 ℃ for the 30 min, 40 cycles of 95 ℃ for 5 s, Specific Annealing

Table 1
Primer sequences and product size of housekeeping genes.

S.No	Gene	Primer Sequence	Tm	Product length
1	Murine β-Actin	F: 5' TGAGAGGGAAATCGTGCGTGACAT3' R: 5' ACCGCTCGTTGCCAATAGTGATGA 3'	58 °C	152 bp
2	Murine B2M	F: 5'TTCTGGTGCTTGTCTCACTGA3' R: 5'CAGTATGTTCGGCTTCCCATTC 3'	60 °C	104 bp
3	Murine18S rRNA	F: 5'GCAATTATTCCCCATGAACG3' R: 5' GGCCTCACTAAACCATCCAA3'	62 °C	123 bp
4	Murine HMBS	F: 5'ATGAGGGTGATTCGAGTGGG3' R: 5'TTGTCTCCCGTGGTGGACATA 3'	60 °C	134 bp
5	Murine FABP4	F: 5'TTTCCTTCAAACTGGGCGTG3' R: 5'GGTCGACTTTCCATCCCACT3'	60 °C	164 bp
6	Murine MCP-1	F: 5'GAGAGCCAGACGGGAGGAAG3' R: 5'TGAATGAGTAGCAGCAGGTGAG 3'	60 °C	197 bp
7	Murine IL-6	F: 5' CCGCTATGAAGTTCCTCTCTGC 3' R: 5' ATCCTCTGTGAAGTCTCCTCTCC 3'	60 °C	119 bp

*Efficiency percentage ranges between 90% and 110% for all the primer pairs.

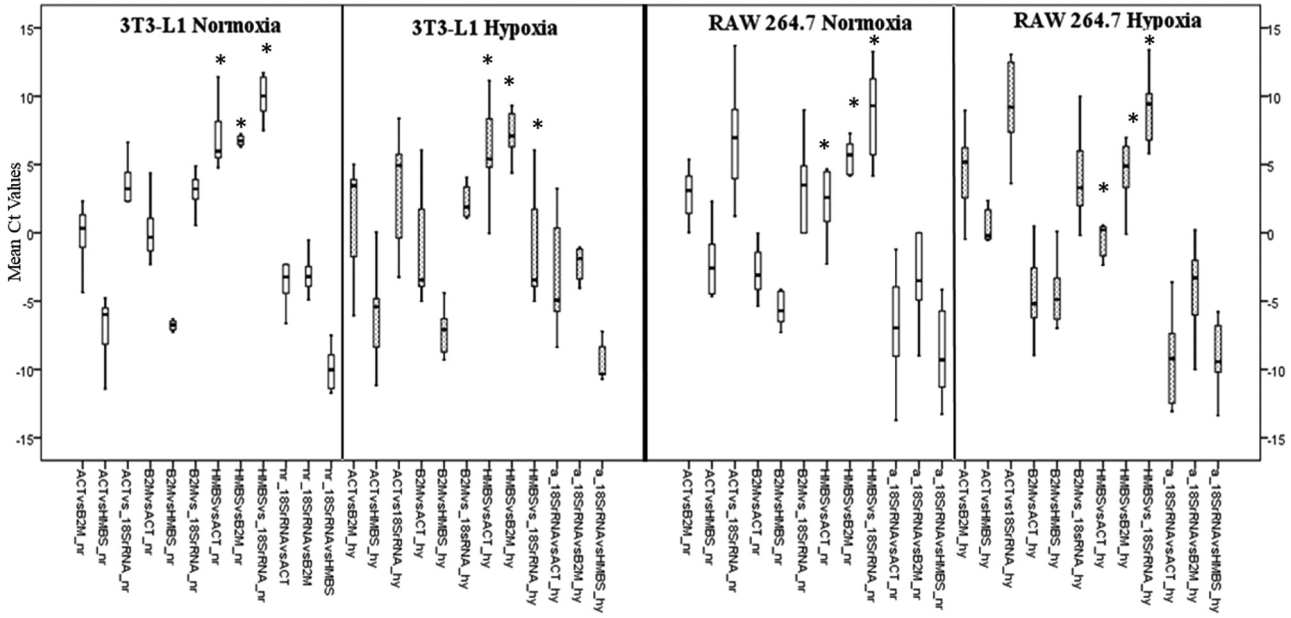

Fig. 1. The range of Ct values for ACTB, B2M, HMBS, and 18S rRNA were evaluated in 3T3-L1 under normoxia (21% O_2), 3T3-L1 hypoxia (1.5% O_2) and RAW 264.7 cells normoxia (21% O_2), RAW 264.7 hypoxia (1.5% O_2). Median Ct values are shown as lines, 25th percentile to 75th percentile (boxes) and ranges (whiskers) for both murine adipocyte and macrophage cell lines The Ct variation for ACTB and 18S rRNA were statistically significant $p \leq 0.01$. (*- shows the least variation when compared to other genes).

Table 2
Expression variability comparisons for four endogenous controls.

Gene Combination	3T3-L1 Normoxia		3T3-L1 Hypoxia		RAW264.7 Normoxia		RAW264.7 Hypoxia	
	Mean ΔCt ± SD	Average SD	Mean ΔCt ± SD	Average SD	Mean ΔCt ± SD	Average SD	Mean ΔCt ± SD	Average SD
Actin vs B2M	− 0.331 ± 2.28	2.65	1.065 ± 3.90	3.79	2.99 ± 2.93	2.9	4.54 ± 2.73	3
Actin vs HMBS	7.109 ± 2.49		− 6.139 ± 3.41		2.19 ± 2.38		0.11 ± 2.04	
Actin vs 18 S rRNA	2.798 ± 3.19		3.345 ± 4.06		5.42 ± 3.65		8.54 ± 4.23	
B2M vs Actin	0.331 ± 2.28	1.4	− 1.065 ± 3.90	2.27	− 2.99 ± 2.93	2.21	− 4.54 ± 2.73	2.81
B2M vs HMBS	− 6.778 ± 0.74		− 7.204 ± 1.71		− 5.58 ± 1.07		− 4.43 ± 2.46	
B2M vs 18 S rRNA	3.129 ± 1.19		2.28 ± 1.19		2.13 ± 2.35		3.99 ± 3.24	
HMBS vs Actin	7.109 ± 2.49	1.53	6.139 ± 3.41	3.01	2.59 ± 2.74	2.14	− 0.11 ± 2.04	2.73
HMBS vs B2M	6.778 ± 0.74		7.204 ± 1.71		5.58 ± 1.07		4.43 ± 2.46	
HMBS vs 18S rRNA	9.907 ± 1.36		− 1.065 ± 3.90		7.72 ± 2.59		8.433.68	
18S rRNA vs Actin	− 2.798 ± 3.19	1.91	− 3.345 ± 4.06	2.18	− 5.06 ± 3.82	2.9	− 8.54 ± 4.23	3.72
18S rRNA vs B2M	− 3.129 ± 1.19		− 2.28 ± 1.19		− 2.13 ± 2.30		− 3.99 ± 3.24	
18S rRNA vs HMBS	− 9.907 ± 1.36		− 9.484 ± 1.29		− 7.72 ± 2.59		− 8.43 ± 3.68	

temperature for different primer pairs for 30 s. Melt curve analysis was done at 65–95 ℃ for 5 s with 0.5 ℃ increment and absence of primer dimers was confirmed. All reactions were run in duplicates.

2.9. Data analysis

RT-qPCR data were analyzed by calculating the fold difference independently for each housekeeping gene. Number of cycles via fluorescence signal cross is defined as Cycle threshold (Ct) which is inversely proportional to the quantity of template present in the reaction. Ct values of test genes under differential oxygen concentration were compared against each housekeeping gene and the fold difference was calculated using the equation:

$$FoldDifference = 2 - \Delta\Delta Ct, where \Delta\Delta Ct = \Delta Cthypoxia - \Delta ctcontrol.$$

Stability value for each gene was determined by calculating Ct covariance percentage (CtCV%) as: CtCV% = Standard Deviation(SD)/ Mean * 100. The results were validated using the $2^{-\Delta\Delta Ct}$ method and NormFinder and BestKeeper software.

2.10. Data analysis using NormFinder and BestKeeper software

Best combination of housekeeping genes was determined using manual calculations and validated using software's to determine the best endogenous controls. NormFinder (v0.953) and the BestKeeper (v1.0) are freely available software and their add-ins can be incorporated into Microsoft Excel. NormFinder and BestKeeper software's utilize inbuilt calculations to produce the stability value (M) and SD (Standard Deviation) and CV (Coefficient of Variance) individually for NormFinder, showing a lower M value indicates better steadiness. The BestKeeper genes with the least variations are shown to be generally firm. According to BestKeeper genes, SD values more than 1 are indicated as conflicting. The BestKeeper Index (BI) from the stable genes are expressed and analyzed by calculating Geometric Mean (GM) of the candidate genes. Crossing Point (CP) values (Eq. (1)) indicates the total number of housekeeping genes included.

$$BestKeeperIndex(BI) = z\sqrt{CP1 \times CP2 \times CP3 \times ..... \times CPz}. \quad (1)$$

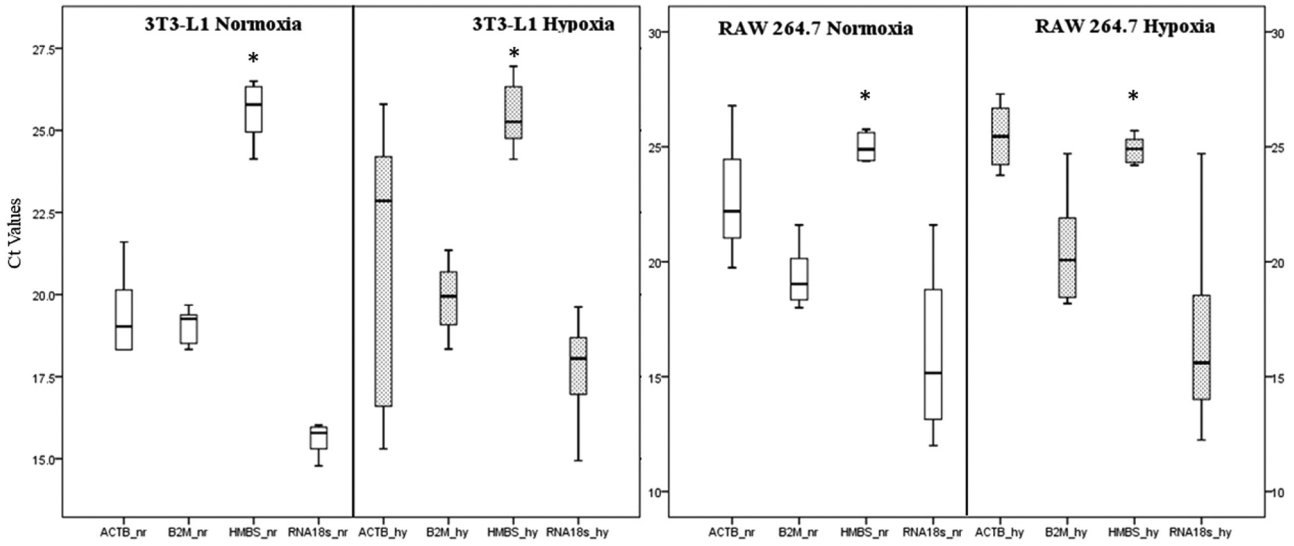

Fig. 2. ΔCt approach to select endogenous controls. ΔCt variables of candidate gene comparisons are shown as medians (lines), 25th percentile to 75th percentile (boxes) and ranges (whiskers) for 3T3-L1 (21% O_2), 3T3-L1 (1.5% O_2) and RAW 264.7 cells normoxia (21% O_2), RAW 264.7 hypoxia (1.5% O_2) cells murine adipocyte and macrophage cell line. The figure shows the evaluation of the complete set of housekeeping genes in all combination. The significance of variation was calculated using one-way ANOVA and difference was considered to be significant at $p \leq 0.01$. (*- shows the least variation when compared to other genes).

Table 3
Ct Coefficient of Variation percentage (CtCV%) for candidate endogenous controls.

Housekeeping genes	3T3-L1 Normoxia			3T3-L1 Hypoxia			RAW264.7 Normoxia			RAW264.7 Hypoxia		
	Ct Mean	SD	CtCV%	Ct Mean	SD	CtCV%	Ct Mean	SD	CtCV%	Ct Mean	SD	CtCV%
Actin	18.73	2.65	12.66	21.01	3.79	19.4	22.82	2.9	10.37	25.01	3	13.96
B2M	19.06	1.4	**2.53**	19.94	2.27	**2.36**	19.43	2.12	**4.03**	20.46	2.81	**4.75**
HMBS	25.14	1.53	**2.18**	25.75	2.73	**1.97**	25.01	2.14	**2.5**	24.9	2.73	**2.3**
18SrRNA	15.9	1.91	6.91	17.67	2.18	9.27	17.29	2.93	16.64	16.47	3.72	22.32

Table 4
Stability values (M) of housekeeping genes determined using NormFinder.

S.No	Genes	Stability value (M) 3T3-L1 Normoxia	*Rank Based on M value	Stability value (M) 3T3-L1 Hypoxia	*Rank Based on M value
1	ACTIN	1.037	4	ND	NA
2	B2M	0.154	2	0.411	2
3	HMBS	0.121	1	0.429	1
4	18SrRNA	0.517	3	0.367	3
S.No	**Genes**	**Stability value(M) RAW264.7 Normoxia**	***Rank Based on M value**	**Stability value(M) RAW264.7 Hypoxia**	***Rank Based on M value**
1	ACTIN	1.036	4	ND	NA
2	B2M	0.151	2	0.409	2
3	HMBS	0.517	1	0.370	1
4	18SrRNA	0.116	3	0.429	3
Best combination of genes predicted by NormFinder					
Cell lines		**Best combination of genes**	**M Value**		
3T3-L1 Normoxia		HMBS and B2M	0.099		
3T3-L1 Hypoxia		HMBS and B2M	0.284		
RAW264.7 Normoxia		HMBS and B2M	0.097		
RAW264.7 Hypoxia		HMBS and B2M	0.286		

ND- Not Defined.
NA- Not Applicable.
*- Ranks of increasing order indicates the most stable gene to the least stable gene.

2.11. Statistical Analysis

All the experiments were run in duplicates. Statistical values were expressed as mean ± standard deviation (SD). Statistical significance was defined by two-tailed *t*-test and the significance of variance was calculated by one-way ANOVA $p \leq 0.01$ was considered to be significant.

3. Results

3.1. Relative stability of candidate housekeeping genes for macrophages and adipocytes exposed to high concentrations of FFAs, under a differential oxygen tension

In this study, we analyzed the expression pattern of the four most commonly used housekeeping genes using RT-qPCR. Fig. 1 shows all

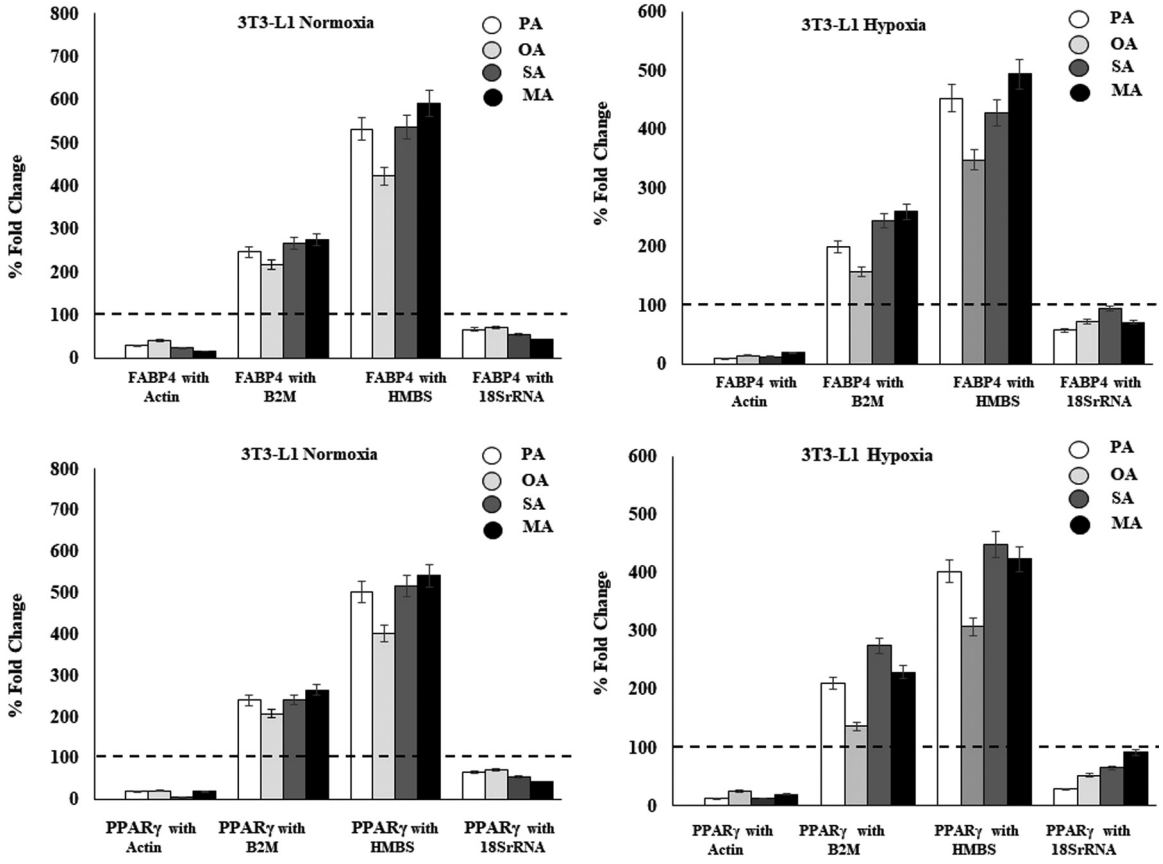

Fig. 3. Gene expression varies with the choice of endogenous control used. In 3T3-L1 cells subjected to different free fatty acids, under differential oxygen concentrations. FABP4 and PPARγ expression were up-regulated when normalized with HMBS and B2M while ambiguous results were obtained when normalized with ACTB and 18S rRNA ($p \leq 0.01$). (dotted lines represent the baseline value).

tested housekeeping genes in RAW264.7 macrophage cell line and 3T3-L1 adipocyte exposed to high FFAs under normoxic and hypoxic conditions. The box plots show that the Ct values varied widely for ACTB and 18 S rRNA under differential oxygen concentration indicating their unstable expression profile under hypoxic and normoxic conditions. Whereas, HMBS and B2M gene expression patterns in differential oxygen tension resulted in narrow box plots, indicating that these two genes had comparatively stable expression under both conditions. Stability of gene expression was evaluated by calculating standard deviation (Table 2) from the ΔCt values of all the genes separately as well in all possible combinations (Fig. 2). The lower deviation was observed when HMBS and B2M were used as housekeeping genes for ACTB and 18S rRNA expression analysis in 3T3-L1 cells under normoxia (Average SD: 2.65 and 1.91 respectively) as compared to hypoxia (Average SD: 3.79 and 2.18 respectively). Similar results were obtained when the study was conducted using RAW264.7 cells under normoxia (Average SD: 2.9 and 2.91 respectively) and hypoxia (Average SD: 3 and 3.72 respectively).

To evaluate the HMBS and B2M expression, using ACTB and 18S rRNA as endogenous control a significant increase in the SD values of 3T3-L1 cells was observed under normoxic (Average SD: 1.53 and 1.40 respectively) and hypoxic conditions (Average SD: 3.01 and 2.27 respectively). However, a similar pattern was also observed in RAW 264.7 cells under normoxic (Average SD: 2.14 and 2.21 respectively) and hypoxic conditions (Average SD: 2.73 and 2.81 respectively). Thus the relative stability of candidate housekeeping gene (ACTB and 18S rRNA) expression in adipocytes and macrophages appeared to be significantly modulated by oxygen tension ($p \leq 0.01$).

In addition, the coefficients of variations for Ct values (CtCV%) were calculated for each housekeeping gene (Table 3). In adipocytes, CtCV% varied widely for ACTB (12.6% at normoxia, 19.4% in hypoxia) and 18S rRNA (6.9% at normoxia, 9.2% at hypoxia). Similar high variations were observed for ACTB (10.6% at normoxia, 13.9% in hypoxia) and 18S rRNA (16.6% at normoxia, 22.2% at hypoxia) in RAW 264.7 cells. Interestingly, HMBS and B2M were stably expressed in adipocytes and macrophage cell lines as observed by their lower CtCV% (Table 3).

3.2. Validation of best housekeeping genes using NormFinder and Bestkeeper

The data generated were analyzed using Microsoft Excel and the findings were validated using the software such as NormFinder and BestKeeper. Our results from manual calculations of ΔCt correlated with those from NormFinder and Bestkeeper softwares. HMBS was found to be the most stable housekeeping gene under differential oxygen tension in adipocytes (M value:0.099 and 0.284, SD:0.68 and 0.53, CV:3.47 and 2.08) $p \leq 0.01$ and macrophages (M value:0.097 and 0.286, SD: 0.71 and 0.63, CV: of 3.54 and 2.42) $p \leq 0.01$ (Table 5). However, the combination of HMBS and B2M were found to be the best housekeeping genes for studying gene expression analysis under varying oxygen concentrations. The ranking of housekeeping genes based on the M value and the best combination of housekeeping gene are given in Table 4.

3.3. Choice of housekeeping genes affects the relative quantification of the genes of interest leading to erroneous results

RT-qPCR was performed on RNAs extracted from RAW 264.7 cells and 3T3-L1 cells grown and differentiated in the presence of high concentrations of FFAs under normoxic and hypoxic conditions. From

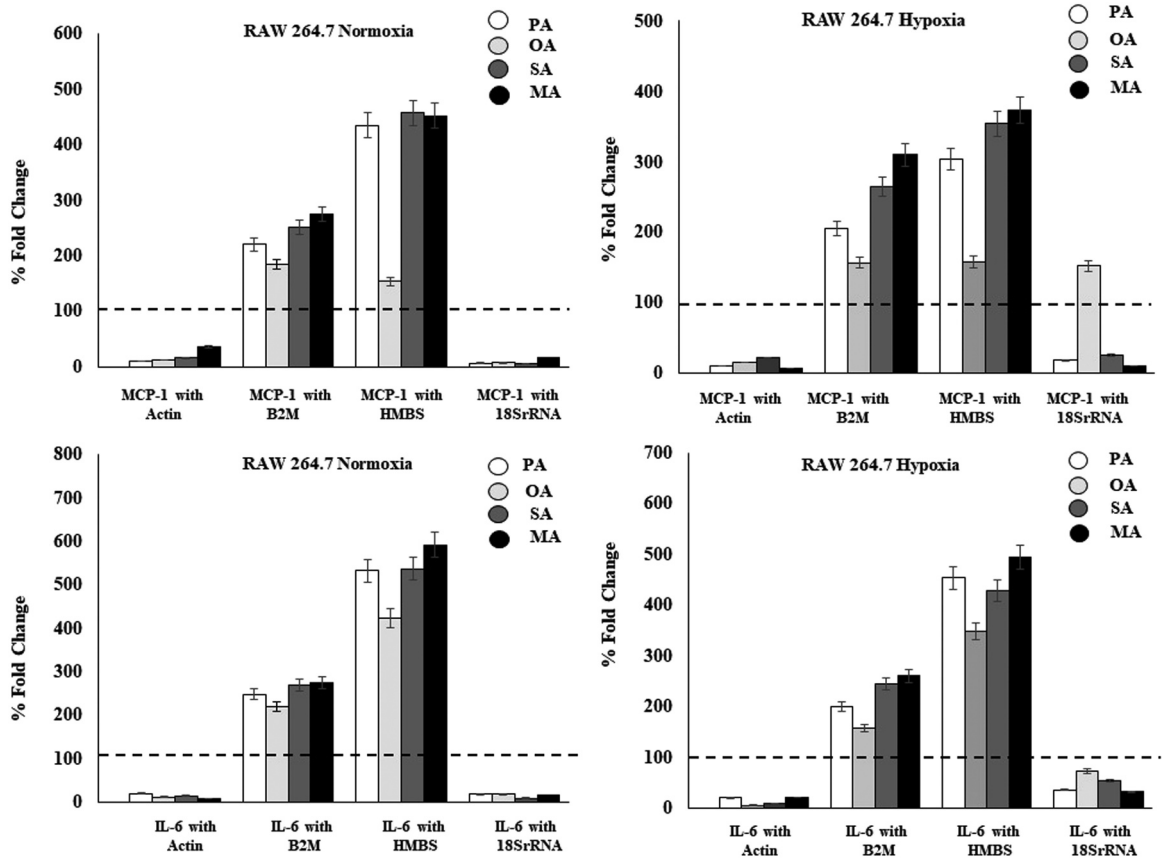

Fig. 4. Gene expression varies with the choice of endogenous control used. In RAW 264.7 cells subjected to different free fatty acids, under differential oxygen concentrations. MCP-1 and IL-6 expression were up-regulated when normalized with HMBS and B2M while ambiguous results were obtained when normalized with ACTB and 18S rRNA ($p \leq 0.01$). (dotted lines represent the baseline value).

the derived cDNAs, the expression level of FABP4 and PPARγ, known markers for adipocyte differentiation and MCP-1 and IL-6, known markers for macrophages inflammation were normalized with tested endogenous controls. Interestingly, the expression profile of all four markers (FABP4, PPARγ, MCP-1, and IL-6) matched with the treatment conditions when HMBS and B2M were used as the endogenous controls. On the other hand, the expression levels were found to be ambiguous when normalized with lesser stable ACTB and 18S rRNA (Figs. 3 and 4).

4. Discussion

RT-qPCR has become the method of choice for gene expression analysis because of its simple, sensitive, accurate, reproducible and high-throughput features (Schmittgen and Livak, 2008). However, RT-qPCR requires accurate data normalization strategies to eliminate non-biological variations. Using endogenous controls is one of the methods to achieve this. However, accumulating evidence suggests that there is no ideal endogenous control with steady expression levels across different physiological and pathological conditions (Rienzo et al., 2013). At the same time, there is an increased emphasis on using more than one reference gene to validate the experimental results as per MIQE guidelines (Bustin et al., 2009). Therefore, a precise evaluation of stable endogenous controls is warranted to achieve definite results in studies carried out under any experimental conditions.

The present study reports a systematic evaluation of four most commonly used endogenous controls for their effectiveness as normalizers in RAW 264.7, a murine macrophage cell line and 3T3-L1, a murine pre-adipocytes cell line cultured in the presence of high concentration of free fatty acids and differential oxygen tension. The RT-qPCR expression data was evaluated for stability analysis of the candidate endogenous controls.

We evaluated the expression stability of the four most commonly used housekeeping genes (ACTB, HMBS, B2M and 18S rRNA) for their suitability as endogenous controls. The evaluation was done by manually calculating fold difference using $2^{-\Delta\Delta Ct}$ and the results were validated using two different software's (NormFinder and Bestkeeper). Our results show that: 1) Expression of ACTB and 18S rRNA were found to be differentially regulated in both cell lines under normoxic and hypoxic conditions, 2) HMBS and B2M have a stable expression profile under differential oxygen tension.

The expression of reference genes may vary under different experimental conditions or in different tissues (Zhang et al., 2016). Recently, (Julian et al., 2014) expression of 18S rRNA in the brains of rats subjected to intermittent hypoxia is regulated by the oxygen tension. Another study reported, that GAPDH and ACTB to be regulated in human chondrocytes cultured in hypoxia (Foldager et al., 2009).

For this work, we first compared the Ct values of all four candidate reference genes in murine adipocyte and macrophage cell lines cultured in differential oxygen tension. The data obtained was changed to fold changes using the $2^{-\Delta\Delta Ct}$ method and the results indicated that HMBS and B2M showed the least expression variation under hypoxic and normoxic conditions. Since each algorithm is one-sided, evaluating the expression of stability of reference genes, many statistical approaches should be integrated to determine the best reference genes (Bruckert et al., 2016). In the present study, we used two most commonly used software (NormFinder and Bestkeeper) to validate our data. Unlike other software, the algorithms of NormFinder and Bestkeeper are less sensitive to co-regulation (Andersen et al., 2004; Pfaffl et al., 2004). NormFinder integrates intra- and inter-group variation into a stability value (M value). This model-based approach provides a more precise

Table 5
Crossing Point (CP) based on the descriptive statistics of four housekeeping genes generated by BestKeeper software. The BestKeeper Index (BI) is computed together with the same CP descriptive for four genes (ACTB, B2M, HMBS,18SrRNA). Abbreviations: n: number of samples; GM [CP]: Geometric Mean of CP; AM [CP]: Arithmetic Mean of CP; Min [CP]: Minimum value of CP; Max [CP]: Maximum value of CP; SD [± CP]: Standard Deviation of CP; CV [%CP]: the coefficient of variance expressed as a percentage on the CP level; Min [x-fold] and Max [x-fold]: the extreme values of expression levels expressed as an absolute x-fold over- or under-regulation coefficient; SD [± x-fold]: standard deviation of the absolute regulation coefficients.

Factors	3T3-L1 Normoxia					3T3-L1 Hypoxia				
	HMBS	**B2M**	**18 S rRNA**	**ACTIN**	**BI (n = 4)**	**HMBS**	**B2M**	**18S rRNA**	**ACTIN**	**BI (n = 4)**
GM [CP]	19.48	25.58	19.04	16.79	20.03	25.39	19.67	19.94	18.57	20.77
AM [CP]	19.5	25.59	19.17	16.86	20.05	25.39	19.7	20.17	18.97	20.8
min [CP]	18.33	24.67	15	14.78	18.94	24.33	18.33	15	14.94	19
max [CP]	21.35	27.13	22.3	19.62	21.86	26.5	22.38	28.79	27.66	23.31
SD [± CP]	**0.68**	**0.52**	1.82	1.45	0.56	**0.53**	**0.84**	2.31	3.21	1
CV [% CP]	3.47	2.01	9.54	8.6	2.82	2.08	4.24	11.49	16.93	4.8
min [x-fold]	−2.23	−1.89	−16.47	−4.02	2.13	−2.08	−2.54	−30.88	−12.39	3.41
max [x-fold]	3.63	2.9	9.56	7.1	3.55	2.15	6.5	458.68	544.12	5.83
SD [± x-fold]	± **1.59**	± **1.43**	± 3.55	± 2.74	± 1.47	± **1.44**	± **1.78**	± 4.99	± 9.27	± 1.99
Factors	**RAW 264.7 Normoxia**					**RAW 264.7 Hypoxia**				
	HMBS	**B2M**	**ACTIN**	**18S rRNA**	**BI (n = 4)**	**HMBS**	**B2M**	**ACTIN**	**18S rRNA**	**BI (n = 4)**
GM [CP]	20.03	25.07	18.8	17.01	20.08	25.98	19.52	19.77	18.77	20.85
AM [CP]	20.05	25.09	18.97	17.12	20.09	25.99	19.55	20.02	19.23	20.88
min [CP]	18.51	23.33	15	14.78	19	24.67	17.33	15	14.94	19
max [CP]	21.98	27.13	22.3	21.83	22.14	27.5	21.38	28.7	27.66	23.31
SD [± CP]	**0.711**	**0.73**	2.04	1.72	0.6	**0.62**	**0.88**	2.45	3.72	0.95
CV [% CP]	3.54	2.91	10.75	10.09	3.01	2.42	4.52	12.25	19.37	4.57
min [x-fold]	−2.88	−3.36	−14.16	−4.71	2.11	−2.49	−4.58	−27.4	−14.24	3.62
max [x-fold]	3.84	4.14	11.12	28.09	4.16	2.85	3.61	516.8	473.5	5.5
SD [± x-fold]	± **1.63**	± **1.66**	± 4.11	± 3.31	± 1.52	± **1.54**	± **1.84**	± 5.47	± 13.2	± 1.94

S. No	Abbreviation	Full-Form
1	ACTB	Beta Actin
2	B2M	Beta2Microglobulin
3	HMBS	Hydroxymethylbilane Synthase
4	18S rRNA	18S ribosomal RNA
5	FABP4	Fatty Acid binding Protein 4
6	MCP-1	Monocyte Chemoattractant Protein 1
7	IL-6	Interleukin 6

and robust estimative of expression variation among different sample types (Omodei and Fontana, 2011). BestKeeper software ranks reference genes according to the ranking of the standard deviation (ΔCt ± SD) and coefficient of variance (CtCV %) of Ct values, which is inversely proportional to the stability of expression (Mitchell et al., 2016). Here, we calculated the comprehensive ranking by the geometric mean of results by both software programs. The analysis using both the software's (Tables 4 and 5) corroborated with our findings of the manual calculation (Figs. 1 and 2 and Table 3). A lower M value by NormFinder indicates better stability of gene expression (Lv et al., 2017; Marques et al., 2013). In the present study, HMBS and B2M had significantly lower M values in both the cell line under differential oxygen tension, independently as well as in combination, when compared with 18S rRNA and ACTB (Table 4). When analyzed by BestKeeper, HMBS showed significantly lower SD and CV values indicating lower variability in their expression profile (Table 5). Thus, the results by $2^{-\Delta\Delta Ct}$ method and those by the two software's were in concurrence.

Further, the results were validated by analyzing the relative gene expression of marker genes for adipocytes and macrophages (FABP4, PPARγ, MCP-1, and IL-6) (Brunmeir et al., 2016; Liu et al., 2018; Xue et al., 2018). Through our work, we demonstrate that the expression of marker genes under varying oxygen tension showed erroneous results when normalized against less stably expressed ACTB and 18S rRNA in contrast to results obtained when normalized with more stably expressed HMBS and B2M. In the latter case, data validated the test conditions as evident from the gene expression pattern. Therefore, our work shows that, in 3T3-L1 and RAW 264.7, HMBS and B2M exhibit stable gene expression profile which is not modulated by differential oxygen tension. We acknowledge the limitations of the present study as our choice of reference genes analyzed was limited to four candidate genes and some other widely used genes, such as Ubiquitin, may warrant consideration in the future. Also, the results of this study may not be applicable to other cell lines, tissues or treatments. Nevertheless, our results show that B2M and HMBS could be considered in future studies in similar experimental set-ups.

5. Conclusion

To conclude, our results indicate that HMBS and B2M exhibit stable expression profile in murine adipocytes and macrophages under hypoxic and normoxic conditions mimicking obesity. Our results show that these genes can be considered as reliable endogenous controls for the studies estimating differential gene expression in inflammation, with hypoxia associated obesity. Though, ACTB and 18S rRNA has been previously shown to be a stable housekeeping gene in obesity studies, which is not validated in our work, emphasizing the need for the careful evaluation of endogenous gene for each experimental conditions.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2019.01.006.

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