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Validation of Reference Genes for Normalizing Gene Expression in Real-Time Quantitative Reverse Transcription PCR in Human Thyroid Cells in Primary Culture Treated with Progesterone and Estradiol

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Abstract The use of appropriately chosen reference genes for normalizing gene expression in real-time quantitative reverse transcription polymerase chain reaction is an important step in the analysis of gene expression, compensating for several technical factors. As female sex hormones have been shown to influence growth and differentiation of thyroid follicular cells, the establishment of normalizer genes in human thyroid cells in primary culture, treated with progesterone, and estradiol, is important to evaluate their effect on gene expression in these cells, so candidate reference genes were studied. β-Actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β2-microglobulin (B2M), and TATA box binding protein (TBP) were evaluated in thyroid cells treated with estradiol, progesterone, and their inhibitors. Normfinder software was used to assess the stability of the genes and identified β-actin as the gene with adequate stability and lower inter-group variations, when compared to TBP, B2M, and GAPDH.

Keywords Real time qRT-PCR · Primary cell culture · Thyroid · Gene expression · Estradiol · Progesterone

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Introduction

Estradiol has been shown to affect both growth and function in rat thyroid cells or abnormal human thyroid cells by modulating the expression of genes such as the sodium/iodide symporter (NIS) [1, 2], thyroglobulin (TG) [3], and thyroperoxidase (TPO) genes [4, 5]. The signaling mechanisms involved in these actions are still unknown [6, 7]. Estradiol-induced thyroid cell proliferation is probably mediated by genes responsible for regulating one or more stages of this process, as cyclin D1 [8], and proto-oncogene c-fos [6]. So far, there are no data regarding the effects of estradiol, and progesterone in vitro in human normal thyroid cells, although their receptors have been identified in these cells [9, 10].

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) is the most sensitive and specific method for assessing gene expression [11], although normalizing this method has been widely discussed recently [12–16]. Normalization of a target gene expression is performed to compensate for several technical factors, such as quality of RNA extraction, RNA purity, poor efficiency of the synthesis of complementary DNA (cDNA), inaccurate quantification of RNA sample, and variability of pipetting [17]. Thus, the choice of an appropriate reference gene for each experiment is a crucial point in the analysis of gene expression.

Software programs, Normfinder [12], GeNorm [18], and Bestkeeper [19], have been used for choosing reference genes. In this context, this study aimed to evaluate the most stable reference gene in human thyroid cells in primary culture treated with progesterone and estradiol, and its inhibitors.

Materials and Methods

Tissue Acquisition

Normal human thyroid tissue was obtained from patients who underwent total thyroidectomy as part of treatment for differentiated thyroid cancer in the Hospital de Clínicas de Porto Alegre (HCPA). After macroscopic and frozen sections evaluation of surgical specimens by two pathologists, some of the tissue was treated to obtain thyroid cells. This study was approved by the Ethics Committee of HCPA (GPPG: 08-454).

Isolation of Primary Epithelial Human Thyroid Cells

Thyroid tissue was cut in fragments of about 1 mm^3 and digested by 3 mg/ml collagenase type I in Hank's solution (GIBCO, Grand Island, NY, USA), for 2 h at 37 °C with gentle shaking. The suspension of cells was sequentially filtered through nylon meshes with 250, 150, and 60 μm pore size. The filtered fraction, containing epithelial thyroid cells, was resuspended in Ham's F-12 Coon's modification medium and seeded in 35-mm Petri dishes at a density of 1×10^6 cells/cm^2. Cells were maintained in the same medium supplemented with 10 % fetal bovine serum, 10 μg/ml insulin, 5 μg/ml transferrin, 1 mU/ml TSH, and 100 U/ml kanamycin (3H medium). Cells were kept at 37 °C in 5 % CO$_2$, with a medium change every 48 h. All reagents were obtained from Sigma Aldrich Co, St. Louis, MO, USA, unless stated otherwise.

When cells were approximately 80 % confluent, they were deprived of TSH for 48 h (3H medium without TSH: 2H medium), and treated with progesterone or estrogen in the presence or absence, respectively, of mifepristone (progesterone antagonist, Sigma Aldrich) or ICI 182780 (antagonist of estradiol, I.C.I. Pharmaceuticals, Macclesfield Cheshire, UK). As there is no published study evaluating the effect of progesterone in human thyroid cells in vitro, three different concentrations of this hormone were tested: 1, 10, and 100 nM. 17β-Estradiol was studied at 10 nM, as described previously [1]. Thus, eight groups were treated, as follows: G1, 2H medium; G2, 2H medium + 20 μU/ml TSH; G3, G4, and G5, 2H medium + 20 μU/ml TSH and, respectively, 100, 10, and 1 nM progesterone; G6, 2H medium + 20 μU/ml TSH + 10 nM progesterone +

100 nM mifepristone; G7, 2H medium + 20 μU/ml TSH and 10 nM 17β-estradiol; and G8, 2H medium + 20 μU/ml TSH + 10 nM 17β-estradiol + 100 nM ICI 182780. Experiments were repeated five times in different culture cells to test reproducibility.

RNA Extraction

RNA extraction was performed with Trizol® (Invitrogen, Life Technologies, Karlsruhe, Germany) following the manufacturer instructions and stored at −80 °C. RNA concentration and purity were assessed by Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE, USA). RNA purity was considered appropriate when the ratio of measurements at A260:A280 was from 1.8 to 2.1.

Synthesis of Complementary DNA (cDNA)

1 μg total RNA was transcribed into cDNA using oligo-dT primers and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer instructions. cDNA was diluted to 1:10 in diethyl pyrocarbonate (DEPC) water and stored at −20 °C.

Selection of Reference Genes and Primers Design

Based on commonly used reference genes in cultured cells, and considering different pathways and functions for each gene, four genes were selected as candidate for reference gene, as shown in Table 1.

The primer sequences, product length, and mean melting temperature (T_m) for each gene are shown in Table 2. PCR primers were kindly supplied by Molecular, Endocrine and Tumor Biology Laboratory, Universidade Federal do Rio Grande do Sul. Annealing temperature of 60 °C was used for amplification.

Table 1 Name and function of candidate reference genes for normalizing gene expression in normal thyroid cells treated with estradiol and progesterone

Symbol	Gene name	Function
β-Actin	β-Actin	Cytoskeletal structural protein
B2M	β-2-microglobulin	Component of MHC-I
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	Glycolysis and gluconeogenesis
TBP	TATA box binding protein	Transcription factor

MHC-I major histocompatibility complex class I

Table 2 Product length, mean melting temperature (T_m), and sequence primers for candidate reference genes for normalizing gene expression in normal thyroid cells treated with estradiol and progesterone

Gene	Primer sequence	Product length (bp)	Mean T_m (°C)
β-Actin	Forward: 5′-TGCCGACAGGATGCAGAAG-3′	106	82.0
	Reverse: 5′-GCCGATCCACACGGAGTACT-3′		
B2M	Forward: 5′-CTATCCAGCGTACTCCAAAG-3′	165	78.5
	Reverse: 5′-ACAAGTCTGAATGCTCCACT-3′		
GAPDH	Forward: 5′-CTTTGTCAAGCTCATTTCCTGG-3′	133	84.2
	Reverse: 5′-TCTTCCTCTTGTGCTCTTGC-3′		
TBP	Forward: 5′-GGGTTTTCCAGCTAAGTTCTTG-3′	150	79.1
	Reverse: 5′-CTGTAGATTAAACAGGAAATAAC-3′		

bp Base pairs, *°C* Celsius degree, *B2M* β-2-microglobulin, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *TBP* TATA box binding protein

cDNA Amplification

qPCR reactions for cDNA amplification were performed on Applied Biosystems® StepOne™ Real-Time PCR System using Kit Platinum SYBR® Green qPCR SuperMix-UDG (Invitrogen Life Technologies, Carlsbad, CA, USA). Duplicate measurements were performed to determine reproducibility, with the following protocol: reaction mixtures were initially incubated at 95 °C for 2 min, followed by 40 cycles of 15 s denaturation step at 95 °C, 30 s at 60 °C annealing step and a 30-s elongation step at 72 °C. For each primer pair, dissociation curve analyses were performed by running a gradient of 60–95 °C to confirm the specificity of the PCR amplification, and the absence of primer dimers.

cDNA standard curves were constructed using the threshold cycles with five successive tenfold dilution points of a pool of cDNA samples.

Statistical Analysis

The variability of gene expression of candidate genes was evaluated by the Normfinder algorithm, which automatically calculates the average expression stability for each candidate gene.

Results

The efficacy of isolation of the epithelial thyroid cells, and the growth conditions were assessed by phase-contrast microscopy. The confluence in monolayers and typical phenotype of normal human thyroid epithelial cells are shown in Fig. 1.

The values of stability of the candidate genes, and ranking obtained from NormFinder analysis are shown, respectively, in Fig. 2 and Table 3. The most stable combination was β-actin plus TBP (0.034).

Discussion and Conclusion

In the present study β-actin gene was shown to be suited for RT-qPCR gene expression data normalization in primary culture cells obtained from normal human thyroid, when treated with progesterone or estradiol. TBP, B2M, and GAPDH genes had a greater variation in gene expression indicating a low stability of these genes in the different groups studied.

Currently, it is accepted that there is no universal reference gene for RT-qPCR data normalization. Each experiment is unique because of its individual characteristics and,

Fig. 1 Phase-contrast microscopy of primary human thyroid cells isolated by collagenase. **a** Thyroid cell in suspension after enzymatic dissociation, and **b** thyroid cells clumps 48 h after seeding. *Scale* **a** 20 μm and **b** 100 μm
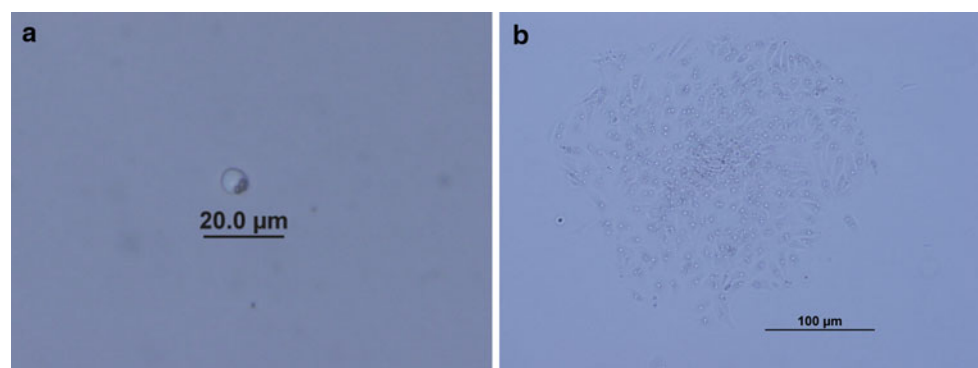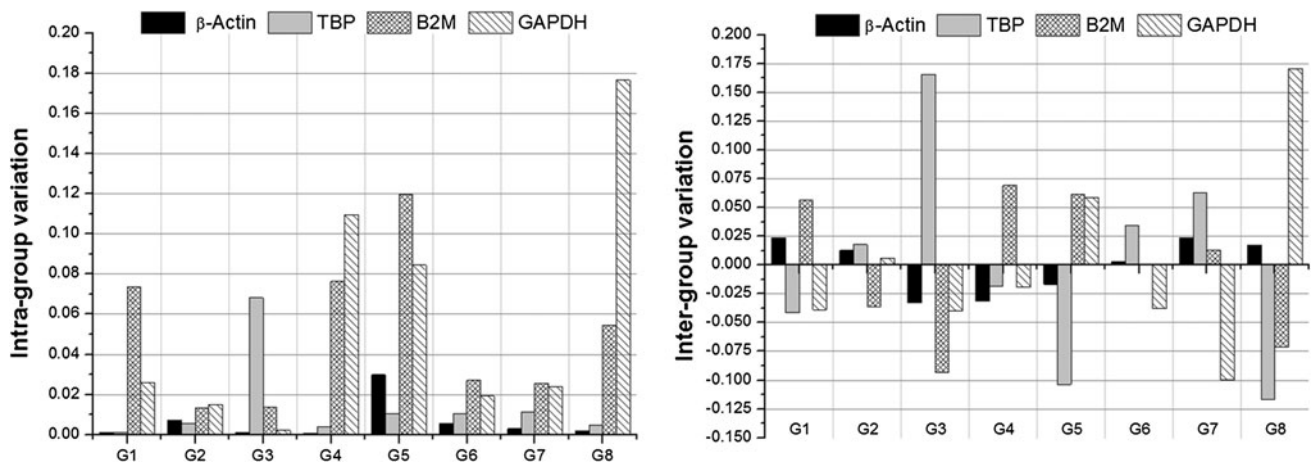
Fig. 2 Intra- and inter-group variation for candidate reference genes in five cultures of normal thyroid cells, according to treatment, as evaluated by NormFinder. The most stable gene is the one that has values closer to zero. GADPH, B2M, and TBP had higher instability than β-actin. *B2M* β-2-microglobulin, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *TBP* TATA box binding protein,

G1 no TSH, *G2* TSH 20 μU/ml, *G3* TSH 20 μU/ml + progesterone 100 nM, *G4* TSH 20 μU/ml + progesterone 10 nM, *G5* TSH 20 μU/ml + progesterone 1 nM, *G6* TSH 20 μU/ml + progesterone 10 nM + mifepristone 100 nM, *G7* TSH 20 μU/ml + estradiol 10 nM, *G8* TSH 20 μU/ml + estradiol 10 nM + 100 nM ICI 182780

Table 3 Candidate reference genes for normalization of RT-qPCR in human thyroid cells, according to their stability, as calculated by NormFinder

Ranking order	Gene	Stability value
1	β-Actin	0.033
2	TBP	0.054
3	GAPDH	0.108
4	B2M	0.108

Genes are listed from the most stable gene (β-actin) to the least stable one (B2M)

B2M β-2-microglobulin, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *TBP* TATA box binding protein

therefore, it is important to validate the candidate reference gene for every experimental situation. The importance of choosing the appropriate reference gene cannot be overstated, because the choice of an unsuitable one could lead to misinterpretation of biological effects in studies of gene expression [16]. The ideal reference gene should provide a transcript constant under all experimental conditions at any time of the cell cycle or cellular differentiation [16].

In the present study, the culture of primary cells obtained from normal human thyroid tissue was characterized by cell growth in adherent monolayer. The differentiation of thyrocytes was confirmed by measuring TG in the supernatant of each study group (data not shown).

NormFinder was chosen to compare candidate genes due to its ability to estimate inter- and intra-group variability separately (Fig. 2) and then combine them into a stability value (Table 3), representing the estimated systematic error [12]. The gene expression is more stable when this value is closer to zero. The stability value less

than 0.15 is the cut-off for an acceptable reference gene [18].

In Table 3, stability values obtained are shown, already considering both variations; the most stable to the least stable genes were β-actin > TBP > GAPDH = B2M. Unfortunately, there are no studies in normal thyroid cells to compare with our results. B2M and GAPDH genes are the most often used as reference genes in several human tissues but today there is emerging evidence that both these genes could vary its expression in different conditions [20].

Stability values were calculated separately for intra- and inter-groups. Although TBP has shown a good stability value (Table 3), due its low intra-group variation (0.068), its high inter-group variation (0.166), as observed in Fig. 2, does not allow us to suggest this gene as a reference gene for the experimental conditions used.

Also through the graphical representations in Fig. 2, it can be seen that the expression of β-actin had a low variation both intra- and inter-group. Nevertheless, it is important to point out that some variation is expected intergroup, when working with primary cells culture, mainly due to tissue conditions in vivo, surgical details, and the various steps for obtaining cells from dissociated tissue. Because of the importance of evaluating these inter- and intra-group expression variations, allowing to evaluate the heterogeneity between different cultures, other softwares, such as GeNorm and Bestkeeper, were not used to assess candidate reference genes.

Although some studies in immortalized cell lines from rat thyroid or human abnormal thyroid have shown that steroid hormones could influence the function and growth of the thyroid [1–3, 6–8, 21] there are no reports on the

effects of estradiol or progesterone in normal human thyroid cells. So these results could be potentially helpful for future studies to assess changes in the pattern of gene expression in normal human thyroid cells when exposed to these steroid hormones.

In conclusion, the results of the present study suggest that β-actin gene is more stable than TBP, GAPDH, and B2M genes to evaluate the response of primary cells culture from normal human thyroid to treatment with progesterone or estradiol.

Conflict of interest The authors have no conflicts of interest.

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