

Selection of reference genes for quantitative real-time polymerase chain reaction in porcine embryos

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Abstract. To study gene expression and to determine distinctive characteristics of embryos produced by different methods, normalisation of the gene(s) of interest against reference gene(s) has commonly been employed. Therefore, the present study aimed to assess which reference genes tend to express more stably in single porcine blastocysts produced *in vivo* (IVO) or by parthenogenetic activation (PA), *in vitro* fertilisation (IVF) and somatic cell nuclear transfer (SCNT) using different analysis programs, namely geNorm, Normfinder and Bestkeeper. Commonly used reference genes including *18S rRNA* (*18S*), *H2A histone family, member Z* (*H2A*), *hypoxanthine phosphoribosyltransferase 1* (*HPRT1*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), *ribosomal protein 4* (*RPL4*), *peptidylprolyl isomerase A* (*PPIA*), *beta actin* (*ACTB*), *succinate dehydrogenase complex, subunit A* (*SDHA*) and *hydroxymethylbilane synthase* (*HMBS2*) were analysed; most of them resulted in significantly ($P < 0.05$) different cycle threshold (CT) values in porcine embryos except for *SDHA* and *H2A*. In evaluation of stable reference genes across *in vivo* and *in vitro* porcine blastocysts, three kinds of programs showed slightly different results; however, there were similar patterns about the rankings of more or less stability overall. In conclusion, *SDHA* and *H2A* were determined as the most appropriate reference genes for reliable normalisation in order to find the comparative gene expression in porcine blastocysts produced by different methods, whereas *18S* was regarded as a less-stable reference gene. The present study has evaluated the stability of commonly used reference genes for accurate normalisation in porcine embryos to obtain reliable results.

Additional keywords: single blastocyst, normalisation, qRT-PCR.

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Introduction

Quantitative analysis of gene expression is an indispensable part of biology. The exploration of the change of gene expression is expected to provide understanding about the gene regulatory network (Nailis *et al.* 2006). The quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a valuable and commonly used technique for quantitative analysis because of its sensitivity, specificity and reproducibility (Radonić *et al.* 2004; Davoren *et al.* 2008). The sensitivity of this technique allows working with a minimal amount of starting material from

only a small number of cells (Fink *et al.* 1998), hence qRT-PCR has been widely used in embryo research (Goossens *et al.* 2005; Kuijk *et al.* 2007; Mamo *et al.* 2007; Llobat *et al.* 2012; Gu *et al.* 2014; Luchsinger *et al.* 2014).

Embryos can be generated not only by *in vivo* (IVO) development in the female uterus after natural mating or artificial insemination but also by *in vitro* culture in the incubator after embryo manipulation such as parthenogenetic activation (PA), *in vitro* fertilisation (IVF) and somatic cell nuclear transfer (SCNT). These embryos are important models for understanding

embryology, reproduction, innate disease and for the generation of transgenic animals. Differences between embryos produced by different *in vivo* and *in vitro* methods have revealed their distinctive characteristics including developmental efficiency, total cell number and gene expression (Kumar *et al.* 2007, 2012; Mamo *et al.* 2007; Magnani and Cabot 2008; McElroy *et al.* 2008; du Puy *et al.* 2011). In particular, compared with others, SCNT embryos have revealed extensively different characteristics caused by the process of manipulation attributable to incomplete and abnormal epigenetic reprogramming (Somers *et al.* 2006; Beyhan *et al.* 2007; Suzuki *et al.* 2008; Zhao *et al.* 2010; Deshmukh *et al.* 2011; Kumar *et al.* 2013). Differences in gene expression were also caused by *in vitro* culture conditions (Niemann and Wrenzycki 2000; Lazzari *et al.* 2002; Rizos *et al.* 2002; Rinaudo and Schultz 2004; McElroy *et al.* 2008) and the methodological procedures used to generate the embryos (Zhou *et al.* 2008; Ross *et al.* 2010).

Although qRT-PCR is commonly employed for analysis of mRNA expression levels, there are several factors causing variation in overall transcriptional activity, such as amount of starting material, enzymatic efficiencies, diversities in protocols and instruments used (Bustin 2002). Furthermore, another distinct variable in embryo study that has to be considered is extremely low transcript mRNA yield from a single embryo for reliable quantification as starting material (Robert *et al.* 2002; Luchsinger *et al.* 2014). One way to minimise these variables for a well-conducted study in qRT-PCR assays is by normalisation of the gene of interest against reference genes (housekeeping genes), which should be expressed consistently and stably in all the samples regardless of experimental conditions and treatments (Bustin 2002; Vandesompele *et al.* 2002; Dheda *et al.* 2004; Huggett *et al.* 2005). The expression of reference genes plays a vital role in maintenance of cellular function and production, which are minimal essential necessities for normal physiology (Butte *et al.* 2001).

Gene expression studies rely on the use of reference genes, yet there has not been any attempt to identify the suitability of any reference gene under various experimental or culture conditions (Radonić *et al.* 2004; Huggett *et al.* 2005). The possible effects of such variation on commonly used reference genes such as *beta actin* (*ACTB*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *18S rRNA* (*18S*) are, consequently, mostly unknown (Uddin *et al.* 2011). The use of these genes was evaluated in different studies and criticised due to observation of variation between treatment groups (Thellin *et al.* 1999; Tricarico *et al.* 2002; Radonić *et al.* 2004). Moreover, the use of inappropriate reference genes for normalisation could result in false conclusions and misleading interpretation of gene expression (Bas *et al.* 2004; Dheda *et al.* 2004; Haller *et al.* 2004). Evaluation for stability of reference genes, therefore, is required in each experimental condition to guarantee more reliable results of biological significance.

Several studies have focussed on evaluation of reference genes from pre-implantation embryos in various species including mouse (Mamo *et al.* 2007; Llobat *et al.* 2012; Gu *et al.* 2014), rabbit (Mamo *et al.* 2008), horse (Smits *et al.* 2009; Paris *et al.* 2011) and cattle (Goossens *et al.* 2005; Luchsinger *et al.* 2014). Although several studies of porcine reference genes have been

investigated under various conditions such as different tissues, age and treatments (Nygard *et al.* 2007; Uddin *et al.* 2011; McCulloch *et al.* 2012), information on suitable reference genes from porcine embryos is available only from IVF embryos (Kuijk *et al.* 2007). However, evaluation of stable reference genes is a necessity for accurate analysis of porcine embryos, because the stability of reference genes can also vary depending on the method used to produce the embryos. Therefore, the aim of the present study was to assess the stable expression of nine widely used reference genes in several kinds of porcine pre-implantation embryos that were produced by IVO, PA, IVF and SCNT to select the most-stable ones by analysis of different algorithms including geNorm, Normfinder and Bestkeeper.

Material and methods

Chemicals and media

All chemicals and media, unless stated otherwise, were purchased from Sigma-Aldrich Chemical Inc. (St. Louis, MO, USA) and Gibco (Grand Island, NY, USA).

Preparation of donor cells for SCNT

All animal samples were collected and handled following the approval of the research ethical committee of the animal centre for biomedical experimentation, Gyeongsang National University, under set guidelines (GNU-140305-P0016). Porcine ear skin tissue from ~1-year-old pigs was collected, minced and explanted onto dishes. The isolated cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (pen-strep; 10 000 IU and 10 000 µg mL⁻¹, respectively) at 38.5°C in a humidified incubator at 5% CO₂ in air. Cells were further passaged to obtain a proliferating homogenous mixture under aseptic conditions. Fibroblasts at Passage 3 were used as SCNT donor cells.

In vitro maturation of oocytes

Cumulus-oocyte complexes (COCs) were collected from pre-pubertal pig ovaries obtained from a local slaughterhouse and *in vitro* maturation (IVM) performed as previously described (Kumar *et al.* 2007, 2013; Lee *et al.* 2014). Briefly, COCs were aspirated from ovarian follicles of 3–5 mm in diameter using a 19-gauge needle attached to a 10-mL syringe. Sets of 100 COCs with even cytoplasm and multilayered cumulus cells were matured in 500 µL tissue culture medium (TCM)-199 medium supplemented with 5% FBS, 1% pen-strep, 0.57 mM cysteine, 10 ng mL⁻¹ epidermal growth factor (EGF), 2.5 mM sodium pyruvate, 1 mM L-glutamine, 0.5 µg mL⁻¹ luteinising hormone (LH) and 0.5 µg mL⁻¹ follicle-stimulating hormone (FSH) for 22 h and then further cultured for an additional 20 h in the same medium without LH and FSH at 38.5°C in a humidified atmosphere of 5% CO₂ in air. After 42 h of IVM, cumulus cells were denuded from oocytes by pipetting in Dulbecco's phosphate-buffered saline (DPBS) with 0.1% hyaluronidase for 1 min. Oocytes having the first polar body (PB1), even cytoplasm and intact membranes were selected for the production of PA, IVF and SCNT embryos.

Preparation of pre-implantation embryos

For the production of *in vivo* and *in vitro* embryos, IVO and embryo manipulations for PA, IVF and SCNT were performed as previously described (Kumar *et al.* 2007, 2012, 2013; Lee *et al.* 2014). For preparation of IVO embryos, oestrous sows were fertilised by artificial insemination twice with a 12-h interval between inseminations. On Day 7 after the first insemination, the sows were shipped to a local slaughterhouse and withdrawn genital tracts were transported to the laboratory at optimal temperature conditions of 35–39°C within 1 h. The uterine horns and uteri were flushed with DPBS supplemented with 0.1% bovine serum albumin (BSA) for embryo recovery of IVO blastocysts (Kumar *et al.* 2007).

For production of PA embryos, MII oocytes were pulsed twice with 2.0 kV cm^{-1} DC for $30 \mu\text{s}$ using a BTX Electro Square Porator and a BTX Electro chamber (ECM 830; BTX Inc., San Diego, CA, USA) filled with 0.28 M mannitol solution containing 0.5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1 mM MgSO_4 , 0.05 mM CaCl_2 and 0.01% BSA. This was followed by culture in porcine zygote medium 5 (PZM5) containing $7.5 \mu\text{g mL}^{-1}$ cytochalasin B (CCB) for 3 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air to activate PA embryos (Kumar *et al.* 2012).

For SCNT embryo production, MII oocytes containing polar body1 (PB1) with small amount of cytoplasm was enucleated in HEPES-buffered TCM-199 supplemented with $7.5 \mu\text{g mL}^{-1}$ CCB, 0.3% BSA and 12 mM sorbitol and replaced with fibroblast. The couplets were fused and activated using the same activation parameters as were used for the PA embryos.

To generate IVF embryos, sets of 20 MII oocytes were fertilised using Percoll (Pharmacia, Uppsala, Sweden) density-gradient-treated spermatozoa (5×10^3 spermatozoa mL^{-1} final concentration) in 50- μL droplets of modified Tris-buffered medium (mTBM) consisting of 113.1 mM NaCl, 3.0 mM KCl, 7.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20.0 mM Tris crystallised free base, 11.0 mM glucose and 5.0 mM sodium pyruvate, supplemented with 2 mM caffeine and 4 mg mL^{-1} BSA for 5 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air (Kumar *et al.* 2013). All embryos produced *in vitro*, including IVF, PA and SCNT embryos, were then cultured in sets of 30 embryos per 30- μL droplet of PZM5 medium supplemented with 3 mg mL^{-1} BSA, $20 \mu\text{g mL}^{-1}$ Eagle's amino acids in basal medium (BME), $10 \mu\text{g mL}^{-1}$ non-essential amino acids (NEAA) for 4 days at 38.5°C in a humidified atmosphere of 5% CO_2 in air and then further cultured in the same medium supplemented with 10% FBS for an additional 2 days, in the same way as previously described (Kumar *et al.* 2012; Lee *et al.* 2014). Day-7 blastocysts were frozen immediately by liquid nitrogen with small volume of DPBS and stored at -80°C until RNA extraction.

RNA extraction, cDNA synthesis and qRT-PCR

Blastocysts produced *in vivo* ($n = 11$) and *in vitro* (each $n = 20$) were employed for all molecular work. Total RNA was extracted from single embryos using the RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and residual genomic DNA was removed by using RNase Free DNase (Qiagen) treatment for 15 min. Due to the

low amounts of total RNA extracted from a single blastocyst, quantification of total mRNA yield could not be measured by UV spectrophotometer. First-strand cDNA was synthesised from the 12 μL of total RNA extracted using 8 μL of mixture with Sensiscript Reverse Transcription Kit (Qiagen), 10 units of RNaseOUT Recombinant Ribonuclease Inhibitor (Invitrogen, Grand Island, NY, USA) and 1 μM oligo dT primer (Invitrogen) at 37°C for 1 h. Quantitative RT-PCR was performed using a Rotor Gene Q qRT-PCR machine (Qiagen) with Rotor-Gene 2X SYBR Green mix (Qiagen) including 2 μL cDNA per reaction and 0.5 μM forward and reverse primers. All experiments were executed one reaction for each single embryo because of limited amounts of cDNA. Quantitative RT-PCR program settings were comprised of pre-denaturation at 95°C for 10 min, 45 PCR cycles at 95°C for 10 s, 60°C for 6 s and 72°C for 4 s, melting curve from 60°C to 95°C by 1°C s^{-1} and cooling at 40°C for 30 s with minor modifications in the manufacturer's qRT-PCR program protocol. Rotor-Gene Q Series Software (Qiagen, Hilden, Germany) was used for analysis of amplification curves, melting curves and cycle threshold values (CT values). All amplicons from qRT-PCR were verified by electrophoresis using 1% agarose gel with 0.1 mg mL^{-1} ethidium bromide for checking non-specific amplification. Images were analysed using zoom browser EX5.7 software (Canon, Tokyo, Japan).

Candidate reference genes and primer efficiency

Nine commonly used reference genes were selected based on different intracellular functional activity. Candidates of reference genes were selected on the basis of previously described articles examining the stability of porcine reference genes under several experimental conditions (Nygard *et al.* 2007; Uddin *et al.* 2011; McCulloch *et al.* 2012; Lee *et al.* 2015) and evaluated according to cases where they were applied as embryo reference genes (Goossens *et al.* 2005; Bettegowda *et al.* 2006; Mamo *et al.* 2007; Smits *et al.* 2009; Gu *et al.* 2014; Luchsinger *et al.* 2014). Consequently, nine widely used reference genes including *18S*, *H2A histone family, member Z (H2A)*, *hypoxanthine phosphoribosyltransferase1 (HPRT1)*, *GAPDH*, *ribosomal protein 4 (RPL4)*, *peptidylprolyl isomerase A (PPIA)*, *ACTB*, *succinate dehydrogenase complex, subunit A (SDHA)* and *hydroxymethylbilane synthase (HMBS2)*, were selected and sequences of all reference genes were taken from Nygard *et al.* (2007) or Lee *et al.* (2015). Their annealing temperatures were confirmed as 60°C by a primer searching program (Primer 3 Plus). The complete information is presented in Table 1. To check PCR efficiencies of reference genes for embryos, a standard curve of each primer of reference gene was generated from the CT values by a four-fold serial dilution of cDNA from PA blastocysts. Standard curve parameters including slope (M), intercept (B), PCR efficiencies (E) and correlation (R^2) calculated by Rotor-Gene Q Series Software (Qiagen) are presented in Table 2.

Evaluation of stable reference gene expression

CT values of each reference gene were log-transformed and analysed by three kinds of widely used algorithmic tools – geNorm, Normfinder and Bestkeeper – to assess the stability of the selected reference genes. By using these methods, we ranked the stability of the reference genes and confirmed the most-

Table 1. Information about primers

Gene name (symbol)	Primer sequences	Amplicon size (bp)	Annealing temp. (°C)	Reference
<i>18S rRNA (18S)</i>	F: CGCGGAAGGATTAAAGTG R: AAACGGCTACCACATCCAAG	141	60	Lee <i>et al.</i> (2015)
<i>H2A histone family, member Z (H2A)</i>	F: GGTAAGGCTGGGAAGGACTC R: CATGGCTGGTCGTCCTAGAT	124	60	Lee <i>et al.</i> (2015)
<i>Hypoxanthine phosphoribosyltransferase1 (HPRT1)</i>	F: AAGCTTGCTGGTGAAGGA R: GTCAAGGGCATAGCCTACCA	100	60	Lee <i>et al.</i> (2015)
<i>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</i>	F: ACACTCACTCTTCTACCTTTG R: CAAATTCATTGTCGTACCAG	90	60	Nygard <i>et al.</i> (2007)
<i>Ribosomal protein 4 (RPL4)</i>	F: CAAGAGTAACTACAACCTTC R: GAACTCTACGATGAATCTTC	122	60	Nygard <i>et al.</i> (2007)
<i>Peptidylprolyl isomerase A (PPIA)</i>	F: AAAACTTCCGTGCTCTGAGC R: TTATGGCGTGTGAAGTCACC	112	60	Lee <i>et al.</i> (2015)
<i>Beta actin (ACTB)</i>	F: TCAACACCCAGCCATGTAC R: AGTCCATCACGATGCCAGTG	84	60	Lee <i>et al.</i> (2015)
<i>Succinate dehydrogenase complex, subunit A (SDHA)</i>	F: CACACGCTTTCCTATGTCGATG R: TGGCACAGTCAGCTTCATTC	94	60	Lee <i>et al.</i> (2015)
<i>Hydroxymethylbilane synthase (HMBS2)</i>	F: TTCATTCCCTCAAGGACCTG R: GGGTGAAAGACAACAGCAT	101	60	Lee <i>et al.</i> (2015)
<i>POU class 5 homeobox 1 (OCT4)</i>	F: ACAAGGAGAAGCTGGAGCCGA R: AGGAGCTTGGCAAATTGTTTCGAGAT	85	59	Lee <i>et al.</i> (2014)

Table 2. Standard curve parameters for candidate reference genes

Gene	Correlation (R^2)	Slope (M)	Intercept (B)	Efficiency (E)
<i>18S</i>	0.999	-3.506	25.450	0.93
<i>H2A</i>	0.989	-3.493	36.641	0.93
<i>HPRT1</i>	0.999	-3.231	40.036	1.04
<i>GAPDH</i>	0.997	-3.526	35.339	0.92
<i>RPL4</i>	0.985	-3.537	39.044	0.92
<i>PPIA</i>	0.989	-3.465	34.454	0.94
<i>ACTB</i>	0.994	-3.511	33.737	0.93
<i>SDHA</i>	0.993	-3.253	37.322	1.03
<i>HMBS2</i>	0.996	-3.234	39.665	1.04

stable reference genes in porcine blastocysts produced by different methods. The geNorm program is based on a Visual Basic application (VBA) tool for Microsoft Excel and provides a measure of gene expression stability (M value). A gene presenting the highest M value is excluded from tested genes and the new M values from the remaining genes are calculated continuously until the last two genes presenting the lowest M value are left. The gene with the highest M value corresponds to the least-stable gene expression, whereas the two genes with the lowest M value determine the most-stable ones. In geNorm, the geometric average of expression of the most-stable reference genes generates normalisation factors (NF) and pairwise variation for investigating the optimal number of reference genes was calculated and presented $V_{n/n+1}$ by stepwise inclusion between two sequential NFs (Vandesompele *et al.* 2002). Normfinder is also a VBA program and is based on an ANOVA-based model to estimate intra- and inter-group variation to deduce the single most-stable reference gene. The values of stability and standard

errors are calculated by the transcriptional variation of the reference genes. This program exhibits the most-stable gene, presenting the low stability values and best combination of two genes (Andersen *et al.* 2004). Bestkeeper, an Excel-based tool, analyses variability in expression of reference genes by calculating comparative analysis based on numerous pairwise correlations of all reference genes against each other (Pfaffl *et al.* 2004).

The application of normalisation to reference genes with different stability

The effect of most- or least-stable reference genes was examined by applying different reference genes against relative gene expression for the gene of interest. *OCT4* (octamer-binding transcription factor 4) expression in each of 20 blastocysts from PA and IVF embryos was normalised against several reference genes to evaluate most-, moderately and least-stable reference genes by using three different types of programs. The primer sequence of *OCT4* has been presented in Table 1.

Statistical analysis

The CT values of reference genes from embryos produced by different *in vivo* and *in vitro* methods were presented as mean \pm s.e.m. and analysed using one-way ANOVA with Tukey's *post hoc* test (PASW Statistics 18; SPSS Inc., IBM SPSS, Armonk, NY, USA) and Student's *t*-test was applied to assess the application of normalisation. Significant differences were considered at $P < 0.05$. Moreover, correlation of the normalisation factor (NF_n) between the optimal number of reference genes (NF_{opt}) from pairwise variation of geNorm and the three most-stable reference genes (NF_3) was analysed by Pearson's correlation in

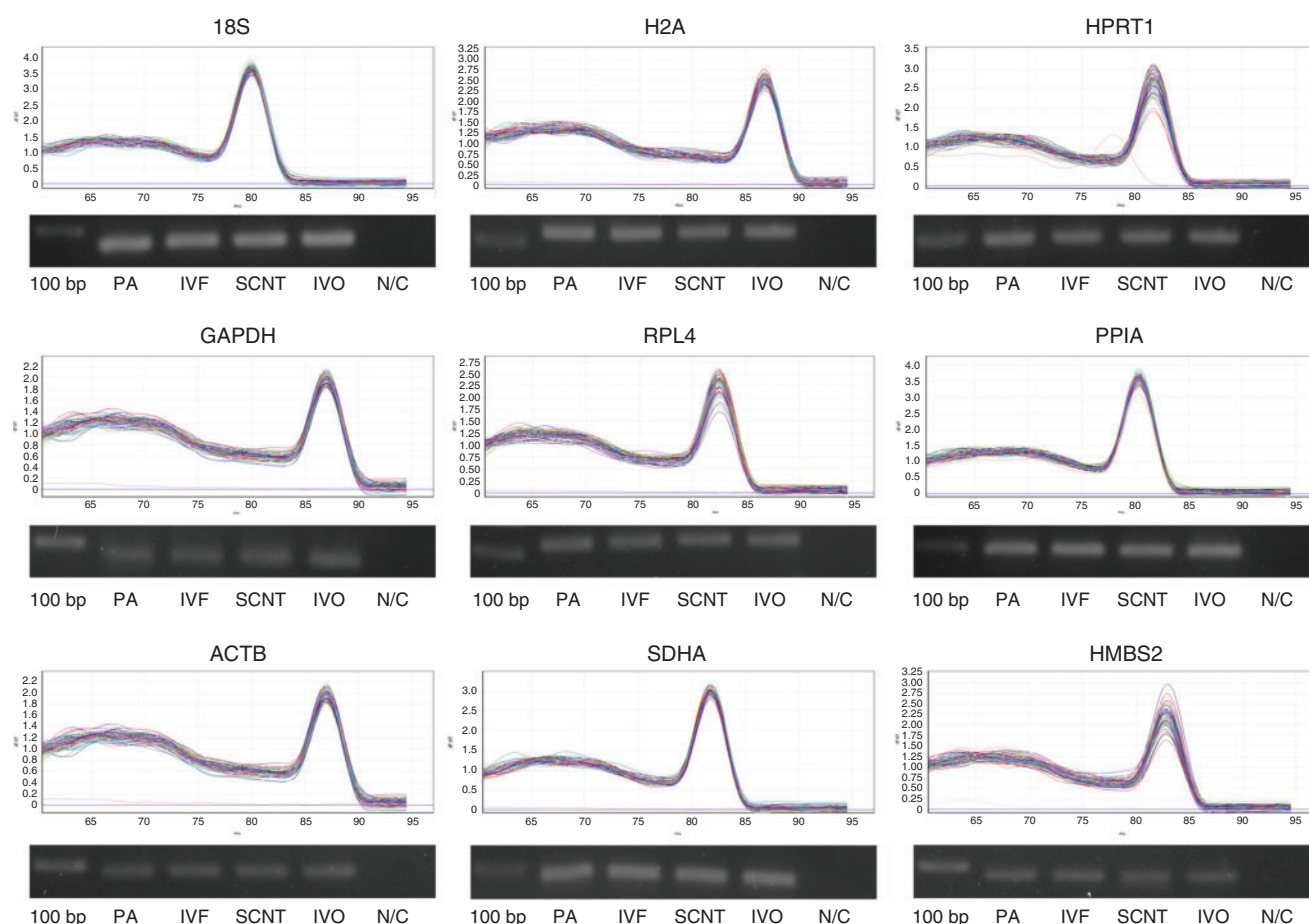


Fig. 1. Confirmation of primer specificity and expected amplicon size in reference genes. Each melting curve analysis of nine selected reference genes is presented and each amplified product is shown by agarose gel electrophoresis. 100bp, 100-bp ladder; PA, parthenogenetic activation blastocysts; IVF, *in vitro* fertilisation blastocysts; SCNT, somatic cell nuclear transfer blastocysts; IVO, *in vivo* blastocysts; N/C, negative control.

PASW Statistics 18, in accordance with a previous article (Lee *et al.* 2015).

Results

Examination of primer specificity and amplicon size

For the nine reference genes used in this study, melting curve analysis was performed at the end of the PCR reactions. All reference genes showed specifically high amplifications of a single peak without non-specific dimer bindings in melting curve analysis. In addition, all amplicons were confirmed by expected product sizes presenting a single band in all samples by agarose gel electrophoresis. Agarose gel images are displayed at the bottom of each melting curve graph (Fig. 1).

To check primer efficiency, the standard curves were generated and measured by using four-fold serial dilutions of cDNA preparations from a single PA embryo. The assays for the candidate reference genes were displayed in a linear form and correlation coefficient (R^2) indicated from 0.985 to 0.999. PCR efficiencies were found to be between 0.92 and 1.04 (Table 2). Based on the correlation coefficient and PCR efficiency results, the assays can be regarded as acceptable and valid for the quantification of transcripts and further experiments.

Average of CT values of reference genes in blastocysts produced by different methods

To investigate the RNA transcription levels across blastocysts produced by different *in vitro* and *in vivo* methods, the CT values from each reference gene were compared and presented in Fig. 2. Cycle threshold values of *H2A* and *SDHA* did not differ across different classes of blastocysts. However, the values of the other seven reference genes were significantly ($P < 0.05$) different. In particular, of the traditionally used reference genes such as *ACTB*, *GAPDH* and *18S*, *ACTB* showed significantly ($P < 0.05$) higher CT values in IVO than IVF and SCNT embryos, while *18S* showed significantly ($P < 0.05$) lower CT values under the same comparison. Further, significantly ($P < 0.05$) higher CT values of *GAPDH* were revealed in IVO and IVF than PA and SCNT embryos.

Examination of stable reference genes geNorm analysis

Using this program, we ranked the nine candidate reference genes following M values in blastocysts produced by different *in vivo* and *in vitro* methods according to their expression stability

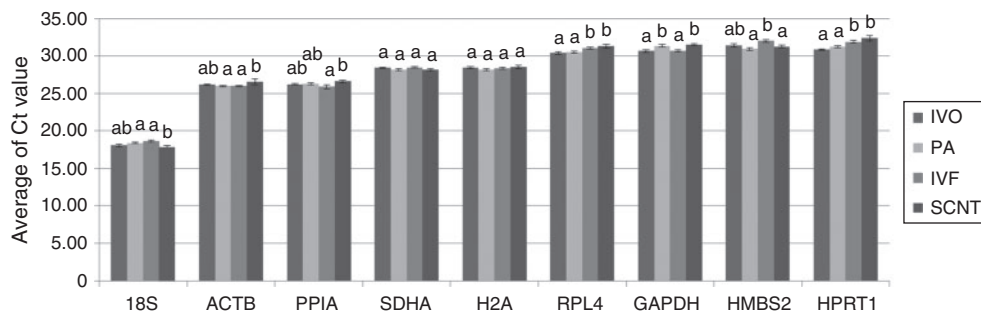


Fig. 2. Average of CT values of reference genes from embryos produced by different *in vivo* and *in vitro* methods. The CT value of each set of primers was analysed by ANOVA with Tukey's *post hoc* test. ^{a,b}Significant differences are indicated by different letters above bars ($P < 0.05$). Graphs are presented as mean \pm s.e.m.

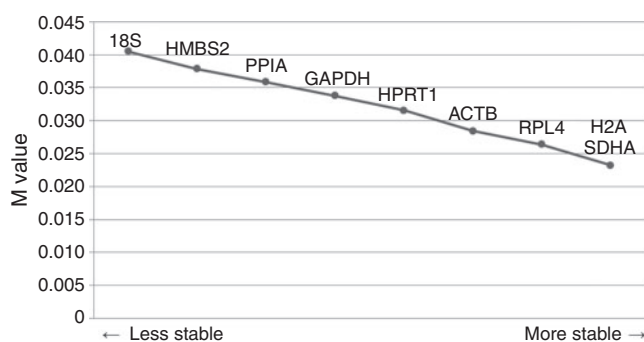


Fig. 3. Ranking of stability (M values) of reference genes by using geNorm analysis. M values of nine reference genes were analysed by the geNorm program from each CT value from qRT-PCR using embryos produced by different *in vivo* and *in vitro* methods. The left side of the graph indicates high M value, which means low stability, and the right side of graph presents high stability with low M value.

(Fig. 3). The gradual stepwise exclusion of the least-stable genes revealed that *H2A*, *SDHA* and *RPL4* were the three most-stable genes; however, expression of *18S* showed the least stability and *GAPDH* revealed moderately stable expression among the nine candidate reference genes. Moreover, pairwise variations showed that the use of seven reference genes ($V_{7/8}$) was regarded as the optimal number of reference genes (Fig. 4a). However, because it is hard to apply seven reference genes to study embryo gene expression, correlation of normalisation factors between the optimal number (NF_7) and three of the most-stable reference genes (NF_3) was analysed to remove excessive usage of reference genes (Fig. 4b). High correlation ($r = 0.905$, $P < 0.01$) was determined between NF_7 and NF_3 by Pearson's correlation.

Normfinder analysis

In Normfinder analysis, the most-stable reference gene in embryos produced by different *in vivo* and *in vitro* methods was determined as *H2A* and the best combination of two genes from the nine candidate reference genes was revealed as being *RPL4* and *SDHA* (Fig. 5). This analysis displayed similar results to the geNorm analysis on *18S* and *GAPDH*, which presented as the least-stable reference gene and having moderately stable expression, respectively.

Bestkeeper analysis

The result of reference gene evaluation by Bestkeeper is presented in Table 3. The most-stable reference gene can be identified as presenting the lowest coefficient of variance ($CV \pm$ s.e.m.). Several reports have mentioned that presenting a s.e.m. higher than 1 should be considered unacceptable (Pfaffl *et al.* 2004). In this analysis, all of the nine candidate genes were available to be accepted as reliable reference genes because the estimation of the s.e.m. (\pm CT) of the CV (% CT) values for all reference genes was lower than cut-off. *SDHA* was determined to be the most-stable reference gene in embryos produced by different *in vivo* and *in vitro* methods, whereas *18S*, *GAPDH* and *ACTB* showed moderately stable expression.

The application for normalisation of evaluated reference genes

Taken together, both *SDHA* and *H2A* were evaluated as the most-stable reference genes, whereas *GAPDH* and *18S* showed moderate or the least stability, respectively, as analysed by three kinds of programs. Therefore, their effect as reference genes for normalisation purpose was examined and the levels of relative quantification are presented in Fig. 6. *OCT4* expression in PA and IVF blastocysts was normalised against *SDHA* and *H2A* as the most-stable reference genes and those results showed significantly ($P < 0.05$) upregulated *OCT4* expression in IVF blastocysts compared to PA blastocysts. In contrast, down-regulation of *OCT4* expression with no significant differences were observed when *GAPDH* or *18S* were applied in normalisation, respectively.

Discussion

Given the dynamic changes in pre-implantation embryos, it is hard to figure out embryonic development clearly because of limited amounts of starting material and due to little information about gene expression and species differences (Bettgowda *et al.* 2006; Kuijk *et al.* 2007; Mamo *et al.* 2008; Luchsinger *et al.* 2014). Prior to the fertilisation of embryos, the first meiotic prophase division of oocytes characterises transcription and translation of numerous genes, resulting in storage of mRNA and proteins for preparing early embryonic development. In contrast, transcription is halted and translation of mRNA is

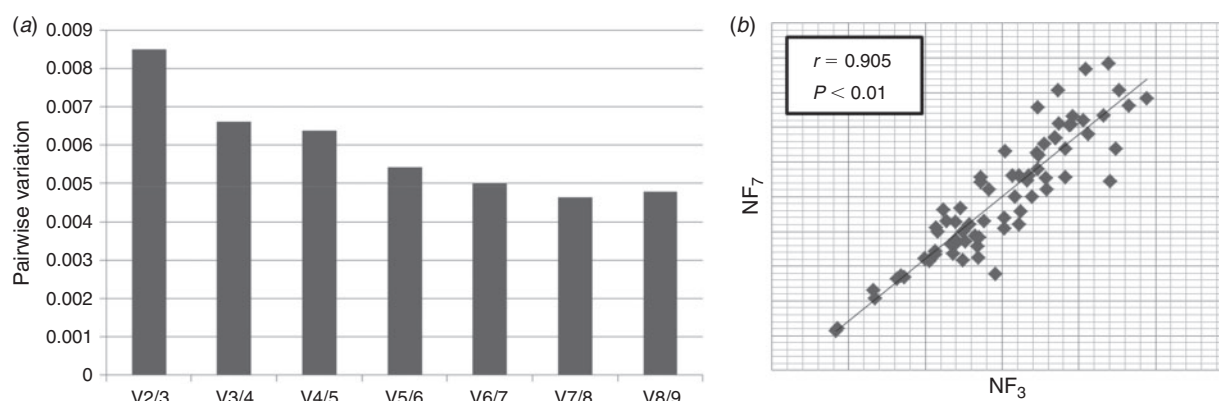


Fig. 4. (a) Optimal number of reference genes for normalisation and (b) correlation between NF_{opt} and NF_3 by using Pearson's correlation. (a) Determining optimal number of reference genes is based on pairwise variation $V_{n/n+1}$ by calculating each two sequential normalisation factors from geNorm. The low V value indicates optimal number of reference genes normalised to target genes. (b) Pearson's correlation was employed between optimal number of reference genes (NF_{opt}) and three of the most-stable reference genes with the lowest M value (NF_3).

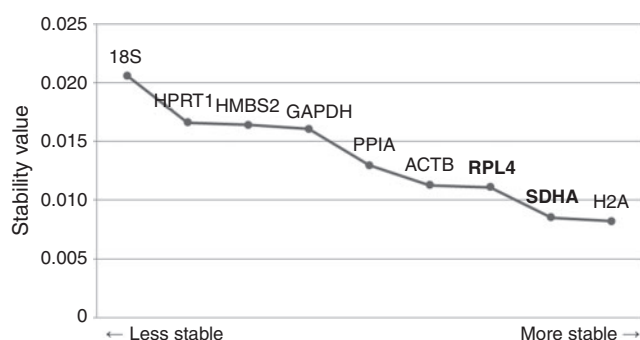


Fig. 5. Values of stability of reference genes by using Normfinder analysis. Stability values of nine reference genes were analysed by estimating intra- and inter-group variation to deduce the single most-stable reference gene. The left side of the graph indicates low stability and the right side of the graph presents high stability. Gene symbols in bold letters indicate the best combination of two genes.

reduced at the second meiotic metaphase division of oocytes (Arya *et al.* 2005). Further, pre-implantation embryos attain distinct shapes due to morphological changes, including cleavage division, compact morula and blastocyst with activation of the embryonic genome after the fertilisation. During these processes, differential expression of genes related to embryonic development is regulated in a stage- and time-dependent manner (Niemann and Wrenzycki 2000).

Likewise, different methods of embryo production also result in drastic changes including gene expression, developmental rate, total cell number and apoptosis (Kumar *et al.* 2007; Magnani and Cabot 2008; McElroy *et al.* 2008; du Puy *et al.* 2011). Compared with embryos produced *in vitro*, *in vivo* embryos show increased patterns of pluripotent gene expression and total cell number at the blastocyst stage, lower levels of chromosomal abnormalities, decreased trends of imprinting and apoptosis (Kumar *et al.* 2007). Thus, embryos produced by different methods show a similar pattern, but not identical expression of pluripotent genes. The highest abundance of

Table 3. Expression stability of reference genes evaluated by Best-keeper software

	<i>HMBS2</i>	<i>HPRT1</i>	<i>GAPDH</i>	<i>RPL4</i>	<i>18S</i>	<i>PPIA</i>	<i>ACTB</i>	<i>H2A</i>	<i>SDHA</i>
s.e.m.	0.71	0.71	0.64	0.59	0.54	0.51	0.51	0.50	0.37
[\pm CT]									
CV	2.27	2.26	2.07	1.92	2.94	1.93	1.79	1.93	1.30
[% CT]									

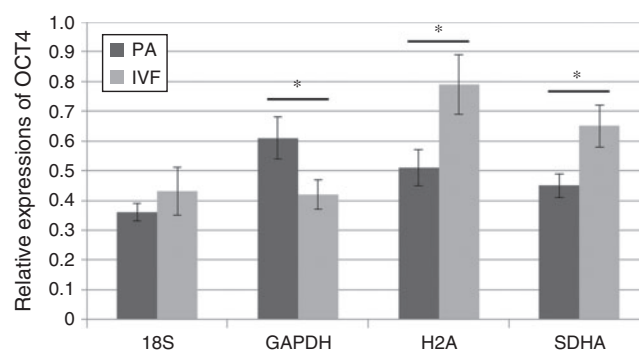


Fig. 6. The application to normalisation of evaluated reference genes. Relative expression level of *OCT4* was normalised against *SDHA*, *H2A*, *GAPDH* and *18S* in IVF and PA embryos. Significant ($P < 0.05$) differences in *OCT4* expression level between IVF and PA are presented as an asterisk.

Nanog expression from *in vivo* embryos was observed at the four-cell and blastocyst stage (Magnani and Cabot 2008). Furthermore, an investigation focussed on the inefficiency of porcine SCNT embryos has revealed that abnormal patterns of developmental efficiency and gene expression were caused by incomplete reprogramming of the donor cell genome as well as aberrant epigenetic mechanisms related to DNA methylation and histone de-acetylation (Kumar *et al.* 2007, 2013; Zhao *et al.*

2010). It has been reported that reference genes in early embryonic developmental stages were differentially expressed and hence the accurate assessment of gene expression could not be performed (Robert *et al.* 2002; Mamo *et al.* 2007, 2008; Gu *et al.* 2014). Gene expression of different embryos reflects their own characteristics, therefore, investigations should be focussed on increasing the accuracy of reliable reference genes for appropriate comparative gene expression studies.

The validation of candidate reference genes is critical for accurate analysis of embryo gene expression to prevent false conclusions from invalidated reference genes (Bas *et al.* 2004; Dheda *et al.* 2004; Haller *et al.* 2004). Differences in relative quantification of IL-4 mRNA expression after normalisation against different reference genes, *GAPDH* and *human acidic ribosomal protein (HuPO)*, were present in blood sample of patients with pulmonary tuberculosis and in healthy individuals. In case of normalisation against *HuPO*, there was a significant ($P < 0.0001$) difference between groups but it was not the same when data was normalised against *GAPDH* (Dheda *et al.* 2004). In agreement with these results, Fig. 6 showed that evaluation of reference genes was a highly essential step for a particular experiment. Relative quantification of *OCT4* after normalisation against *SDHA* and *H2A* (evaluated as stable reference genes) presented significant ($P < 0.05$) upregulation of *OCT4* in IVF blastocysts; however, *GAPDH* and *18S* were assessed as less-stable genes and showed different results from those of *SDHA* and *H2A*. Therefore, these differences by normalisation against non-evaluated or less-stable reference genes can lead to false conclusions.

Several previous studies have provided evidence that transcription levels of reference genes from embryos did not express constantly in different developmental stages and under different production methods (Mamo *et al.* 2007, 2008; Smits *et al.* 2009; Gu *et al.* 2014). In the present study, the differences in CT values of reference gene expression were observed in blastocysts produced by different *in vivo* and *in vitro* methods (Fig. 2). The CT values were inversely proportional to the amount of template present in the PCR amplification (Walker 2002). The majority of reference genes showed CT values in the range of 26 to 31 but *18S* showed a lower value of 18, which means more-abundant transcripts in any kind of blastocyst. However, most of the reference genes presented significant differences between different types of embryo, except for expression of *H2A* and *SDHA*, and there were no constant patterns that can be explained for all blastocysts produced by different *in vivo* and *in vitro* methods. Mouse and rabbit embryos also showed drastic changes at blastocyst stages presenting higher transcript copy numbers in the case of *in vivo* embryos compared with *in vitro* ones, and the variations were different in most of the reference genes (Mamo *et al.* 2007).

There are clear differences in morphological and molecular biological diversities within different mammalian embryos. Accordingly, even if some reference genes could be evaluated as having stable expression in certain embryos, there have been different results depending upon different species. Several studies have been performed to answer this issue of validating stable reference genes in embryos of different species, including mouse (Jeong *et al.* 2005; Mamo *et al.* 2007; Llobat *et al.* 2012;

Gu *et al.* 2014), cow (Robert *et al.* 2002; Goossens *et al.* 2005; Luchsinger *et al.* 2014), rabbit (Mamo *et al.* 2008), horse (Smits *et al.* 2009; Paris *et al.* 2011) and chicken (Yang *et al.* 2013). *PPIA*, *H2A* and *HPRT1* were determined as the most-stable reference genes in mouse embryos produced *in vivo* and *in vitro* by geNorm analysis (Mamo *et al.* 2007; Gu *et al.* 2014). In addition, the *H2A* gene was also suggested as a stable reference gene in single mouse eggs and pre-implantation embryos with the use of a simple freezing–thawing method for RNA collection and economic SYBR-green dye system for quantitative RT-PCR (Jeong *et al.* 2005). In rabbit pre-implantation embryos, the use of *H2A*, *YWHAZ* (tyrosine 3-monooxygenase/ Tryptophan 5-monooxygenase activation protein, zeta) and *HPRT1* was preferable for normalisation (Mamo *et al.* 2008). While working with chicken embryo fibroblasts infected with avian leucosis virus, commonly used *ACTB* and *GAPDH* were found to be unsuitable reference genes with varied expression as compared with the most-stably expressed *RPL30* (ribosomal protein L30) and *SDHA* with the lowest M value by geNorm analysis (Yang *et al.* 2013). In addition, *SDHA*, *PGK1* (phosphoglycerate kinase 1) and *TBP* (TATA box binding protein) have been shown as the most suitable genes among the analysis of 14 internal control genes in case of embryonic (ES), trophectoderm (TS) and extra-embryonic endoderm (XEN; Veazey and Golding 2011). These results support our findings that *H2A* and *SDHA* can be a better choice for porcine embryology to get the most-stable expression in quantitative real-time PCR for normalisation of interest genes (Fig. 3 and 5; Table 3).

Earlier, in one porcine embryo study, only IVF embryos were tested for selection of reference genes by using a single-analysis program. The study determined that *GAPDH* was the most-stable reference gene in porcine IVF embryos. Furthermore, *H2A* and *18S* were revealed as moderately stably expressed genes (Kuijk *et al.* 2007). In the last two previous articles about bovine embryos, *GAPDH* was investigated as a stable reference gene in bovine embryos regardless of not only different production procedures such as IVF, SCNT and intracytoplasmic sperm injection (ICSI) but also different pre-implantation stages (Goossens *et al.* 2005; Luchsinger *et al.* 2014); our results are not in agreement with those findings. We concluded that *H2A* expression is the most stable, whereas *18S* or *GAPDH* were found to be expressed as least- or moderately stable genes, respectively. These differences in our results compared with other reported studies may be due to different species, composition of candidate reference genes (Table 1) and different experimental groups including IVO, PA, IVF and SCNT embryos.

The expression of reference genes in embryos produced by different *in vivo* and *in vitro* methods was compared using three different commonly used analysis programs for investigating stable expression. geNorm, Normfinder and Bestkeeper were programmed by different algorithms and analytical principles (Vandesompele *et al.* 2002; Andersen *et al.* 2004; Pfaffl *et al.* 2004). In agreement with other studies resulting in an optimal number of reference genes from geNorm analysis (Uddin *et al.* 2011; Lee *et al.* 2015), it is not easy to use many reference genes for normalisation in embryo studies because of insufficient starting material and unnecessarily excessive experiments. For

this reason, the correlation of normalisation factors by Pearson's correlation was analysed between the optimal number (NF₇) from pairwise variation and three of the most-stable reference genes (NF₃; Fig. 4). The analysis showed high correlation between NF₇ and NF₃ ($r = 0.905$) and this result demonstrated that three of the most-stable reference genes by geNorm analysis should be satisfactory to use for normalisation. Although rankings from analysis by the three programs presented slightly different results, there were similar patterns about the stability orders of more- or less-stable reference genes by each program despite the variations of embryo sources. *SDHA*, *H2A* and *RPL4* were found to be three of the most-stable reference genes by geNorm and Normfinder analysis, whereas *SDHA*, *H2A* and *ACTB* presented stable gene expression by Bestkeeper analysis (Fig. 3 and 5; Table 3). Taken together, all analysis programs comprehensively concluded that *SDHA* and *H2A* were expressed stably across porcine blastocysts produced by different *in vivo* and *in vitro* methods. On the other hand, *18S* and *GAPDH* were determined as the least-stable or moderately stable reference genes, respectively. Our results using various analysis programs are in agreement with other published studies. To verify appropriate reference genes to investigate both diploid and polyploid mouse embryos, the stability of twelve reference genes was compared and *UBC* (ubiquitin C), *PPIA* and *PGK1* were determined to be the most-stable reference genes by four different programs (Gu *et al.* 2014). By using same sets of analysis programs as the present study, *RPL4*, *PPIA* and *YWHAZ* were determined as the most appropriate combination of reference genes for normalisation of different porcine tissues at different ages (Uddin *et al.* 2011) and the use of *PPIA*, *ACTB*, *GAPDH* and *SDHA* was recommended as appropriate reference genes for the study of porcine articular cartilage (McCulloch *et al.* 2012).

In conclusion, genomic DNA-free total RNA extracted from single blastocysts produced by different *in vivo* and *in vitro* methods was evaluated for stable expression of reference genes for reliable normalisation by qRT-PCR analysis. Three kinds of analysis programs displayed similar results that the *SDHA* and *H2A* genes were suggested as the appropriate reference genes across various porcine blastocysts and *18S* was revealed as the least-stable reference gene. The present study suggests that appropriate evaluation of reference genes for normalisation guarantees more-reliable results of gene expression studies in porcine embryos.

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