J Anim Reprod Biotechnol 2022;37:96-105
pISSN: 2671-4639 • eISSN: 2671-4663
https://doi.org/10.12750/JARB.37.2.96

JARB
Journal of Animal Reproduction and Biotechnology
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Original Article

Selection of suitable reference gene for gene expression studies of porcine ovaries under different conditions in quantitative reverse transcription polymerase chain reaction assay

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Received June 11, 2022
Accepted June 14, 2022

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ABSTRACT The ovary undergoes substantial physiological changes along with estrus phase to mediate negative/positive feedback to the upstream reproductive tissues and to play a role in producing a fertilizable oocyte in the developing follicles. However, the disorder of estrus cycle in female can lead to diseases, such as cystic ovary which is directly associated with decline of overall reproductive performance. In gene expression studies of ovaries, quantitative reverse transcription polymerase chain reaction (qPCR) assay has been widely applied. During this assay, although normalization of target genes against reference genes (RGs) has been indispensably conducted, the expression of RGs is also variable in each experimental condition which can result in false conclusion. Because the understanding for stable RG in porcine ovaries was still limited, we attempted to assess the stability of RGs from the pool of ten commonly used RGs (18S, B2M, PPIA, RPL4, SDHA, ACTB, GAPDH, HPRT1, YWHAZ, and TBP) in the porcine ovaries under different estrus phase (follicular and luteal phase) and cystic condition, using stable RG-finding programs (geNorm, Normfinder, and BestKeeper). The significant ($p < 0.01$) differences in Ct values of RGs in the porcine ovaries under different conditions were identified. In assessing the stability of RGs, three programs comprehensively agreed that TBP and YWHAZ were suitable RGs to study porcine ovaries under different conditions but ACTB and GAPDH were inappropriate RGs in this experimental condition. We hope that these results contribute to plan the experiment design in the field of reproductive physiology in pigs as reference data.

Keywords: cystic ovary, estrus phase, porcine ovary, qPCR, reference gene

INTRODUCTION

Pigs (sus scrofa) are the most important animals in the field of livestock industry, toxicology, preclinical study, and biotechnology in Korea (Choi et al., 2020; Han et al., 2020; Kim et al., 2021). In normal and non-pregnant condition, the estrus cycle in pubertal female pigs repeats every 21 days with morphological changes in the ovaries

such as developing follicles to ovulation with luteolysis during the follicular phase (proestrus and estrus period for approximately 6 days) and small follicle waves with mature corpus luteum (CL) in the luteal phase (metestrus and diestrus period for approximately 15 days) by a complex regulatory network of excitatory and inhibitory hormones from the hypothalamic-pituitary-gonadal axis (Yang et al., 2018). Of note, the healthy and normal ovaries complicatedly mediate negative and positive feedback to the upstream reproductive tissues, using several steroidal hormones (estrogen and progesterone). However, the disorder of estrus cycle in female can lead to diseases, such as cystic ovary. According to the previous articles, the cystic ovary was derived from non-ovulatory but continually growing follicles until it exceeded a diameter of 11 mm, and its incidence in the female pigs was found to be 2.4-40.0%; high occurrence of cystic ovary in pig herds was directly associated with decline of overall reproductive performance due to anestrus, anovulation, lesser conception rates, irregular/prolonged estrus cycles, and metabolic disorders (Castagna et al., 2004; Cech and Dolezel, 2007). Therefore, the understandings for morphological and functional changes of ovaries under normal estrus cycle or abnormal condition including cystic ovary are essential to achieve better reproductive performance, resolve reproductive problems such as infertility, and help to develop effective husbandry plans (Cech and Dolezel, 2007; Kim et al., 2018; Hwangbo et al., 2019).

Several reports have addressed that the ovary undergoes substantial physiological changes along with inherent condition related with estrus cycle (e.g. follicle growth, follicle atresia during follicular wave, ovulation, formation of CL, luteolysis, and pregnancy) and other external factors. When global gene expressions in the porcine ovaries between proestrus and estrus stages or granulosa cells between normal and atretic stages were analyzed using a microarray, a number of differentially expressed genes (DEGs) were found at 2,167 or 1,684 genes, respectively (Terenina et al., 2017; Yang et al., 2018). In addition, in response to fluctuation of follicle-stimulating hormones (FSH) and lutenizing hormone (LH) to ovarian follicles, increased production of estradiol in the steroid-producing cells, elevated expression of receptors for LH and prolactin in the granulosa cells, up-regulated leptin in the mature follicles, and increased cytochrome P450 1A2 (CYP1A2) expression in the atretic follicles during follicular wave could be observable (Archanco et al., 2003; Picazo et al., 2004; De los Reyes et al., 2017; Toms et al., 2018; Hwang et al., 2020). And it has been known that pregnant ovaries turn hormonal production from estradiol at heat to progesterone from CL during gestation (Martínez-Giner et al., 2013). As external factors, immobilization stress could alter CYP1A2 expression in the ovaries and supplementation of reagent (butyric acid) to the granulosa cells or hormones (LH, oxytocin, and noradrenaline) to the luteal cells induced the changed pattern of hormonal secretion and receptors in the ovary-related tissues (Rekawiecki and Kotwica, 2007; Lu et al., 2017; Hwang et al., 2021). Therefore, it can be concluded that the morphology and function of the ovary are directly influenced by several inherent and external factors.

In particular, during these findings, quantitative reverse transcription polymerase chain reaction (qPCR) assay has been widely applied to reveal fluctuation of target gene expression in the ovary (Picazo et al., 2004; Rekawiecki and Kotwica, 2007; De los Reyes et al., 2017; Lu et al., 2017). Since qPCR assay has advantage of convenience, accuracy, sensitivity, reproducibility, and reliability, it has been widely applied in gene expression studies in the current days (Lee et al., 2017). However, because the results from qPCR can be influenced by sample quality, poor reverse transcription, mRNA quality, cDNA concentration, and others, the normalization step of target genes against reference genes (RGs) is indispensably conducted to adjust for these variations and to correct sample-to-sample or run-to-run variations; the RGs are believed as constant expression regardless of experimental conditions (Li et al., 2009; Lee et al., 2017; Hassanpour et al., 2019). Unfortunately, it has been identified that the expression of RGs is also variable depending on each experimental condition including various types of tissues, cells, phases of disease, and treatment (Lee et al., 2015; Katarzyńska-Banasik et al., 2017; Hassanpour et al., 2019). Of note, the application of unstable RGs to normalization step resulted to false conclusions (Lee et al., 2017). Therefore, a number of previous studies have paid attention to discover the most stable RGs, optimized for each experimental condition, as a prerequisite to obtain reliable results at the further study (Kolkova et al., 2013; Lee et al., 2015; Katarzyńska-Banasik et al., 2017; Lee et al., 2017; Hassanpour et al., 2019).

In human, the most stable RGs in the normal/diseased

ovaries or ovary-derived tissues with different treatments have been identified (Li et al., 2009; Kolkova et al., 2013; Lv et al., 2017). In pigs, although several studies evaluated the most stable RGs from several combinations of different tissues, the ovary was unfortunately excluded from these pools of tissues (Nygard et al., 2007; Svobodová et al., 2008; Li et al., 2011; Martínez-Giner et al., 2013). Because the normal ovary undergoes substantial physiological changes along with estrus cycle and the cystic ovary presents the hormonal imbalance, evaluation of the most stable RGs that can encompass porcine ovaries under different conditions is believed as a prerequisite step for obtaining the reliable results from qPCR assay in the further study. Therefore, we attempted to assess the most stable RGs in the normal ovaries under the follicular and luteal phases, and cystic ovaries from pigs, using stable RG-finding programs (geNorm, Normfinder, and BestKeeper).

MATERIALS AND METHODS

Ethics statement

All procedures were approved by the Institutional Animal Care Use Committee at Kyungpook National University (approval number: 2021-0098).

Chemicals and media

Unless otherwise specified, the chemicals and reagents were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

Sampling from pigs

The samples were collected from healthy sows who were raised in pig farms located near Daegu-si, republic of Korea (latitude: 128.63°E; longitude: 35.87°N), approximately two-year-old (pubertal), threeway crossbred [(Landrace × Yorkshire) × Duroc sows], weighed approximately 200 kg, and non-pregnant after slaughtering at the local abattoir. After slaughtering, normal ovaries under the follicular phase (n = 10) and luteal phase (n = 10) as well as cystic ovaries (n = 5) showing abnormally enlarged (> 1.5 cm), fluid-filled, and non-ovulatory follicles than normal follicles were collected; from both ovaries per a pig, one was snap-frozen for RNA extraction and the other was fixed with 4% paraformaldehyde (Duksan Chemical, Korea) for determination of the estrus phase (follicular or luteal phase). And during slaughtering process, fresh whole bloods from the sows for normal ovary groups were collected in the plain tubes and allowed to clot at room temperature (RT) to further confirm the estrus phases of each ovary by enzyme-linked immunoabsorbent assay (ELISA); the bloods from pigs with cystic ovary were not obtained because assessing hormonal imbalance in diseased ovaries was not the main purpose in the present study. Then, sera were isolated by centrifugation at 4,000 rpm for 15 min in 4℃. The isolated sera were immediately transferred into new tubes and stored at a deep freezer at -80℃ until further analysis.

Enzyme-linked immunoabsorbent assay (ELISA) to confirm estrus phase

To further confirm the estrus phase of sampled normal ovaries between the follicular and luteal phase, the estradiol and progesterone concentration in the corresponding serum were evaluated by ELISA (Cayman Chemical Company, MI, USA). In brief, thawed serum sample, enzyme-immunoassay buffer, tracer, and antiserum (estradiol or progesterone) were co-incubated for 60-90 min in RT, which was followed by reacting with development reagents (Ellman's Reagent) for 60 min in the incubator. Then the reacted 96-well plate was read at a wavelength in 414 nm using a microplate reader (Epoch, Biotek, VT, USA). The concentrations of each hormone in the serum were calculated by a 4-parameter logistic fit using free software (www.myassay.com).

Candidate of reference genes (RGs)

Given that the candidates of RGs in the present study were necessary to be selected on the basis of commonly used ones in the previous researches with different intracellular biological pathways, the total number of ten RGs (18S, B2M, PPIA, RPL4, SDHA, ACTB, GAPDH, HPRT1, YWHAZ, and TBP) was selected to further analyze their stability in porcine ovaries under different conditions (Lee et al., 2015; Lee et al., 2017). The detailed information of each gene with respect to full name, symbol, sequence, and accession number were described in Table 1.

RNA extraction, cDNA preparation, and qPCR running

The ovaries were evenly cut with blades (1 × 1 cm) and minced with a homogenizer in the ice box. The total RNA was then extracted from homogenized ovaries using a QIA shredder column and RNeasy mini Kit (Qiagen, CA, USA)

Table 1. Information on primers used in the present study

Gene name	Gene symbol	Primer sequences	Accession number
18S ribosomal RNA	18S	F: cgcggaaggatttaaagtg R: aaacggctaccacatccaag	NR_046261.1
Beta-2-microglobulin	B2M	F: tccgccccagattgaaattg R: tccttgctgaaagacaggtctg	NM_213978.1
Peptidylprolyl isomerase A	PPIA	F: aaaacttccgtgctctgagc R: ttatggcgtgtgaagtcacc	NM_214353.1
Ribosomal protein 4	RPL4	F: caagagtaactacaaccttc R: gaactctacgatgaatcttc	XM_005659862.3
Succinate dehydrogenase complex, subunit A	SDHA	F: cacacgctttcctatgtcgatg R: tggcacagtcagcttcattc	XM_021076931.1
Beta actin	ACTB	F: tcaacaccccagccatgtac R: agtccatcacgatgccagtg	XM_003124280.5
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	F: acactcactcttctacctttg R: caaattcattgtcgtaccag	NM_001206359.1
Hypoxanthine phosphoribosyltrasnfrase1	HPRT1	F: aagcttgctggtgaaaagga R: gtcaagggcatagcctacca	NM_001032376.2
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	YWHAZ	F: tgcttcctttgcttgcatcc R: tcagggtaggcagggtttatag	XM_001927228.7
TATA box binding protein	TBP	F: aacagttcagtagttatgagccaga R: agatgttctcaaacgcttcg	DQ845178

in accordance with manufacturer's instruction. The quantified RNAs within 2 ± 0.2 range at the optical density ratio A260/A280 using an UV-Vis spectrophotometer (Nabi, MicroDigital Co., Ltd., Korea) were only collected. The cDNA was synthesized with 0.5 μg total RNA using Maxime RT PreMix Kit (iNtRON Biotechnology, Korea) at 45℃ for 60 min in a thermal cycler (Qiagen), which was followed by obtaining 20 μL cDNA. To obtain cycle threshold (Ct) values of each RG in porcine ovaries, the qPCR was conducted using a Step One Plus qPCR machine (Applied Biosystem, MA, USA) with 1 μL cDNA per reaction, 0.5 mM forward and reverse primers of RGs, and AccuPower® 2× GreenStar™ qPCR Master Mix (BIONEER, Korea) by means of predenaturation (95℃ for 10 min) and 45 PCR cycles (95℃ for 10 s and 60℃ for 10 s). Upon completing amplification by qPCR, the presence of gene-specific peaks and absence of nonspecific amplification were verified by melting curve analysis (60℃ to 95℃ at 1℃/s).

Assessment of stability of RGs in the porcine ovaries under different conditions

The stability of the raw Ct values of each RG in the normal and cystic ovaries of pigs was assessed by means of well-known and excel-based algorithms, geNorm, Normfinder, and BestKeeper (Kolkova et al., 2013; Martínez-Giner et al., 2013; Lee et al., 2017; Hassanpour et al., 2019). The geNorm suggests the stability ranking from the pool of RGs as M value, indicated that lower value is more stable than others and 1.5 of M value is considered as the cut-off value for further analysis. Similar with geNorm, the lower value in Normfinder is also considered as more stable. BestKeeper creates the stability ranking by standard deviation (SD) and coeffcient of variance (CV) of Ct values in the pool of RGs; lower value of SD is regarded as more stability and > 1 of SD value is considered as unacceptable RGs (Lee et al., 2017).

Statistical analysis

Student's t-test or one-way ANOVA with Tukey's post hoc was applied using SPSS 12.0 (SPSS Inc. Chicago, IL, USA) to analyze ELISA data or Ct values of each RG in porcine ovaries under different conditions, respectively. Significant differences were considered at $p < 0.01$.

RESULTS

Confirmation of estrus phases in the normal ovaries and morphology of cystic ovaries

First, the estrus phases of the normal ovaries were classified as the follicular and luteal phases by means of

morphologically observing for growing follicles and/or presence of CL in the sectioned surface of ovaries. The ovaries in the follicular (Fig. 1A) or luteal phase (Fig. 1B) presented developing follicles with luteolysis or small follicle waves with mature CL, respectively. In the further analysis by ELISA to confirm their estrus phases, the significantly ($p < 0.01$) higher estradiol (Fig. 1D) and lower progesterone (Fig. 1E) concentrations in the corresponding serum were identified in the follicular phase than luteal phase; the luteal phase showed the opposite pattern. The cystic ovaries in the present study morphologically had non-ovulatory, abnormally enlarged (> 1.5 cm), and fluid-filled follicles (Fig. 1C).

Specificity of RGs in qPCR

The melting curve analysis was performed at the end of the qPCR amplification program to evaluate the specificity of the ten RGs in the present study (Fig. 2). All RGs could amplify their PCR products with a high peak and without any nonspecific amplification, indicated that the primers of RGs in the present study were regarded as valid for the quantification of transcripts in porcine ovary samples.

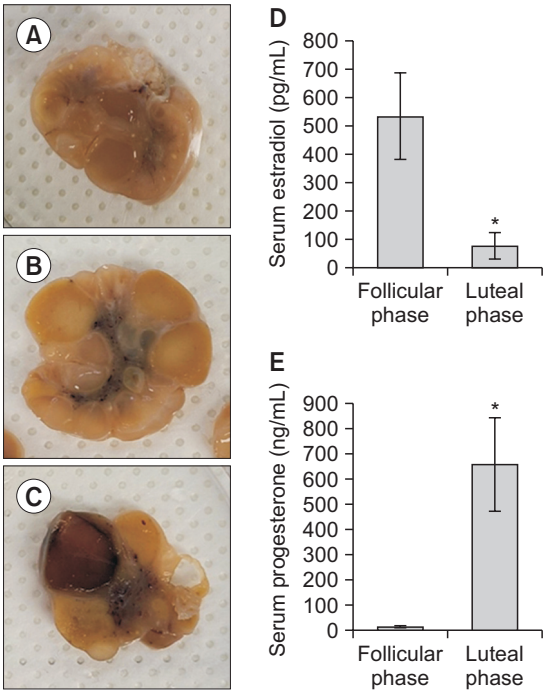

Fig. 1. Confirmation of estrus phases in the normal ovaries and morphology of cystic ovaries. Representative images were displayed for the follicular phase (A) developing follicles with luteolysis, luteal phase (B) with small follicle waves with mature corpus luteum, and cystic ovary with non-ovulatory, abnormally enlarged (> 1.5 cm), and fluid-filled follicle. While the follicular phase exhibited significantly ($p < 0.01$) higher estradiol and lower progesterone level, the luteal phase presented the opposite results.

Ct values of each RG in porcine ovaries under different conditions

As shown in Table 2, the majority of Ct values of RGs (ACTB, B2M, YWHAZ, GAPDH, RPL4, PPIA, and SDHA) were in the range of 20 to 24. While 18S showed a lower Ct value of about 18 (most abundant transcripts in the porcine ovaries), Ct values in TBP and HPRT1 were higher than 26 (less abundant transcripts). In addition, several RGs exhibited significantly ($p < 0.01$) different transcripts level among samples. Transcripts of 18S, B2M, and GAPDH were significantly ($p < 0.01$) abundant in the luteal phase and cystic ovaries than follicular ones. In addition, the mRNA levels of RPL4 and HPRT1 were significantly ($p < 0.01$) higher in the cystic ovaries than others. Because an equal amount of total RNA was used in qPCR procedure, these results implied that the transcript levels of RGs in the porcine ovaries were directly influenced by estrus phase and abnormal condition.

Assessment of stability of each RG in porcine ovaries

The raw Ct values of each RG in porcine ovaries under different conditions were assessed by geNorm, Normfinder, and BestKeeper. Both geNorm (Fig. 3A) and Normfinder (Fig. 3B) suggested that TBP, YWHAZ, and PPIA were the three most stable RGs in the porcine ovaries under different conditions and traditional RGs including ACTB, 18S, and GAPDH were interestingly the least stable ones. TBP, RPL4, and YWHAZ were the three most stable RGs but ACTB and GAPDH were unstable ones in accordance with BestKeeper analysis (Fig. 3C). In case of cut-off value in geNorm and BestKeeper, although all M values were lower than 1.5 in geNorm, indicated that the expressions were considered as valid as RG, three RGs (ACTB, GAPDH, and B2M) were determined as unsuitable for RGs (> 1 of SD) in porcine ovaries under different conditions in BestKeeper analysis. Although detailed rankings from three different programs showed slightly different results, they comprehensively agreed that TBP and YWHAZ were suitable RGs to study porcine ovaries under different conditions but ACTB and GAPDH did not.

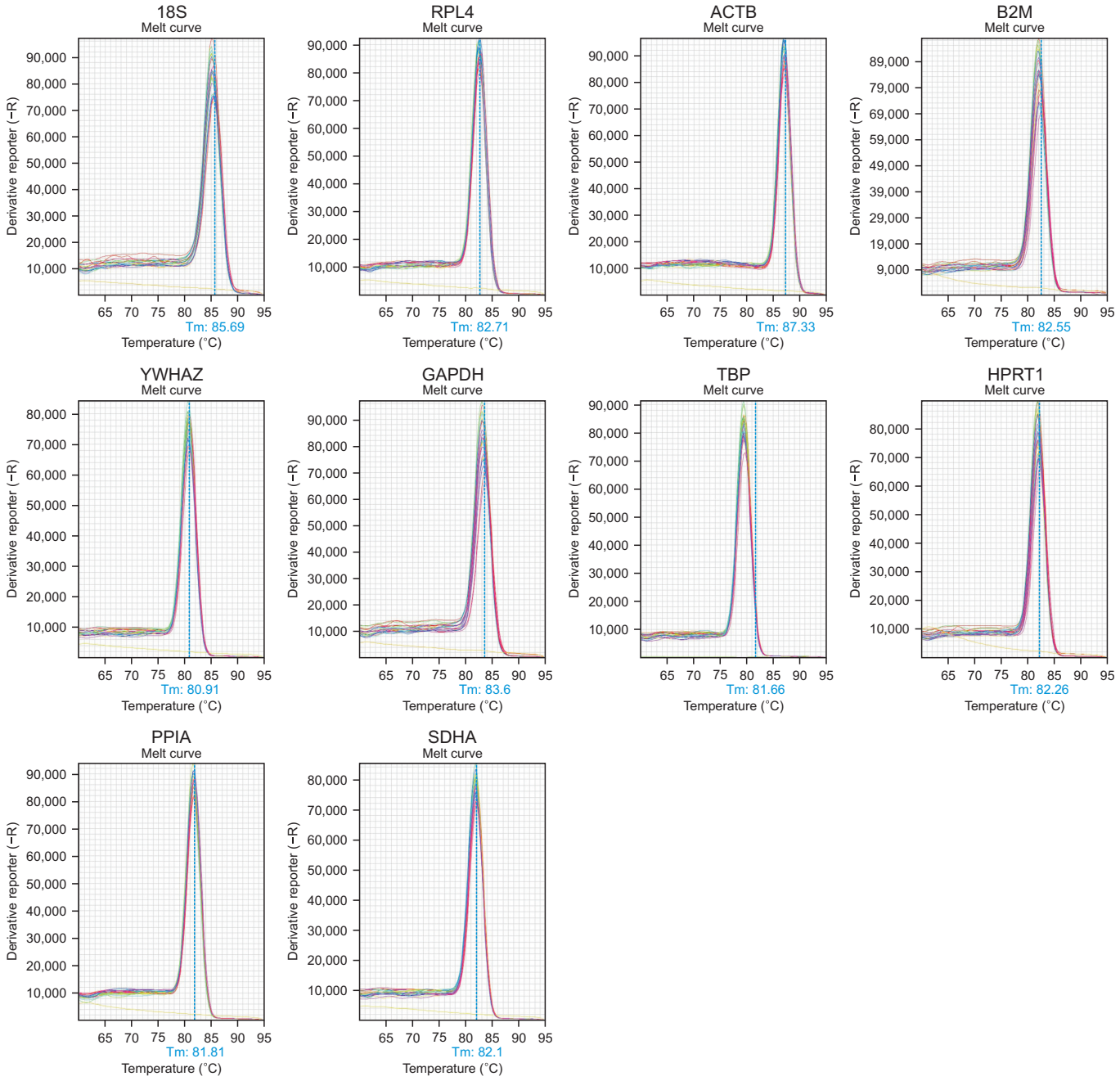

Fig. 2. Specificity of RGs in qPCR. The melting curve analysis showed that all RGs amplified their PCR products with a high peak and without any nonspecific amplification in the porcine ovaries.

DISCUSSION

In the normal condition, the ovary-relevant tissues such as the granulosa cells, follicular cells, theca cells, and luteal cells play a pivotal role in producing a fertilizable oocyte in the developing follicles, appearing estrus signs, and signaling to prepare for pregnancy by complex network of reproductive hormones (Toms et al., 2018; Yang et al., 2018). Meanwhile, etiopathogenesis of cystic ovaries in pigs remains elusive but it may involve hormonal imbalances during the follicular development in terms of high level of stress-related hormone (adrenocorticotrophic hormone), reduction of LH, increased intraovarian androgens, and circulating anti-Müllerian hormone levels; with-

Table 2. Ct values of each gene in different ovary condition

Gene	Follicular phase	Luteal phase	Cystic ovary
18S	18.55 ± 0.23	17.25 ± 0.25*	17.46 ± 0.28*
ACTB	23.59 ± 0.51	22.70 ± 0.16*	24.12 ± 1.16*
B2M	22.36 ± 0.38	20.43 ± 0.11*	20.37 ± 0.24*
YWHAZ	22.84 ± 0.40	21.96 ± 0.16*	21.78 ± 0.20*
GAPDH	22.76 ± 0.49	20.85 ± 0.18*	20.41 ± 0.20*
RPL4	22.64 ± 0.26	21.96 ± 0.21*	21.17 ± 0.12*
TBP	28.24 ± 0.32	27.76 ± 0.15*	27.54 ± 0.27*
HPRT1	27.58 ± 0.40	26.39 ± 0.23*	25.70 ± 0.42*
PPIA	22.05 ± 0.49	20.97 ± 0.18*	20.61 ± 0.29*
SDHA	24.54 ± 0.39	23.04 ± 0.24*	23.09 ± 0.36*

The values are presented as mean ± SEM. The asterisks indicate the significant ($p < 0.01$) differences from follicular phase to other groups (luteal phase or cystic ovary) of each gene.

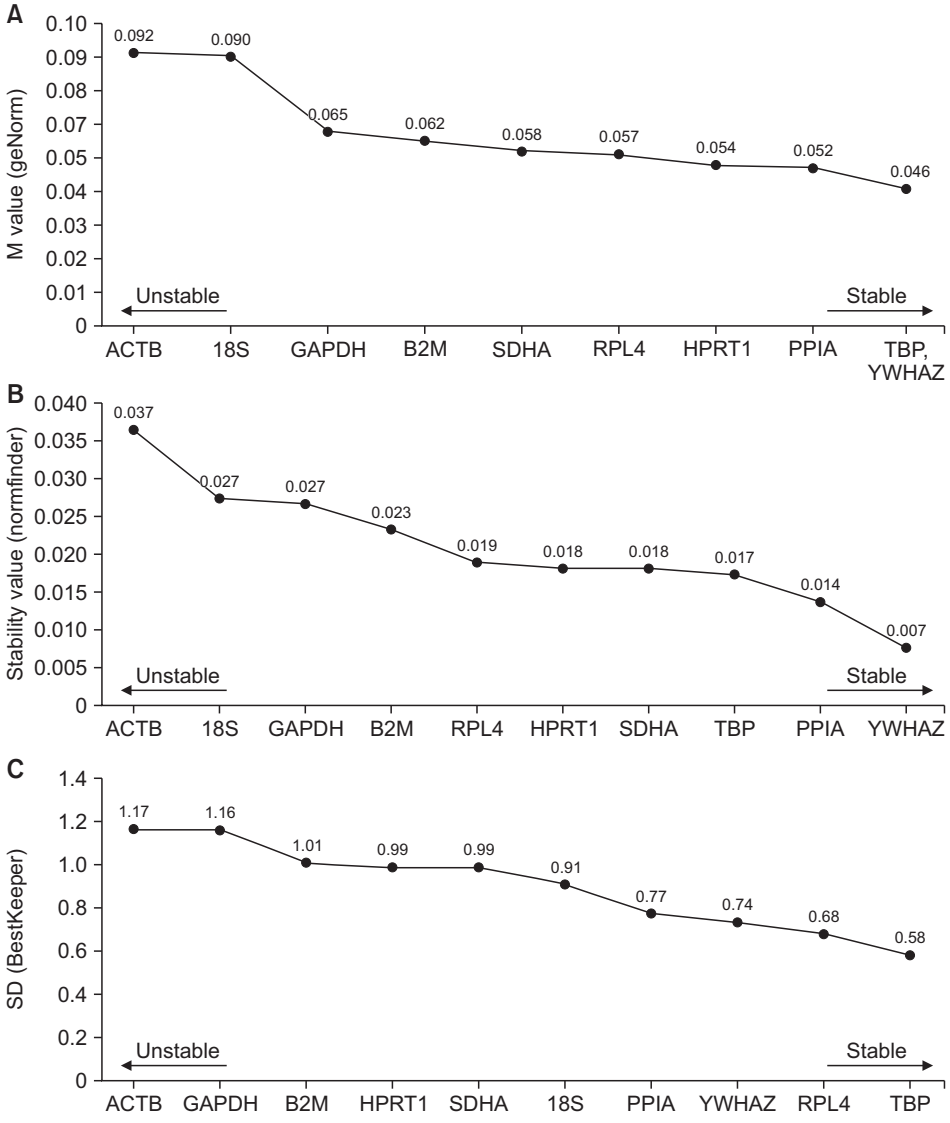

Fig. 3. Assessment of stability of each RG in porcine ovaries. The ranking of stability of RGs in porcine ovaries under different conditions were assessed by geNorm (A), Normfinder (B), and BestKeeper (C) analysis. In the all panels, the left to right side of the graph indicated lower to the most stable RG.

out proper treatment, favorable results in insemination rate and conception rate could not be expected in the sows with cystic ovaries (Cech and Dolezel, 2007; Lv et al., 2017). Likewise, because the changes of gene expression profile including RGs in the ovary is highly associated with their different conditions such as estrus phase and disease, a specific evaluation of suitable RGs in the porcine ovaries under different conditions is considered as a prerequisite step to make sure to obtain the reliable conclusion and to develop effective husbandry plan (Castagna et al., 2004; Cech and Dolezel, 2007; Terenina et al., 2017; Yang et al., 2018). Therefore, we attempted to identify the suitable RGs in porcine ovaries under several conditions during qPCR. As expected, the significant ($p < 0.01$) differences in Ct values of RGs in the ovaries obtained from different conditions were identified (Table 2). Thereafter, the Ct values were assessed for their stability by three different programs (geNorm, Normfinder, and BestKeeper) to evaluate the stability ranking from the pool of ten RGs (Fig. 3); because there has been no standard consensus method for the assessment of stability of RGs, we derived a conclusion for the suitable RGs in porcine ovaries under different conditions by intersection results obtained from each program. As a result, three programs comprehensively agreed that TBP and YWHAZ were suitable RGs to study porcine ovaries under different conditions but ACTB and GAPDH were inappropriate RGs in this experimental condition; the slightly different rankings among programs might be derived due to their different algorithms (Martínez-Giner et al., 2013; Lee et al., 2017). As far as we are aware, there is still no published research in the suitable RGs for porcine ovaries under different estrus phases and diseased condition.

In the previous study, it has been addressed that most molecular studies by qPCR use the traditional RGs including GAPDH, ACTB, and 18S for normalization (Lee et al., 2015). However, the expressions of these traditional RGs are also variable along with each experimental condition. The expression of GAPDH was changed in accordance with cell types and advanced stages of various kinds of solid tumors (Kolkova et al., 2013). The expression of ACTB gene, encoding a cellular structure for cytoskeleton, was fluctuated following cell migration and cell division. In case of stable RGs (TBP and YWHAZ) in the present study, their expressions are also variable depending on the experimental conditions. The expression of TBP was also affected depending on cell types such as normal and tumor tissues. YWHAZ was highly expressed in multiple types of cancers as well as can act as an oncogene (Gan et al., 2020). These findings suggest that identification of stable RGs in their experimental condition is important prior to qPCR assay because the expressions of RGs including traditional ones can be changed depending on internal and external factors.

Consistent with the present results, TBP and YWHAZ were suitable RGs for qPCR in other porcine specimens. TBP was determined as the most suitable RG in the pool of 17 kinds of porcine tissues (Nygard et al., 2007), porcine peripheral blood mononuclear cells (PBMC) with polyinosinic:polycytidylic acid (poly I:C) stimulation (Wang et al., 2014), and heat-stressed porcine PBMC (Xiang-Hong et al., 2011). Expression of YWHAZ was the most stable in porcine alveolar macrophages (AM) in response to lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Cinar et al., 2012), porcine PBMCs in response to LPS or LTA (Cinar et al., 2013), the pool of 13 kinds of tissues from newborn and adult pigs (Uddin et al., 2011), and the set of different fats and muscles (Gu et al., 2011). In addition, similar with the present results, traditional RGs (GAPDH and ACTB) were demonstrated to be the least stable in several studies. GAPDH was the least stable RG in the pool of 17 kinds of pig tissues (Nygard et al., 2007), in the pool of 13 kinds of tissues from newborn and adult pigs (Uddin et al., 2011), porcine AM treated with LPS and LTA (Cinar et al., 2012), and porcine PBMCs with LPS and LTA stimulations (Cinar et al., 2013). ACTB was also unstable in poly I:C-stimulated porcine PBMC (Wang et al., 2014).

In pig, only an article demonstrates the stable RGs in the ovary; the TBP was one of stable RG in the pregnant ovary during different physiological time points (heat, and 15, 30, 45, and 60 days of pregnancy) (Martínez-Giner et al., 2013). Likewise, although limited information is available for the stable RG in case of porcine ovaries, several articles with normal and/or diseased ovaries in other species have investigated the stability of RGs. Consistent with the present results, the expression of TBP in the human ovary-related tissue was determined as the most stable RG, with respect to the set of serous ovarian cancer and normal ovarian epithelial tissue (Li et al., 2009), the pool of benign, borderline, and malignant ovarian tumors (Kolkova et al., 2013) as well as GAPDH in hu-

man ovary was the most unstable RG in the set of serous ovarian cancer and normal ovarian epithelial tissue from human (Li et al., 2009), and in the pool of several types of ovarian tumors (Kolkova et al., 2013). In case of chicken, TBP or YWHAZ was stably expressed in the ovarian tissues treated with silver nanoparticles in geNorm analysis (Katarzyńska-Banasik et al., 2017) or in the set of heat-stress ovary and uterine, respectively (Hassanpour et al., 2019).

CONCLUSION

Despite RGs are believed as constantly stable regardless of each experimental condition, their expressions are also affected by inherent and/or external stimuli. Therefore, evaluation for specific RG in each experiment is required as an essential prerequisite step for further study. Here, we suggest that TBP and YWHAZ are stable RGs in porcine ovaries under different estrus phase and cystic condition, but GAPDH and ACTB are regarded as unsuitable RGs in this experimental design. We hope that these results contribute to plan the experiment design in the field of reproductive physiology in pigs as reference data.

Author Contributions: Conceptualization, H-D.K., H-J.J.; data curation, H-D.K., H-J.J.; formal analysis, H-D.K., H-J.J., M.J.; funding acquisition, W-J.L.; investigation, H-D.K., H-J.J., J-E.H.; methodology, H-D.K., H-J.J., J-E.H.; project administration, W-J.L.; resources, H-D.K., H-J.J.; software, S-G.B., S-H.Y.; supervision, S.J.K.; validation, S-J.K.; visualization, H-D.K., H-J.J.; writing original draft, H-D.K., H-J.J.; review & editing, S-J.K., W-J.L.

Funding: This work was supported by a grant from the National Research Foundation (NRF) of Korea, funded by the government of the Republic of Korea (NRF-2020R1F1A1076723).

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent to Publish: Not applicable.

Availability of Data and Materials: The data used to support the findings of this study are available from the corresponding author upon request.

Acknowledgements: None.

Conflicts of Interest: No potential conflict of interest relevant to this article was reported.

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