Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2026.070

RESEARCH ARTICLE

TBP and *HPRT1* are the Most Suitable Reference Genes for Normalization of mRNA Expression in Feline Gonadal Tissue-Derived Mesenchymal Stem Cells

Sang-Yun Lee[1,†,‡], Sanghyeon Park[2,†], Tae-Hyun Park[1,†], Chan-Hee Jo[2], Seong-Ju Oh[2], Chae-Yeon Hong[2], Yong-Ho Choe[2], Yeon-Woo Jeong[3], Hyeon-Jeong Lee[2], Eun-Yeong Bok[4], Won-Jae Lee[5], Sung-Lim Lee[2] and Young-Bum Son[1,*]

[*1]Department of Obstetrics, College of Veterinary Medicine, Chonnam National University, 300 Yonbongdong, Buk-gu, Gwangju 61186, Republic of Korea; [2]Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Gyeongsang National University, Jinju, 52828, Republic of Korea; [3]Department of Companion Animal and Animal Resources Science, Joongbu University, Geumsan 32713, Republic of Korea; [4]Division of Animal Diseases & Health, National Institute of Animal Science, Rural Development Administration, Wanju 55365, Republic of Korea; [5]Department of Obstetrics, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Republic of Korea. ‡Current affiliation: Department of Biology Education, Korea National University of Education, Cheongju 28173, Republic of Korea.
*Corresponding author: ybson@jnu.ac.kr

ARTICLE HISTORY (25-648)

Received: December 07, 2025
Revised: March 16, 2026
Accepted: March 22, 2026
Published online: March 25, 2026

Key words:
Feline
Mesenchymal stem cells
Ovarian tissue
Reference genes
Testicular tissue

ABSTRACT

Mesenchymal stem cells (MSCs), especially those derived from gonadal tissues that are easy to collect during routine sterilization procedures, provide an ethically viable source of feline MSCs. Considering MSCs are potential candidates for regenerative medicine, reporting therapeutic effects suggests that further research to develop more effective therapy using feline gonadal MSCs is needed for clinical application. Gene expression analysis and therapeutic potential of MSCs can be analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). Reliable interpretation of results requires accurate normalization. Hence, this study focused on determining the highest stability of candidate reference genes and accurate gene expression determination in ovary-derived MSCs (O-MSCs) and testis-derived MSCs (T-MSCs). O-MSCs and T-MSCs were retrieved from three female and three male felines, and then their stemness properties, which included spindle-like morphology, specific surface markers, and trilineage differentiation capacity, were characterized. To rank the nine candidate reference gene stability and pairwise variation stability, geNorm and NormFinder were used. In terms of overall analysis, *TBP* and *HPRT1* had the lowest stability values (<0.03), whereas *GUSB*, *RPL7*, and *ACTB* had higher stability values (>0.04). When stable reference genes were utilized for normalization, statistically significant differences in *OCT4* expression were observed between O-MSCs and T-MSCs ($P<0.05$), which were not observed with unstable reference genes. This provides a methodological framework by which future qRT-PCR-based studies can be conducted on feline gonadal MSCs. The potential therapeutic applications of these cells can also be studied.

To Cite This Article: Lee SY, Park S, Park TY, Jo CH, Oh SJ, Hong CY, Choe YH, Jeong YW, Lee HJ, Bok EY, Lee WJ, Lee SL and Son YB, 2026. *TBP* and *HPRT1* are the most suitable reference genes for normalization of mRNA expression in feline gonadal tissue-derived mesenchymal stem cells. Pak Vet J, 46(4): 930-939. http://dx.doi.org/10.29261/pakvetj/2026.070

INTRODUCTION

Mesenchymal stem cells (MSCs) have been receiving significant attention in regenerative medicine for their in vitro self-renewal ability, trophic properties, and immunomodulatory functions (Song *et al*., 2020). In veterinary medicine, therapeutic MSC applications have been documented in treating orthopedic, inflammatory, and immune-mediated diseases in canines and felines (Pérez-Merino *et al*., 2015; Harman *et al*., 2016; Arzi *et al*., 2020). Isolation of MSCs for clinical and research applications commonly uses adipose tissue and bone marrow (Russell *et al.*, 2016; Voga *et al*., 2020). However, they require invasive procedures to obtain the tissue (Alderete *et al.*,

2015; Ridgeway *et al*., 2017). Because gonadal tissues such as ovarian and testicular tissues were obtained during routine sterilization surgeries (Diagone *et al*., 2012; Binder *et al*., 2021), they are regarded as easily accessible and ethically acceptable sources of feline MSCs. The use of gonadal tissues enables efficient cell acquisition without requiring additional surgical procedures and allows autologous cell banking (Jeung *et al.*, 2024), serving as a practical source of MSCs in veterinary medicine. Moreover, gonadal tissue-derived MSCs were observed to be useful in therapeutic applications with a superior safety profile compared with adipose-derived MSCs, implying potential of regenerative therapy (Jeung *et al.*, 2024). Although MSC therapies for feline diseases such as gingivostomatitis have shown potential applicability (Arzi *et al.*, 2020), the molecular and therapeutic characteristics of feline MSCs remain underexplored. Progress in feline MSC research has been hindered by inconsistent methodologies and a lack of standardized validation within veterinary medicine (Morawska-Kozłowska *et al*., 2025). In addition, no MSC therapies have been approved for use in felines by authorities such as the Food and Drug Administration (FDA), and existing studies remain largely preliminary (Webb and Webb, 2024). Therefore, further investigation of molecular characteristics of feline MSCs are needed to establish future translational applications.

To analyze comprehensive molecular alteration and cellular functions, such as therapeutic and disease mechanisms, gene expression plays an important role (Erkin *et al*., 2022). Quantitative real-time polymerase chain reaction (qRT-PCR) possess great sensitivity, specificity, and simplicity of use. It is frequently used to analyze gene expression (Bustin, 2002). Accurate interpretation of gene of interest (GOI) expression across various studies, normalization with suitable reference gene is necessary. However, many factors including species, cell type, and experimental conditions affect the expression of reference gene (Kozera and Rapacz, 2013). Among different tissues, the reference gene expressions can vary. Therefore, confirming gene stability is essential to ensure reliable results when working with different MSC sources, including ovarian and testicular tissues. MSCs derived from porcine tissues sources have been analyzed to identify stable reference gene for qRT-PCR (Lee *et al.*, 2015). Although reference genes have been assessed in MSCs from different species, it is unclear feline gonadal tissue-derived MSCs reference gene expression stability.

Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β−actin (*ACTB*) have long been used as conventional reference genes and are often applied as internal controls for qRT-PCR interpretation (Suzuki *et al*., 2000). These traditional reference genes, however, may not be appropriate for qRT-PCR analysis (Lee *et al*., 2015; Jeon *et al*., 2019). Therefore, in every experiment, verifying gene reference stability is necessary. Reference gene stability was assessed using the complementary methods geNorm and NormFinder (Vandesompele *et al*., 2002; Andersen *et al*., 2004; Derveaux *et al*., 2010). NormFinder ranks gene stability by examining intra- and intergroup variation, while geNorm ranks the candidate genes by estimating the average pairwise variance and identifying the most stable combinations. GeNorm, however, does not differentiate groups in its analysis (Lanoix *et al*., 2012). In this case, comparison groups may provide different stability results depending on the design of the research. Integrating pairwise variation analysis with group-based systematic error correction allows geNorm and NormFinder to assess gene stability more comprehensively. The current study aimed to determine the most stable reference genes for qRT-PCR in feline MSCs. For this purpose, a combination of the two algorithms was chosen for a complete and precise evaluation of reference gene stability. We hypothesized that some reference genes would show consistent stability in gonadal tissue-derived MSCs, which would provide reliable qRT-PCR interpretation. Therefore, this study assessed reference gene stability for qRT-PCR analysis using MSCs isolated from feline gonadal tissues. For gene candidates, nine commonly used reference genes were selected: *ACTB*, *GAPDH*, TATA-box binding protein (*TBP)*, beta-2-microglobulin (*B2M*), glucuronidase beta (*GUSB*), hypoxanthine phosphoribosyl transferase 1 (*HPRT1*), ubiquitin C (*UBC*), ribosomal protein L7 (*RPL7*), and tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). Ultimately, this validation would improve the clinical translation of feline MSC-based regenerative therapies by making it easier to implement standardized protocols and quality control measures.

MATERIALS AND METHODS

Chemicals and media: All chemicals, unless otherwise specified, were purchased from Sigma (St. Louis, MO, USA), and all media were obtained from Gibco (Gibco Life Technologies, Gaithersburg, MD, USA).

Ethics approval: All experiments were approved by the Gyeongsang National University Institutional Animal Care and Use Committee (IACUC) (GNU-250326-T0058-01), and all IACUC guidelines were followed.

Collection and culture of MSCs from ovarian and testicular tissues: For this study, ovarian and testicular tissues were obtained from a total of six felines, consisting of three females and three males aged between one and three years, during routine ovariohysterectomy or castration procedures. The purpose of this experiment was to validate an exploratory methodology, by assessing the stability of reference gene rather than estimating the associated effect size. Each feline gonadal tissue was processed and analyzed separately to maintain biological variability, avoiding pooling the samples. The isolation and culture of MSCs from ovarian and testicular tissues were conducted following previously reported methods with minor modifications (Jeon *et al.*, 2019). Sterilized scissors were used to mince each tissue sample, which was then digested at 37°C in 0.1% collagenase I with gentle shaking. Following enzymatic digestion, the cell suspension was strained through a 100μm cell strainer and centrifuged at 300 ×g for 5minutes. Resulting cells were resuspended in Advanced Dulbecco's Modified Eagle's medium (ADMEM) supplemented with 1% penicillin-streptomycin (Pen-Strep), 1% GlutaMAX, and 20% fetal bovine serum (FBS), and were cultured in the same medium at 37°C in a humidified atmosphere of 5% CO_2. At 80–90% confluence, the cells were detached using 0.25% trypsin-ethylene-diamine-tetra-acetic acid (trypsin-EDTA) and collected by centrifugation at 300 ×g for

5minutes. The cells were cultured to passage 3 for each experiment.

Cell surface marker analysis using flow cytometry: To confirm the MSC-specific surface marker expression, cells were harvested by trypsinization and fixed in a 4% paraformaldehyde solution for 1 hour. The cells were stained with FITC mouse IgG1 κ isotype control (1:200; BD Biosciences, NJ, USA, cat. 550616), APC mouse IgG1 κ isotype control (1:200; BD Biosciences, NJ, USA, cat. 554681), FITC mouse anti-human CD34 (1:200; BD Biosciences, NJ, USA, cat. 560942), FITC rat anti-mouse CD44 (1:200; BD Biosciences, NJ, USA, cat. 553133), FITC rat anti-dog CD45 (1:200; ThermoFisher, Rockford, IL, USA, cat. MA1-80304), and APC mouse anti-human CD105 (1:200; ThermoFisher, Rockford, IL, USA, cat. 17-1057-42) at 4°C for 30 minutes in the dark. After staining, the cells were analyzed using FACSLyric™ flow cytometer (BD Biosciences, NJ, USA). Gating was based on the fluorescence profiles of the isotype control. To evaluate the proportion of cells expressing specific markers, the acquired data were processed using flow cytometry software (FlowJo™ v10; BD Biosciences).

***In vitro* trilineage differentiation of MSCs**: To assess their potential for trilineage differentiation, MSCs were induced into adipocytes, chondroblasts, and osteoblasts. The protocol followed that of a previous study with minor modifications (Jeon *et al.*, 2019). For adipocyte differentiation, MSCs were cultured for 21 days in DMEM supplemented with 1% Pen-Strep, 10% FBS, 1μM dexamethasone, 100μM indomethacin, and 10μM insulin. Adipogenic differentiation was verified by Oil Red O staining, which revealed intracellular lipid droplet accumulation (Sigma, St. Louis, MO, USA, cat. O0625). Differentiation into chondroblasts was induced for 21 days in STEMPRO™ chondrogenesis medium (STEMPRO™ chondrogenesis differentiation kit; Invitrogen, CA, US). Proteoglycan accumulation was evaluated using 1% Alcian blue staining (Sigma, St. Louis, MO, USA, cat. A3157). Osteoblast differentiation was conducted for 21 days with DMEM supplemented with 1% Pen-Strep, 10% FBS, 200μM ascorbic acid, 0.1μM dexamethasone, and 10mM sodium ß-glycerophosphate. Mineralization and calcium deposition were confirmed by Alizarin Red S staining (Sigma, St. Louis, MO, USA, cat. A5533). Trilineage differentiation was qualitatively confirmed by observing specific staining under bright-field microscopy.

Selection of candidate reference genes: Nine candidate reference genes were chosen based on prior studies and their diverse intracellular functions (Ho and Patrizi, 2021; Song *et al.*, 2022; Lee *et al.*, 2024). Primer sequences were designed using Primer3Plus software and verified with OligoAnalyzer 3.1 software to avoid secondary structures such as hairpins, homodimers, or heterodimers. To validate the amplification performance of each primer pair, standard curves were generated using a four-fold dilution series of cDNA from 1:10 to 1:10,000. The slope (M), intercept (B), PCR efficiency (E), and correlation coefficient (R^2) for each primer were calculated using Rotor-Gene Q v2.1.0 software (Qiagen, Hilden, Germany). Detailed primer information is provided in Table 1.

Table 1: Lists of candidate reference genes and their information

	Information of primers			Standard curve parameters			
Gene name (Symbol)	Sequence	Base pair	Accession	R^2	M	B	E
Beta-actin (*ACTB*)	F: GGACTTCGAGCAGGAGATGG	186	ON164672.1	0.984	-3.519	35.152	1.02
	R: ATGATGGAGTTGAAGGTAGTTTCG						
Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)	F: CTGGAGAAAGCTGCCAAATACG	117	XM_006933438.4	0.989	-3.488	34.814	0.99
	R: GTTAAAGTCGCAGGAGACAACC						
TATA-box binding protein (*TBP*)	F: AGCTTGACCTAAAGACCATTGC	80	JQ424890.1	0.995	-3.423	34.857	1.02
	R: TCTCATGATAACAGCAGCAAACC						
Beta-2-microglobulin (*B2M*)	F: CTCCAAAGGTTCAGGTTTACTCC	184	NM_001009876.1	0.992	-3.412	38.941	1.01
	R: ACCAGAAGATAGAAAGTCCAGTCC						
Glucuronidase beta (*GUSB*)	F: GTGTATTGCATGATCCTGAGACC	155	NM_001009310.1	0.991	-3.481	38.915	1.02
	R: CCCATCCTTCATAGCTGTATGC						
Hypoxanthine phosphoribosyl transferase 1 (*HPRT1*)	F: GCTCCTCTATACCACACCTACC	185	XM_023248905.2	0.993	-3.461	37.115	0.99
	R: CGACTTTGCCTTCCTCATCC						
Ubiquitin C (*UBC*)	F: CGCTCGATCTCTTGGTAAATACG	84	XM_045041876.1	0.999	-3.495	36.022	1.01
	R: GGGTGGTCTTCTTCTTCATTCC						
Ribosomal protein L7 (*RPL7*)	F: CTGCTGGTGATGACAAGAAAGG	152	XM_023248286.2	0.997	-3.512	35.172	0.98
	R: CTGATAGGGTGTGTTGGTTGC						
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*)	F: TCAGACTGAAGAGCTACTGTAACG	103	XM_006943327.5	0.992	-3.416	36.955	0.99
	R: TGCAACCTTGACCATCTTTGG						
POU class 5 homeobox 1 (*OCT4*)	F: CACCATCGAGAATGTCAAGGC	160	NM_001173441.1				
	R: TGTTTCCCAGCAAAGATCAACC						

RNA isolation, reverse transcription, and qRT-PCR: Total RNA was isolated from MSCs derived from each animal, without pooling, using the easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Seongnam, Korea) following the instructions provided by the manufacturer. The quantity and purity of RNA were confirmed with a spectrophotometer (OPTIZEN™ NanoQ Lite; K LAB, Daejeon, Korea). For reverse transcription, 500ng of total RNA was used, and the HiSenScript™ RH(-) RT PreMix Kit (iNtRON Biotechnology, Seongnam, Korea) was utilized to produce cDNA. For qRT-PCR, 50ng of cDNA, 0.2µM forward and reverse primers, and RealMOD™ Green AP 5x qPCR mix (iNtRON Biotechnology, Seongnam, Korea) were combined, and the reaction mixture was placed into a Rotor-Gene Q cycler (Qiagen, Hilden, Germany). The conditions for the reaction were 95°C for 12 minutes of initial activation, with 40 subsequent PCR cycles (95°C for 15 seconds, 60°C for 25 seconds, and 72°C for 25 seconds). Cycle threshold (Ct) values and melting curves were acquired and processed using Rotor-Gene Q software. PCR products were additionally validated through gel electrophoresis to confirm the amplification of intended targets.

Evaluation of reference gene stability: To assess the expression stability of the nine candidate reference genes, the geNorm and NormFinder algorithms were used, which are among the most commonly employed methods. With the geNorm algorithm, the expression stability of genes is measured using the M value, which is the average standard deviation of the log-transformed expression ratios of one gene and all other genes. Genes with the highest M values, reflecting low stability, were removed step by step until the two most stable ones remained. In addition, geNorm determined the optimal number of reference genes by continuously assessing the pairwise variation ($V_{n/n+1}$) between sequential normalization factors (NFs) (Vandesompele *et al.*, 2002). To prevent excessive reference genes, the Pearson correlation between NF_2, which is calculated from the two most stable genes, and NF_{opt}, which is based on the optimal number of genes, was analyzed using SPSS. Using ANOVA, NormFinder was used to calculate stability values, which reflect intra- and intergroup variation. NormFinder orders all the candidates based on their stability values and from this identifies the single most stable reference gene, which is the one with the lowest stability value. The most suitable pair of genes for normalization was also determined (Andersen *et al.*, 2004).

Normalization of GOI against different reference genes: To evaluate the impact of reference gene stability, the relative mRNA levels of the GOI, *OCT4*, were normalized using both the most stable and least stable reference genes identified through stability analysis. qRT-PCR was performed as detailed above, and expression levels were calculated using the Rotor-Gene Q software.

Statistical analysis: All experimental data were examined through SPSS version 23. Student's t-test was used to determine statistical differences. Prior to performing the t-test, data normality was assessed. Data are presented as the mean ± standard deviation (SD) of three biological replicates, each analyzed in seven technical replicates. A value of $P<0.05$ was considered statistically significant.

RESULTS

Derivation and phenotyping of gonadal tissue-derived MSCs: Feline MSCs were successfully isolated from feline ovarian and testicular tissues. The ovary-derived MSCs (O-MSCs) and testis-derived MSCs (T-MSCs) were cultured in standard growth media and exhibited a spindle-like morphology (Fig. 1A). To confirm MSC-specific characteristics, surface markers of O-MSCs and T-MSCs were analyzed using flow cytometry. Both O-MSCs and T-MSCs were positive for MSC-specific markers CD44 and CD105 but negative for hematopoietic markers CD34 and CD45 (Fig. 1B). Under appropriate differentiation conditions, O-MSCs and T-MSCs gave rise to adipocytes, chondrocytes, and osteocytes, confirming their multipotent nature. Lipid droplet formation was confirmed through staining with the Oil Red O dye, indicating successful adipogenic differentiation. Proteoglycan extracellular matrices were confirmed through staining with Alcian blue dye, indicating successful chondrocytic differentiation. Alizarin Red S staining confirmed that cells differentiated into osteoblasts, as verified by the mineral nodules. (Fig. 1C).

Evaluation of Ct values, amplicon size, and primer efficiency of candidate reference genes: Standard curves for primer evaluation were generated using a four-fold serial dilution of cDNA. Amplification efficiency (E) values ranged from 0.98 to 1.02. Linearity coefficients (R^2) were between 0.984 and 0.999, indicating stable amplification across all candidate primer sets. Specificity was verified by melt-curve profiling, which produced a single discrete peak for each transcript, with no detectable signals indicative of hairpin or primer-dimers (Fig. 2A). Amplicon integrity was assessed by gel electrophoresis, confirming that band sizes matched the anticipated amplicon length and that nonspecific products, including self-dimers or hetero-dimers, were not detected (Fig. 2B). Ct analysis revealed significant expression differences between O-MSCs and T-MSCs for *ACTB* ($P<0.05$), *GUSB* ($P<0.05$), *B2M* ($P<0.001$), and *UBC* ($P<0.001$) (Fig.2C).

Reference gene stability profiling using geNorm-based ranking: Expression stability of the nine candidate reference genes in O-MSCs and T-MSCs was assessed using the geNorm algorithm. Using stepwise elimination, M values were calculated, and as all reference genes were below the threshold of 1.5, they were all indicated appropriate for normalization. With O-MSCs, *TBP*, *HPRT1*, and *RPL7* were validated as the highest stability, while *GUSB* and *ACTB* were found to be the most unstable. Similarly, in T-MSCs, *HPRT1*, *YWHAZ*, and *TBP* showed the highest stability, while *RPL7* and *B2M* were the remaining reference gene candidates with the least stability. In combined, both O-MSCs and T-MSCs were analyzed. *TBP* and *HPRT1* were identified as the highest stability, whereas *B2M* and *GUSB* showed the lowest stability (Fig. 3A). Pairwise variation (V value)

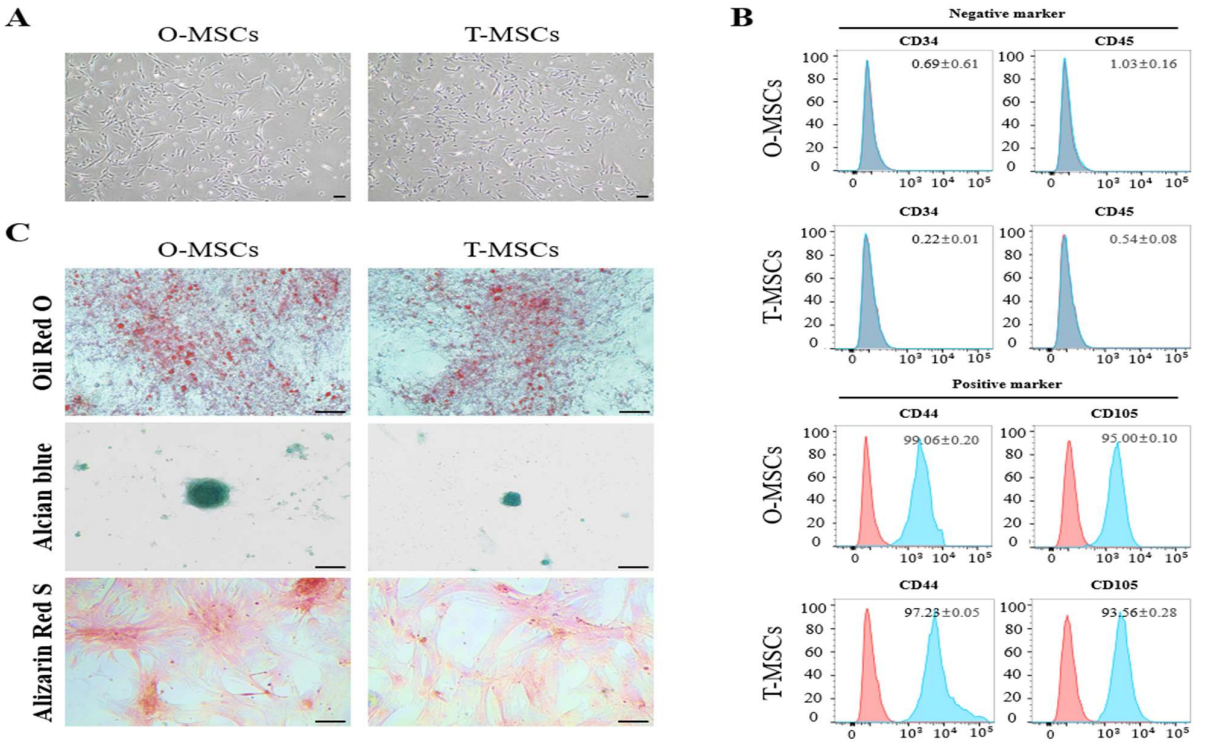

Fig. 1: Characterization of O-MSCs and T-MSCs. (A) Cellular morphology of O-MSCs and T-MSCs. O-MSCs and T-MSCs showed a spindle-like morphology (scale bar=100μm). (B) Analysis of cell surface marker expression in O-MSCs and T-MSCs using flow cytometry. Red peaks show isotype IgG expression as a control. The expression of each CD marker is represented by blue peaks. Analysis of flow cytometry demonstrated positive expression of mesenchymal markers including CD44 and CD105 and negative expression levels of the hematopoietic markers CD34 and CD45. (C) Mesenchymal lineage differentiation was confirmed by lineage-specific cytochemical staining. The accumulation of intracellular lipid droplets in adipocytes was verified by Oil Red O. The sulfated proteoglycan depositions in chondroblasts were confirmed by Alcian blue staining. The mineralization and calcium deposits in osteoblasts were verified by Alizarin Red S. (scale bar=100μm).

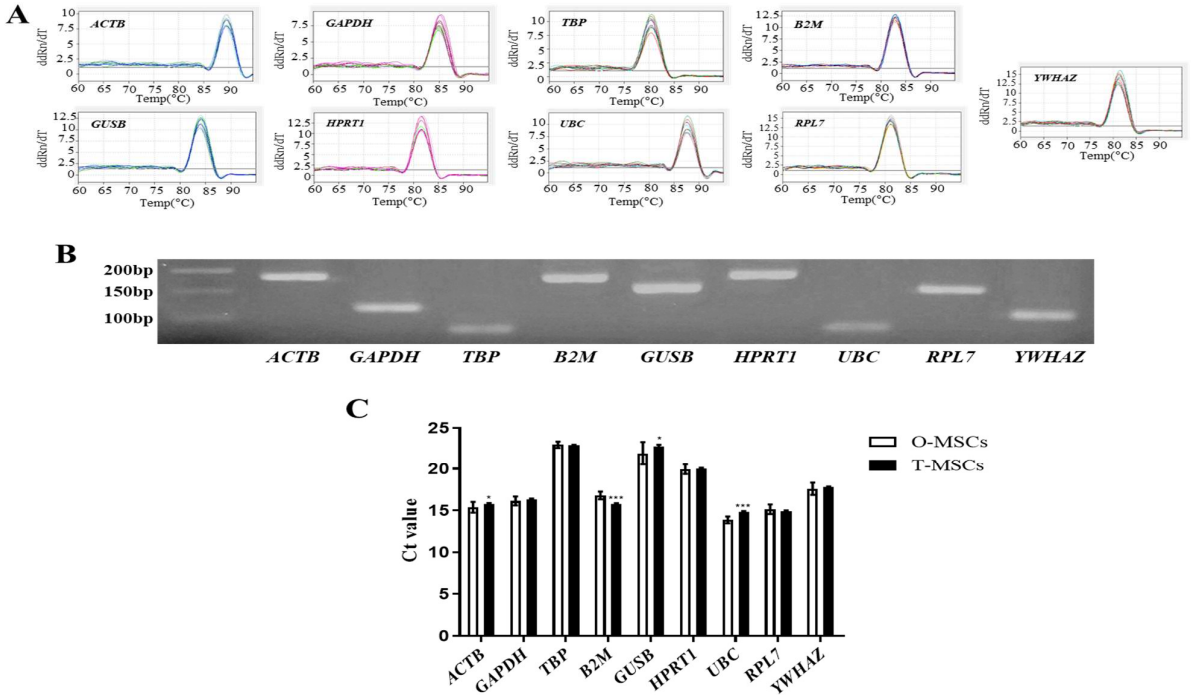

Fig. 2: Evaluation of primer specificity, amplicon size, and Ct values for candidate reference genes in O-MSCs and T-MSCs. (A) To confirm the specificity of primers for nine candidate reference genes, melting curve analysis was performed. Each gene showed a single peak, indicating specific amplification without primer-dimer or nonspecific products. (B) PCR amplicons were visualized on a 1% agarose gel. A single band of the anticipated size was formed by each primer pair, and nonspecific bands were not observed, confirming amplicon specificity. Images were captured at ×1.0 magnification using a 50 bp DNA ladder. (C) The nine reference genes were quantified by Ct value analysis in O-MSC and T-MSC samples to evaluate their expression stability. Data are reported as the mean ± SD. Differences between O-MSCs and T-MSCs that were statistically significant are highlighted with asterisks. (*P<0.05, *** P<0.001).

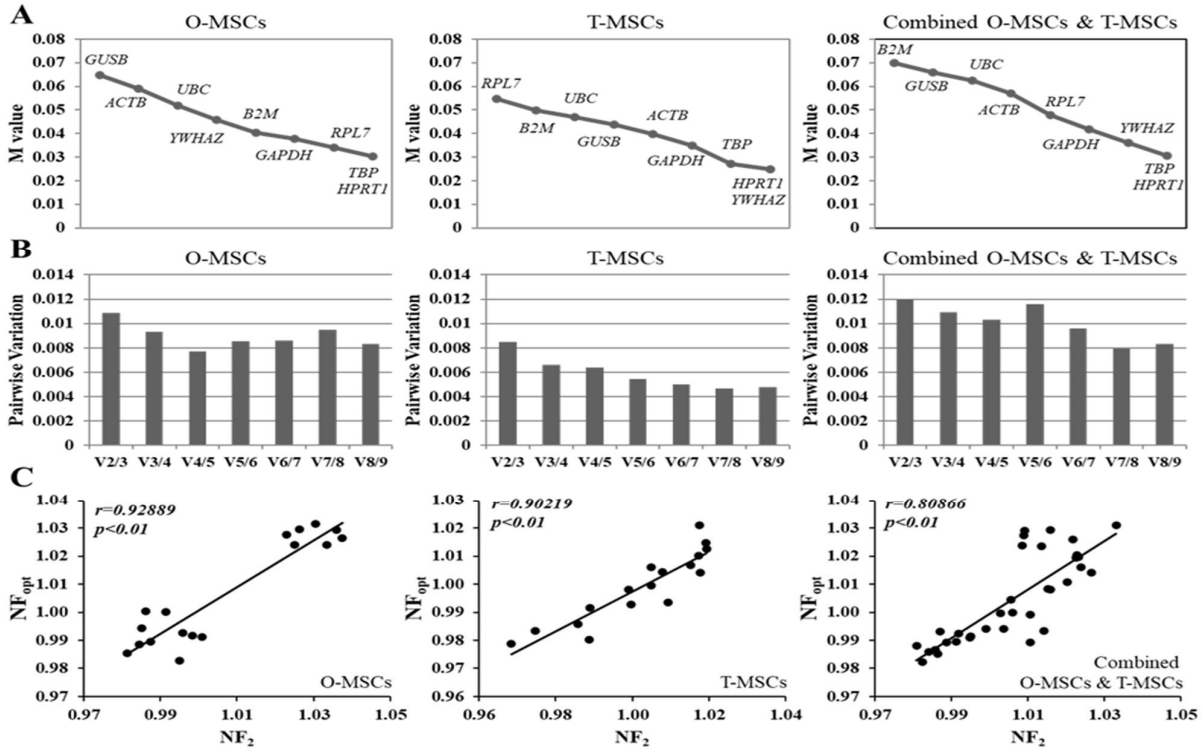

Fig. 3: GeNorm analysis to evaluate stability and establish the ideal number of reference genes. (A) GeNorm was used to determine the M value of nine potential reference genes. On the graph, reference genes are arranged from least stable on the left to most stable on the right. (B) Determining the optimal number of reference genes for accurate normalization involved analyzing pairwise variation (V values) among the reference genes. The analysis indicated that four genes are sufficient for O-MSCs and seven genes for T-MSCs. (C) Pearson correlation analysis was conducted to examine the consistency between normalization factors calculated using three (NF_2) and optimal (NF_{opt}) reference genes. Strong positive correlations were observed in both O-MSCs (r=0.92889, P<0.01), T-MSCs (r=0.90219, P<0.01), and combined O-MSCs and T-MSCs (r=0.80866, P<0.01).

analysis was performed to determine the optimal number of reference genes required for reliable normalization. The lowest V values for O-MSCs, T-MSCs, and combined O-MSCs and T-MSCs were observed at $V_{4/5}$ (V=0.00772), $V_{7/8}$ (V=0.00645), and $V_{7/8}$ (V=0.00791), respectively. The lowest V values were observed at $V_{4/5}$ (V=0.00772) for O-MSCs, $V_{7/8}$ (V=0.00645) for T-MSCs, and $V_{7/8}$ (V=0.00791) for the combined dataset, indicating that a minimum of four reference genes for O-MSCs and seven for both T-MSCs and the combined both O-MSCs and T-MSCs would ensure adequate normalization accuracy (Figure 3B). To examine whether fewer reference genes could maintain comparable normalization performance, the correlation between the normalization factor derived from the two most stable genes (NF_2) and the optimal gene set (NF_{opt}) was examined. Strong positive correlations were observed in O-MSCs (r=0.92889, P<0.01), T-MSCs (r=0.90219, P<0.01), and combined both O-MSCs and T-MSCs (r=0.80866, P<0.01), supporting that two carefully selected reference genes may be sufficient for reliable qRT-PCR normalization (Fig. 3C).

Reference gene stability profiling using NormFinder-based ranking: NormFinder was applied to quantify expression stability among nine normalization targets. Across O-MSCs, T-MSCs, and combined both O-MSCs and T-MSCs, *TBP* and *HPRT1* consistently exhibited the lowest stability values. In O-MSCs, *ACTB* and *GUSB* were identified as the least stable genes (Figure 4A). Similarly, *RPL7* and *UBC* showed low stability in T-MSCs (Figure 4B). In the combined O-MSCs and T-MSCs, *TBP* and *HPRT1* remained the most stable pair, while *B2M* and *ACTB* ranked among the least stable (Fig. 4C). Furthermore, the combination of *TBP* and *HPRT1* was identified as the optimal reference pair for qRT-PCR interpretation in both O-MSCs, T-MSCs, and combined both O-MSCs and T-MSCs. Our results showed that *TBP* and *HPRT1* were identified as the most suitable reference genes across all groups based on stability rankings evaluated by both NormFinder and geNorm. In contrast, traditional reference genes such as *GAPDH* and *ACTB* exhibited low stability in our study, indicating that they are not suitable for reliable normalization.

Normalization based on reference gene stability: Both algorithms suggested that *HPRT1* and *TBP* were the highest stable reference genes among the candidate reference genes. Especially, *ACTB,* a widely used traditional reference gene, showed relatively lower stability than other candidate genes, including *GAPDH,* in both O-MSCs and T-MSCs. Furthermore, less stable reference genes, such as *GUSB*, *RPL7,* and *ACTB,* were also utilized to normalize *OCT4*, a commonly used marker for MSC identity, to assess the impact of different reference genes on GOI. When normalized to *TBP* or *HPRT1*, *OCT4* expression was significantly lower in T-MSCs than in O-MSCs (P<0.05 for both). However, normalization using *ACTB*, *GUSB*, or *RPL7* failed to detect significant differences between the groups (Fig. 5).

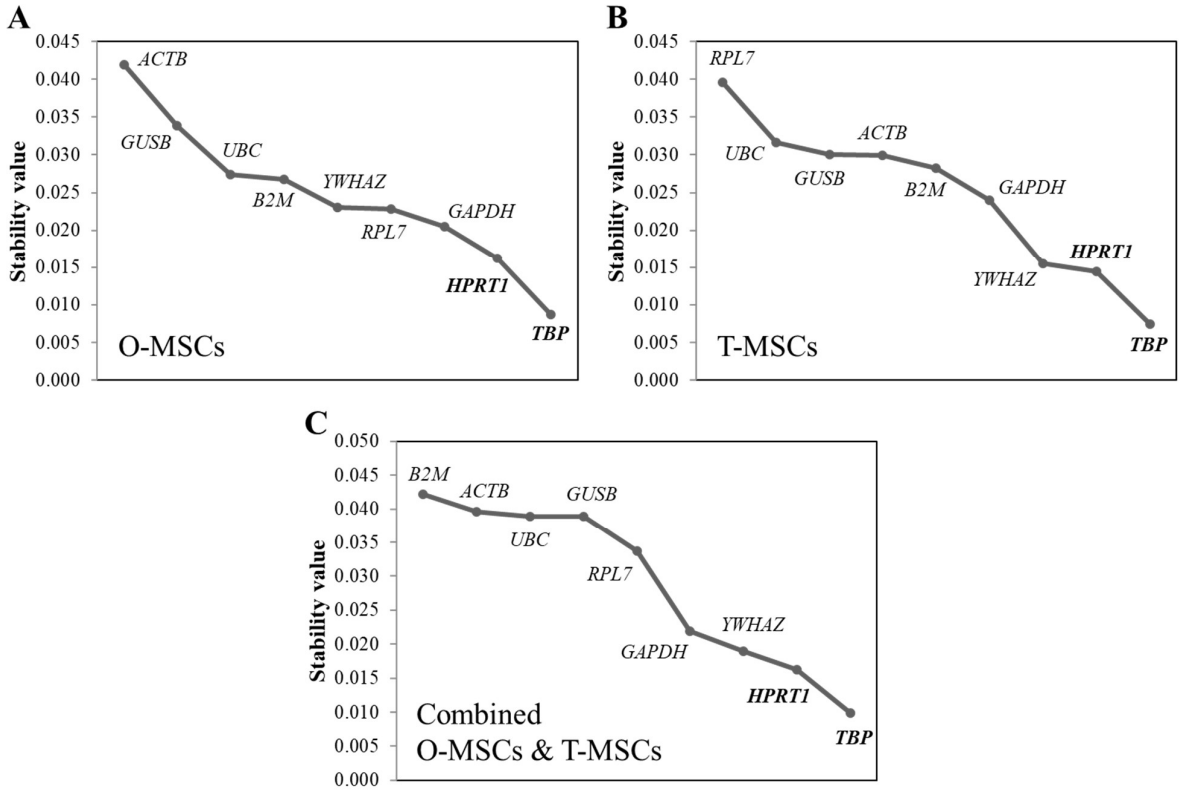

Fig. 4: NormFinder analysis to evaluate candidate reference genes according to expression stability. (A) With the least stable gene on the left and the most stable gene on the right, the graph displays the stability analysis of candidate reference genes in O-MSCs. The gene pair with the highest overall stability is indicated at the end of the curve in bold. (B) The stability ranking of potential reference genes in T-MSCs is displayed in the graph, with genes ordered from left to right in order of increasing stability. At the end of the curve, the most stable gene pair is bolded. (C) The most stable reference gene ranked on the right side and the most unstable reference gene on the left side of the graph in combined O-MSCs and T-MSCs. The best combination of two reference genes was shown in bold letters.

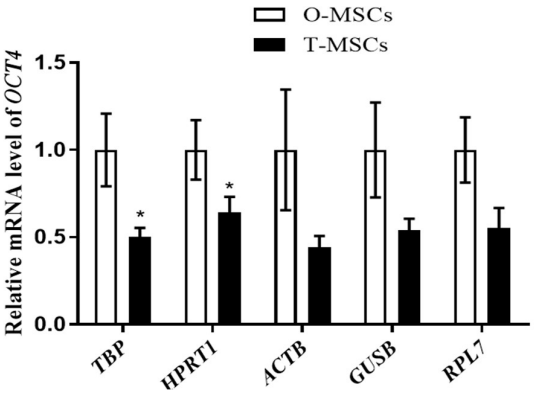

Fig. 5: Comparative analysis of *OCT4* mRNA expression levels normalized by different reference genes. To evaluate the influence of reference gene selection on normalization accuracy, *OCT4* expression was normalized using two stable (*TBP* and *HPRT1*) and three less stable (*ACTB*, *GUSB*, and *RPL7*) reference genes. Relative expression levels were compared between O-MSCs and T-MSCs. Data are reported as the mean±SD. Differences between O-MSCs and T-MSCs that were statistically significant are highlighted with asterisks. (*P<0.05).

DISCUSSION

Currently, reference gene stability in human, porcine, and bovine MSCs has been reported (Lee *et al*., 2015; Jeon *et al*., 2019; Jang *et al*., 2020). Our results showed that the reference gene stability was the highest in *TBP* and *HPRT1*, whereas widely used internal controls such as *GUSB*, *RPL7*, and *ACTB* showed comparatively low stability. The absence of universally stable reference genes across experimental conditions emphasizes the risk of misleading interpretation caused by improper normalization (Palombella *et al*., 2017; Jeon *et al*., 2019). Reference gene selection and validation should be guided by biological context and experimental design. Therefore, we focused on validating the highest stability of candidate reference genes for accurate qRT-PCR interpretation in feline MSCs.

Under defined culture conditions, feline MSCs obtained from ovarian and testicular tissues displayed spindle-like morphology, MSC-specific marker expression, and the capacity for trilineage differentiation. It is consistent with the International Society for Cell & Gene Therapy guidelines (Viswanathan *et al.*, 2019). In addition, gonadal tissue-derived MSCs were demonstrated that extended proliferation capacity and possessed potential for effective MSC therapy sources (Gonzalez *et al.*, 2009). However, the molecular basis of the therapeutic efficacy using feline gonadal tissue-derived MSCs is unclear. Accordingly, we focused on the assessment of reference gene expression stability in feline gonadal tissue-derived MSCs for molecular biological analysis. To obtain accurate interpretation in preclinical and translational studies, identifying the stability of reference genes as internal controls is needed.

When evaluating reference gene stability, the consistency of gene expression could be impacted by

primer and amplicon length (Brinkhof *et al.*, 2006). In order to avoid primer and amplicon length effects, primers generating between 80 and 190 base pairs amplicon length were designed and used in this study. Among the available algorithms, the two most popular algorithms utilized to evaluate reference gene stability are geNorm and NormFinder (Expósito-Rodríguez *et al.*, 2008). The analysis using geNorm and NormFinder demonstrated comparable results, with both algorithms identifying *TBP* and *HPRT1* as the highest stability. The minor differences in the reference gene stability rankings derived from geNorm and NormFinder can be explained by the differences in their algorithmic approaches (Lee *et al.*, 2024). Even if geNorm recommends the number of reference genes for optimal normalization depending on V value, using multiple reference genes is frequently impractical and expensive. The combination of *TBP* and *HPRT1* was suggested enough to precise normalization due to the lowest M value. This approach is consistent with a previous study, which using two to three verified reference genes offers sufficient normalization accuracy (Vandesompele *et al.*, 2002).

Ct values were analyzed in O-MSCs and T-MSCs to determine suitable reference genes. We calculated Ct values using the formula [XT=X0 × (1 + E)^Ct]. In this formula, XT refers to the amount of amplified product at the threshold cycle, X0 means the number of molecules, and E means the amplification efficiency of the PCR reaction (Schmittgen and Zakrajsek, 2000). Variation in Ct values was observed among nine candidate reference genes when comparing with O-MSCs and T-MSCs. In addition, some reference genes including *ACTB*, *GUSB*, *B2M*, and *UBC* showed statistically significant differences between O-MSCs and T-MSCs. These reference genes may exhibit tissue-specific expression patterns and should be carefully validated prior to use in normalization (Penning *et al.*, 2007). Therefore, reference genes need to be confirmed for gene stability under the given experimental conditions to interpret the qRT-PCR results.

Among the nine candidate reference genes in O-MSCs and T-MSCs, *TBP* and *HPRT1* were identified as the highest expression stability. In contrast, *GUSB*, *RPL7*, and *ACTB* were estimated to be less stable than *TBP* and *HPRT1*. TATA-box binding protein (*TBP*) is essential for eukaryotic transcription initiation. *TBP* is essential for core promoter recognition and assembly of the pre-initiation complex (Akhtar and Veenstra, 2011). Hypoxanthine-guanine phosphoribosyl transferase, an enzyme of the purine salvage pathway that aids in nucleotide synthesis by catalyzing the conversion of hypoxanthine and guanine into inosine monophosphate and guanine monophosphate, is encoded by *HPRT1*. This function is essential for cell cycle progression and proliferation (Townsend *et al*., 2018; Wu *et al.*, 2022). *GUSB* is a pivotal lysosomal enzyme involved in the catabolic processing of glycosaminoglycans containing glucuronic acid residues (Naz *et al.*, 2013). *RPL7* is a ribosomal protein traditionally known to have a molecular weight of 27 kDa (Meyuhas and Klein, 1990). *ACTB* functions as a fundamental cytoskeletal component essential for cell survival and plays a vital role in maintaining various cellular processes (Jeon *et al.*, 2019). In previous studies, it was demonstrated that *HPRT1*, a non-ribosomal reference gene, identifies stable expression in various feline organs, including liver, lung, and adrenal gland tissues (Kessler *et al.*, 2009; Penning *et al.*, 2007). Similarly to our study, in gonadal tissues such as the ovary and testis, *TBP* has also been validated to be a reliable reference gene (Li *et al*., 2009; Katarzyńska-Banasik *et al*., 2017; Zhou *et al*., 2020). For normalization of data, *ACTB* and *GAPDH* have traditionally been the most commonly used reference genes. Nonetheless, its expression has been shown to be unstable in feline pancreatic tissue, spinal tissue, and genomic DNA (Kessler *et al*., 2009; Helfer-Hungerbuehler *et al*., 2013; Ashwell *et al*., 2019). Moreover, in feline degenerative joint disease, *GUSB* has also been shown to be unstable (Ashwell *et al.*, 2019). In contrast, other studies have demonstrated that *ACTB,* along with some ribosomal proteins are reliable reference gene in the feline endometrium and also in dental tissues (Penning *et al*., 2007; Jursza *et al*., 2014). The differences in the reference gene stability rankings across studies may be due to differences in the type of tissue and its condition, along with the differences in the experimental design. Therefore, a reliable interpretation of qRT-PCR bases on prior verification of reference genes employed under specific experimental conditions (Lee *et al.*, 2024).

The influence of reference gene stability on target gene expression, *OCT4*, was estimated using both stable and unstable reference genes as internal controls. In stem cells, *OCT4* plays the role of a pluripotency regulator, allowing the cell to maintain self-renewal and remain undifferentiated (Boyer *et al.*, 2005). Gene expression of *OCT4* has been observed in MSCs from various tissues, contributing to the preservation of their cell functions (Tsai and Hung, 2012). Even though both normalization results showed identical trends, statistical analysis revealed that reference gene stability significantly influenced the interpretation. Consequently, *TBP* and *HPRT1* were regarded as the most reliable reference genes for evaluating the gene expression of feline O-MSCs and T-MSCs. Previous study on human embryonic gonadal tissues has shown that *OCT4* expression is predominant in ovarian tissue cells, including oocyte and early follicular cells, but declines throughout spermatogenesis (Mamsen *et al*., 2017). This is consistent with the increased *OCT4* expression seen in O-MSCs compared to T-MSCs. It was suggested that *OCT4* expression plays an essential role in preserving oocyte pluripotency and follicle development. Conversely, expression of *OCT4* level is downregulated during the process of testicular germ cell differentiation. Therefore, higher expression *OCT4* levels in O-MSCs may reflect origin tissue characterization. The findings emphasized the importance of validating reference genes, showing that normalization based on validation of their stability more accurate result than normalization based on unstable reference genes. Using traditional reference genes without validation of their stability could affect the reliability of the interpretation (Jeon *et al.*, 2019). The *TBP* and *HPRT1* stability in both gonadal tissues-derived MSCs suggests that these genes are minimally influenced by sex- or tissue-specific variation. The *TBP* and *HPRT1*, which have been established as playing important roles in fundamental cellular processes, were consistently expressed for their suitability as universal reference genes.

Considering the three biological replicates and seven technical replicates, the sample size was still small, and the studies were done exclusively in one laboratory, which might restrict the ability to generalize the results. To further validate the stability of *TBP* and *HPRT1*, experiments using large samples were needed.

Conclusions: *TBP* and *HPRT1* were identified as the most stable for precise interpretation of GOI in feline ovarian- and testicular-derived MSCs. Normalization of *OCT4* expression revealed significant differences based on the stability of the reference gene, emphasizing the necessity of validation prior to qRT-PCR analysis. The reliable reference genes revealed that *OCT4* expression level was significantly lower in T-MSCs compared to O-MSCs, indicating tissue-specific variations in stemness potential. These results offer a reliable foundation for future qRT-PCR-based studies focused on feline MSC biology and the potential therapeutic applications of feline MSCs.

Disclosure statement: No potential conflict of interest was reported by the author(s).

Acknowledgements: This study was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, and Forestry (IPET) through the Agriculture and Food Convergence Technologies Program for Research Manpower Development (RS-2024-00398561) and H-BION, Republic of Korea (2024-2107-01).

Authors contribution: Conceptualization: S-YL, SP, T-HP and Y-BS methodology: S-JO, C-YH and W-JL software: C-HJ, S-LL, E-YB and H-JL validation: Y-WJ, Y-HC and E-YB investigation: S-YL, SP, and T-HP data curation: C-HJ, H-JL, and W-JL writing—original draft preparation: S-YL, SP, T-HP and Y-BS writing—review and editing: Y-BS and S-LL visualization: C-YH, Y-WJ, Y-HC and S-JO supervision: Y-BS.

REFERENCES

Alderete TL, Sattler FR, Sheng X, *et al*., 2015. A novel biopsy method to increase yield of subcutaneous abdominal adipose tissue. International Journal of Obesity 39:183-186.

Andersen CL, Jensen JL and Ørntoft TF, 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64:5245-5250.

Arzi B, Peralta S, Fiani N, *et al*., 2020. A multicenter experience using adipose-derived mesenchymal stem cell therapy for cats with chronic, non-responsive gingivostomatitis. Stem Cell Research and Therapy 11:115.

Ashwell M, Freire M, O'Nan AT, *et al*., 2019. Characterization of gene expression in naturally occurring feline degenerative joint disease-associated pain. Veterinary Journal 243:42-47.

Binder C, Reifinger M, Aurich J, *et al*., 2021. Histopathological findings in the uteri and ovaries of clinically healthy cats presented for routine spaying. Journal of Feline Medicine and Surgery 23:770-776.

Boyer LA, Lee TI, Cole MF, *et al*., 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122:947-956.

Brinkhof B, Spee B, Rothuizen J, *et al*., 2006. Development and evaluation of canine reference genes for accurate quantification of gene expression. Analytical Biochemistry 356:36-43.

Bustin SA, 2002. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. Journal of Molecular Endocrinology 29:23-39.

Derveaux S, Vandesompele J and Hellemans J, 2010. How to do successful gene expression analysis using real-time PCR. Methods 50:227-230.

Diagone KV, Feliciano MAR, Pacheco MR, *et al*., 2012. Histology and morphometry of the testes of adult domestic cats (Felis catus). Journal of Feline Medicine and Surgery 14:124-130.

Erkin ÖC, Cömertpay B and Göv E, 2022. Integrative analysis for identification of therapeutic targets and prognostic signatures in non-small cell lung cancer. Bioinformatics and Biology Insights 16:11779322221088796.

Expósito-Rodríguez M, Borges AA, Borges-Pérez A, *et al*., 2008. Selection of internal control genes for quantitative real-time RT-PCR studies during tomato development process. BMC Plant Biology 8:131.

Gonzalez R, Griparic L, Vargas V, *et al*., 2009. A putative mesenchymal stem cells population isolated from adult human testes. Biochemical and Biophysical Research Communications 385:570-575.

Harman R, Carlson K, Gaynor J, *et al*., 2016. A prospective, randomized, masked, and placebo-controlled efficacy study of intraarticular allogeneic adipose stem cells for the treatment of osteoarthritis in dogs. Frontiers in Veterinary Science 3:81.

Helfer-Hungerbuehler AK, Widmer S and Hofmann-Lehmann R, 2013. GAPDH pseudogenes and the quantification of feline genomic DNA equivalents. Molecular Biology International 2013:587680.

Ho KH and Patrizi A, 2021. Assessment of common housekeeping genes as reference for gene expression studies using RT-qPCR in mouse choroid plexus. Scientific Reports 11:3278.

Jang SJ, Jeon RH, Kim HD, *et al*., 2020. TATA box binding protein and ribosomal protein 4 are suitable reference genes for normalization during quantitative polymerase chain reaction study in bovine mesenchymal stem cells. Asian-Australasian Journal of Animal Sciences 33:2021-2030.

Jeon RH, Lee WJ, Son YB, *et al*., 2019. PPIA, HPRT1, and YWHAZ genes are suitable for normalization of mRNA expression in long-term expanded human mesenchymal stem cells. BioMed Research International 2019:3093545.

Jeung SY, An JH, Kim SS, *et al*., 2024. Safety of gonadal tissue-derived mesenchymal stem cell therapy in geriatric dogs with chronic disease. Animals 14:2134.

Jursza E, Skarzynski DJ and Siemieniuch MJ, 2014. Validation of reference genes in the feline endometrium. Reproductive Biology 14:302-306.

Katarzyńska-Banasik D, Grzesiak M and Sechman A, 2017. Selection of reference genes for quantitative real-time PCR analysis in chicken ovary following silver nanoparticle treatment. Environmental Toxicology and Pharmacology 56:186-190.

Kessler Y, Helfer-Hungerbuehler AK, Cattori V, *et al*., 2009. Quantitative TaqMan real-time PCR assays for gene expression normalisation in feline tissues. BMC Molecular Biology 10:106.

Kozera B and Rapacz M, 2013. Reference genes in real-time PCR. Journal of Applied Genetics 54:391-406.

Lanoix D, Lacasse AA, St-Pierre J, *et al*., 2012. Quantitative PCR pitfalls: the case of the human placenta. Molecular Biotechnology 52:234-243.

Lee SY, Jeong YW, Choe YH, *et al*., 2024. Identification of reference gene for quantitative gene expression in early-term and late-term cultured canine fibroblasts derived from ear skin. Animals 14:2722.

Lee WJ, Jeon RH, Jang SJ, *et al*., 2015. Selection of reference genes for quantitative gene expression in porcine mesenchymal stem cells derived from various sources along with differentiation into multilineages. Stem Cells International 2015:235192.

Li YL, Ye F, Hu Y, *et al*., 2009. Identification of suitable reference genes for gene expression studies of human serous ovarian cancer by real-time polymerase chain reaction. Analytical Biochemistry 394:110-116.

Mamsen LS, Ernst EH, Borup R, *et al*., 2017. Temporal expression pattern of genes during the period of sex differentiation in human embryonic gonads. Scientific Reports 7:15961.

Meyuhas O and Klein A, 1990. The mouse ribosomal protein L7 gene. Its primary structure and functional analysis of the promoter region. Journal of Biological Chemistry 265:11465-11473.

Morawska-Kozłowska M, Pitas M and Zhalniarovich Y, 2025. Mesenchymal stem cells in veterinary medicine: still untapped potential. Animals 15:1175.

Naz H, Islam A, Waheed A, *et al*., 2013. Human β-glucuronidase: structure, function, and application in enzyme replacement therapy. Rejuvenation Research 16:352-363.

Palombella S, Pirrone C, Cherubino M, *et al*., 2017. Identification of reference genes for qPCR analysis during hASC long culture maintenance. PLoS One 12:e0170918.

Penning LC, Vrieling HE, Brinkhof B, *et al*., 2007. A validation of 10 feline reference genes for gene expression measurements in snap-frozen tissues. Veterinary Immunology and Immunopathology 120:212-222.

Pérez-Merino EM, Usón-Casaús JM, Duque-Carrasco J, *et al*., 2015. Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: endoscopic and histological outcomes. Veterinary Journal 206:391-397.

Ridgeway JA, Tinsley S and Kurtin SE, 2017. Practical guide to bone marrow sampling for suspected myelodysplastic syndromes. Journal of the Advanced Practitioner in Oncology 8:29-39.

Russell KA, Chow NHC, Dukoff D, *et al*., 2016. Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells. PLoS One 11:e0167442.

Schmittgen TD and Zakrajsek BA, 2000. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. Journal of Biochemical and Biophysical Methods 46:69-81.

Song J, Cho J, Park J, *et al*., 2022. Identification and validation of stable reference genes for quantitative real time PCR in different minipig tissues at developmental stages. BMC Genomics 23:585.

Song N, Scholtemeijer M and Shah K, 2020. Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential. Trends in Pharmacological Sciences 41:653-664.

Suzuki T, Higgins PJ and Crawford DR, 2000. Control selection for RNA quantitation. BioTechniques 29:332-337.

Townsend MH, Robison RA and O'Neill KL, 2018. A review of HPRT and its emerging role in cancer. Medical Oncology 35:89.

Tsai CC and Hung SC. 2012. Functional roles of pluripotency transcription factors in mesenchymal stem cells. Cell Cycle 11:3711-3712.

Vandesompele J, De Preter K, Pattyn F, *et al*., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3:RESEARCH0034.

Viswanathan S, Shi Y, Galipeau J, *et al*., 2019. Mesenchymal stem versus stromal cells: International Society for Cell and Gene Therapy Mesenchymal Stromal Cell committee position statement on nomenclature. Cytotherapy 21:1019-1024.

Voga M, Kovač V and Majdic G, 2020. Comparison of canine and feline adipose-derived mesenchymal stem cells with regard to cell surface marker expression, viability, proliferation, and differentiation potential. Frontiers in Veterinary Science 7:610240.

Webb TL and Webb CB, 2024. Scoping review of the use of mesenchymal stem and stromal cell products in cats, Part 1: current logistics and safety. Journal of the American Veterinary Medical Association 262:S16-S23.

Wu T, Jiao Z, Li Y, *et al*., 2022. HPRT1 promotes chemoresistance in oral squamous cell carcinoma via activating MMP1/PI3K/Akt signaling pathway. Cancers 14:855.

Zhou X, Wu X, Chu M, *et al*., 2020. Validation of suitable reference genes for gene expression studies on yak testis development. Animals 10:182.