

Aging-Associated Transcriptomic Changes in the Bovine Endometrium

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ABSTRACT

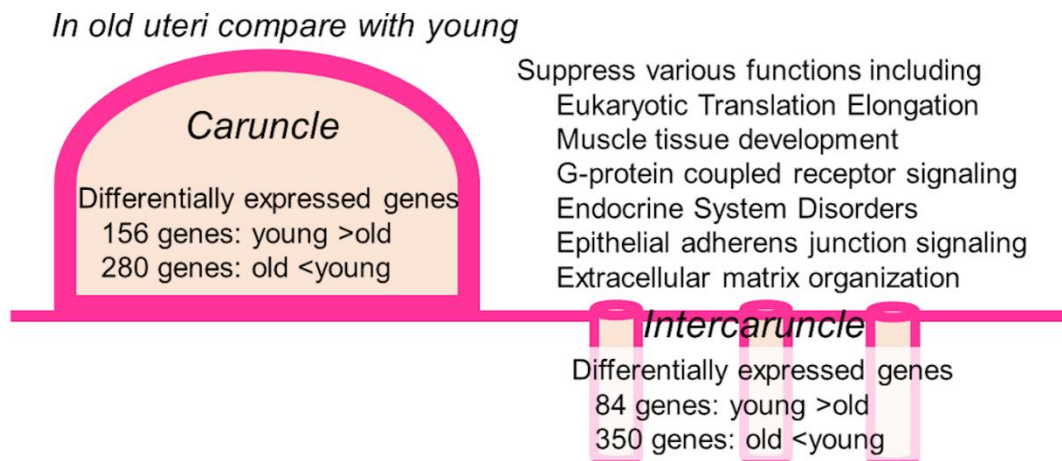
Proper endometrial function is essential for fertility, but aging impairs endometrial activity through mechanisms that remain unclear in cattle. This study aimed to identify age-related differentially expressed genes (DEGs) in the endometrium of approximately 121-month-old multiparous Japanese Black cows (N = 5) compared with approximately 24-month-old nulliparous heifers (N = 5), using RNA sequencing. A total of 20,285 and 26,241 annotated genes were expressed in the intercaruncular (ICAR) and caruncular (CAR) regions, respectively. In aged cows, 350 genes showed higher and 84 lower expression in the ICAR, while 280 were higher and 156 higher in the CAR ($P < 0.01$, fold change $> 20\%$). In total, 434 and 436 genes were identified as DEGs in ICAR and CAR respectively, including growth factors, receptors, and orphan G-protein-coupled receptors. Metascape analysis of ICAR DEGs revealed enrichment in pathways related to cell growth, growth factor response, lipid biosynthetic, negative regulation of cell adhesion and differentiation, and embryonic morphogenesis. CAR DEGs were enriched in collagen-associated and epithelial differentiation pathways. Ingenuity Pathway Analysis (IPA) of ICAR DEGs highlighted networks involving oxidative stress-induced senescence, arachidonic acid metabolism, epithelial adherens junction signaling, and tight junction signaling, as well as canonical pathways of G-protein coupled receptor

signaling, and glycosaminoglycan metabolism ($P < 0.01$). IPA of CAR DEGs indicated enrichment in nonsense mediated decay, collagen chain trimerization, and wound healing signaling pathway. These findings identify DEGs and signaling pathways associated with aging in the bovine endometrium.

Keywords: Aging; Bovine Endometrium; Cellular senescence; G-protein-coupled receptor; Japanese Black cattle; RNA-seq; Ruminants;

Highlights

1. Aging alters gene expression in bovine caruncular and intercaruncular endometrium.
2. RNA-seq identified DEGs between young heifers and aged multiparous cows.
3. Enriched pathways include senescence, GPCR signaling, and cell adhesion.
4. ICAR and CAR regions showed distinct transcriptomic aging signatures.



1. Introduction

The endometrium plays a critical role in successful reproduction by supporting sperm transport, embryo implantation, and early embryonic development, while also providing defence against microbial invasion despite the multiple damages and stresses acting upon it around delivery. Aging negatively impacts endometrial function, contributing to reduced fertility in cattle ([Osoro and Wright, 1992](#)); however, the molecular mechanisms underlying this decline remain largely undefined.

Cattle are the important source of food and dairy products worldwide. The maximum recorded lifespan of dairy and beef cattle is approximately 20 years ([Hoffman and Valencak, 2020](#)); however, their average lifespan is only approximately 25% of this value due to culling when fertility is suppressed ([Moreira et al., 2021](#)). Importantly, embryonic loss rather than fertilization failure is the major cause of reproductive inefficiency in aged cows ([Warnick and Hansen, 2010](#)). The health of the endometrium, particularly in the

caruncular (CAR) and intercaruncular (ICAR) regions, is essential for maintaining fertility due to their respective roles in maternal recognition and placental development (Poock et al., 2020). The endometrium secretes 34 putative embryokines, supporting embryogenesis (Tribulo et al., 2018).

While studies in humans have revealed age-related alterations in endometrial gene expression, including changes in inflammatory, metabolic, endometrial epithelial growth, receptivity, and hormone-responsive pathways (Kawamura et al., 2021; Loid et al., 2024), comparable data in cattle remain limited. *In vitro* investigations indicate that aged bovine endometrial cells show increased inflammatory and interferon signalling and reduced proliferative capacity (Tanikawa et al., 2017); nevertheless, comprehensive *in vivo* transcriptomic analyses are still lacking.

RNA sequencing (RNA-Seq) offers high-resolution, global gene expression profiling and has significantly advanced reproductive biology research. Several studies used microarrays or RNA-Seq to investigate bovine endometrial gene expression under various physiological conditions and stages of the estrous cycle (Mitko et al., 2008; Forde et al., 2011; Sakumoto et al., 2015; Mesquita et al., 2015; Musavi et al., 2018). Despite this, few studies have utilized RNA-Seq to elucidate age-associated transcriptomic changes in the bovine endometrium *in vivo*. In the present study, we employed RNA-Seq to compare

endometrial transcriptomes between young heifers and aged multiparous cows, with the goal of identifying genes and signaling pathways implicated in reproductive aging.

2. Materials and Methods

2.1 Sample collection

All experiments complied with the *Guiding Principles for the Care and Use of Experimental Animals in the Field of Physiological Sciences* (Physiological Society of Japan) and were approved by the Committee on Animal Experiments of the School of Veterinary Medicine, Yamaguchi University (approved number 301).

Both intercaruncular (ICAR) and caruncular (CAR) endometrial samples were obtained from cattle raised by contract farmers in western Japan. Since 2003, all cattle born in Japan have been registered at birth with a unique individual identification number in the National Livestock Breeding Centre database. We utilized these identification numbers alongside information provided by contract farmers to identify cattle for this study. All animals were slaughtered at local abattoirs for beef production under regulations from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

Among 2600 Japanese Black heifers, the minimum age at first calving was 21.4 months (Inoue et al., 2020), indicating sexual maturity occurs around 15 months of age. Fig. 1 shows the flow chart that illustrates the workflow used for RNA-seq data analysis. Both ICAR and CAR endometrial samples were collected according to previously described methods (Ferdousy et al., 2020a, 2020b; Ferdousy and Kadokawa, 2022) from healthy, post-pubertal, young nulliparous Japanese Black heifers (n = 5; 24.4 ± 1.5 months old, “young” group) and old multiparous Japanese Black cows (n = 5; 120.8 ± 6.2 months old, 8.0 ± 0.3 parities, slaughtered at least 3 months after their last parturition, “old” group). Animals were sampled between days 8 and 12 of the estrous cycle (day 0 = day of estrus), as determined by macroscopic examination of the ovaries and uterus (Miyamoto et al., 2000). Due to slaughterhouse regulations, it was not possible to collect blood from the cattle; therefore, progesterone and estradiol concentrations in the blood could not be measured.

All animals were non-lactating, non-pregnant, and free from follicular cysts, luteal cysts, endometritis, pyometra, or other ovarian or uterine disorders, confirmed by macroscopic examination (Kamomae, 2012). Old cows were slaughtered after completing the planned number of parturitions by farmers for beef harvesting, generally after 84

months of age. Samples were collected from the side ipsilateral to the corpus luteum, immediately frozen in liquid nitrogen, and stored at -80°C until RNA extraction.

2.2 Preparation of Transcriptome Libraries for Deep Sequencing

Transcriptome library preparation followed a previously described protocol ([Abdillah et al., 2022](#)) with slight modifications. Total RNA was extracted from CAR or ICAR tissues using QIAzol ([Qiagen](#), Hilden, Germany) and the RNeasy Plus Universal Kit (Qiagen). Samples were treated with ribonuclease-free deoxyribonuclease (Qiagen) to eliminate possible genomic DNA contamination. RNA quality was assessed by agarose gel electrophoresis and capillary electrophoresis using an Agilent 4200 TapeStation system ([Agilent Technologies](#), Santa Clara, CA, USA) with a High Sensitivity RNA assay, after RNA quantification by NanoDrop ONE ([Thermo Fisher Scientific](#), Wilmington, DE, USA). All samples exhibited RNA integrity numbers (RIN) > 7 , indicating high-quality RNA.

RNA-seq libraries were prepared using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina ([New England Biolabs](#), Ipswich, MA, USA). Briefly, 100 ng total RNA was reverse transcribed into cDNA and amplified with primers supplied in the kit. Unique barcodes were added to amplicons, and adapter sequences were ligated to

both ends using the NEBNext Adaptor Kit (New England Biolabs). Libraries were PCR-amplified for 13 cycles (denaturation at 98°C for 10 s; annealing and extension at 65°C for 75 s) using NEBNext multiplex oligos. The quality of the products was verified using a Bioanalyzer, and concentrations were measured with a Qubit 2.0 Fluorometer (ThermoFisher Scientific).

Sequencing was performed on an Illumina NovaSeq 6000 system (Illumina Inc., San Diego, CA, USA) using the NovaSeq 6000 S1 Reagent Kit v1.5. Each sample yielded approximately 54 million reads and 2.6 gigabases of data.

2.3 Analysis of read information

Short sequence reads (FASTQ files) generated using the Illumina NovaSeq 6000-were processed and aligned to the *Bos taurus* (Hereford) ARS-UCD1.3 reference genome (National Center for Biotechnology Information [NCBI] assembly accession GCF_002263795.2; https://genome.ucsc.edu/cgi-bin/hgTracks?db=hub_6542057_GCF_002263795.2) using CLC Genomics Workbench v24.0.1 (QIAGEN, Aarhus, Denmark). The mapping ratio exceeded 96.3%, with read counts over 41 million per sample. The analysis procedure, including RNA-seq data

processing and generation of interpretable results using CLC Genomics Workbench was described previously ([Liu and Di, 2020](#)).

2.4 Quantitative Reverse Transcription PCR (RT-qPCR)

Quantitative reverse transcription PCR ([RT-qPCR](#)) was performed to validate the RNA-seq results by measuring five genes (*GATA6*, *GPR183*, *CD27*, *IHH*, and *FGFR4*). These genes were chosen for their biological relevance to endometrial function and early pregnancy: *GATA6* regulates steroidogenic programs in endometrial stromal cells ([Bernardi et al., 2019](#)); *GPR183* directs oxysterol-guided migration of B cells and dendritic cells that organize mucosal immunity ([Liu et al., 2011](#); [Hannedouche et al., 2011](#)); *CD27* marks activated/memory B cells present in mid-luteal human endometrium, supporting a role for adaptive immunity at implantation ([Shen et al., 2021](#)); *IHH* is a progesterone-responsive mediator of epithelial–stromal signaling essential for implantation ([Lee et al., 2006](#)); and *FGFR4* represents the FGF signaling axis across the bovine endometrium–conceptus interface during the pre-implantation period ([Okumu et al., 2014](#); [Ozawa et al., 2012](#)). Genes were also prioritized because they showed large fold-changes and small P-values in our RNA-seq dataset, indicating robust, statistically significant differential expression suitable for validation.

Complementary DNA (cDNA) was synthesized from 2 µg total RNA using random hexamer primers and the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) in 20-µL reactions. Primers for RT-qPCR were designed using Primer Express Software v3.0 (Thermo Fisher Scientific) based on reference sequences (Table 1). RT-qPCR was performed in duplicate using 20 ng cDNA per reaction on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific). Each run included a six-point relative standard curve, no-template controls, and no-reverse transcription controls. Standard curves were generated using 10-fold serial dilutions of purified, amplified DNA fragments. Five cDNA samples per tissue group were analyzed. PCR cycling conditions were as follows: 95°C for 10 min (pre-denaturation); 5 cycles of 95°C for 15 s and 66°C for 30 s; 40 cycles of 95°C for 15 s and 60°C for 60 s. Melting curve analyses were performed at 95°C for each amplicon and each annealing temperature to ensure the absence of smaller non-specific products such as dimers.

To optimize the real-time PCR assay, serial dilutions of a cDNA template were used to generate a standard curve by plotting the log of the starting quantity of the dilution factor against the C_q value obtained during amplification of each dilution. Reactions with a coefficient of determination (R²) >0.98 and efficiency between 95 and 105% were

considered optimized. The concentration of PCR products was calculated by comparing Cq values of unknown samples with the standard curve using appropriate software (CFXmanagerV3.1, Bio-Rad).

Gene expression levels were normalized to the geometric mean of two housekeeping genes, *C2orf29* (NCBI accession XM_002691150.6) and *SUZ12* (NM_001205587.3), which were previously validated as stable references in bovine CAR and ICAR (Nahar and Kadokawa, 2017; Ferdousy et al., 2020a).

2.5 Statistical Analysis and Gene Selection for Pathway Analysis

Statistical comparison of transcripts per million (TPM) values between young and old endometria followed the approach described by (Abdillah et al., 2022). Briefly, the binary logarithm of each TPM value was calculated following the addition of 1. Differences in gene expression were assessed by unpaired *t*-tests. We calculated the average TPM values for old ICAR/CAR and divided them by the average TPM values of the young ICAR/CAR (old/young ratio) to show the differences in the TPM values for each gene. We also calculated the fold change, which was calculated as: [binary logarithm of (old ICAR/CAR TPM + 1)] – [binary logarithm of (young ICAR/CAR TPM + 1)]. Positive fold-change values were defined as indicating higher expression levels in old

cows compared with heifers, whereas negative fold-change values indicated lower expression levels in old cows compared with heifers. In addition, a ratio greater than 1 indicates higher expression in old cows than in heifers, whereas a ratio less than 1 indicates lower expression in old cows than in heifers. Data are expressed as the mean $\pm$ SEM.

Differentially expressed genes (DEGs) were selected based on the following criteria: (1) an average binary logarithm ($\log_2$) of $(\text{TPM} + 1) \geq 0.01$ in both young and old CAR/ICAR samples; (2) a significant expression difference between the groups ($p < 0.01$); and (3) a fold change of greater than a 20% increase ($\log_2$ fold change > 0.263) or greater than a 20% decrease ($\log_2$ fold change < -0.263). Genes without assigned Ensembl gene identifiers ([ENSBTAG](#)) were excluded. The significant DEGs selected were subsequently used for pathway analysis.

In addition, a Venn diagram was generated to visualize the distribution of expressed genes among the four experimental groups (young CAR, old CAR, young ICAR, and old ICAR) using Venny v2.1, an interactive tool for comparing lists with Venn diagrams (Oliveros, J.C., 2007–2015, BioinfoGP, CNB-CSIC; available at: <https://bioinfoGP.cnb.csic.es/tools/venny/>; RRID:SCR_016561).

Functional pathway analyses were performed using Metascape

228 (<https://metascape.org>) ([Zhou et al. 2019](#)) on May 8, 2025, and Ingenuity Pathway
229 Analysis ([IPA; Qiagen](#), Valencia, CA, USA; content version 134816949, release date
230 February 19, 2025) on May 9, 2025, with default parameters. The Molecular Activity
231 Predictor function in IPA was used to infer upstream regulators and downstream effectors
232 of canonical pathways. Protein localization and functions were annotated based on IPA
233 databases, although each expressed protein may have various locations and multiple roles.
234 The analysis procedure, including Ingenuity Pathway Analysis, was described previously
235 ([Liu and Di, 2020](#)).

236 For functional annotation, DEGs identified in CAR and ICAR were analyzed using
237 the Diseases & Functions module of IPA. Categories showing significant enrichment
238 were extracted separately for CAR and ICAR. To improve biological relevance,
239 categories associated with unrelated organs (e.g., eye) or conditions (e.g., developmental
240 disorders) were excluded prior to further interpretation.

241 RT-qPCR data were analyzed using StatView v5.0 ([SAS Institute](#), Cary, NC, USA).
242 Normality was assessed by Shapiro–Wilk’s W test or Kolmogorov–Smirnov Lilliefors
243 test. *GATA6*, *FGFR4*, *GPR183*, and *IHH* mRNA expression values were normally
244 distributed, whereas *CD27* expression showed a log-normal distribution and was
245 therefore log-transformed. Variance homogeneity was confirmed by F-tests, and Grubb’s

test detected no outliers. Differences in gene expression between groups were evaluated by unpaired *t*-tests.

3. Results

3.1 Annotated genes expressed in endometrium from heifers and old cows

Figure 2A shows a volcano plot of 20,285 annotated genes in the ICAR, of which 18,194 genes had a P-value ≥ 0.05 . Among the remaining 2,091 genes, 434 DEGs were identified, including 84 genes expressed at significantly lower levels and 350 genes expressed at significantly higher levels ($P < 0.01$, fold change $> 20\%$) in the ICAR of older cows compared with heifers. Conversely, these correspond to 84 genes expressed at significantly higher levels and 350 genes expressed at significantly lower levels in the ICAR of heifers compared with older cows. **Figure 2B** shows a volcano plot of 26,241 annotated genes in the CAR, with 23,663 genes having a P-value ≥ 0.05 . Among the remaining 2,578 genes, 436 DEGs were identified, including 156 expressed at significantly lower levels and 280 expressed at significantly higher levels ($P < 0.01$, fold change $> 20\%$) in the CAR of older cows compared to heifers.

Supplementary Tables 1 and 2 show the top 50 genes with increased or decreased expression in old cow ICAR compared with young ICAR, with heat maps illustrating expression across five samples per group. Supplementary Tables 3 and 4 provide the corresponding results for CAR, comparing old cows with heifers and reflecting age-related differences. Heat maps are also included to visualize expression profiles.

Among the top 50 genes expressed at significantly lower levels in the ICAR of old cows, only eight were unnamed (annotated as “ENSBTAG” and marked with a green “+” in Figure 2A; Supplementary Table 1), whereas 35 unnamed genes (marked with a red “+” in Figure 2A; Supplementary Table 2) were found among the top 50 genes expressed at significantly higher levels. In the CAR of old cows, only five unnamed genes (green “+” in Figure 2B; Supplementary Table 3) were among the top 50 genes expressed at significantly lower levels, whereas 39 unnamed genes (red “+” in Figure 2B; Supplementary Table 4) were among the top 50 genes expressed at significantly higher levels.

Of the 434 DEGs between the ICAR of heifers and old cows, 134 were unnamed (Supplementary Table 5; Figure 3A). Excluding these unnamed genes, 300 genes remained differentially expressed ($P < 0.01$), with 295 genes recorded in the IPA database (Supplementary Table 6). Four genes not recorded in IPA were expressed at higher levels

282 in old ICAR: *BREH1* (Breast cancer overexpressed gene H1), *C17H5orf52* (Chromosome
283 17 Open Reading Frame 52), *CCDC96* (Coiled-Coil Domain Containing 96), *gzmA*
284 (Granzyme A). In contrast, one gene not recorded in IPA, *SMIM15_1* (Small Integral
285 Membrane Protein 15, isoform 1), was expressed at lower levels in old ICAR.

286 Of the 436 DEGs in the CAR, 131 were unnamed ([Supplementary Table 7; Figure](#)
287 [3B](#)), leaving 305 named genes differentially expressed ($P < 0.01$), with 298 genes
288 recorded in IPA ([Supplementary Table 8](#)). All seven genes not recorded in IPA were
289 expressed at higher levels in young CAR: *AOX4* (Aldehyde Oxidase 4), *C14H8orf34*
290 (Chromosome 14 Open Reading Frame 34), *RNaseP_nuc_3* (Ribonuclease P nuclear
291 RNA subunit 3), *SNORD22* (Small Nucleolar RNA, C/D Box 22), *SNORD39* (Small
292 Nucleolar RNA, C/D Box 39), *U3_21* (U3 Small Nucleolar RNA, snoRNA 21 variant),
293 and *Y_RNA_7*.

294 [Table 2A](#) summarizes the subcellular locations of proteins encoded by the 295 ICAR
295 and 298 CAR DEGs, and [Table 2B](#) summarizes their respective protein families.

296 In addition, to visualize gene distribution among the experimental groups, a Venn
297 diagram was constructed ([Figure 4](#)). A total of 26,338 genes were identified in bovine
298 endometrial tissues. Of these, 19,865 genes (75.4%) were expressed in all four groups
299 (young CAR, old CAR, young ICAR, and old ICAR). Group-specific expression included

943 genes in young CAR, 1,009 in old CAR, 16 in young ICAR, and 33 in old ICAR. Smaller subsets were shared by two or three groups; for example, 4,101 genes were shared between young and old CAR, and 48 between young and old ICAR. Each detailed gene list corresponding to the unique and shared categories is provided in [Supplementary Tables 9](#). Furthermore, the results of *t*-tests comparing the expression of all genes in the bovine genome across all four tissues (young ICAR, old ICAR, young CAR, and old CAR) in all six pairwise combinations (young ICAR vs. old ICAR, young CAR, or old CAR; young CAR vs. old ICAR or old CAR; old ICAR vs. old CAR) are presented as [Supplementary Table 10](#).

3.2 Annotation of growth factor genes

Among the 295 ICAR DEGs, three encoded growth factors—*angiotensinogen* (*AGT*; fold change 2.598), *fibroblast growth factor 21* (*FGF21*; fold change 0.276), and *growth differentiation factor 1* (*GDF1*; fold change 1.065)—and 14 encoded receptors. These three growth factors were expressed in both heifers and old cows. Among the 298 CAR DEGs, two encoded growth factors—*neuregulin 1* (*NRG1*; fold change -0.693) and *neudesin neurotrophic factor* (*NENF*; fold change -0.419). Both factors were expressed in both heifers and old cows.

3.3 Annotation of receptor genes

Table 3 lists the TPM values of established, well-studied receptor genes expressed in both the ICAR and CAR of heifers and old cows. Only *anti-Müllerian hormone receptor type 2* (*AMHR2*) was differentially expressed between the ICAR of heifers and old cows ($P < 0.05$), and only *estrogen receptor 2* (*ESR2*) was differentially expressed between the CAR of heifers and old cows.

Table 4 presents 15 differentially expressed less-well studied receptor genes in the ICAR, including *adhesion G protein-coupled receptor F5* (*ADGRF5*), *intercellular adhesion molecule 3* (*ICAM3*), *endothelin receptor type A* (*EDNRA*), *retinoic acid receptor beta* (*RARB*), and two orphan G-protein-coupled receptors (*GPCRs*), *GPR63* and *GPR82*. Table 5 shows 12 differentially expressed receptor genes in the CAR that have not been well-studied in the bovine uterus, such as *tumor necrosis factor receptor superfamily member 17* (*TNFRSF17*), *CD27 molecule* (*CD27*), *corticotropin-releasing hormone receptor 1*, and *GPR183*.

3.4 Annotation of putative embryokine genes

Table 6 presents TPM values of 34 putative embryokine genes expressed in ICAR and CAR. Among these, *insulin like growth factor 2 (IGF2)*, *C-X-C motif chemokine ligand 16 (CXCL16)*, *C-C motif chemokine ligand 14 (CCL14)*, and *interleukin 33 (IL33)* were significantly upregulated in the ICAR of old cows compared to heifers ($P < 0.05$). *Wnt family member 5A (WNT5A)* and *C-C motif chemokine ligand 21 (CCL21)* were the only putative embryokine gene differentially expressed in the CAR, showing upregulation in old cows ($P < 0.05$).

3.5 Results of Metascape analysis

Metascape analysis of the 434 ICAR DEGs revealed 20 significantly enriched biological pathways (Table 7), including “regulation of cell growth”, “response to growth factor”, “lipid biosynthetic process”, “negative regulation of cell adhesion”, “negative regulation of cell differentiation”, and “embryonic morphogenesis.” Similarly, analysis of the 436 CAR DEGs identified such as 20 enriched pathways (Table 8), such as “glucose homeostasis”, “positive regulation of secretion by cell”, and “epithelial cell differentiation.” The most significant pathway was “Eukaryotic Translation Elongation”.

3.6 Results of IPA

357

358 IPA of the 295 ICAR DEGs recorded in the IPA database (69 expressed at higher
359 levels in heifers than in cows, and 226 expressed at higher levels in cows than in heifers,
360 as shown in [Fig. 3A](#)) revealed three important canonical pathways. [Supplementary figure](#)
361 [1](#) shows the “G-protein coupled receptor signaling” pathway ($P = 3.63 \times 10^{-4}$), with 2.8%
362 (20/713) gene overlap, highlighting key genes such as *adhesion G protein-coupled*
363 *receptor F4 (ADGRF4)*, *adhesion G protein-coupled receptor F5 (ADGRF5)*,
364 *cannabinoid receptor 2 (CNR2)*, *5-hydroxytryptamine receptor 2B (HTR2B)*, *vasoactive*
365 *intestinal peptide receptor 1 (VIPR1)*, *G-protein coupled receptor 63 (GPR63)*, and *G-*
366 *protein coupled receptor 82 (GPR82)*. [Supplementary figure 2](#) the “glycosaminoglycan
367 metabolism” pathway ($P = 5.37 \times 10^{-4}$) with 5.5% (7/128) gene overlap, including
368 *carbohydrate sulfotransferase 6 (CHST6)*, *chondroitin sulfate N-*
369 *acetylgalactosaminyltransferase 1 (CSGALNACT1)*, *decorin (DCN)*, *galactosidase beta*
370 *1 like 3 (GLB1L3)*, *hyaluronidase 1 (HYAL1)*, *lumican (LUM)*, and *ST3 beta-galactoside*
371 *alpha-2,3-sialyltransferase 4 (ST3GAL4)*. [Supplementary figure 3](#) shows “oxidative
372 stress-induced senescence” ($P = 2.82 \times 10^{-3}$) with 5.7% (5/88) gene overlap, featuring
373 *cyclin dependent kinase inhibitor 2A (CDKN2A)*, *cyclin dependent kinase inhibitor 2C*
374 (*CDKN2C*), *Jun proto-oncogene, AP-1 transcription factor subunit (JUN)*, *mitogen-*

375 *activated protein kinase kinase kinase 5 (MAP3K5)*, and *mitogen-activated protein kinase*
376 *12 (MAPK12)* as central genes.

377 IPA of the 295 ICAR DEGs also revealed four functional networks, including
378 “arachidonic acid metabolism” (Supplementary figure 4), “epithelial adherens junction
379 signaling” (Supplementary figure 5), “tight junction signaling” (Supplementary figure 6),
380 and “production of nitric oxide and reactive oxygen species in macrophages”
381 (Supplementary figure 7). Seven upstream regulators with no predicted activation state
382 were identified: *TGFB1* ($P = 1.91 \times 10^{-5}$), *IGF1* ($P = 1.26 \times 10^{-3}$), *PGR* ($P = 2.50 \times 10^{-3}$),
383 *GATA6* ($P = 2.94 \times 10^{-3}$), *FGF2* ($P = 4.48 \times 10^{-3}$), *EGF* ($P = 0.0153$), and *VEGF* (P
384 $= 0.0211$).

385 IPA of the 298 CAR DEGs recorded in the IPA database (106 expressed at higher
386 levels in heifers than in cows, and 192 expressed at higher levels in cows than in heifers,
387 as shown in Fig. 3B) revealed three important pathways. Supplementary figure 8 shows
388 “nonsense-mediated decay” ($P = 5.01 \times 10^{-17}$) with 16.2% (19/117) gene overlap,
389 highlighting *ribosomal protein L10a (RPL10A)*, *ribosomal protein L11 (RPL11)*,
390 *ribosomal protein L22 (RPL22)*, *ribosomal protein L24 (RPL24)*, *ribosomal protein S12*
391 (*RPS12*), *ribosomal protein S13 (RPS13)*, *ribosomal protein S15 (RPS15)* as the central
392 genes. Supplementary figure 9 shows “Collagen chain trimerization” ($P = 1.38 \times 10^{-3}$)

with 9.09% (4/44) gene overlap, highlighting *collagen type XVIII alpha 1 chain* (COL18A1), *collagen type XXIV alpha 1 chain* (COL24A1), *collagen type XXVI alpha 1 chain* (COL26A1), and *collagen type II alpha 1 chain* (COL2A1) as the central genes. [Supplementary figure 10](#) shows the “wound healing signaling pathway” ($P = 6.61 \times 10^{-3}$) with 3.21% (8/249) gene overlap, including *bone morphogenetic protein receptor type 2* (BMP2), *collagen type XVIII alpha 1 chain* (COL18A1), *collagen type XXIV alpha 1 chain* (COL24A1), *collagen type XXVI alpha 1 chain* (COL26A1), *collagen type II alpha 1 chain* (COL2A1), and *junctional cadherin 5 associated* (JUN). [Table 9](#) lists some of the pathways highlighted by IPA in ICAR and CAR of heifers and old cows.

Several upstream regulators were also identified for the CAR DEGs. *ESR2* ($P = 7.57 \times 10^{-6}$), *TNF* ($P = 3.51 \times 10^{-4}$), and *CX3CL1* ($P = 3.53 \times 10^{-4}$) showed no predicted activity, while *NOTCH3* ($P = 2.44 \times 10^{-3}$) had a predicted activity of activation.

Using the Disease & Functions feature of IPA, we identified 76 significant categories based on ICAR DEGs and 81 significant categories based on CAR DEGs. After excluding categories associated with unrelated organs or irrelevant conditions the remaining 56 categories were summarized in [Table 10](#). Interestingly, the following categories were significant in CAR but not in ICAR: Antimicrobial Response, Cellular Response to Therapeutics, Humoral Immune Response, Organismal Functions, Protein Trafficking,

and RNA Post-Transcriptional Modification. Conversely, the following category was significant in ICAR but not in CAR: Energy Production.

3.7 Results of RT-qPCR

To validate the RNA-seq findings, five selected DEGs (*GATA6*, *CD27*, *FGFR4*, *GPR183*, and *IHH*) were analyzed using qPCR in ICAR and CAR. The expression patterns observed by qPCR were consistent with those obtained from RNA-seq, confirming higher or lower expression levels in aged cows compared with young heifers (Figure 5). Among these, *GATA6* and *CD27* mRNA levels were higher in both ICAR and CAR of old cows than in heifers. *FGFR4* expression was lower in the ICAR of old cows compared to heifers. *GPR183* expression was elevated in the CAR of old cows relative to heifers. *IHH* expression was higher in the ICAR of old cows compared to heifers.

4. Discussion

Gene expression profiling of endometrial tissues from young nulliparous heifers and aged multiparous cows was conducted using RNA-Seq to identify genes and pathways associated with aging. The number of DEGs identified in this study provides an important

indication of the reliability of the RNA-seq data and the robustness of the analytical pipeline. In ICAR, 434 DEGs were detected, whereas in CAR, 436 DEGs met the stringent selection criteria (average $\log_2(\text{TPM} + 1) \geq 0.01$, $p < 0.01$, and fold change $> 20\%$). Notably, these numbers are comparable to those reported by [Sakumoto et al. \(2015\)](#), who analyzed global gene expression profiles in the bovine endometrium between summer and autumn using transcriptomic analysis, thereby reinforcing the accuracy of our methodology. Subsequent Metascape and IPA analyses of DEGs identified several novel pathways and regulatory networks not previously reported in RNA-Seq studies of cultured bovine endometrial cells ([Tanikawa et al., 2017](#)) or human clinical samples ([Kawamura et al., 2021](#); [Loid et al., 2024](#)). The most significant pathway identified by Metascape in CAR was Eukaryotic Translation Elongation, a core process of protein synthesis. Its enrichment in aged bovine endometrium may reflect age-related changes in translational efficiency and proteostasis, potentially contributing to impaired uterine function and reduced fertility in older cows. These findings warrant further discussion of the implicated pathways, networks, and upstream regulators.

The endometrium harbors stem/progenitor cells ([Gargett et al., 2016](#)), and its thickness is a critical factor in implantation success. In women, a thin endometrium is associated with reduced embryo implantation and clinical pregnancy rates ([Mouanness et](#)

al., 2021; Wang et al., 2024). Also in cows, endometrium thickness has been reported as a good predictor of pregnancy success (Souza et al., 2011). In the current study, Metascape and IPA analyses of DEGs in the ICAR or CAR region revealed enrichment in biological processes such as “regulation of cell growth”, “response to growth factor”, “negative regulation of cell differentiation”, and “epithelial cell differentiation” suggesting age-related alterations in cellular proliferation and differentiation dynamics in the endometrium.

Epithelial-to-mesenchymal transition (EMT) is a process whereby epithelial cells lose polarity and adhesion, acquiring mesenchymal characteristics that facilitate migration. EMT plays essential roles in physiological processes like wound healing and tissue regeneration, as well as in pathological contexts (Bilyk et al., 2017). In ruminants, EMT is believed to be necessary for successful embryo implantation into the endometrium (Yamada et al., 2022). However, repeated pregnancies, deliveries, and postpartum infections in older cows often lead to endometrial inflammation (Pascottini et al., 2023), and chronic inflammation caused by pathogens or lipopolysaccharides has been shown to induce EMT (Guo et al., 2014; Kandikattu et al., 2021; Sun et al., 2024). EMT prior to implantation may, therefore, impair fertility in aged cows.

IPA analysis identified seven upstream regulators—*TGFB1*, *IGF1*, *PGR*, *GATA6*, *FGF2*, *EGF*, and *VEGF*—that are associated with endometrial remodeling, regeneration, angiogenesis, and immune modulation. Several of these, including *TGFB1*, *IGF1*, and *PGR*, exhibit stage-specific expression during the estrous cycle or peri-implantation (Sakumoto et al., 2015; Forde et al., 2014; Bauersachs et al., 2006). *GATA6*, *FGF2*, *EGF*, and *VEGF* are implicated in tissue remodeling, vascular development, and inflammatory responses (Shimizu et al., 2010; Bauersachs et al., 2012; Ibrahim et al., 2016; Ulbrich et al., 2009). The therapeutic potential of these growth factors for infertility remains largely unexplored. In the CAR, *ESR2*, *TNF*, *CX3CL1*, and *NOTCH3* were predicted as upstream regulators, mediating estrogen signaling, immune responses, leukocyte recruitment, and vascular or stromal development (Hayashi et al., 2017; Fischer et al., 2010; Sheldon et al., 2009; Sasnauskienė et al., 2014). These overlaps suggest that the upstream regulators identified in this study are part of core regulatory networks active across diverse reproductive and pathological contexts in the bovine endometrium.

The IPA Disease & Functions analysis revealed distinct functional profiles between the CAR and ICAR regions. Notably, categories such as antimicrobial response, cellular response to therapeutics, humoral immune response, organismal functions, protein trafficking, and RNA post-transcriptional modification were significantly enriched in

CAR, but not in ICAR. These functions may reflect tissue-specific adaptations of the CAR endometrium, which plays a central role in maternal–fetal interactions and immune modulation. In contrast, energy production was identified only in ICAR, suggesting that metabolic activity in this compartment may contribute to local regulation of uterine physiology. Although the precise biological implications of these differences remain to be clarified, our results highlight region-dependent functional specialization within the bovine endometrium that may be relevant to reproductive performance.

The Venn diagram analysis revealed that a large proportion of genes were commonly expressed across both CAR and ICAR regions, indicating the presence of a core transcriptome essential for uterine function. At the same time, a greater number of region-specific genes were observed in the CAR compared with the ICAR, suggesting more dynamic transcriptional regulation in the CAR. Notably, aged CAR tissues contained a larger number of unique genes, which may reflect age-related changes in pathways related to tissue remodeling and immune modulation. In contrast, the ICAR exhibited relatively stable gene expression profiles, consistent with its primarily structural and supportive roles. These findings suggest that aging exerts a stronger impact on the transcriptome of the CAR region, which could contribute to reduced uterine receptivity and fertility in older cows.

Metascape and IPA analyses identified pathways related to arachidonic acid metabolism, lipid and glycosaminoglycan biosynthesis, growth factor response, and GPCR signaling—key processes for synthesizing and secreting uterine steroids, cytokines, chemokines, and hormones (Bonsale et al., 2018; Campanile et al., 2021; Wang et al., 2024). Notably, the GPCR pathway included upregulated cannabinoid receptor 2 (*CNR2*) in the old cows, potentially as a response to endometrial injury to regulate nitric oxide-driven inflammation, as a study has done previously (Bonsale et al., 2018). IPA also identified nitric oxide and reactive oxygen species production in macrophages as a relevant pathway. *CNR2* is known for its anti-inflammatory effects to protect human endometrial tissue (Iuvone et al., 2008), thus it may have similar roles in bovine endometrium. These findings suggest that aged cows may develop subclinical endometritis which may lead to reduced fertility rates. Additionally, dysregulation of these pathways may compromise the uterine secretory environment.

The network “Oxidative stress-induced senescence” emerged as another significant pathway in the IPA analysis. While cellular senescence contributes to normal embryonic and placental development through the senescence-associated secretory phenotype (SASP) (Gong et al., 2023), excessive senescence during the proliferative phase has been linked to impaired implantation and decreased stemness in human endometrial stromal

cells (Tomari et al., 2020). The enrichment of SASP-related pathways in the current study suggests that senescence may negatively impact fertility in aged cows. Further investigation is needed to clarify the roles of oxidative stress and SASP in age-related infertility in older cows.

The endometrial architecture must undergo dynamic remodeling to support implantation and pregnancy (Grund and Grummer, 2018). The DEGs were enriched in pathways such as “negative regulation of cell adhesion”, “epithelial adherens junction signaling”, and “tight junction signaling”. Disruption of these processes may lead to implantation failure, abnormal embryo development, and endometrial disorders (Grund and Grummer, 2018). Altered intercellular communication mediated by tight junctions or gap junctions has been implicated as one of the characteristics of biological aging (López-Otín et al., 2023). Further mechanistic studies are warranted to understand how aging affects these adhesion-related processes.

Abnormal expression of major constituents of the extracellular matrix, such as collagens and matrix metalloproteinases, may be associated with impaired or failed implantation and subsequent embryo death (Killeen et al., 2016). IPA analysis of the CAR genes identified two key pathways: “collagen chain trimerization” and the “wound healing signaling pathway.” Notably, several collagen genes involved in these pathways;

COL18A1, *COL24A1*, *COL26A1*, and *COL2A1* are upregulated in older cows. While the expression of most known receptor genes remained unchanged, *AMHR2* was an exception as it was significantly upregulated in old CAR. Bovine endometria express the AMHR2 protein, and AMH has been shown to stimulate a collagen-specific chaperone, 47 kDa heat shock protein, thereby promoting extracellular matrix formation in bovine endometrial cells ([Ferdousy and Kadokawa, 2022](#)). Further research is needed to determine whether abnormal collagens negatively affect implantation in aged cows.

We identified *GPR183*, *GPR63* and *GPR82*, among the differentially expressed receptor genes. However, little is known about these GPCRs. *GPR183*, upregulated in CAR, disrupts circadian signalling by inhibiting period circadian regulator 1, promoting endothelial senescence and dysfunction ([Chu et al., 2024](#)), though its endometrial function remains unknown. GPCRs are pharmaceutical targets, thus, further research is needed to determine whether they play important roles in age-related infertility.

Among the biological processes highlighted by Metascape analysis, embryonic morphogenesis was of particular interest. Of the 34 proposed embryokine candidates ([Tribulo et al., 2018](#)), only *IGF2*, *CXCL16*, *CCL14*, and *IL33* were differentially expressed in the ICAR region, while *CCL21* and *WNT5A* were altered in the CAR region. These molecules are known to influence trophoblast proliferation, invasion, and

endometrial remodeling ([Harris et al., 2011](#); [Huang et al., 2006](#); [Hannan and Salamonsen, 2008](#); [Mei et al., 2019](#); [Liu et al., 2024](#); [Bae et al., 2019](#); [Zhou et al., 2021](#)). These findings indicate that aging may disrupt the expression of critical embryokines, warranting further investigation into their therapeutic potential, including strategies for local delivery in older animals.

RT-qPCR confirmed mRNA expression of key genes across various pathways. *GATA6*, upregulated in both ICAR and CAR of older cows, has been linked to impaired decidualization in human endometrial stromal cells from adenomyosis patients ([Pavlovic et al., 2023](#)). Interestingly, *CD27* was upregulated in both ICAR and CAR. *CD27* marks uterine NK cells that promote immune tolerance and pregnancy by suppressing T helper 17 cells via interferon gamma secretion ([Kitazawa et al., 2020](#)). *FGFR4*, downregulated in ICAR, binds several FGFs ([Ozawa et al., 2012](#)) and is crucial in bovine embryos for interferon tau expression and outgrowth ([Okumu et al., 2014](#)), although its endometrial role is unclear. *IHH*, upregulated in ICAR, supports epithelial proliferation and fertility through hedgehog signalling, even under ethanol-induced damage ([Nakajima et al., 2012](#); [Luo et al., 2025](#)).

It is important to interpret these results with caution. The older cows in this study were multiparous (average eight parities), and due to practical limitations, age-matched

nulliparous cows were unavailable, as infertile heifers are not typically retained by farmers. Therefore, extrapolating these findings to aged but nulliparous females, including women, should be approached with care.

It should be noted that the present study compared young nulliparous Japanese Black heifers with old multiparous Japanese Black cows, and therefore the observed transcriptomic differences may reflect the combined effects of chronological aging and reproductive history. Multiparity is known to influence endometrial structure and function through repeated cycles of pregnancy, parturition, and postpartum uterine involution, which can result in long-term transcriptional changes ([Dickson, 2022](#); [Merenda et al. 2020](#)). Aging has also been associated with alterations in uterine physiology and gene expression in mammals ([Tanikawa et al., 2017](#); [Pathare et al., 2023](#)). While some of the identified DEGs are consistent with aging-related alterations reported in other mammalian tissues, others could be attributable to cumulative reproductive events. Because age and parity are inherently confounded in this experimental design, the present data cannot fully separate their respective contributions. Future studies including age-matched multiparous and nulliparous cows, or longitudinal sampling of the same animals across reproductive life stages, will be required to disentangle the relative effects of aging and fertility on the bovine endometrial transcriptome.

Due to slaughterhouse regulations, it was not possible to collect blood from the cattle; therefore, progesterone and estradiol concentrations in the blood could not be measured. Although hormonal measurements were unavailable, gene expression patterns in our dataset share similarities with those reported for the luteal-phase endometrium in previous studies ([Mitko et al., 2008](#); [Mesquita et al., 2015](#); [Sakumoto et al., 2015](#)). Notably, several genes involved in progesterone-mediated modulation of immune responses and endometrial remodeling, such as *TGFB1*, *IGF1*, *PGR*, *GATA6*, *FGF2*, *EGF*, and *VEGF*, showed expression patterns consistent with a luteal-phase–like profile. These genes have been implicated in processes including angiogenesis, tissue remodeling, and inflammatory regulation during the luteal phase, supporting the possibility that the observed expression profiles partly reflect a luteal-phase–like hormonal environment. Given that the timing of sample harvesting and the hormonal milieu are critical determinants of endometrial gene expression, this hormonal context should be considered when interpreting our findings.

A notable proportion of DEGs in our dataset (e.g., [Supplementary Table 4](#)) lacked an Entrez gene name or any associated functional annotation. This is likely because the bovine genome is still incompletely annotated, with some transcripts corresponding to predicted loci, uncharacterized proteins, or non-coding RNAs for which orthologous

relationships to human or model organism genes have not yet been established. Many of these unannotated transcripts may represent species-specific genes or poorly conserved elements, which could play unique roles in bovine endometrial physiology. Although their functions remain unknown, their differential expression suggests potential relevance to the biological processes underlying our findings, and further functional characterization may reveal novel pathways involved in reproductive aging in cattle.

In conclusion, this study identified differentially expressed genes and aging-associated pathways in the endometrial tissues of heifers and older cows. These findings suggest that age-related alterations in gene expression, cellular processes, and signaling pathways may contribute to reduced fertility in aged animals. Future studies should aim to elucidate the functional roles of the identified upstream regulators, growth factors, and receptors to enhance our understanding of endometrial aging and potentially mitigate its impact on fertility.

Declarations of competing interests

The authors declare that they have no known competing financial interest or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The datasets of the present study are available from the corresponding authors upon reasonable request.

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926

927

928 **Table 1.** Name and details of the primers used for RT-qPCR

Gene name	Primer	Sequence 5'-3'	Size (bp)
<i>C2orf29</i> ^a	Forward	TCAGTGGACCAAAGCCACCTA	169
	Reverse	CTCCACACCGGTGCTGTTCT	
<i>SUZ122</i> ^b	Forward	CATCCAAAAGGTGCTAGGATAGATG	160
	Reverse	TTGGCCTGCACACAAGAATG	
<i>GATA6</i> ^c	Forward	TGTGCAATGCCTGTGGACTC	61
	Reverse	TTCATAGCAAGCGGTCGAGG	
<i>GPR183</i> ^d	Forward	TTGGGCGAATACTGACTGCT	65
	Reverse	GTGTTTCAGGGTCTCTCTCAGT	
<i>CD27</i> ^e	Forward	CCACAACCCTCCCACCTTACC	213
	Reverse	GAACAGGGCTCCGACTATGG	
<i>IHH</i> ^f	Forward	ACCGCGACCGCAATAAGTA	119
	Reverse	GGCTGAGTGCTCGGACTTG	
<i>FGR4</i> ^g	Forward	GAGCTGTTCTCGCTGCTACG	79
	Reverse	GCATCAGCCCGTATAGCTCTGG	

929

930 ^aXM_002691150.6, ^bNM_001205587.3, ^cXM_024984607.2, ^dNM_001046472.2,931 ^eNM_001082434.2, ^fNM_001076870.2, ^gNM_001192584.2

932

Table 2.

(A) Summary of the subcellular localization of ICAR and CAR proteins encoded by 295 and 298 differentially expressed genes, respectively, between heifers and old cows (details provided in [Supplementary Table 6 and 8](#)).

(B) Summary of the protein types encoded by the same differentially expressed genes in ICAR and CAR, respectively, between heifers and old cows.

(A)

Location	Number of genes	
	ICAR	CAR
Cytoplasm	107	123
Extracellular Space	26	23
Nucleus	64	78
Plasma Membrane	62	52
Other	36	22
Total	295	298

(B)

Type	Number of genes	
	ICAR	CAR
Enzyme	60	45

G-protein coupled receptor	7	6
Growth factor	3	2
Ion channel	6	5
Kinase	10	17
Ligand-dependent nuclear receptor	1	1
Peptidase	9	6
Phosphatase	8	6
Transcription regulator	35	35
Transmembrane receptor	6	5
Transporter	16	16
Translation regulator	0	3
Others	134	151
Total	295	298

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944

945 **Table 3.** TPM values (mean $\pm$ SEM) of the annotated receptor genes in the ICAR and
946 CAR of young and old cows. A ratio > 1 indicates higher expression in old cows than in
947 heifers, whereas a ratio < 1 indicates lower expression.

Gene	Description	ICAR		CAR	
		TPM of Young	Ratio	TPM of Young	Ratio
		TPM of Old		TPM of Old	
<i>OXTR</i> ¹	oxytocin receptor	240.4 $\pm$ 104.4 62.0 $\pm$ 55.9	0.26	96.5 $\pm$ 60.3 26.2 $\pm$ 20.1	0.27
<i>IGF1R</i> ²	insulin like growth factor 1 receptor	44.8 $\pm$ 5.1 34.8 $\pm$ 6.5	0.78	37.6 $\pm$ 4.1 44.3 $\pm$ 4.4	1.18
<i>ESR1</i> ³	estrogen receptor 1	115.8 $\pm$ 23.5 105.0 $\pm$ 22.7	0.91	104.2 $\pm$ 13.6 128.7 $\pm$ 12.7	1.23
<i>PGR</i> ⁴	progesterone receptor	40.6 $\pm$ 13.6 37.2 $\pm$ 14.2	0.92	33.4 $\pm$ 9.2 34.3 $\pm$ 2.5	1.03
<i>PGRMC1</i> ⁵	progesterone receptor membrane component 1	177.3 $\pm$ 14.7 173.4 $\pm$ 19.1	0.98	161.3 $\pm$ 2.0 164.4 $\pm$ 9.4	1.02
<i>PGRMC2</i> ⁶	progesterone receptor membrane component 2	89.7 $\pm$ 5.7 92.2 $\pm$ 2.3	1.03	92.7 $\pm$ 6.5 99.8 $\pm$ 5.9	1.08
<i>IGF2R</i> ⁷	insulin like growth factor 2 receptor	20.4 $\pm$ 2.2 22.0 $\pm$ 2.6	1.08	22.7 $\pm$ 2.2 28.8 $\pm$ 2.0	1.27
<i>IFNAR1</i> ⁸	interferon alpha and beta receptor subunit 1	39.1 $\pm$ 5.8 45.1 $\pm$ 12.8	1.15	37 $\pm$ 4.5 36.9 $\pm$ 3.6	1.00

<i>IFNAR2</i>⁹	interferon alpha and beta receptor subunit 2	22.2 ± 3.3 32.9 ± 8.4	1.48	23.3 ± 2.2 26.6 ± 2.0	1.14
<i>IGFLR1</i>¹⁰	IGF like family receptor 1	8.8 ± 1.5 14.6 ± 2.3	1.66	11.2 ± 2.4 13.5 ± 1.6	1.21
<i>AMHR2</i>¹¹	anti-Mullerian hormone receptor type 2	1.2 ± 0.4 2.7 ± 0.5	2.32*	1.7 ± 0.4 3.1 ± 0.5	1.78
<i>GPER1</i>¹²	G protein- coupled estrogen receptor 1	0.1 ± 0.0 0.3 ± 0.1	2.73	0.2 ± 0.1 0.2 ± 0.1	1.28
<i>ESR2</i>¹³	estrogen receptor 2	8.4 ± 3.1 34.2 ± 14.2	4.09	7.8 ± 3.1 32.2 ± 12.3	4.12*

948

949 * Indicates significant (P < 0.05) differences between the TPM values of the genes in the
950 heifers and old cows.

951 ¹NM_174134; ²NM_001244612; ³NM_001001443; ⁴NM_001205356; ⁵NM_001075133;

952 ⁶NM_001099060; ⁷NM_174352; ⁸NM_174552; ⁹NM_174553; ¹⁰NM_001099167;

953 ¹¹NM_001205328; ¹²XM_024985174; ¹³NM_174051

954

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Table 4. TPM values (mean $\pm$ SEM) of receptor genes differentially ($P < 0.01$) expressed between the ICAR of heifers and old cows, ranked by the ratio of average TPM in old cows to that in heifers. A ratio > 1 indicates higher expression in old cows than in heifers, whereas a ratio < 1 indicates lower expression.

Gene	Description	Accession No.	TPM value		Ratio
			Young	Old	
ADGRF4	adhesion G protein-coupled receptor F4	NM_001143875	7.4 $\pm$ 2.6	0.5 $\pm$ 0.2	0.07
GPR82	G protein-coupled receptor 82	XM_005228303	0.4 $\pm$ 0.1	0.1 $\pm$ 0.0	0.33
CD4	CD4 molecule	NM_001103225	3.8 $\pm$ 0.6	7.1 $\pm$ 0.5	1.89
GPR63	G protein-coupled receptor 63	XM_005210862	1.2 $\pm$ 0.3	2.4 $\pm$ 0.2	1.98
EDNRA	endothelin receptor type A	NM_174308	2.1 $\pm$ 0.3	5.0 $\pm$ 0.7	2.40
ICAM3	intercellular adhesion molecule 3	NM_174349	10.4 $\pm$ 1.2	31.0 $\pm$ 8.2	2.99
RARB	retinoic acid receptor beta	XM_059882386	0.8 $\pm$ 0.2	2.4 $\pm$ 0.3	3.02
ADGRF5	adhesion G protein-coupled receptor F5	NM_001193243	4.7 $\pm$ 0.7	16.5 $\pm$ 2.9	3.50
SLAMF1	signaling lymphocytic activation molecule family member 1	NM_174184	0.7 $\pm$ 0.2	2.7 $\pm$ 0.6	3.64
TNFRSF17	TNF receptor superfamily member 17	XM_059881681	0.6 $\pm$ 0.1	2.1 $\pm$ 0.4	3.66
CD27	CD27 molecule	NM_001082434	1.9 $\pm$ 0.4	8.3 $\pm$ 1.8	4.37
CNR2	cannabinoid receptor 2	NM_001192303	0.1 $\pm$ 0.0	0.3 $\pm$ 0.1	4.54

<i>CHRNA2</i>	cholinergic receptor nicotinic beta 2 subunit	NM_001192474	0.0 ± 0.0	0.2 ± 0.0	7.19
<i>HTR2B</i>	5- hydroxytryptamine receptor 2B	NM_001205448	0.0 ± 0.0	0.3 ± 0.1	12.00
<i>VIPR1</i>	vasoactive intestinal peptide receptor 1	NM_001081607	0.0 ± 0.0	0.4 ± 0.1	15.37

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961

Table 5. TPM values (mean $\pm$ SEM) of receptor genes differentially (P < 0.01) expressed between the CAR of heifers and old cows, ranked by the ratio of average TPM in old cows to that in heifers. A ratio > 1 indicates higher expression in old cows than in heifers, whereas a ratio < 1 indicates lower expression.

Gene	Description	Accession	TPM Values		Ratio
			Young	Old	
CRHR1	corticotropin releasing hormone receptor 1	NM_174287.1	1.1 $\pm$ 0.2	0.2 $\pm$ 0.1	0.16
GFRA3	GDNF family receptor alpha 3	NM_001083747.1	1.7 $\pm$ 0.2	0.5 $\pm$ 0.2	0.31
B2M	beta-2-microglobulin	NM_173893.3	1025.6 $\pm$ 131.9	533.9 $\pm$ 46.5	0.52
NR2C2	nuclear receptor subfamily 2 group C member 2	NM_001192061.1	12.5 $\pm$ 1.1	17.5 $\pm$ 1.0	1.40
CYSLTR2	cysteinyl leukotriene receptor 2	NM_001102240.2	1.2 $\pm$ 0.1	2.6 $\pm$ 0.3	2.05
NPTXR	neuronal pentraxin receptor	NM_001435297.2	2.6 $\pm$ 0.6	5.6 $\pm$ 0.7	2.14
ADORA2A	adenosine A2a receptor	XM_024977856.2	2.4 $\pm$ 0.4	5.4 $\pm$ 0.7	2.29
GALR1	galanin receptor 1	NM_001192241.1	0.4 $\pm$ 0.2	1.5 $\pm$ 0.2	3.55

<i>TNFRSF17</i>	TNF receptor superfamily member 17	XM_059881681.1	0.7 ± 0.1	2.8 ± 1.0	0.6
<i>ADCYAP1R1</i>	ADCYAP receptor type I	NM_175715.2	0.3 ± 0.1	1.1 ± 0.3	4.02
<i>CD27</i>	CD27 molecule	NM_001082434.2	1.9 ± 0.3	9.1 ± 3.1	4.70
<i>GPR183</i>	G protein-coupled receptor 183	NM_001046472.2	2.1 ± 0.4	14.7 ± 5.0	7.07

966

Table 6. TPM values (mean $\pm$ SEM) of 34 putative embryokine genes in the ICAR and CAR of heifers and old cows. A ratio > 1 indicates higher expression in old cows than in heifers, whereas a ratio < 1 indicates lower expression.

Gene	Description	ICAR		CAR	
		TPM of Young TPM of Old	Ratio	TPM of Young TPM of Old	Ratio
<i>IGF1</i> ¹	insulin like growth factor 1	41.7 $\pm$ 15.6 15.4 $\pm$ 5.3	0.37	45.6 $\pm$ 10.6 68.3 $\pm$ 7.2	1.50
<i>BMP7</i> ²	bone morphogenetic protein 7	7.4 $\pm$ 3.7 3.7 $\pm$ 1.5	0.50	4.6 $\pm$ 3.4 2.8 $\pm$ 0.7	0.61
<i>IL15</i> ³	interleukin 15	6.5 $\pm$ 1.8 3.7 $\pm$ 0.8	0.57	5.8 $\pm$ 1.1 3.9 $\pm$ 1.1	0.67
<i>CCL26</i> ⁴	C-C motif chemokine ligand 26	0.8 $\pm$ 0.3 0.5 $\pm$ 0.1	0.60	1.0 $\pm$ 0.3 0.6 $\pm$ 0.3	0.60
<i>FGF13</i> ⁵	fibroblast growth factor 13	12.5 $\pm$ 2.3 9.5 $\pm$ 1.3	0.76	15.4 $\pm$ 1.9 15.6 $\pm$ 6.0	1.01
<i>HGF</i> ⁶	hepatocyte growth factor	6.9 $\pm$ 2.7 5.6 $\pm$ 2.9	0.81	8.4 $\pm$ 2.4 5.1 $\pm$ 0.7	0.61
<i>HDGF</i> ⁷	heparin binding growth factor	188.2 $\pm$ 15.1 155.9 $\pm$ 18.2	0.83	149.7 $\pm$ 16.0 113.7 $\pm$ 7.1	0.76
<i>WNT5A</i> ⁸	Wnt family member 5A	120.1 $\pm$ 11.9 110.5 $\pm$ 20.5	0.92	135.5 $\pm$ 12.2 179.4 $\pm$ 12.1	1.32*
<i>WNT16</i> ⁹	Wnt family member 16	16.3 $\pm$ 5.4 16.6 $\pm$ 4.1	1.01	48.0 $\pm$ 10.9 47.1 $\pm$ 7.0	0.98
<i>WIF1</i> ¹⁰	WNT inhibitory factor 1	14.6 $\pm$ 2.1 16.0 $\pm$ 5.7	1.09	10.2 $\pm$ 3.1 10.5 $\pm$ 3.2	1.03
<i>CXCL12</i> ¹¹	C-X-C motif chemokine ligand 12	38.9 $\pm$ 9.7 44.1 $\pm$ 10.6	1.13	69.5 $\pm$ 14.1 63.9 $\pm$ 9.9	0.92
<i>SFRP4</i> ¹²	secreted frizzled related protein 4	19.0 $\pm$ 5.2 23.6 $\pm$ 7.4	1.24	62.6 $\pm$ 31.8 73.5 $\pm$ 30.8	1.17

VEGFB ¹³	vascular	32.1 ± 5.6	1.27	44.8 ± 7.9	1.10
	endothelial	40.8 ± 3.0		49.2 ± 4.3	
	growth factor B				
SFRP1 ¹⁴	secreted frizzled	13.0 ± 2.3	1.33	41.8 ± 9.8	1.11
	related protein 1	17.3 ± 3.0		46.4 ± 4.9	
FGF2 ¹⁵	fibroblast	8.9 ± 2.1	1.33	14.3 ± 2.1	1.24
	growth factor 2	11.9 ± 2.4		17.7 ± 1.7	
FGF1 ¹⁶	fibroblast	9.9 ± 2.5	1.35	7.1 ± 2.3	1.65
	growth factor 1	13.4 ± 1.7		11.6 ± 3.5	
IL16 ¹⁷	interleukin 16	3.5 ± 0.6	1.51	4.8 ± 0.7	1.28
		5.3 ± 0.5		6.2 ± 0.3	
IGF2 ¹⁸	insulin like	269.9 ± 34.8	1.59*	488.6 ± 65.3	1.32
	growth factor 2	429.6 ± 44.0		645.4 ± 67.8	
CSF2 ¹⁹	colony	0.2 ± 0.1	1.61	0.4 ± 0.3	0.35
	stimulating	0.3 ± 0.2		0.1 ± 0.1	
	factor 2				
SFRP2 ²⁰	secreted frizzled	52.4 ± 17.7	1.84	116.1 ± 28.4	1.16
	related protein 2	96.4 ± 28.6		135.0 ± 20.2	
CXCL16 ²¹	C-X-C motif	62.9 ± 2.4	2.29*	55.0 ± 6.7	1.53
	chemokine	143.8 ± 33.0		84.1 ± 15.7	
	ligand 16				
CX3CL1 ²²	C-X3-C motif	17.1 ± 2.0	2.74	36.4 ± 7.8	1.03
	chemokine	46.9 ± 21.8		37.5 ± 5.4	
	ligand 1				
WNT7A ²³	Wnt family	5.9 ± 2.0	2.75	3.0 ± 0.8	5.21
	member 7A	16.2 ± 6.0		15.5 ± 10.5	
CCL14 ²⁴	C-C motif	13.1 ± 2.5	3.15*	25.1 ± 2.6	0.81
	chemokine	41.3 ± 12.2		20.3 ± 2.3	
	ligand 14				
IL33 ²⁵	interleukin 33	3.8 ± 0.7	3.85*	5.1 ± 0.9	1.45
		14.7 ± 6.1		7.4 ± 1.7	
WNT11 ²⁶	Wnt family	7.4 ± 2.1	5.61	4.7 ± 1.7	5.47
	member 11	41.4 ± 19.8		26.0 ± 10.2	
CXCL3 ²⁷	C-X-C motif	14.1 ± 4.4	9.08	12.6 ± 2.4	0.51
	chemokine	128.3 ± 109.1		6.5 ± 1.8	
	ligand 3				

GRO1²⁸	chemokine (C-X-C motif) ligand 1	2.6 ± 1.0 39.0 ± 35.7	14.79	2.0 ± 0.9 1.0 ± 0.5	0.52
CCL21²⁹	C-C motif chemokine ligand 21	0.1 ± 0.1 2.0 ± 1.3	26.20	0.1 ± 0.1 1.2 ± 0.6	20.88*
IL1B³⁰	interleukin 1beta	2.1 ± 0.2 62.8 ± 61.5	30.44	3.6 ± 1.0 2.7 ± 0.6	0.75
CTGF³¹	connective tissue growth factor	Not detected Not detected		Not detected Not detected	
HDGFRP2³²	hepatoma-derived growth factor-related protein 2	Not detected Not detected		Not detected Not detected	
IL8³³	interleukin 8	Not detected Not detected		Not detected Not detected	
TDGF1³⁴	teratocarcinoma-derived growth factor 1	Not detected Not detected		Not detected Not detected	

971

972 * Indicates significant (P < 0.05) differences between the TPM values of the genes in the
973 young and old cows.

974 ¹NM_001077828; ²NM_001206015; ³NM_174090; ⁴NM_001205635;

975 ⁵NM_001098892; ⁶NM_001031751; ⁷NM_175832; ⁸NM_001205971;

976 ⁹NM_001014949; ¹⁰NM_001075996; ¹¹NM_001113174; ¹²NM_001075764;

977 ¹³NM_174487; ¹⁴NM_174460; ¹⁵NM_174056; ¹⁶NM_001314006; ¹⁷NM_001075253;

978 ¹⁸NM_174087; ¹⁹NM_174027; ²⁰NM_001034393; ²¹NM_001046095;

979 ²²XM_002694885; ²³NM_001192788; ²⁴NM_001046585; ²⁵NM_001075297;
980 ²⁶NM_001082456; ²⁷NM_001046513; ²⁸NM_175700; ²⁹NM_001038076;
981 ³⁰NM_174093; ³¹NM_174030; ³²NM_001105361; ³³NM_173925; ³⁴NM_001080358.

982 **Table 7.** Top 20 pathways revealed by Metascape analysis in ICAR with their
 983 representative enriched terms.

Gene ontology classification	Description	N ^a	% ^b	Log10(P) ^c	Log10(q) ^d
61061	muscle structure development	23	7.77	-8.50	-4.16
1558	regulation of cell growth	17	5.74	-6.14	-2.34
70848	response to growth factor	20	6.76	-6.08	-2.34
71466	cellular response to xenobiotic stimulus	11	3.72	-5.37	-1.98
48872	homeostasis of number of cells	13	4.39	-5.13	-1.86
7162	negative regulation of cell adhesion	13	4.39	-4.85	-1.66
WP422	MAPK cascade	5	1.69	-4.79	-1.65
8610	Lipid biosynthetic process	18	6.08	-4.51	-1.47
90066	Regulation of anatomical structure size	16	5.41	-4.33	-1.31
3228	Atrial cardiac muscle tissue development	4	1.35	-4.24	-1.25
35239	Tube morphogenesis	19	6.42	-4.21	-1.25
Hsa05200	Pathways in cancer	16	5.41	-4.09	-1.16
R-HSA- 109582	Hemostasis	16	5.41	-4.07	-1.16
48598	embryonic morphogenesis	17	5.74	-4.03	-1.16
6790	Sulfur compound metabolic process	12	4.05	-4.00	-1.14
48863	stem cell differentiation	9	3.04	-3.96	-1.13
36119	response to platelet-derived growth factor	4	1.35	-3.88	-1.09
M195	PID CMYB PATHWAY	6	2.03	-3.75	-1.02
71320	Cellular response to cAMP	5	1.69	-3.73	-1.02
1903510	Mucopolysaccharide metabolic process	6	2.03	-3.72	-1.02

984

Table 8. Top 20 pathways revealed by Metascape analysis in CAR with their representative enriched terms.

Gene ontology classification	Description	N ^a	% ^b	Log10(P) ^c	Log10(q) ^d
R-HSA-156842	Eukaryotic Translation Elongation	20	6.69	-20.85	-16.50
R-HSA-416572	Sema4D induced cell migration and growth-cone collapse	4	1.34	-4.70	-2.10
60537	muscle tissue development	13	4.35	-4.22	-1.63
42593	glucose homeostasis	10	3.34	-4.12	-1.54
3012	muscle system process	11	3.68	-3.71	-1.16
1903532	positive regulation of secretion by cell	11	3.68	-3.71	-1.16
7507	heart development	16	5.35	-3.64	-1.10
1901798	positive regulation of signal transduction by p53 class mediator	4	1.34	-3.62	-1.09
9415	response to water	3	1.00	-3.60	-1.07
16310	phosphorylation	13	4.35	-3.38	-0.90
6091	generation of precursor metabolites and energy	12	4.01	-3.34	-0.87
M272	PID CD8 TCR DOWNSTREAM PATHWAY	5	1.67	-3.34	-0.87
32228	regulation of synaptic transmission, GABAergic	4	1.34	-3.32	-0.86
2000369	regulation of clathrin-dependent endocytosis	3	1.00	-3.23	-0.80
2456	T cell mediated immunity	4	1.34	-3.19	-0.77
48871	multicellular organismal-level homeostasis	16	5.35	-3.17	-0.76
55123	digestive system development	7	2.34	-3.14	-0.74
30855	epithelial cell differentiation	16	5.35	-3.12	-0.72
44057	regulation of system process	15	5.02	-3.11	-0.72
61035	regulation of cartilage development	5	1.67	-3.10	-0.72

989 ^a number of genes in the provided lists with membership in the given ontology term.

990 ^b percentage of all provided genes found in the given ontology term (only input genes

991 with at least one ontology term annotation are included in the calculation).

992 ^c P-value in log base 10.

993 ^d multi-test adjusted P-value in log base 10.

994

995

996 **Table 9.** List of canonical pathways identified by IPA in ICAR and CAR endometrial
 997 regions.

ICAR

G-protein coupled receptor signaling
 Glycosaminoglycan metabolism
 Tight junction signaling
 Arachidonic acid
 Oxidative stress induced senescence
 Acute response phase signaling
 Production of nitric oxide and reactive oxygen species in macrophages
 Epithelial adherens junction signaling
 Senescence-associated secretory phenotype (SASP)
 Extracellular matrix organization

CAR

Eukaryotic translation elongation
 Nonsense-mediated decay
 Response of EIF2AK4 (GCN2) to amino acid deficiency
 SRP-dependent cotranslational protein targeting to membrane
 Ribosomal quality control signaling pathway
 Collagen chain trimerization
 wnt/ β -catenin signaling pathway
 Collagen biosynthesis and modifying enzymes
 Wound healing signaling pathway

999
 1000

Table 10. Summary of significant Disease & Functions categories identified by IPA using DEGs from CAR and ICAR. Categories unrelated to the uterus (e.g., eye, auditory system) or irrelevant to the study context (e.g., developmental disorders) were excluded. For each category, the range of P-values is shown separately for ICAR and CAR. “N. S.” indicates that no significant association was detected in that comparison.

Category	P-value in ICAR	P-value in CAR
Amino Acid Metabolism	7.07E-04-3.21E-03	2.46E-03-8.73E-03
Antimicrobial Response	N. S.	1.1E-02-1.1E-02
Cancer	1.37E-31-5.57E-03	3.54E-31-1.1E-02
Carbohydrate Metabolism	1.16E-04-4.09E-03	6.21E-04-1.1E-02
Cell Cycle	1.2E-04-5.1E-03	1.21E-04-1.1E-02
Cell Death and Survival	4.51E-09-5.58E-03	8.41E-08-1.1E-02
Cell Morphology	9.02E-08-5.25E-03	5.18E-04-1.1E-02
Cell Signaling	4.01E-03-5.26E-03	3.31E-03-1.07E-02
Cell-mediated Immune Response	1.54E-04-3.48E-03	4.39E-03-1.1E-02
Cell-To-Cell Signaling and Interaction	8.54E-05-5.57E-03	1.06E-03-1.1E-02
Cellular Assembly and Organization	4.24E-05-5.17E-03	2.77E-05-1.1E-02
Cellular Compromise	4.32E-03-4.32E-03	1.29E-03-1.1E-02
Cellular Development	6.27E-07-5.08E-03	5.88E-05-1.1E-02
Cellular Function and Maintenance	6.96E-07-5.17E-03	7.95E-06-1.1E-02
Cellular Growth and Proliferation	1.78E-05-4.99E-03	2.67E-05-1.1E-02
Cellular Movement	1.3E-07-4.66E-03	1.57E-04-1.1E-02
Cellular Response to Therapeutics	N. S.	4.67E-03-7.44E-03
Connective Tissue Development and Function	6.61E-05-5.1E-03	1.21E-04-1.1E-02
Connective Tissue Disorders	2.9E-06-5.08E-03	1.3E-04-1.1E-02
DNA Replication, Recombination, and Repair	2.78E-04-5.08E-03	2.55E-03-1.1E-02
Drug Metabolism	3.56E-04-3.56E-04	1.79E-04-1.1E-02
Embryonic Development	2.59E-06-4.93E-03	1.21E-04-1.1E-02
Endocrine System Development and Function	1.35E-05-4.25E-03	2.1E-03-1.1E-02
Endocrine System Disorders	2.4E-22-5.47E-03	8.76E-18-1.1E-02

Energy Production	7.07E-04-4.09E-03	N. S.
Free Radical Scavenging	2.59E-04-4.53E-03	1.12E-03-1.19E-03
Gene Expression	1.87E-06-4.5E-03	6.39E-08-1.09E-02
Hematological Disease	3.95E-09-5.25E-03	2.06E-12-1.1E-02
Humoral Immune Response	N. S.	9.22E-04-1.77E-03
Immune Cell Trafficking	3.85E-04-4E-03	1.84E-03-1.1E-02
Immunological Disease	3.23E-07-5.25E-03	2.32E-07-1.09E-02
Infectious Diseases	2.16E-05-5.55E-03	2.67E-04-7.53E-03
Inflammatory Disease	1.26E-04-4.29E-03	1.52E-04-1.1E-02
Inflammatory Response	4.35E-04-5.08E-03	1.63E-04-1.1E-02
Lipid Metabolism	4.14E-05-4.53E-03	2.1E-03-6.25E-03
Lymphoid Tissue Structure and Development	4.59E-05-3.85E-03	9.22E-04-1.1E-02
Metabolic Disease	8.37E-07-4.09E-03	4.04E-04-1.1E-02
Molecular Transport	1.35E-05-5.08E-03	1.79E-04-1.1E-02
Nucleic Acid Metabolism	5.08E-03-5.08E-03	1.01E-02-1.01E-02
Nutritional Disease	1.29E-04-1.49E-03	8.8E-04-8.8E-04
Organ Development	2.59E-06-4.66E-03	1.21E-04-1.1E-02
Organ Morphology	3.44E-07-5.08E-03	6.18E-04-1.1E-02
Organismal Functions	N. S.	1.63E-03-2.46E-03
Organismal Injury and Abnormalities	1.37E-31-5.58E-03	3.54E-31-1.1E-02
Organismal Survival	4.7E-08-2.97E-03	2.74E-07-3.26E-03
Post-Translational Modification	4.25E-03-5.26E-03	4.1E-03-1.07E-02
Protein Synthesis	3.56E-04-5.58E-03	6.93E-05-1.09E-02
Protein Trafficking	N. S.	6.47E-04-5.15E-03
Reproductive System Development and Function	1.49E-03-2.97E-03	1.77E-03-1.77E-03
Reproductive System Disease	2.55E-14-3.62E-03	6.02E-10-1.1E-02
RNA Post-Transcriptional Modification	N. S.	1.37E-03-1.37E-03
Small Molecule Biochemistry	1.35E-05-5.08E-03	6.21E-04-1.1E-02
Tissue Development	6.27E-07-5.08E-03	2.67E-05-1.1E-02
Tissue Morphology	7.1E-10-5.08E-03	1.39E-04-1.1E-02
Tumor Morphology	8.36E-04-5.1E-03	3.51E-07-1.1E-02
Vitamin and Mineral Metabolism	4.01E-03-4.53E-03	2.1E-03-1.01E-02

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1008

Flow chart of RNA-seq bioinformatics and statistical analysis

Study design

- Heifer ICAR vs Old cow ICAR
- Heifer CAR vs Old cow CAR

Compare gene expression levels (Heifer ICAR vs Old cow ICAR; Heifer CAR vs Old cow CAR)

- $\log_2(\text{TPM} + 1)$ transformation
- Unpaired *t*-test for group comparison
- Calculate old/young TPM ratio
- Calculate fold change: $[\log_2(\text{old TPM} + 1)] - [\log_2(\text{young TPM} + 1)]$

Select DEGs between (Heifer ICAR vs Old cow ICAR; Heifer CAR vs Old cow CAR)

- (1) Average of $\log_2(\text{TPM} + 1) \geq 0.01$ in both groups
- (2) $p < 0.01$ (unpaired *t*-test)
- (3) fold change > 0.263 or < -0.263
- (4) Exclude genes without ENSBTAG

Metascape

Ingenuity Pathway Analysis

- Canonical pathways
- Upstream regulators
- Downstream effectors
- Protein localization/functions
- Disease and functions

1009

1010 **Fig. 1.** The flow chart illustrates the workflow used for RNA-seq data analysis. The study
1011 design compared heifers and old cows in both intercaruncular (ICAR) and caruncular
1012 (CAR) regions of the endometrium. Gene expression values were normalized as
1013 $\log_2(\text{TPM} + 1)$ and compared between groups using unpaired *t*-tests. Fold change was
1014 calculated as the difference between $\log_2$ -transformed TPM values of old and young cows.
1015 Differentially expressed genes (DEGs) were selected according to the following criteria:
1016 (1) average $\log_2(\text{TPM} + 1) \geq 0.01$ in both groups, (2) $p < 0.01$, (3) fold change > 0.263 or
1017 < -0.263 , and (4) exclusion of genes lacking ENSBTAG annotation. DEGs were further
1018 analyzed using Metascape for functional enrichment, and Ingenuity Pathway Analysis

1019 (IPA) was conducted to identify canonical pathways, upstream regulators, downstream
1020 effectors, protein localization/functions, and disease-related functions.
1021

(A) ICAR

P-value	Fold Change	Number of genes
● $P < 0.01$	Young/Old > 1.2	84 genes
● $P < 0.01$	Old/Young > 1.2	350 genes
● $P < 0.01$	Others	34 genes
● $0.01 \leq P < 0.05$	All	1,623 genes
● $0.05 \leq P$	All	18,194 genes

(B) CAR

P-value	Fold Change	Number of genes
● $P < 0.01$	Young/Old > 1.2	156 genes
● $P < 0.01$	Old/Young > 1.2	280 genes
● $P < 0.01$	Others	56 genes
● $0.01 \leq P < 0.05$	All	2,086 genes
● $0.05 \leq P$	All	23,663 genes

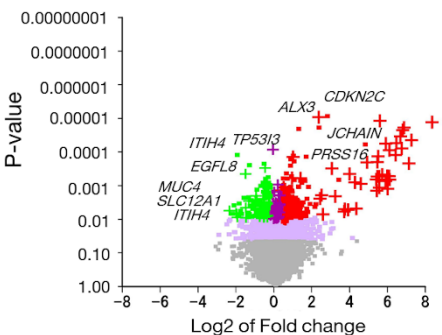
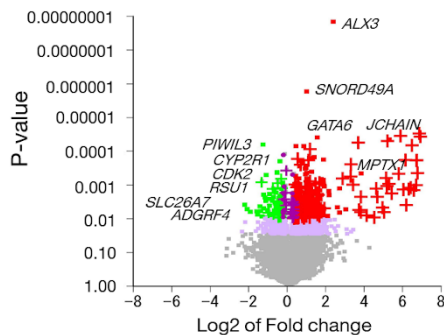
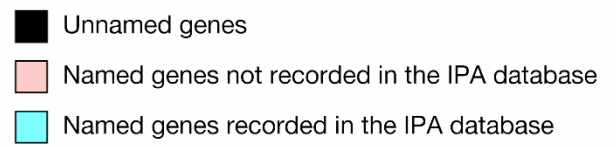


Fig. 2. (A) Volcano plot of 20,285 annotated genes comparing old ICAR with young ICAR. The X-axis shows the binary logarithm ($\log_2$) of fold change; genes on the right indicate higher expression in old ICAR (old $>$ young), whereas genes on the left indicate higher expression in young ICAR (young $>$ old). The Y-axis shows the statistical significance as the P-value, with smaller P-values plotted higher. Gray dots (18,194 genes) represent non-significant changes ($P \geq 0.05$). Green dots (84 genes) indicate genes with significantly lower expression in old ICAR compared to young ICAR ($P < 0.01$, $\log_2$ fold change < -0.263), and red dots (350 genes) indicate genes with significantly higher expression in old ICAR compared to young ICAR ($P < 0.01$, $\log_2$ fold change > 0.263) in old ICAR. In total, 434 genes (84 lower and 350 higher) were identified as differentially

expressed genes (DEGs) in ICAR. Dark purple dots (34 genes) represent genes with $P < 0.01$ but fold changes within ± 0.263 (i.e., not meeting the $>20\%$ threshold). Light purple dots (1,623 genes) represent genes with $0.01 \leq P < 0.05$. Genes shown in italic are highlighted as top candidates and listed in Tables 1 and 2. Genes marked with “+” lack gene names and are identified only by Ensembl gene IDs.

(B) Volcano plot of 26,241 annotated genes comparing old CAR to young CAR. Gray dots (23,663 genes) represent non-significant changes ($P \geq 0.05$). Green dots (156 genes) indicate significantly lower expression in old CAR ($P < 0.01$, $\log_2$ fold change < -0.263), and red dots (280 genes) indicate significantly higher expression in old CAR ($P < 0.01$, $\log_2$ fold change > 0.263) in old CAR. In total, 436 genes (156 lower and 280 higher) were identified as DEGs in CAR. Dark purple dots (56 genes) represent genes with $P < 0.01$ but fold changes within ± 0.263 . Light purple dots (2,086 genes) represent genes with $0.01 \leq P < 0.05$. Genes shown in italic are top candidates listed in Tables 3 and 4. Genes marked with “+” lack gene names and are identified only by Ensembl gene IDs.



(A) DEGs in ICAR

(B) DEGs in CAR

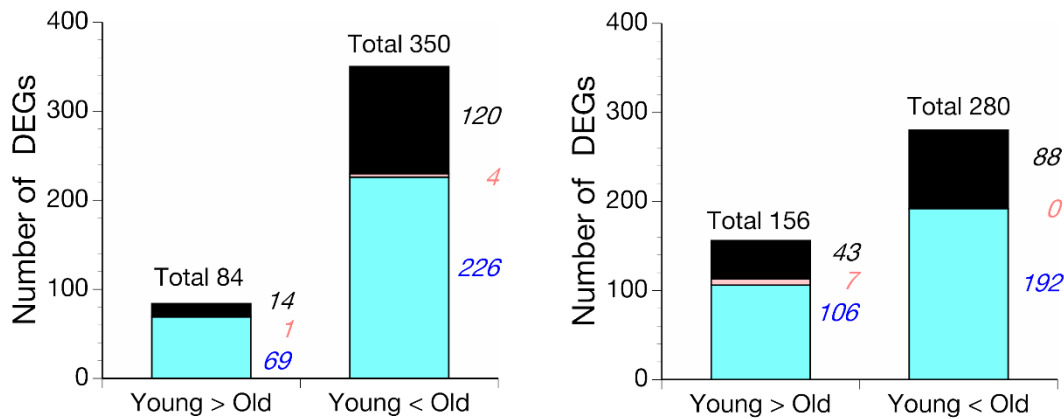


Fig. 3. Bar charts comparing differentially expressed genes (DEGs) in ICAR (A) and CAR (B). Italic numbers indicate detailed counts. Among the 434 DEGs identified between the ICAR of heifers and old cows, 134 were unnamed (black), 295 were named genes recorded in the IPA database (blue), and 5 were named genes not recorded in the IPA database (red). Among the 436 DEGs identified in the CAR, 131 were unnamed (black), 298 were named genes recorded in the IPA database (blue), and 7 were named genes not recorded in the IPA database (red).

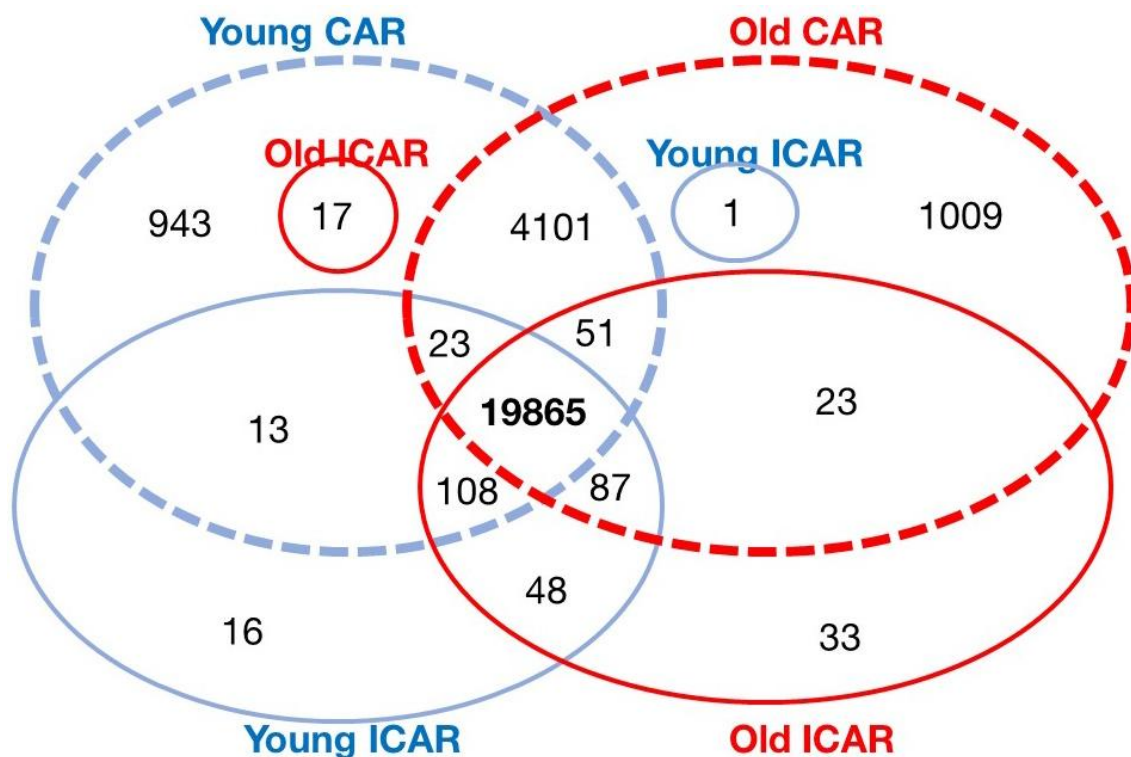


Fig. 4. Venn diagram of expressed genes in bovine endometrial tissues. The Venn diagram illustrates the overlap and unique distribution of expressed genes across four experimental groups: young CAR, old CAR, young ICAR, and old ICAR tissues. In total, 26,338 genes were identified, of which 19,865 (bold) were commonly expressed in all four groups. Unique gene sets were detected in each group (943 in young CAR, 1,009 in old CAR, 16 in young ICAR, and 33 in old ICAR). Smaller subsets of genes were shared between two or three groups, such as 4,101 genes shared between young and old CAR.

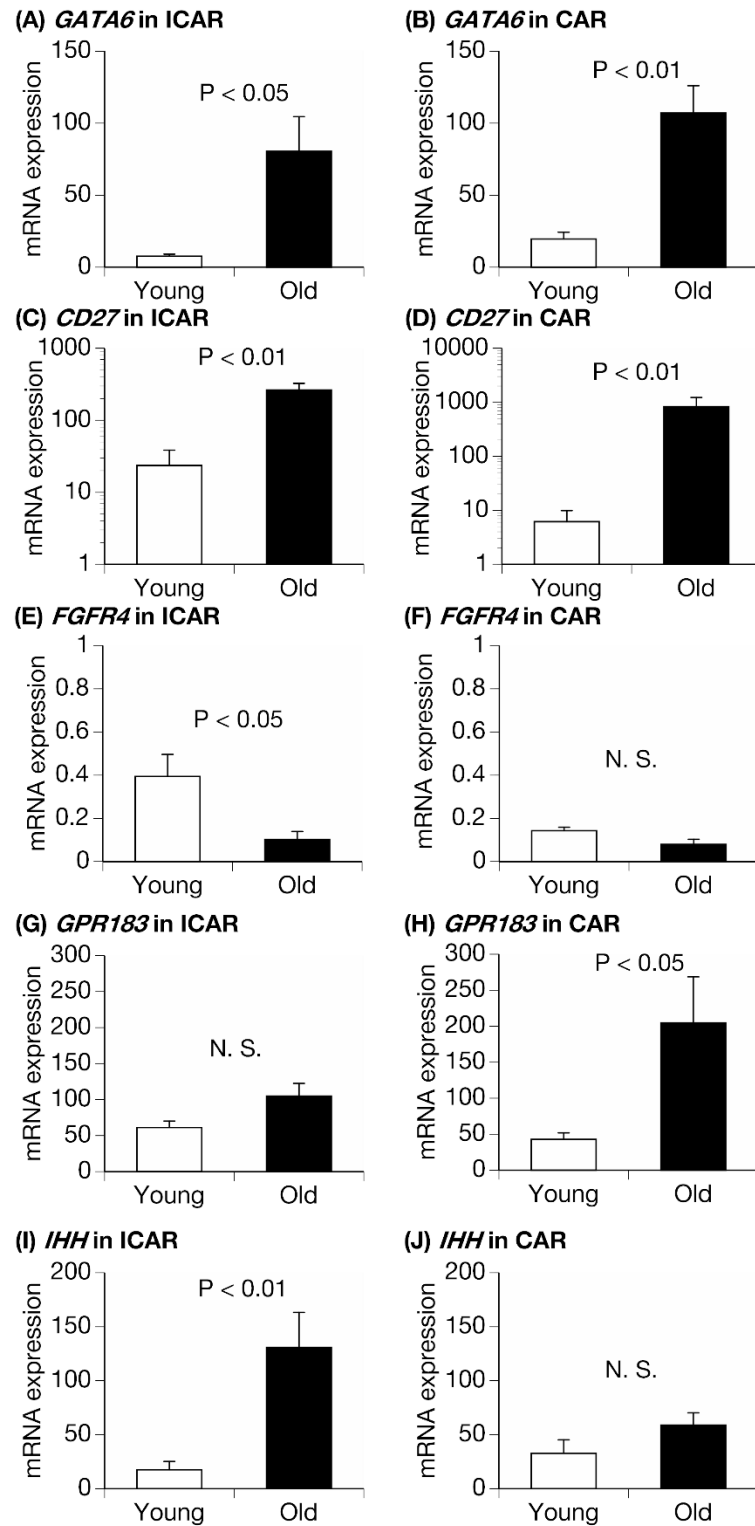


Fig. 5. Validation of RNA-seq data by qPCR. Expression levels of selected DEGs in the CAR and ICAR regions of the endometrium from heifers ($n = 5$) and old cows ($n = 5$)

1070 were measured using RT-qPCR. Data are shown as mean $\pm$ SEM (n=5 per group). The
1071 values are expressed as relative expression (dimensionless), and thus no units are
1072 indicated. *P*-values shown in each graph represent comparisons between groups using an
1073 unpaired *t*-test.