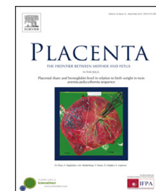
Placenta xxx (2014) 1–6

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta



Evaluation of reference genes for expression studies in leukocytes from term human pregnancy

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ARTICLE INFO

Article history:
Accepted 14 November 2014

Keywords:
Pregnancy
Labor
Leukocytes
Housekeeping genes
Stability
geNorm

ABSTRACT

Introduction: Human labor is considered an inflammatory process modulated by systemic and local leukocytes that infiltrate into the maternal–fetal interface. The putative roles of these leukocytes are currently being studied with gene expression assays. Such assays are normalized by the expression of housekeeping genes. However, expression of housekeeping genes may vary depending on the cell type and/or the experimental conditions. The aim of this study was to analyze the expression stability of several housekeeping genes in leukocytes from term human pregnancies, considering both anatomical origin and presence of labor.
Methods: We analyzed the gene expression of *ACTB*, *B2M*, *GAPDH*, *GUSB*, *PGK1*, *RN18S1*, *TBP* and *UBC* in leukocytes from maternal peripheral blood, placental blood and choriodecidua in women delivering at term with or without the presence of labor through real-time qPCR. Then we used geNorm to evaluate expression stability and pairwise variation.
Results: The expression of all tested genes showed to be stable independent of the anatomical compartment and the absence or presence of labor. However, *PGK1*, *GUSB* and *TBP* showed to be the most stable and *RN18S1* the least stable. Pairwise variation analyses showed that only two genes are needed for normalization yet the inclusion of a third improves its accuracy.
Discussion: *PGK1*, *GUSB* and *TBP* are the most adequate reference genes for gene expression normalization in leukocytes from term pregnancies, regardless of their anatomical origin (maternal peripheral blood, placental blood or choriodecidua) or the presence or absence of labor. Our study is the first report on housekeeping gene stability in leukocytes from healthy pregnant women.
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1. Introduction

Human parturition has been described as the result of an inflammatory process that takes place within the intrauterine milieu when pregnancy comes to term [1], which seems to be modulated by specific leukocyte subsets that infiltrate into the choriodecidual tissues [2] and show an increased proinflammatory capacity when compared to peripheral blood leukocytes [3,4]. The characterization of these infiltrating leukocytes has been recently undertaken using cellular and molecular techniques, including gene expression assays.

Housekeeping gene expression is used to normalize the expression of target genes in every expression assay. Over the last years, several studies have shown that housekeeping gene expression may vary depending on the cell type and/or the experimental conditions [5–8], stressing the importance of analyzing their expression in particular experimental conditions.

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http://dx.doi.org/10.1016/j.placenta.2014.11.010
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Furthermore, it has been demonstrated that using a single reference gene or an unstable gene rather than using two or more reference genes for normalizing the expression of a target gene, will yield different results [9–11].

Despite of these evidences, studies analyzing the stability of housekeeping genes in leukocytes from healthy individuals are scarce, even with contradictory results partly due to different analytical strategies [9,12,13], and none of the available studies included pregnant women. Furthermore, there is not a single study with leukocytes involved in pregnancy-related events addressing the stability of these genes. In this study, we report for the first time the expression stability of several housekeeping genes in leukocytes from healthy women with term pregnancies, analyzing whether or not the anatomical compartment (peripheral blood, placental blood or choriodecidua) or the physiologic event (no labor or active labor) have any effects on said stability.

2. Methods

2.1. Participating women

This project was approved by the Internal Review Board of the Instituto Nacional de Perinatología in Mexico City (register 212250-02191) and written informed consent was obtained from all participating women. We included healthy women with term pregnancies (>37 weeks of gestation) who underwent elective cesarean section ($n = 25$) or labor ($n = 19$). Labor was considered to be present when women presented signs of uterine activity and cervical dilation was >2 cm. Women with diabetes, autoimmune diseases, multiple pregnancy or clinical signs of infection were excluded.

2.2. Isolation of leukocytes

We isolated leukocytes from maternal peripheral blood (collected immediately after delivery by venous puncture of the forearm) and from intervillous placental blood (collected by manually compressing the cotyledons) by density gradient using Polymorphprep (Axis-Shield, Oslo, Norway), recovering both the mononuclear and polymorphonuclear bands. Leukocytes were washed with 1× PBS, incubated with erythrocyte lysis buffer (150 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) for 15 min and washed again with 1× PBS.

Additionally, to isolate choriodecidual leukocytes, the chorion of fetal membranes (cut from the placenta) was manually separated from the amnion, cut into small pieces (approximately 3 × 3 cm) and incubated at 37 °C for 5–10 min with low agitation in 100 ml of DMEM containing 0.1% trypsin for enzymatic digestion of the choriodecidua. Media was recovered, diluted with 1× PBS and centrifuged. Pelleted cells were incubated in erythrocyte lysis buffer for 15 min and washed with 1× PBS. Choriodecidual leukocytes were then isolated from other cells by positive-selection magnetic cell sorting (MACS) using an anti-CD45 microbead-conjugated antibody and MS columns, following the manufacturer's protocol (Miltenyi Biotec, Germany). All buffers were ice-cooled to minimize RNA degradation.

2.3. RNA isolation and cDNA synthesis

We isolated total RNA from leukocytes of the three anatomical compartments using Trizol reagent following the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and analyzed RNA integrity on half of the samples in an Agilent 2100 Bioanalyzer using RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). All samples showed RNA integrity numbers (RIN) over 7. Complementary DNA was synthesized from approximately 500 ng of total RNA with the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Mannheim), using random hexamers, at 25 °C-10 min/55 °C-30 min/85 °C-5 min. cDNA was stored at −20 °C until further used.

2.4. Quantitative real-time PCR

We analyzed the housekeeping genes *ACTB*, *B2M*, *GAPDH*, *GUSB*, *PGK1*, *RNA18S1*, *TBP* and *UBC* by high throughput gene expression analysis using DNA binding dye (Evagreen) for detection and highly specific primers designed for each gene (DELTAgene Assays, Fluidigm Corporation, San Francisco, CA, USA). We selected these genes over other housekeeping genes (like *YWHAZ*, *HMBS*, *HPRT* or *SDHA*) because predesigned, validated assays were already available from the manufacturer. Primer sequences and amplification efficiencies are shown in Table 1.

First we performed a specific target preamplification reaction on each cDNA using 2× TaqMan Preamp Master Mix (Applied Biosystems, Foster City, CA, USA) and 500 nM pooled primer mixture. Preamplification was carried at 95 °C–10 min/14 cycles of 95 °C–15 s and 60 °C–4 min. A cleanup step of preamplification products was performed with 8 U of Exonuclease I (New England Biolabs, Ipswich, MA, USA) at 37 °C–30 min/80 °C–15 min. The final clean-up products were diluted 1:5 with TE buffer.

For the qPCR, we followed the Fast Gene Expression Analysis Using EvaGreen on the Biomark System Protocol from Fluidigm to prepare assay mixes (100 μM of each pair of primers, 2X Assay Loading Reagent, 1× TE buffer) and sample mixes (diluted preamplified Exonuclease-treated cDNA, 2× SsoFast Master mix (BioRad, Hercules, CA, USA), 20× DNA Binding Dye Sample Loading Reagent). Assay and sample mixes were loaded into the 48.48 Dynamic Array using the IFC Controller MX and then transferred to the BioMark for qPCR (95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 60 s). Each assay was run in duplicate and the results averaged. All samples showed Ct values <30 and inter-plate coefficient of variation was 9.83 (average of the ten samples with the lowest Ct values for every gene).

2.5. Analysis of housekeeping gene stability and variability

We used the geNorm VBA applet for Microsoft Excel (available at http://medgen.ugent.be/genorm/) to analyze expression stability and variability. It measures gene expression stability (M) for a reference gene, as the average pairwise variation for that gene against all other tested reference genes. Genes with the smallest M value (lower than 1.5) are considered the most stable.

In order to analyze whether or not the anatomical compartment (peripheral blood, placental blood or choriodecidua) or the physiological event (no labor or labor) affects the stability of housekeeping genes, we performed two sets of analyses. The first set tested the stability between compartments and was comprised of two different analyses, one with samples from women without labor and the other with samples from women in labor. The second set tested the stability of genes in the absence or presence of labor and was comprised of three different analyses, one for each anatomical compartment. In order to confirm our results, we performed an analysis including all samples.

Moreover, we also calculated the pairwise variation ($Vn/n + 1$) in order to evaluate the minimum number of genes required for normalization in comparative gene expression analyses. According to Vandesompele et al., a pairwise variation of 0.15 is considered to be the cut-off value below which the inclusion of an additional reference gene is not required [9].

Since geNorm requires expression quantities (not Ct values), all Ct values were transformed into said quantities using the comparative Ct method. Briefly, the highest Ct value for each gene was set to 1 and values for the rest of the samples were calculated relative to this value. These expression quantities were the input data for geNorm.

3. Results

Fig. 1 shows the distribution of Ct values of all the tested genes. All samples showed Ct values <30. From this graph, genes can be roughly grouped according to their median expression levels into three groups: high abundance genes with median Ct values ≤5, comprising *RNA18S1* and *B2M*; medium abundance genes with median Ct values between 5 and 15, comprising *ACTB*,

Table 1
Primer sequences and amplification efficiencies.

Gene symbol	RefSeq	Forward primer	Reverse primer	Amplification efficiency
ACTB	NM_001101.3	ccaaccgcgagaagatgac	tagcacagcctggatagcaa	1.075
B2M	NM_004048.2	tccgtggccttagctgtg	cccagacacatagcaattcagg	1.030
GAPDH	NM_002046.3	acaccatggggaaggtgaag	gtgaccaggcgcccaata	1.043
GUSB	NM_000181.3	catcgatgacatcaccgtcac	acaggttactgcccttgaca	0.985
PGK1	NM_000291.3	gtggaatggcttttaccttcc	cttggctccctcttcatcaa	0.970
RNA18S1	NR_003286.2	acgagactctggcatgctaa	aacgccacttgtccctctaa	1.053
TBP	NM_001172085.1	tgcccgaaacgccgaatata	cgtggttcgtggctctctta	1.127
UBC	NM_021009.5	tcggccttagaaccccagta	gaaaaccagtgccctagagtca	1.067

Amplification efficiencies were calculated from the slopes of serial dilution curves using the equation $E = 10^{(-1/m)} - 1$.

Please cite this article in press as: Vega-Sanchez R, et al., Evaluation of reference genes for expression studies in leukocytes from term human pregnancy, Placenta (2014), http://dx.doi.org/10.1016/j.placenta.2014.11.010

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