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Identification of suitable reference genes in the mouse placenta

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ABSTRACT

Introduction: Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) is a reliable tool to analyse gene expression profiles. The expression of housekeeping genes generally serves as a reference for mRNA amount, assuming that it remains stable under pathophysiological and experimental conditions. To date, an empirical validation of reference genes suitable for RT-qPCR-based studies in the mouse placenta is missing.

Methods: We used NormFinder and BestKeeper statistical software to analyse the expression stability of candidate housekeeping genes quantified by RT-qPCR in mouse placentas.

Results: Fifteen of 32 potential candidate housekeeping genes analysed on gestation day (gd) 16.5 in mouse placentas exhibited an optimal cycle threshold (Ct). Among them *B2m*, *Polr2a*, *Ubc*, and *Ywhaz* genes showed the highest expression stability in placentas from control, but also experimentally-challenged mice. These genes as well as the currently widely used housekeeping genes *Hprt1*, *Actb*, and *Gapdh* were selected for further quality assessments. We quantified the Ct values of these selected genes in placental samples obtained from wild-type or genetically engineered dams at different gds, or upon selected experimental interventions known to affect placental phenotype. Among all housekeeping genes analysed, *Polr2a* was the most stably expressed and its expression stability excelled in combination with *Ubc*.

Discussion: Polr2a, especially in combination with *Ubc*, can be proposed as highly suitable endogenous reference for gene expression analysis in mouse-derived placental tissue. Moreover, the validation of both genes as a stable reference gene in human placenta-derived tissue strengthens the translational relevance of RT-qPCR findings using mouse placenta.

1. Introduction

Quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) is a reliable and highly sensitive tool to assess gene expression profiles in biological samples [1]. In order to reliably identify changes of mRNA transcripts for the gene(s) of interest among different samples, it is pivotal to control for differences in enzymatic efficiency and in the overall transcriptional activity. Also, the sample to sample variation of mRNA content, introduced e.g. by erroneous mRNA quantification, must be considered to obtain reliable data using RT-qPCR [2,3]. Reference genes, such as housekeeping genes, are commonly utilized as endogenous controls for the mRNA amount in the respective sample. In this regard, an essential prerequisite for a suitable reference gene is that the sensitivity range of its expression is similar to the gene(s) of interest [4]. Another pivotal requirement is that endogenous control gene expression remains stable under different conditions, such as pathophysiological settings or upon experimental interventions. The expression of housekeeping genes has been described to vary among tissues and species [5,6]. Therefore, it is now recommended to identify and validate the most suitable reference gene for each particular tissue source and also distinct experimental conditions. To date, a number of computer programs such as Genorm [2], BestKeeper [3], and NormFinder [7] have been developed in order to facilitate the statistical analysis of potential endogenous control genes [8]. These programs use algorithms for identifying the stability of gene expression among a set of candidates [6,9]. Among them, NormFinder algorithm estimates systematic intergroup

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variations of gene expression whereas BestKeeper indexes the stability in the expression of reference genes in a set of samples irrespective of intergroup variations.

In the mammalian placenta, the possibility to quantify gene expression by means of RT-qPCR has shed light into multiple physiological and pathological processes, such as the vascularization of healthy and pre-eclamptic human placentas [10]. These analyses have been supported by extensive comparative expression studies on healthy and pathological human placental samples, aiming to identify housekeeping genes suitable to be employed as reference for RT-qPCR (Supplementary Table 1). Moreover, the clear understanding of pathophysiological mechanisms associated with human pregnancies could be greatly advanced by experimental research involving rodent animal models, despite significant species-specific placental differences. Here, the determination of gene expression by RT-qPCR is also an essential tool to identify biomarkers. Surprisingly, a comprehensive validation of the most suitable reference gene for the mouse placenta, based on its gene expression stability is still largely missing. To date, reference genes have only been studied in trophectoderm progenitor stem cells cultured *in vitro*. The comparison of housekeeping genes for trophectoderm, embryonic and extraembryonic endoderm stem cells lead to recommend the use of Phosphoglycerate kinase 1 (*Pgk1*), succinate dehydrogenase complex, subunit A, flavoprotein (Fp) (*Sdha*) and TATA-binding protein (*Tbp*) as reference [11]. In contrast, in undifferentiated or differentiating trophectoderm progenitor stem cells cultured with and without ethanol exposition, Mitochondrial ribosomal protein L1 (*Mrpl1*), *Sdha* and Peptidylprolyl isomerase A (*Ppia*) gene expression proved to be the most stable [6]. Noteworthy, these two studies did not analyse the same set of candidate genes, which limits the comparative assessment of potential reference genes and the selection of the most suitable genes.

Thus, we here addressed the limitation of the yet to be identified most suitable reference gene(s) in the mouse placenta, which can then be used as normalization controls in RT-qPCR-based assessments under different experimental conditions. We pursued the analysis in a stepwise approach, in which we first identified suitable candidates and subsequently verified their expression stability [7]. The criteria we applied in selecting the most suitable genes included that 1) the expression of the candidate genes are abundant and in a range comparable to the expression of the gene of interest that shall be assessed in the respective experiment; 2) the transcripts of these genes are stably expressed among different conditions.

2. Methods

2.1. Animals

Animals used in this study were either purchased in Charles Rivers Laboratories or obtained from our in-house breeding colony. Mice were kept under 12 h light/dark cycles (lights turned on at 6 am) and received food and water ad libitum. A summary of the different mouse strains and models as well as the gestational time point selected for placental tissue collection is given in Table 1. Briefly, placentas from DBA/2J-mated Balb/c mice were harvested on gestational day (gd) 16.5 gd (0.5 corresponds to the observation of a vaginal plug the morning after mating). Some dams had been exposed to sound stress on gd 12.5 and 14.5 for 24 h respectively in order to induce IUGR [12]. Sound stress was emitted at a frequency of 460 Hz and at an intensity of 88 dB by a rodent repellent device (Conrad Electronics) placed into the animal cage. The sound lasted for 1 s and was emitted randomly four times per minute. Light/dark cycles were maintained during stress exposure [13]. Similarly, placentas were harvested from CBy.SJL(B6)-Ptprc^a/J-mated C57Bl/6J female mice, on gestational days (gd) 11.5, 13.5, 14.5, 16.5 and 18.5. Additional groups of CBy.SJL(B6)-Ptprc^a/J-mated C57Bl/6J female mice had been exposed to prenatal challenges, such as sound stress (as described above) or intraperitoneal (ip) injection of 250 mg/kg acetaminophen (APAP) on gd 12.5 [14]. From these dams, placental tissues were harvested on gd 13.5 or 16.5 respectively. Another group of CBy.SJL(B6)-Ptprc^a/J-mated C57Bl/6J females was ip injected with 0.5 μg/day *Escherichia coli* lipopolysaccharide (LPS, serotype O26:B6, Sigma) daily between gd 8.5 to 12.5 [15] and placentas were harvested on gd 18.5. Further, placentas homozygous for the genetic deletion of progesterone receptor ($PR^{-/-}$) were obtained on gd 16.5 from $PR^{+/-} \times PR^{+/-}$ mating combinations. $PR^{-/-}$ implantations gave rise to smaller fetuses [12], which exhibited smaller placentas (unpublished observations) than $PR^{+/+}$ implantations. Structural abnormalities were also observed in $PR^{-/-}$ placentas, such as a reduced labyrinth to junctional zone ratio [12,16].

For all experiments, mice were anesthetized by CO_2 inhalation and euthanized at 8 am by cervical dislocation. Placenta tissues were carefully dissected from the gravid uterus. Firstly the decidua parietalis was sectioned and the fetus and fetal membranes were cut off in the base of the chorionic plate. Secondly, the placentas were grasp to detach from the decidua basalis and immediately stored at 4 °C in RNAlater (Ambion) for 24 h to be kept at −20 °C until further use. In order to ensure independence among the samples of each group, only one placenta per litter was included in the subsequent analysis.

All experiments were performed in accordance with the animal ethics approval given by the State Authority of Hamburg (G10/067, G11/094, G11/087, ORG_702).

2.2. Cell cultures

Immortalized trophoblast SM9-1 cells derived from gd 9 Swiss-Webster mouse placenta were grown in RPMI 1640 containing antibiotics and supplemented with 10% fetal bovine serum and 50 μM mercapto-ethanol at 37 °C, 5% CO_2, as previously described [17]. Three separate flasks of low-passage (<10) SM9-1 cells were exposed to lysis buffer (QIAGEN), harvested and frozen at −80 °C for later extraction of mRNA.

2.3. RNA extraction and cDNA synthesis

Tissue homogenization was performed using micro packaging vials with ceramic beads (1.4 mm) in a Precellys® 24 Tissue Homogenizer (Peqlab). Total RNA isolation was conducted by use of RNeasy Plus Universal Mini Kit (QIAGEN) according to manufacturer's instructions. Subsequently, the DNA-free Kit (Applied Biosystems by Life Technologies) was used to minimize potential DNA contamination. The, cDNA synthesis was performed with random primers (Invitrogen by Life Technologies) using 1 μg RNA. Concentration and purity of RNA and cDNA were assessed using the NanoQuant (Tecan). Samples were stored at −20 °C.

2.4. RNA integrity and RNA integrity number (RIN)

Since high RNA integrity is a pivotal prerequisite for reliable RT-qPCR analyses, we firstly validated the method used for RNA isolation from mouse placental tissue. RNA integrity was assessed by using the Agilent 2100 Bioanalyzer. The respective Software and Agilent RNA 6000 Pico Kit was used according to the manufacturer's manual. Agilent RNA kits contain RNA Pico chips and reagents designed for analyzes of RNA fragments by electrophoretical separation. By analyzing the presence of degradation products in the electrophoretic trace the software assigned to each RNA sample a RIN that ranged from 1 (completely degraded RNA) to 10 (intact