

Received: 21 April 2020

Revised: 8 June 2020

Accepted: 9 June 2020

DOI: 10.1096/fj.202000882RR

RESEARCH ARTICLE



The role of CAPG in molecular communication between the embryo and the uterine endometrium: Is its function conserved in species with different implantation strategies?

Haidee Tinning¹ | Alysha Taylor^{1,2} | Dapeng Wang³ | Bede Constantinides² |
Ruth Sutton¹ | Georgios Oikonomou⁴ | Miguel A. Velazquez⁵ | Paul Thompson⁶ |
Achim Treumann⁶ | Mary J. O'Connell^{2,7} | Niamh Forde¹

¹Discovery and Translational Sciences Department, Faculty of Medicine and Health, Leeds Institute of Cardiovascular and Metabolic Medicine, University of Leeds, Leeds, UK

²School of Biology, Faculty of Biological Sciences, University of Leeds, Leeds, UK

³LeedsOmics, University of Leeds, Leeds, UK

⁴Department of Livestock Health and Welfare, Institute of Veterinary Science, University of Liverpool, Liverpool, UK

⁵School of Natural and Environmental Sciences, Newcastle University, Newcastle upon Tyne, UK

⁶Protein and Proteome Analysis (NUPPA), Newcastle University, Newcastle upon Tyne, UK

⁷School of School of Life Sciences, Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham, UK

Correspondence

Niamh Forde, Discovery and Translational Sciences Department, Leeds Institute of

Abstract

During the preimplantation period of pregnancy in eutherian mammals, transcriptional and proteomic changes in the uterine endometrium are required to facilitate receptivity to an implanting blastocyst. These changes are mediated, in part, by proteins produced by the developing conceptus (inner cell mass and extraembryonic membranes). We hypothesized that this common process in early pregnancy in eutheria may be facilitated by highly conserved conceptus-derived proteins such as macrophage capping protein (CAPG). We propose that CAPG may share functionality in modifying the transcriptome of the endometrial epithelial cells to facilitate receptivity to implantation in species with different implantation strategies. A recombinant bovine form of CAPG (91% sequence identity between bovine and human) was produced and bovine endometrial epithelial (bEECs) and stromal (bESCs) and human endometrial epithelial cells (hEECs) were cultured for 24 hours with and without recombinant bovine CAPG (rbCAPG). RNA sequencing and quantitative real-time PCR analysis were used to assess the transcriptional response to rbCAPG (Control, vehicle, CAPG 10, 100, 1000 ng/mL; n = 3 biological replicates per treatment per species). Treatment of bEECs with CAPG resulted in alterations in the abundance of 1052 transcripts (629 increased and 423 decreased) compared to vehicle controls. Treatment of hEECs

Abbreviations: ABAM, anti-biotic anti-mycotic solution; AMP, ampicillin; ANOVA, analysis of variation; bEECs, bovine endometrial epithelial cells; bESCs, bovine endometrial stromal cells; BSA, bovine serum albumin; CAPG, macrophage capping protein; cDNA, complementary DNA; CL, corpus luteum; DEGs, differentially expressed genes; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate-buffered solution; dsDNA, double stranded deoxyribonucleic acid; dTTP, deoxythymidine triphosphate; dUTP, deoxyuridine triphosphate; F12, Ham's F12 nutrient mixture; FBS, fetal bovine serum; FDR, false discovery rate; GO, gene ontology; GSP, penicillin-streptomycin-glutamine solution; HBSS, Hank's balanced salt solution; hCG, human chorionic gonadotrophin; hEECs, human endometrial epithelial cells; HGF, hepatocyte growth factor; IFNT, interferon tau; lncRNA, long noncoding RNA; KEGG, Kyoto encyclopedia of genes and genomes; LB, Luria broth; mRNA, mature RNA; MYA, million years ago; P4, progesterone; PCA, principle component analysis; qRT-PCR, quantitative reverse transcription—polymerase chain reaction; rbCAPG, recombinant bovine CAPG; RNA, ribonucleic acid; roIFNT, recombinant ovine IFNT; RPKM, reads per kilobase of transcript, per Million mapped reads; RPMI, Roswell Park Memorial Institute medium; rRNA, ribosome RNA; RT, room temperature; STRING, search tool for the retrieval of interacting genes/proteins; ULF, uterine luminal fluid.

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Cardiovascular and Metabolic Medicine,
Faculty of Medicine and Health, University
of Leeds, Leeds, West Yorkshire LS2 9JT,
UK.
Email: n.forde@leeds.ac.uk

Present address

Bede Constantinides, Modernising Medical
Microbiology Consortium, Nuffield
Department of Clinical Medicine, John
Radcliffe Hospital, University of Oxford,
Oxford, UK

Funding information

RCUK | Biotechnology and Biological
Sciences Research Council (BBSRC),
Grant/Award Number: BB/R017522/1; N8
AgriFood Pump Priming; QR GCRF

with bovine CAPG increased expression of transcripts previously known to interact with CAPG in different systems (*CAPZB*, *CAPZA2*, *ADD1*, and *ADK*) compared with vehicle controls ($P < .05$). In conclusion, we have demonstrated that CAPG, a highly conserved protein in eutherian mammals, elicits a transcriptional response in the endometrial epithelium in species with different implantation strategies that may contribute to pregnancy success.

1 | INTRODUCTION

In the majority of mammals, most pregnancy loss occurs in the pre- and peri-implantation periods of pregnancy. The contributory factors to this loss are multifactorial and include poor gamete quality, aberrant embryonic development, and a suboptimal uterine environment, all of which can influence the likelihood of pregnancy loss. Advances in assisted reproductive technologies have facilitated the production of large numbers of viable embryos in vitro for transfer into recipient females. However, pregnancy loss still occurs, thought to be due (in part at least) to inappropriate molecular communication between the developing conceptus (ie, embryo and associated extraembryonic membranes) and the maternal environment—specifically the uterine endometrium.^{1,2}

The uterine endometrium is a complex tissue, consisting predominantly of luminal and glandular epithelial cells, underlying stromal cells, alongside immune cells, and endothelial microvasculature. These heterogeneous cells respond spatially and temporally to steroid hormones, the concentrations of which fluctuate throughout the menstrual/estrous cycle in maternal circulation.^{3,4} Following successful fertilization, the embryo enters the uterus on approximately day 4 of pregnancy where it is entirely reliant for its growth and survival on the secretion and transport of molecules from the luminal and glandular epithelial cells of the endometrium. These molecules include growth factors, cytokines, amino acids and ions, and are collectively termed uterine luminal fluid (ULF).^{5,6} ULF supports growth and development of the conceptus until a fully functional placenta is established.⁷ The embryo undergoes a number of key morphological changes in the uterus prior to implantation including transition from a morula to a blastocyst, at which stage differentiation begins between the inner cell mass (that forms the fetus) and the outer cell mass (the trophoblast, that goes on to form the

placenta). Next, the blastocyst hatches from the zona pellucida, following which implantation occurs. However, the timing and type of implantation varies from species to species. For example, in humans, implantation is invasive whereby the blastocyst invades into the uterine endometrium⁸ while in cattle the trophoblast undergoes a rapid period of proliferation to produce an elongated conceptus and implantation does not begin until about Day 19 of pregnancy and is non-invasive in nature.⁹ Irrespective of conceptus morphology in early pregnancy, type of implantation, and type of placentation (hemochorial in human and synepitheliocorial in ungulates) two key conserved processes are required to modify the uterine endometrium to facilitate early pregnancy establishment, namely the actions of maternally derived progesterone (P4) and the species-specific pregnancy recognition signal.

First, P4 drives spatial and temporal changes to the endometrial transcriptome.¹⁰ Paradoxically, continued exposure of the uterine endometrium to P4 downregulates the P4 receptor from the luminal and glandular epithelium, a process required to establish uterine receptivity to implantation in all mammal species studied thus far.^{11,12} Second, the conceptus must secrete sufficient quantities of its pregnancy recognition signal to enhance uterine receptivity to implantation, that is, modify the transcriptome and proteome of the endometrium to prevent luteolysis and thereby maintain P4 production as well as to facilitate implantation. The molecular cue for pregnancy recognition varies across different mammals. In cattle and sheep, a type 1 Interferon (interferon-tau, IFNT) is involved while in humans it is chorionic gonadotrophin (hCG). However, the full extent of the essential interactions between the conceptus and the uterine environment required to support successful pregnancy establishment have yet to be elucidated.

The protracted peri-implantation period of pregnancy in cattle provides an opportunity to investigate the role of selected molecules in the process of pregnancy recognition. Previous

studies by our group and others identified proteins, in addition to IFNT, produced by the peri-implantation conceptus which may play a role in pregnancy recognition.^{13,14} We identified proteins uniquely present in the uterine fluid of pregnant cattle on the day of pregnancy recognition. These proteins were also detected in conceptus-conditioned medium with a large number of spectral counts via nano-liquid column-tandem mass spectrometry.¹³ One of these proteins is Macrophage Capping protein (CAPG)—a calcium-sensitive protein which functions to modulate cell motility through interactions with the cytoskeleton. CAPG, therefore, could function in remodeling the endometrium for successful implantation.¹⁵ CAPG also has known roles in cell migration, has been identified in exosomes from trophoblast cells in vitro, and has been shown to play a role in tumor invasiveness in endometrial cancer in humans.¹⁶ Given its high abundance around maternal recognition of pregnancy in cattle¹³ and its roles in proliferation of human endometrial cells,¹⁶ we hypothesized that CAPG modifies the endometrium during the process of pregnancy recognition. This is particularly important as the transcriptional response of the endometrium to the developing conceptus as a whole, is much greater than its response to the pregnancy recognition signal alone.³ Because of the high levels of sequence conservation of this protein in mammals, we hypothesized that it may also modify the endometrial transcriptome in species with different implantation strategies to cattle, for example, humans. Our specific aims were to (1) assess levels of sequence conservation of CAPG in representative eutherian mammal species, (2) produce a recombinant form of bovine CAPG, and (3) test how the cells of the endometrium from different species respond to this recombinant bovine CAPG (rbCAPG) in vitro.

2 | MATERIALS AND METHODS

2.1 | Analysis of sequence homology for CAPG

Homologs for CAPG were identified across 20 species including 15 placental mammals¹⁷ and five outgroup species,

and extracted from Ensembl¹⁸ (Figure 1). A multiple sequence alignment was generated using MAFFT v7.3¹⁹ for the 19 species with CAPG gene annotation (none available for microbat) and the resultant alignment was visualized in SeaView.²⁰ The Percent Identity Matrices were generated using Clustal Omega.²¹

2.2 | Production of rbCAPG protein

The sequence for bovine CAPG was subcloned into pET3A using Thermo Fisher Scientific's GenArt service, dissolved in 50 μ L dH₂O, and 2 μ L of the plasmid transformed into HB101 pre-competent cells, selecting for transformants on 100 μ L Ampicillin, Luria Agar plates. Luria broth (LB, 200 mL, 100 μ g/mL AMP) was inoculated with a scrape of plasmid, and incubated for 9 hours at 37°C. The pellet was then harvested via centrifugation (9000 rpm, 4°C, 10 minutes) and a Plasmid Midi Kit was used to extract plasmids as per manufacturer's instructions (Qiagen, Crawley, UK) which were dissolved in a final volume of 750 μ L dH₂O. Two μ L of plasmid were transformed into BL21-AI pre-competent cells, selecting for transformants on 100 μ L Ampicillin, Luria Agar plates, and grown overnight at 37°C. Twenty-four 10 mL LB starter cultures (100 μ g/mL AMP), were inoculated with individual colonies picked from expression plates. These were then incubated at 30°C for ~24 hours.

Large scale production of CAPG was undertaken by setup of 20 x 500 mL LB (100 μ g/mL AMP) flasks which were inoculated with the whole of an individual starter culture. These were held at 10°C until growth at 30°C, shaking at 160 rpm. All cultures were induced with a final concentration of 0.2% L-Arabinose 7 hours later and cells were harvested by centrifugation (9000 rpm, 4°C, 10 minutes) 5 hours after that. Each tube of cells was resuspended in 450 mL of 50 mM mixed phosphate, pH 7.2, 1 mM Benzamidine. The resulting cell suspension was sonicated at 14 microns for 42 minutes, while stirring on an ice bath, and then, centrifuged at 9000 rpm, for 42 minutes at 4°C. The supernatant was then carefully decanted and loaded

| | | Mouse | | | | | | | | | | | | | | | | | | | |
|-------------|-----------------------|-------|-------|----------|-------|--------|-------|-----|---------|-----|--------|-----|-------|----------|-----------|---------|---------|----------|---------|-----------|-----------|
| | | Human | Lemur | Bushbaby | Mouse | Rabbit | Sheep | Cow | Dolphin | Pig | Ferret | Dog | Horse | Elephant | Armadillo | Wallaby | Opossum | Platypus | Chicken | Zebrafish | Zebrafish |
| Human | ENSP00000263867 | 100 | 96 | 95 | 93 | 94 | 91 | 91 | 93 | 93 | 95 | 95 | 95 | 95 | 85 | 82 | 85 | 77 | 64 | 56 | 57 |
| Mouse Lemur | ENSMHICP00000013734 | 96 | 100 | 96 | 94 | 94 | 93 | 93 | 96 | 94 | 95 | 95 | 95 | 96 | 85 | 84 | 86 | 79 | 65 | 57 | 58 |
| Bushbaby | ENSCGAP00000000969 | 95 | 96 | 100 | 93 | 93 | 91 | 91 | 94 | 93 | 94 | 95 | 95 | 95 | 84 | 84 | 87 | 78 | 65 | 57 | 58 |
| Mouse | ENSMUSP00000109705 | 93 | 94 | 93 | 100 | 93 | 90 | 90 | 92 | 93 | 93 | 91 | 93 | 93 | 82 | 83 | 84 | 77 | 64 | 57 | 58 |
| Rabbit | ENSCUCP00000002018 | 94 | 94 | 93 | 93 | 100 | 91 | 91 | 93 | 94 | 95 | 93 | 94 | 94 | 84 | 83 | 84 | 77 | 64 | 56 | 57 |
| Sheep | ENSOARP00000022035 | 91 | 93 | 91 | 90 | 91 | 100 | 99 | 93 | 92 | 92 | 90 | 91 | 93 | 82 | 83 | 83 | 76 | 64 | 57 | 57 |
| Cow | ENSBTAP00000007150 | 91 | 93 | 91 | 90 | 91 | 99 | 100 | 92 | 92 | 92 | 90 | 91 | 92 | 82 | 82 | 83 | 76 | 64 | 57 | 56 |
| Dolphin | ENSTTRP00000008502 | 93 | 96 | 94 | 92 | 93 | 93 | 92 | 100 | 94 | 93 | 93 | 94 | 93 | 83 | 83 | 86 | 77 | 64 | 57 | 57 |
| Pig | ENSSPCP00000008796 | 93 | 94 | 93 | 93 | 94 | 92 | 92 | 94 | 100 | 94 | 92 | 94 | 93 | 84 | 84 | 85 | 76 | 65 | 57 | 58 |
| Ferret | ENSMPIUP00000008771 | 95 | 95 | 94 | 93 | 95 | 92 | 92 | 93 | 94 | 100 | 96 | 95 | 95 | 85 | 84 | 85 | 78 | 66 | 57 | 57 |
| Dog | ENSCAFP000000011524 | 95 | 95 | 93 | 91 | 93 | 90 | 90 | 93 | 92 | 96 | 100 | 94 | 94 | 85 | 83 | 84 | 77 | 64 | 57 | 58 |
| Horse | ENSECAP000000012946 | 95 | 95 | 95 | 93 | 94 | 91 | 91 | 94 | 94 | 95 | 94 | 100 | 95 | 85 | 84 | 86 | 78 | 64 | 57 | 58 |
| Elephant | ENSLAFP000000013680 | 95 | 96 | 95 | 93 | 94 | 93 | 92 | 93 | 93 | 95 | 94 | 95 | 100 | 85 | 83 | 86 | 78 | 65 | 57 | 57 |
| Armadillo | ENSNDOP000000015185 | 85 | 85 | 84 | 82 | 84 | 82 | 82 | 83 | 84 | 85 | 85 | 85 | 85 | 100 | 73 | 74 | 70 | 59 | 51 | 52 |
| Wallaby | ENSMIEUP000000012369 | 82 | 84 | 84 | 83 | 83 | 83 | 82 | 83 | 84 | 84 | 83 | 84 | 83 | 73 | 100 | 91 | 77 | 64 | 58 | 58 |
| Opossum | ENSMODP000000019465 | 85 | 86 | 87 | 84 | 84 | 83 | 83 | 86 | 85 | 85 | 84 | 86 | 86 | 74 | 91 | 100 | 81 | 66 | 57 | 58 |
| Platypus | ENSOANP00000001768 | 77 | 79 | 78 | 77 | 77 | 76 | 76 | 77 | 76 | 78 | 77 | 78 | 77 | 78 | 77 | 81 | 100 | 63 | 57 | 58 |
| Chicken | ENSGALP000000045996 | 64 | 65 | 65 | 64 | 64 | 64 | 64 | 64 | 65 | 66 | 64 | 64 | 65 | 59 | 64 | 66 | 63 | 100 | 51 | 53 |
| Zebrafish | ENSDDARP00000001549 | 56 | 57 | 57 | 57 | 56 | 57 | 57 | 57 | 57 | 57 | 57 | 57 | 57 | 51 | 58 | 57 | 57 | 57 | 100 | 72 |
| Zebrafish | ENSDDARP0000000136284 | 57 | 58 | 58 | 58 | 57 | 57 | 56 | 57 | 58 | 57 | 56 | 58 | 57 | 52 | 58 | 58 | 58 | 53 | 72 | 100 |

FIGURE 1 Levels of sequence identity for the CAPG homolog across a range of 20 species: 14 eutherian mammals, 2 marsupials, 1 monotreme, 2 birds, and 1 fish (2 orthologues). Darker cells indicate higher % of sequence identity

onto a pre-equilibrated (Buffer 1) ProBond column. The column was washed with 500 mL of Buffer 1, eluted with a 500 mL gradient of 0.0 to 0.3 M Imidazole, 50 mM mixed phosphate, pH 7.2, and individual 5 mL fractions were collected.

Every third fraction between fractions 3 to 81 was subsequently loaded onto a 10% of SDS-PAGE gel. Those fractions containing the greatest concentration of rbCAPG were pooled and dialyzed into 2 × 5 L with 50 mM mixed phosphate, pH7.2, along with 1 mM DTT to remove Imidazole from the pool. Pooled rbCAPG was then filter sterilized with a 0.45 µm disc filter following which concentration and quality were calculated via UV spectroscopy.

2.3 | Bovine primary endometrial cell culture

Culture of bovine endometrial cells was achieved by isolating endometrial epithelial and stromal cells from late luteal stage bovine reproductive tracts as previously described.^{22,23} Unless otherwise stated, all solutions were maintained at room temperature (RT). Individual tracts were sprayed with 70% of EtOH and the ipsilateral uterine horn (the horn attached to the ovary with the corpus luteum (CL)) was opened longitudinally to expose the uterine endometrium. The endometrium was washed with 25 mL of endometrial wash solution (DPBS, 1% ABAM) and dissected away from the underlying myometrium. The sheets of endometrium were placed into a wash solution (25 mL HBSS (no calcium no magnesium), 1% ABAM) and gently swirled prior to pour off and an additional 25 mL of HBSS was used to further wash endometrial strips. Endometrial tissue was then dissected into 3-5 mm³ pieces using tweezers and scissors and placed into 25 mL of fresh HBSS. HBSS was poured off and digestive solution was added to the tissue to a final volume of 40 mL (filter sterilized, 50 mL HBSS (no calcium no magnesium), 25 mg collagenase II, 50 mg BSA, 125 µL 4% DNase I, 500 µL 0.0175% trypsin in HBSS) and tissue incubated in a rocking hot box for 1 hour at 37°C.

The resulting solution was strained through a 100 µm strainer above a 40 µm strainer into a 50 mL sterile falcon containing 5 mL of stop solution (HBSS with 10% charcoal-stripped FBS). The flow through, containing the stromal cell fraction, was vortexed briefly. The 40 µm of strainer was inverted, flushed with 5 mL complete medium (Gibco RPMI 1640, 10% charcoal-stripped FBS, 1% ABAM) (37°C) and the resulting epithelial-enriched fraction was plated into a T25 flask and cultured at 37°C in 5% CO₂. Stromal cells were centrifuged at 400 g for 5 minutes and 5 mL of sterile H₂O at 37°C was added to the pellet to lyse erythrocytes.

The stromal cell suspension was briefly vortexed and resuspended immediately in 45 mL of stop solution (37°C). The

solution was then centrifuged at 400 g for 5 minutes, 10 mL of complete medium (Gibco RPMI 1640, 10% charcoal-stripped Fetal Bovine Serum [FBS], 1% ABAM) (37°C) added to the pellet, resuspended, and stromal cells were transferred into a T75 flask and placed into a 37°C/5% CO₂ incubator. Twenty-four hours later (following adherence of cells) medium was aspirated, washed 1× with PBS (37°C), and 10 mL of fresh medium added. Once the stromal cells had reached ~70% confluency, they were dislodged by trypsinization (5 mL 0.025% trypsin in PBS) and plated at 150,000 per well into 6-well plates in 2 mL medium for treatment.

Following 2-3 days of culture of epithelial cells at 37°C/5% CO₂ un-adhered cells and medium were aspirated and adhered cells washed gently with 5 mL of DPBS (37°C) and replenished with 5 mL medium. Contaminating stromal cells were removed by trypsinization (0.025% trypsin in DPBS) for 2 min. Cells were plated at 300 000 per well in 6-well dishes in 2 mL medium, cultured at 37°C/5% CO₂ overnight, and medium replenished prior to treatment.

Epithelial and stromal cells were then treated with one of the following for 24 hours: (1) Control, (2) Vehicle, (3) 1000 ng/mL roIFNT, (4) 10 ng/mL rbCAPG, (5) 100 ng/mL rbCAPG, (6) 1000 ng/mL rbCAPG, (7) 1000 ng/mL rbCAPG + 100 ng roIFNT. Following treatment, culture medium was removed, cells were chemically lysed, lysate was snap-frozen in liquid nitrogen and kept at -80°C.

2.4 | Human endometrial cell culture

Human immortalized endometrial epithelial Ishikawa cells (passage 10) were previously frozen in dimethyl sulfoxide (DMSO) and stored at -80°C. Cells were thawed rapidly, centrifuged at 400 g for 5 minutes, and resuspended in 10 mL medium (Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM/F12), 10% FBS, 1% GSP). Cells were then incubated in a T75 flask at 37°C/5% CO₂, cultured until 80%-90% confluent and split into T75 flasks (n = 3). Human Ishikawa cells (n = 3) were seeded at a density of 300 000 cells per well in 2 mL medium, cultured at 37°C/5% CO₂ overnight, and medium replenished prior to treatment. Cells were then treated with one of the following for 24 hours: (1) Control, (2) Vehicle, (3) rbCAPG 10 ng/mL, (4) rbCAPG 100 ng/mL (5) rbCAPG 1000 ng/mL. Cells were chemically lysed, lysate snap-frozen in liquid nitrogen and kept at -80°C.

2.5 | RNA extraction, library preparation, and sequencing

Total RNA was extracted from cell lysate thawed on ice, using the MirVana RNA extraction kit as per manufacturer's

protocol (Life Technologies) and eluted in 50 μL RNase-DNase-free water. RNA was characterized using a Nanodrop and diluted to a standard concentration (Human 40 ng/ μL ; Bovine 50 ng/ μL). Libraries were prepared from approximately 100 ng total RNA in 10 μL of water using the Illumina TruSeq® Stranded Total kit, according to the manufacturer's guidelines. rRNA was removed first by heating the RNA to 68°C for 5 minutes and cooling to room temperature for 1 minute in the presence of 5 μL of rRNA Removal Mix, followed by 1 minute incubation at RT in the presence of 35 μL of rRNA Removal Beads. The beads were pelleted in the presence of a magnetic field and the solution containing mRNA and lncRNA removed and cleaned using Ampure RNAClean XP beads (Beckman Coulter, California, USA) as follows: the RNA was bound to 99 μL of beads which were pelleted under magnetic field and the solution discarded. The beads were gently washed twice with 200 μL 70% of ethanol, air-dried and resuspended in 11 μL of Elution Buffer. Then, 8.5 μL were transferred to a new well to which 8.5 μL of the Elute, Prime, Fragment High Mix containing random hexamers was added prior to incubation at 94°C for 8 minutes. First strand synthesis was performed by the addition of 8 μL SuperScript II with First Strand Synthesis Act D Mix (1 to 9 ratio, respectively), and incubation at 25°C for 10 minutes followed by heating to 42°C for 15 minutes before terminating the reaction at 70°C for 15 minutes. Second strand cDNA synthesis was performed following the addition of 5 μL of resuspension buffer and 20 μL of Second Strand Master Mix containing DNA polymerase I and RNase H and incubated at 16°C for 1 hour. Strand specificity is achieved by replacing dTTP with dUTP within the master mix. The cDNA was purified and size selected for fragments of approximately 300 bp by binding the cDNA to 90 μL of AxyPrep Mag PCR cleanup Kit (Axygen, Corning, New York, USA). The supernatant was discarded and the beads washed twice with 200 μL of 80% ethanol, air-dried, and finally resuspended in 18.5 μL Resuspension Buffer. Following pelleting of the beads with a magnetic field, 17.5 μL of solution was transferred to a new well. Adenylation of 3' ends was performed by adding 12.5 μL of A-Tailing Mix and incubating the samples at 37°C for 30 minutes and terminated by heating to 70°C for 5 minutes, following the addition of 2.5 μL of Resuspension buffer. The samples were indexed by ligating 2.5 μL of indexed Illumina sequencing compatible adapters to each sample in the presence of 2.5 μL Ligation Mix by heating to 30°C for 10 minutes. Five microliters of Stop Ligation Buffer was added to each well to stop the ligation process.

The samples were purified using 42 μL of AxyPrep Mag PCR cleanup Kit and washing twice with 80% of ethanol and air-dried. The samples were resuspended in 52.5 μL of

Resuspension buffer and 50 μL of transferred to a new well. The cleanup was repeated a second time with 50 μL of beads and resuspended in 22.5 μL of Resuspension buffer of which 20 μL was transferred to a new tube. The libraries were then amplified by PCR using 5 μL of PCR Primer Cocktail and 25 μL of PCR Master Mix with an initial 98°C for 30 seconds, followed by 15 cycles of: 98°C 10 seconds, 60°C 30 seconds, 72°C 30 seconds with a final extension time of 72°C for 5 minutes. Finally, the cDNA libraries were purified and size selected to remove adaptor dimers and unincorporated adaptors using 50 μL of AxyPrep Mag PCR cleanup Kit as described above and resuspended in 32.5 μL of Resuspension Buffer with 30 μL of transferred to a new well.

The library quality, size range, and sequencing adaptor dimer contamination was assessed with an Agilent TapeStation 2200 using the DNA broad range kit. Excess sequencing adaptor dimer, if present, was removed by AxyPrep Mag PCR cleanup kit bead mediated size selection. The final libraries were then quantified by measuring fluorescence with the Qubit dsDNA assay kit and Qubit fluorometer (Life Technologies) before creating an equimolar pool of the libraries. The RNA libraries were sequenced by the University of Leeds Next Generation Sequencing Facility using the Illumina NextSeq 500 (Illumina, California, USA) with a single end 75 bp length read.

2.6 | RNA sequencing bioinformatics analysis and string interaction networks

To analyze differences in expression, adapter trimming was done by Cutadapt²⁴ and the reads were filtered using fastq_quality_filter as part of FASTX-toolkit with parameters including “-q 20” and “-p 90”. The bovine (*Bos taurus*) and human (*Homo sapiens*) reference genome and gene annotation files were retrieved from the Ensembl genome database (release 96)²⁵ and GENCODE (release 31),²⁶ respectively. With the help of the Rsubread package,²⁷ read mapping was performed by means of Subread aligners with only uniquely mapped reads reported in the final alignment and read summarization and quantification was carried out using the featureCounts function. Statistical analysis for differential gene expression was conducted via DESeq2²⁸ with the cutoffs of $\log_2\text{FoldChange} > 1$ (or < -1) and $\text{padj} < .05$. After this analysis, only protein-coding genes and lncRNAs were retained based on the gene biotype labels. Overrepresentation enrichment analysis of differential expression protein-coding gene sets was executed using WebGestalt²⁹ for gene ontology terms and KEGG pathways, and protein-protein interaction networks

were predicted using STRING.³⁰ For gene ontology terms, biological process nonredundant data sets were chosen as functional database, and for both types of analyses, significance level was determined by FDR < 0.05. For principal component analysis (PCA) plotting of each group of samples, both protein-coding genes and lncRNAs with RPKM value ≥ 1 in at least one sample were used and subsequently $\log_2(\text{RPKM} + 1)$ transformation and a quantile normalization were applied.

2.7 | Quantitative real-time PCR analysis of selected transcripts in human and bovine endometrial cells

Transcripts analyzed in bovine cells were selected on the basis of being upregulated during the pregnancy recognition process both due to and independent of IFNT. Selected transcripts for analysis in human cells were determined using string interaction network to identify nine human proteins that are known to be related to CAPG either through experimental determination or curated databases.³⁰ These were Cysteine Rich Protein 1 (*CRIP1*), Ubiquitin-Fold Modifier

Conjugating Enzyme 1 (*UFC1*), Pirin (*PIR*), Adenosine Kinase (*ADK*), Ubiquitin-Fold Modifier 1 (*UFM1*), Alpha-Adducin 1 (*ADD1*), Capping Actin Protein of Muscle Z-Line Subunit Alpha 1 (*CAPZA1*), Capping Actin Protein of Muscle Z-Line Subunit Alpha 2 (*CAPZA2*), and Capping Actin Protein of Muscle Z-Line Subunit Beta (*CAPZB*).

RNA was extracted as described above and RNA (500 ng bovine, 400 ng human) was reverse transcribed using the High Capacity cDNA reverse transcription kit (Applied Biosystems) as per manufacturer's protocol. Nine human primers, seven bovine primers, and respective normalizers (Table 1) were supplied by Integrated DNA Technologies (IDT) at 0.5 μM and 5 ng of cDNA per reaction was used alongside 5 μL of 2 \times Roche SYBR green master mix and all samples were analyzed in duplicate using the Roche LightCycler 480 II. For human samples, *ACTB*, *GADPH*, and *PPIA* were used as normalizer genes and for bovine samples the average was calculated from *ACTB* and *GADPH* normalizer genes. The expression of genes of interest was determined using the comparative C_T method ($2^{-\Delta\Delta C_T}$ method)³¹ prior to ANOVA to identify treatment effects on expression. The ANOVA analysis included a Dunnett's multiple comparisons test which allowed all sample data to be individually

TABLE 1 List of primers used for quantitative real-time PCR analysis of selected candidate transcripts in bovine and human endometrial cells treated with and without rbCAPG for 24 hours

| Gene name | Forward | Reverse | Accession number | Product length (bp) |
|----------------|---------------------------|---------------------------|--------------------------------|---------------------|
| <i>hCRIP1</i> | GGTGTACTTCGCCGAGAGGG | GGTTCAGTAGGGTTTGCCT | NM_001311.5 | 131 |
| <i>hUFC1</i> | CCCGAGATCGTGAGTTGTGG | TTCCAAACCACCGAGTTCCT | NM_016406.4 | 138 |
| <i>hPIR</i> | TTTGCACTGGATGACTGCCG | CCTGGTACTGAGGCTCCACC | NM_003662.3 | 131 |
| <i>hADK</i> | TGAGACAGAAGCTGCCACTTT | GATCACGATTCGCTGCCTCT | NM_001123.3 | 121 |
| <i>hUFM1</i> | CCACCATGTCTGAAGGTTTCC | GACTGCTGTGAAAGGTGACT | NM_016617.4 | 101 |
| <i>hADD1</i> | CCACGATTCGCTTCTGAGGAAC | CTCTCCTTGTGAGGGGCTGT | NM_001119.5 | 106 |
| <i>hCAPZA1</i> | CGCCCGGTGATTCCATC | TGCGTACCTTCTCCTCATCCG | XM_017002424 | 125 |
| <i>hCAPZA2</i> | AAGGAGCAGCCATGCATTT | CCCAAGTCGCCATGTTCTGT | NM_006136.3 | 106 |
| <i>hCAPZB</i> | GCCTTGACCTAATGAGGCG | AGACTGGGGACCAGGTCGAT | NM_004930.5 | 80 |
| <i>hACTB</i> | AGAAAATCTGGCACACACC | TAGCACAGCCTGGATAGCAA | NM_001101.4 | 173 |
| <i>hGAPDH</i> | TTTCTATAAATTGAGCCCGCAGC | TGGCTCGGCTGGCGAC | NM_002046.7 | 94 |
| <i>hPPIA</i> | TCATCCTAAAGCATACTGGTCTCTG | ATCCAACCACTCAGTCTTGGCAGTG | NM_021130.5 | 107 |
| <i>bDKK1</i> | CCATTGACAACCACCAGCCGTATC | CCGACAGGCGAGGCAGATTT | NM_001205544.1 | 113 |
| <i>bMX2</i> | GGGCAGCGGAATCATCACCC | AGCTGCTGCGTAATGTTGCGGTA | NM_173941.2 | 102 |
| <i>bISG15</i> | TGACGGTGAAGATGCTAGGG | ACTGCTTCAGCTCGGATACC | NM_174366.1 | 78 |
| <i>bTKT</i> | GGCCGATCAGATCATCCAGG | GCGGGTAGCTATCTTGTCCC | NM_001003906.1 | 157 |
| <i>bFABP3</i> | GTGCGGGAAATGTTGAC | GTAGCCCACTGGCAGAAGAG | NM_174313.2 | 131 |
| <i>bRSAD2</i> | GGTCTGCTGATGCTGAAAGA | GCTCCTGCTTACAGAACTTC | NM_001045941.1 | 118 |
| <i>bGAPDH</i> | TGAACACTGGAGAAACC | CGCCTGCTTACCACCTT | NM_001034034.2 | 57 |
| <i>bACTB</i> | TGGATGATGATATTGC | AAGCCGGCCTTGCACAT | NM_173979.3 | 61 |
| <i>bMX1</i> | TCCATCCGACTACATTTCCA | CTGCCTCCTTCTCTGACC | NM_173940.2 | 94 |

compared to the vehicle control data. Significant differences were determined when $P < .05$.

3 | RESULTS

3.1 | CAPG sequence is highly conserved across eutherian mammals

CAPG demonstrated a high degree of sequence conservation among all placental mammal species investigated (Figure 1). Specifically, bovine CAPG showed > 90% sequence identity when compared to 12 of the 13 other placental mammals investigated, with the exception of the Armadillo CAPG gene (82% amino acid sequence identity). There was 99% similarity between bovine and ovine sequences; this was not surprising given that these two species are the closest relatives among the species sampled (diverging ~18 MYA³²). There was also a high degree of similarity compared to other placental mammals with different implantation strategies, for instance, the human and bovine CAPG amino acid sequences are 91% identical.

3.2 | Treatment of bovine endometrial epithelial cells with rbCAPG induces a transcriptional response that may facilitate the pregnancy recognition process

PCA of bovine endometrial epithelial cell gene expression data showed clear separation of control cells (no treatment) and those treated with +IFNT, +CAPG, or +IFNT&CAPG (Figure 2A). Treatment of epithelial cells for 24 hours with CAPG altered expression of 537 protein-coding transcripts (422 increased and 115 decreased) compared to controls (Table S1). One thousand four hundred and fifty-three transcripts were modified by IFNT alone (1028 increased and 425 decreased: Table S2), while treatment of epithelial cells with a combination of CAPG and IFNT increased expression of 984 and decreased expression of 398 protein-coding transcripts (Table S3). Venn diagram analysis (Figure 3A) showed consistent changes in the expression of 400 transcripts when cells were treated with IFNT or CAPG alone or in combination (Table S4). One hundred and twelve transcripts were modified following treatment with CAPG alone, or CAPG & IFNT, while 14 transcripts were modified by CAPG treatment alone. A string interaction network of differentially expressed transcripts in the epithelial cells demonstrated that the majority of transcripts modified by IFNT alone, or in combination with CAPG, interacted with one another (Figure 4A,C). However, the string interaction network from CAPG treatment formed three distinct clusters of nodes indicating a difference in the transcriptional response

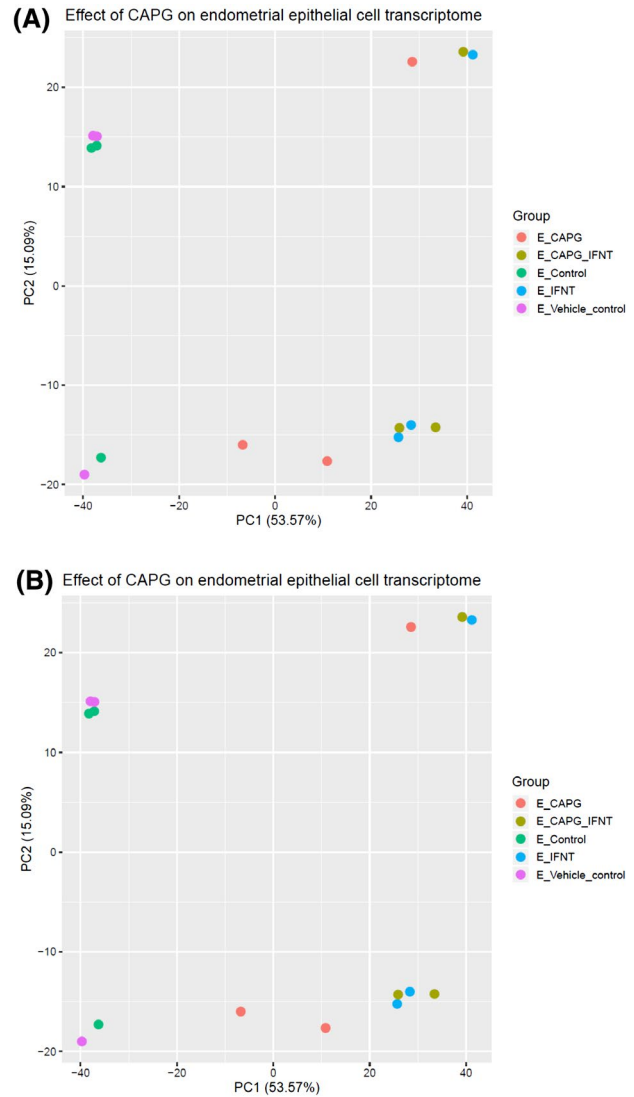


FIGURE 2 Principal component analysis (PCA) plot of overall transcriptional profile following RNA sequencing of (A) bovine endometrial epithelial cells treated with 1000 ng/mL rbCAPG (E_CAPG), 1000 ng/mL rbCAPG + 100 ng/mL roIFNT (E_CAPG_IFNT), media control (E_Control), 1000 ng/mL of roIFNT (E_IFNT), or PBS vehicle control (E_Vehicle_control) for 24 hours or (B) bovine endometrial stromal cells treated with 1000 ng/mL rbCAPG (S_CAPG), 1000 ng/mL rbCAPG + 100 ng/mL roIFNT (S_CAPG_IFNT), media control (S_Control), 1000 ng/mL of roIFNT (S_IFNT), or PBS vehicle control (S_Vehicle_control) for 24 hours

induced by CAPG as compared to IFNT (Figure 4B). String interaction network analysis was further conducted on filtered DEGs following Venn analysis (Figure 3A). The 400 DEGs common among all three treatment groups—CAPG only, IFNT only, and a combination of both—clustered together and did not form any distinct clusters (Figure 4D). However, the 122 DEGs common between CAPG treatment alone and also in combination with IFNT formed two distinct clusters (Figure 4E). String analysis of the 14 DEGs unique to CAPG treatment revealed no interacting nodes.

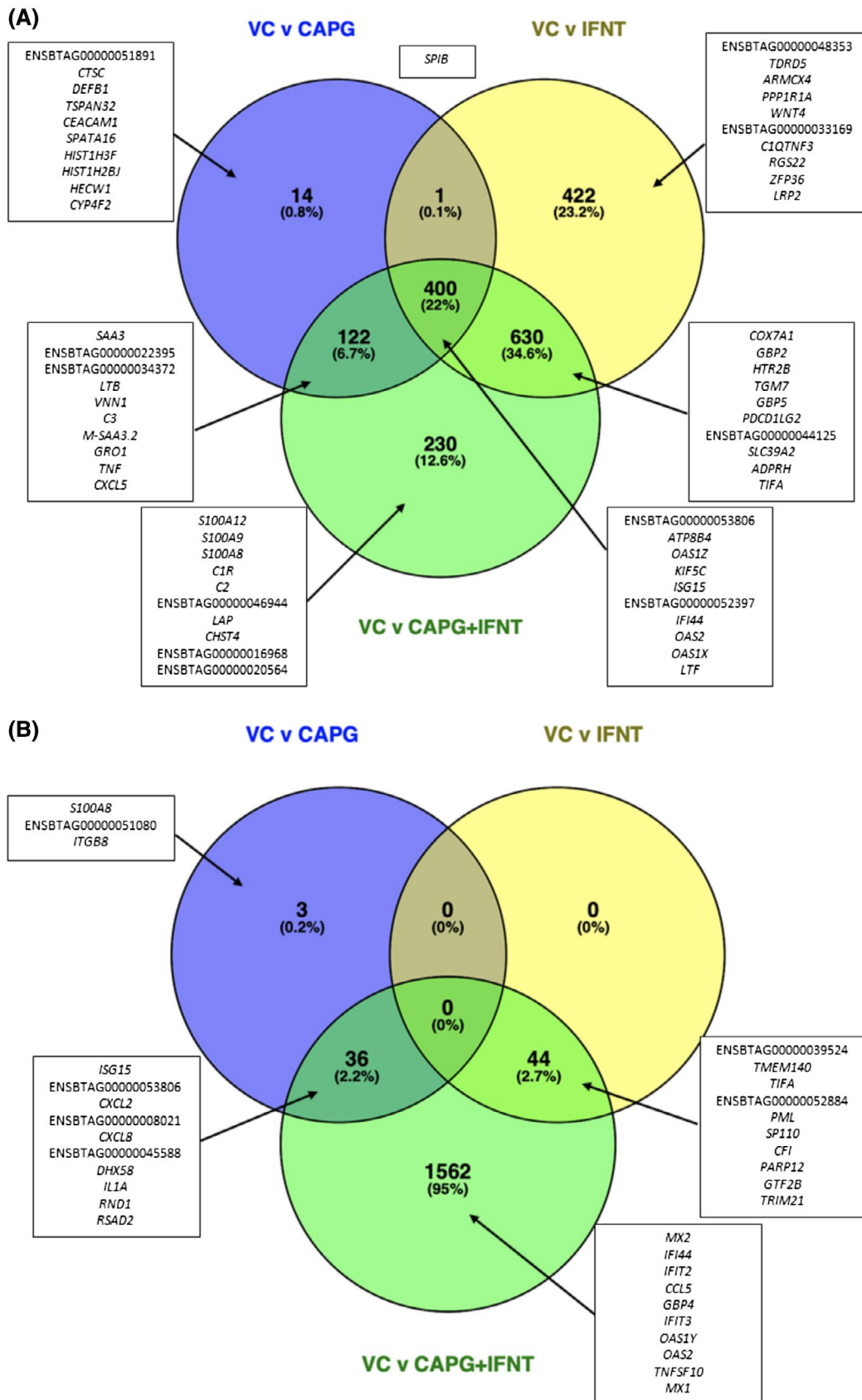


FIGURE 3 Venn diagram of differentially expressed transcripts with designated ensemble transcript identifiers with an adjusted P value of $<.05$ and fold change of greater than two in (A) bovine endometrial epithelial cells, or (B) bovine endometrial stromal cells, treated with recombinant bovine CAPG (1000 ng/mL), recombinant ovine IFNT (1000 ng/mL), or a combination of both. All lists are comparisons between treatment and vehicle control and gene information is presented in Tables S4 and S11. The top 10 up/downregulated transcripts with the greatest fold change are shown in the boxes

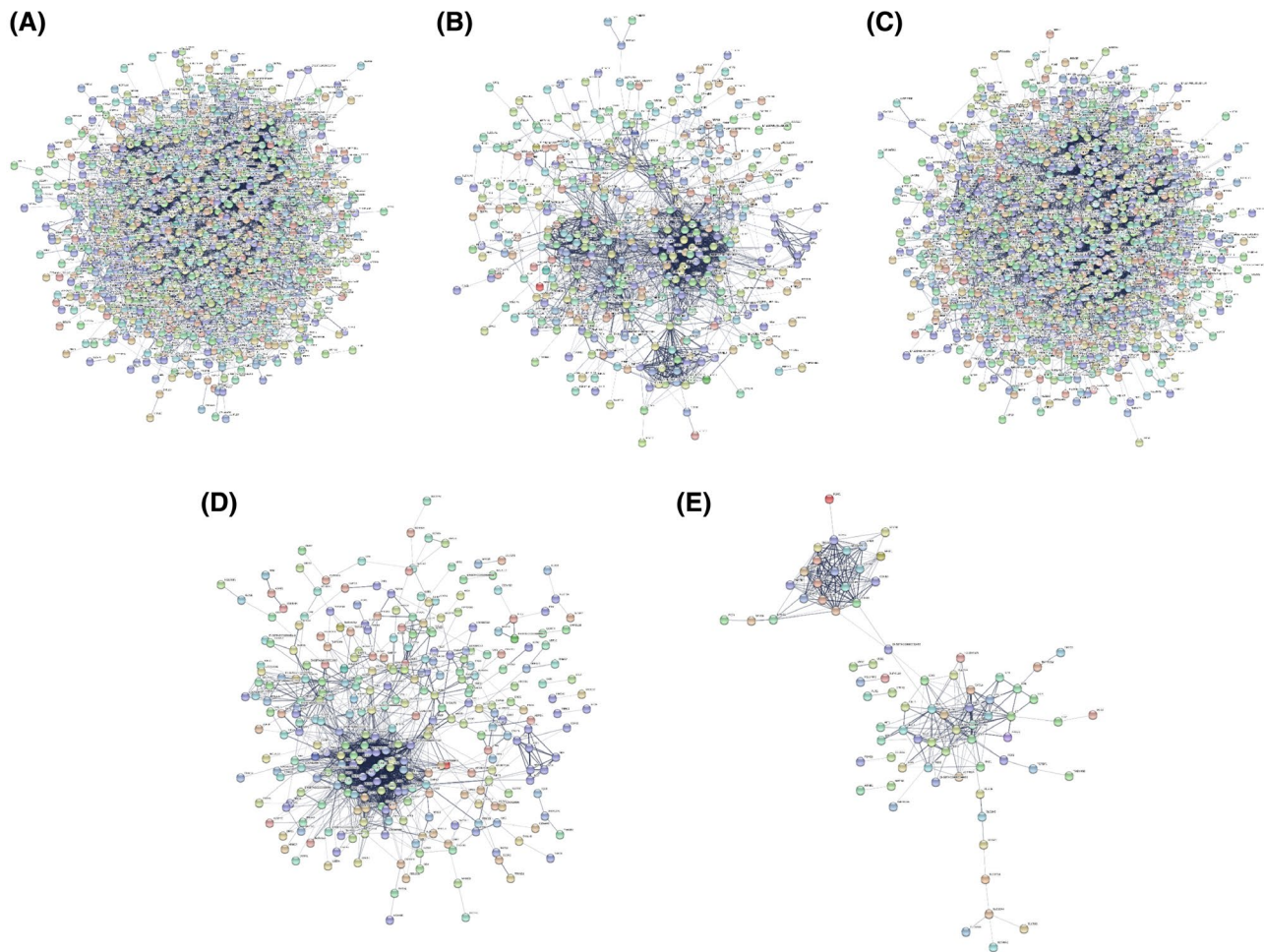


FIGURE 4 String interaction network analysis for those DEGs identified with an adjusted P value of $<.05$ and with a fold change difference of fold change of greater than two following treatment of bovine endometrial epithelial cells for 24 hours with (A) recombinant ovine IFNT (1000 ng/mL) alone, (B) recombinant bovine CAPG alone (1000 ng/mL) alone, or (C) a combination of both for 24 hours. Further string analysis was conducted on DEGs following Venn analysis (Figure 2A), (D) 400 DEGs common between VC vs IFNT, VC vs CAPG, and VC vs CAPG + IFNT and (E) 122 DEGs common between VC vs CAPG and VC vs CAPG + IFNT

3.3 | Specific gene ontologies and pathways were overrepresented following treatment of bovine endometrial epithelial cells with rbCAPG, roIFNT, or a combination of both

Treatment of cells with CAPG for 24 hours resulted in the modification of more transcripts involved in 29 biological processes than one would expect by chance (Table S5). The GO term “response to biotic stimulus” contains 399 genes, 48 of which were altered by CAPG when we would have expected 12.5 DEGs to be changed by chance alone. Similarly, for the GO term “response to cytokine,” 40 DEGs were overrepresented in our analysis from a possible 392 genes. Other GO terms included “innate immune response” (35/273), “inflammatory response” (32/292), “immune effector process” (32/301), “positive regulation of immune system

process” (31/408), “cytokine production” (30/327), “cellular response to oxygen-containing compound” (28/434), “regulation of defence response” (27/256), “regulation of response to external stimulus” (27/325), “regulation of immune response” (26/315), “nucleobase-containing small molecule metabolic process” (26/384), “small molecule biosynthetic process” (25/372), “cell activation” (25/389), “regulation of multi-organism process” (23/179), “drug metabolic process” (23/384), “response to lipid” (22/335), “import into cell” (22/343), “interspecies interaction between organisms” (19/179), “peptide secretion” (19/255), “purine-containing compound metabolic process” (19/300), “adaptive immune response” (17/153), “negative regulation of immune system process” (17/190), “leukocyte migration” (14/160), “response to oxidative stress” (14/191), “I-kappaB kinase/NF-kappaB signalling” (13/118), “antigen processing and presentation” (9/40), “cellular modified amino acid

TABLE 2 Over-represented pathways, that is, those with more differentially expressed transcripts associated with a gene list than one would expect by chance in primary bovine endometrial epithelial cells following treatment with recombinant bovine CAPG (1000 ng/mL) for 24 hours

| Gene set | Pathway | Numbers of genes in set | Number of DEGs in set | Enrichment ratio | P value | False discovery rate |
|----------|---|-------------------------|-----------------------|------------------|----------|----------------------|
| bta04621 | NOD-like receptor signaling pathway | 132 | 22 | 4.559468 | 1.50E-09 | 4.77E-07 |
| bta05169 | Epstein-Barr virus infection | 177 | 24 | 3.709398 | 1.85E-08 | 2.07E-06 |
| bta05164 | Influenza A | 138 | 21 | 4.162992 | 1.95E-08 | 2.07E-06 |
| bta05134 | Legionellosis | 50 | 11 | 6.018498 | 1.28E-06 | 6.99E-05 |
| bta05168 | Herpes simplex infection | 147 | 19 | 3.535914 | 1.30E-06 | 6.99E-05 |
| bta05162 | Measles | 107 | 16 | 4.090738 | 1.31E-06 | 6.99E-05 |
| bta04623 | Cytosolic DNA-sensing pathway | 45 | 10 | 6.079291 | 3.58E-06 | 1.63E-04 |
| bta04064 | NF-kappa B signaling pathway | 81 | 13 | 4.390599 | 6.00E-06 | 2.39E-04 |
| bta04668 | TNF signaling pathway | 100 | 14 | 3.829953 | 1.35E-05 | 4.79E-04 |
| bta04622 | RIG-I-like receptor signaling pathway | 54 | 10 | 5.066075 | 2.02E-05 | 6.43E-04 |
| bta04657 | IL-17 signaling pathway | 78 | 11 | 3.858011 | 1.08E-04 | 0.002881 |
| bta05323 | Rheumatoid arthritis | 78 | 11 | 3.858011 | 1.08E-04 | 0.002881 |
| bta04620 | Toll-like receptor signaling pathway | 81 | 11 | 3.715122 | 1.53E-04 | 0.003764 |
| bta05167 | Kaposi sarcoma-associated herpesvirus infection | 157 | 16 | 2.787955 | 1.76E-04 | 0.004018 |
| bta04060 | Cytokine-cytokine receptor interaction | 227 | 20 | 2.410291 | 2.09E-04 | 0.00425 |
| bta05170 | Human immunodeficiency virus 1 infection | 176 | 17 | 2.642419 | 2.13E-04 | 0.00425 |
| bta04612 | Antigen processing and presentation | 54 | 8 | 4.05286 | 6.82E-04 | 0.012792 |
| bta05144 | Malaria | 45 | 7 | 4.255503 | 0.001097 | 0.019317 |
| bta05160 | Hepatitis C | 102 | 11 | 2.950244 | 0.001151 | 0.019317 |
| bta04062 | Chemokine signaling pathway | 152 | 14 | 2.519706 | 0.001237 | 0.019723 |
| bta04217 | Necroptosis | 120 | 12 | 2.735681 | 0.001353 | 0.020552 |
| bta04625 | C-type lectin receptor signaling pathway | 90 | 10 | 3.039645 | 0.001528 | 0.022149 |
| bta05161 | Hepatitis B | 126 | 12 | 2.60541 | 0.002061 | 0.02859 |
| bta05133 | Pertussis | 67 | 8 | 3.266484 | 0.002845 | 0.037821 |
| bta05322 | Systemic lupus erythematosus | 68 | 8 | 3.218448 | 0.003126 | 0.039886 |
| bta00330 | Arginine and proline metabolism | 41 | 6 | 4.003435 | 0.003425 | 0.040792 |
| bta04145 | Phagosome | 134 | 12 | 2.449863 | 0.003453 | 0.040792 |
| bta04672 | Intestinal immune network for IgA production | 42 | 6 | 3.908115 | 0.003877 | 0.044171 |

metabolic process” (9/93), and “humoral immune response” (8/78).

In contrast, those that were regulated by IFNT treatment alone (Table S6) involved 13 overrepresented biological processes including response to biotic stimulus (71/399), nucleobase-containing small molecule metabolic process (62/384), drug metabolic process (60/384), small molecule biosynthetic process (55/372), response to cytokine (53/392), innate immune response (48/273), purine-containing compound metabolic process (48/300), ribose phosphate metabolic process (45/281), immune effector process (45/301), organophosphate biosynthetic process (42/284), cofactor metabolic process (39/277), regulation of multi-organism process (29/179), and antigen processing and presentation (12/40).

3.4 | Specific pathways had significantly more transcripts modified following treatment of endometrial epithelial cells with rbCAPG

Similar to the overrepresented GO terms, more pathways were overrepresented following treatment with CAPG for 24 hours than were expected by chance (Table 2). These included pathways associated with Epstein-Barr virus infection (24/177), NOD-like receptor signaling pathway (22/132), Influenza A (21/138), Cytokine-cytokine receptor interaction (20/227), Herpes simplex infection (19/147), Human immunodeficiency virus 1 infection (17/176), Kaposi sarcoma-associated herpesvirus infection (16/157), Measles (16/107), Chemokine signaling

pathway (14/152), TNF signaling pathway (14/100), NF-kappa B signaling pathway (13/81), Phagosome (12/134), Hepatitis B (12/126), Necroptosis (12/120), Hepatitis C (11/102), Toll-like receptor signaling pathway (11/81), Rheumatoid arthritis (11/78), IL-17 signaling pathway (11/78), Legionellosis (11/50), C-type lectin receptor signaling pathway (10/90), RIG-I-like receptor signaling pathway (10/54), Cytosolic DNA-sensing pathway (10/45), Systemic lupus erythematosus (8/68), Pertussis (8/67), Antigen processing and presentation (8/54), Malaria (7/45), Arginine and proline metabolism (6/41), and Intestinal immune network for IgA production (6/42).

Cells treated with IFNT for 24 hours had a larger number of transcripts associated with the following pathways than one would expect by chance (Table S7), Metabolic pathways (135/1131), Herpes simplex infection (30/147), Epstein-Barr virus infection (29/177), Influenza A (27/138), NOD-like receptor signaling pathway (26/132), Parkinson disease (24/132), Cytosolic DNA-sensing pathway (12/45), Arginine and proline metabolism (12/41), and Nicotinate and nicotinamide metabolism (10/25).

3.5 | Limited transcriptional response of bovine endometrial stromal cells to rbCAPG following 24 hours of treatment

PCA analysis of stromal cell gene expression data demonstrated less clear separation between controls and treated cells compared with that observed in epithelial cells (Figure 2B). This was reflected in the number of proteins-coding transcripts that were modified with only 38 increased in expression while 1 decreased following treatment with CAPG for 24 hours (Table S8) while treatment with IFNT modified 44 transcripts all of which were increased in expression (Table S9). However, treatment of stromal cells with a combination of CAPG and IFNT modified the expression of 1642 transcripts, of which 1095 increased and 547 decreased in expression (Table S10). Venn diagram analysis (Figure 3B) showed that only three transcripts were modified by CAPG alone (*S100A6*, *ITGB8*, and an uncharacterized transcript) while all those transcripts modified by IFNT were also changed by the combined treatment of CAPG and IFNT (Table S11). Given the limited number of DEGs identified following treatment with CAPG or IFNT alone, no downstream analysis was undertaken.

3.6 | Selected transcripts are modified by rbCAPG in a dose-dependent manner in both human and bovine cells in vitro

RNA sequencing analyses of Ishikawa cells treated with 1000 ng/mL of bovine rbCAPG did not identify any differences in the transcriptional response (data not shown).

However, qRT-PCR analysis of human endometrial epithelial cells exposed to rbCAPG in a dose-dependent manner, resulted in increased expression of *ADD1*, *ADDK*, *CAPZA2*, *CAPZB*, and *CRIP1*, but only at the lower doses of rbCAPG compared to vehicle control ($P < .05$: Figure 5) except for *CAPZB*. In bovine stromal cells, the expression of *MX1*, *MX2*, and *RSAD2* increased in a dose responsive manner but only when treated with rbCAPG in combination with IFNT (Figure 6B) while expression of *ISG15* and *TKT* had a limited response to rbCAPG treatment alone (Figure 6A), whereas the addition of IFNT and rbCAPG enhanced their expression ($P < .05$).

4 | DISCUSSION

Here, we tested the hypothesis that CAPG is highly conserved among placental mammals and facilitates establishment of uterine receptivity and enhances pregnancy recognition in species with different implantation strategies, specifically human and cow. Moreover, we hypothesized that high sequence conservation of this protein would be indicative of a function in early pregnancy/implantation in species with different implantation strategies. We show for the first time that the CAPG protein-coding sequence is highly conserved among the 14 placental mammals investigated. Treatment with a recombinant form of bovine CAPG (produced specifically for this study) modifies the transcriptome of both human and bovine endometrial cells in a species- and cell-specific manner in vitro. In cattle, CAPG not only modifies the transcriptional response when present alone, but also works synergistically with IFNT to enhance the pregnancy recognition process. Addition of recombinant bovine CAPG to human endometrial epithelial cells can also elicit a change in selected transcripts with which it is known to interact in other nonreproductive/implantation physiological systems. We propose that because CAPG has a high degree of sequence homology between cattle and humans, it plays an important role in modifying the endometrium in these species which exhibit markedly different implantation strategies.

4.1 | CAPG protein sequence is highly conserved among eutherian mammals

CAPG is a member of the gelsolin family of proteins all of which are comprised of gelsolin-like repeats in their protein sequences, of which CAPG has three.³³ It is a calcium sensitive protein that reversibly blocks the barbed ends of actin filaments and is predominantly expressed in the cytoplasm of macrophages.³⁴ CAPG null mice appear to be fertile, but have impaired motility function in macrophages derived from bone

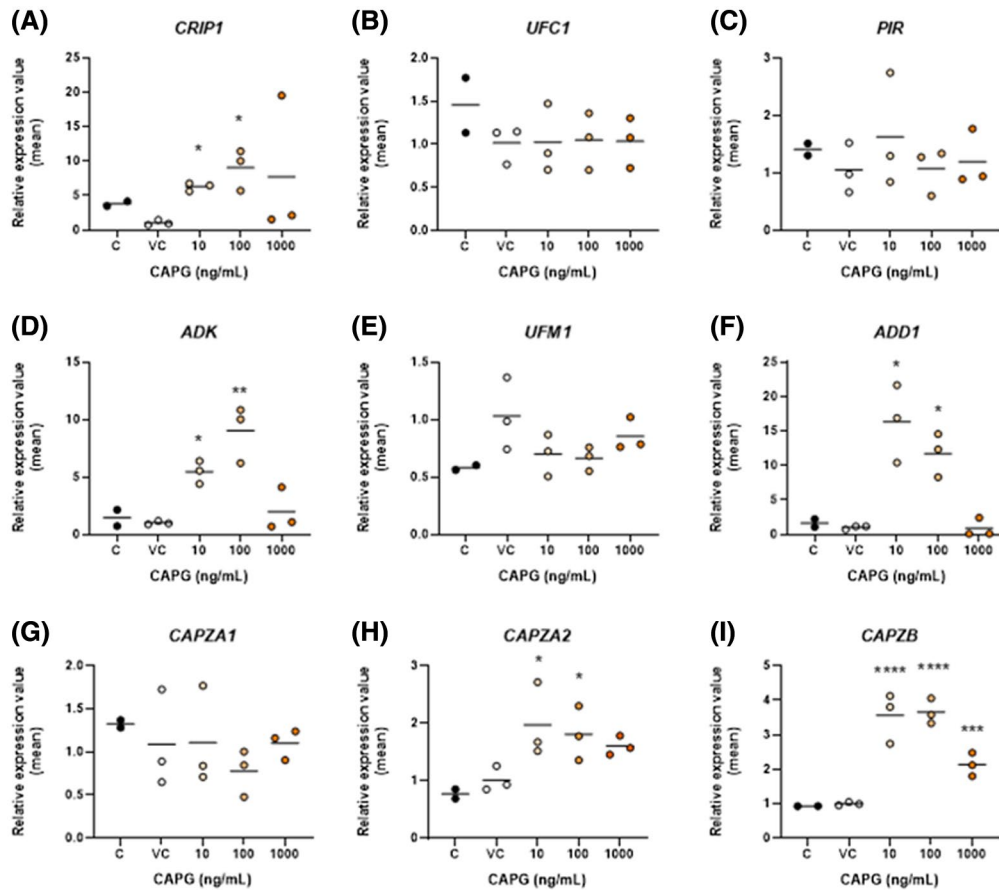


FIGURE 5 Analysis of expression values by qRT-PCR for selected transcripts in human endometrial epithelial cells (Ishikawa cells; $n = 3$ biological replicates) for (A) *CRIP1*, (B) *UFC1*, (C) *PIR*, (D) *ADK*, (E) *UFM1*, (F) *ADD1*, (G) *CAPZA1*, (H) *CAPZA2*, and (I) *CAPZB*. Cells were treated for 24 hours with (i) control (black circles), (ii) vehicle control (open circles), (iii) 10 ng/mL, (iv) 100 ng/mL, or (v) 1000 ng/mL (orange circles) recombinant bovine CAPG. Expression values with bar representing mean (calculated with $2^{-\Delta\Delta Ct}$ method using *ACTB*, *GADPH*, and *PPIA* as normalizer transcripts). Significant differences in expression values were calculated by ANOVA analysis using Prism, where samples were compared with a vehicle control of PBS (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$)

marrow.³⁵ There is evidence for a duplication event within the gelsolin family of proteins leading to some members of the family having six gelsolin subunits rather than the three that are present in CAPG. It is thought that the family underwent this duplication event in the vertebrate lineage which may explain the sequence similarity across members of the family.³⁶ While CAPG null mice are fertile, Campbell et al³⁷ demonstrated that knockout of a close family member (flightless 1: *Fliih*) permitted embryo development but resulted in subsequent postimplantation loss. Interestingly, if the human form of CAPG was introduced, not only were murine cells capable of producing the human form, but it also abrogated the previously reported postimplantation embryo loss.³⁷ These data suggest that members of this family have a high degree of sequence similarity and family members may play a role in compensatory mechanisms in certain knockout mouse models. Moreover, the data presented here demonstrate that CAPG—with its high level of sequence conservation across species—may play a similar role across eutherian mammals with different implantation strategies.

4.2 | CAPG induces a transcriptional response in bovine endometrial epithelial cells that is distinct from that induced by IFNT and may facilitate the pregnancy recognition process

Our data not only support the hypothesis that CAPG facilitates pregnancy recognition by working in synergy with IFNT, but also by modifying two main clusters of interacting transcripts in its own right. Previous studies have demonstrated that there are conceptus-induced changes in the bovine endometrium that occur coordinate with IFNT production but are independent of the actions of IFNT exposure alone. For example, production of prostaglandins by the elongating conceptus primes the endometrium prior to the actions of IFNT.³⁸ Other studies have shown that there are early IFNT-independent changes to the endometrium,^{39,40} as well as during the pregnancy recognition period.³ Of these transcripts that were identified as conceptus-specific but independent of IFNT, we

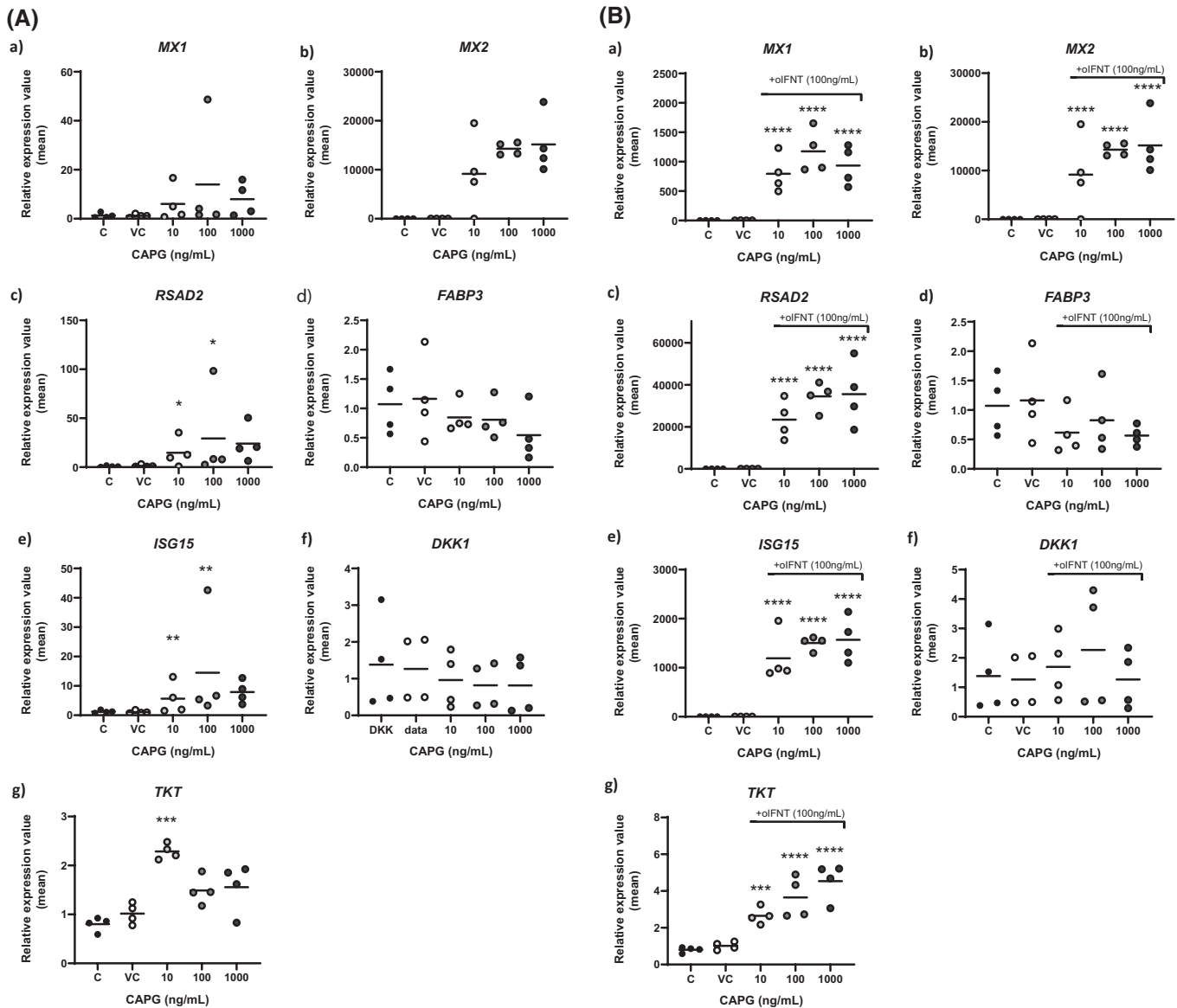


FIGURE 6 Analysis of expression values by qRT-PCR for selected transcripts in primary bovine endometrial stromal cells (late luteal phase: $n = 4$ biological replicates) for (a) *MX1*, (b) *MX2*, (c) *RSAD2*, (d) *FABP3*, (e) *ISG15*, (f) *DKK1*, (g) *TKT*, in cells treated for 24 hours with (A) (i) control (black circles), (ii) vehicle control (open circles), (iii) 10 ng/mL, (iv) 100 ng/mL, or (v) 1000 ng/mL recombinant bovine CAPG (blue circles). (B) (i) control (black circles), (ii) vehicle control (open circles), (iii) 10 ng/mL, (iv) 100 ng/mL, or (v) 1000 ng/mL recombinant bovine CAPG and recombinant bovine IFNT (100 ng/mL) (blue circles). Expression values with bar representing mean (calculated with $2^{-\Delta\Delta Ct}$ method using *ACTB* and *GADPH* as normalizer transcripts). Significant differences in expression values were calculated by ANOVA analysis using Prism, where samples were compared with a vehicle control of PBS (* $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$)

have demonstrated that five are modified by CAPG. The discrepancy may be due to the actions of additional conceptus-derived proteins. Forde *et al* (2015) identified over 1000 proteins in day 16 bovine conceptus-conditioned medium and 85 proteins present in the uterine luminal fluid in only pregnant animals on day 16, whereby CAPG was just 1 of these proteins identified.¹³

Treatment of bovine stromal cells with CAPG revealed few differences in gene expression. This may reflect the in

vivo scenario in which conceptus-derived products first come into contact with the endometrial epithelium and it is possible that any actions of CAPG that may occur in vivo may be indirect via actions on epithelial cells which may produce factors that can themselves act on stromal cells. Similar to the mechanism of the action of stromal-derived growth factors such as HGF which act on the epithelial cells.⁴¹ These distinct effects on epithelial vs stromal cells reflect the different roles these cells play in facilitating uterine receptivity to implantation.

4.3 | High levels of sequence identity between bovine and human CAPG—Bovine CAPG can elicit a response in human endometrial epithelial cells but only at low concentrations in vitro

RNA sequencing showed no effect of bovine CAPG on human endometrial epithelial cells. However, sequencing was only carried out on those cells exposed to 1000 ng/mL of CAPG alongside appropriate controls. Selected transcripts known to interact with CAPG were identified from string interaction networks; qRT-PCR demonstrated a dose response of human cells to recombinant bovine CAPG but only at the lower concentrations, that is, 10 and 100 ng/mL (Figure 5), except for the transcript *CAPZAI*. The transcripts that were altered by CAPG in human endometrial epithelial cells are those that have been previously identified as interacting with CAPG in different systems in humans, thus implicating CAPG in modifying transcripts in the human endometrium. The lack of DEGs in human cells treated with the highest concentration of rbCAPG may simply reflect a dose-specific mechanism of action in humans. Indeed, no data are available to date that demonstrate the concentration of CAPG present in the uterine lumen in humans. CAPG is expressed in the human embryo⁴² as well as in the embryos of other species including bovine⁴³ and porcine⁴⁴ further implicating CAPG in conceptus-maternal interactions in a range of mammals.

In conclusion, we have demonstrated that CAPG exhibits a high degree of sequence conservation among eutherian mammals, and modifies the expression of mRNA transcripts in endometrial epithelial cells in a species-specific manner. In cattle, CAPG modified the expression of transcripts known to be important for pregnancy recognition and implantation, which are also altered by IFNT. Treatment of CAPG in combination with IFNT enhanced the transcriptional response, while CAPG alone elicited a unique transcriptional signature. In humans, it modifies specific interaction partners but only at lower concentrations in vitro. We propose that CAPG plays a facilitative role in establishing uterine receptivity to implantation and contributes to early pregnancy success across species with different early implantation strategies.

ACKNOWLEDGMENTS

We would like to Dr Fuller Bazer from Texas A&M for the kind gift of recombinant ovine IFNT. We acknowledge Dr James Cronin from the University of Swansea for guidance with the bovine cell isolation method. We would like to acknowledge the assistance of Stefania Mountevadi in helping with the bovine cell isolation and culture. We would like to thank Dr Ian Carr, Morag Raynor, and Ummey Hany from the University of Leeds's next generation sequencing facility core for undertaking sequencing analyses. This work was

supported by N8 agri-food pump priming, QR GCRF, as well as BBSRC grant number BB/R017522/1.

CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

N. Forde, M. O'Connell, A. Valazquez, and G. Oikonomou designed the experiments; H. Tinning, A. Taylor, R. Sutton, B. Constantinides, P. Thompson, undertook experimental work; N. Forde, M. O'Connell, H. Tinning, analyzed the data; A. Treuman and P. Thompson generated novel protein; N. Forde and H. Tinning wrote the first draft of the manuscript. All authors contributed to revising, reading, critiquing the manuscript.

REFERENCES

1. Macklon NS, Brosens JJ. The human endometrium as a sensor of embryo quality1. *Biol Reprod.* 2014;91:98.
2. Lonergan P, Forde N. Maternal-embryo interaction leading up to the initiation of implantation of pregnancy in cattle. *Animal.* 2014;8:64-69.
3. Bauersachs S, Ulbrich SE, Reichenbach H-D, et al. Comparison of the effects of early pregnancy with human interferon, alpha 2 (IFNA2), on gene expression in bovine endometrium1. *Biol Reprod.* 2012;86:1-15.
4. Forde N, Beltman ME, Duffy GB, et al. Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. *Biol Reprod.* 2011;84:266-278.
5. Forde N, Simintiras CA, Sturmey R, et al. Amino acids in the uterine luminal fluid reflects the temporal changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle. *PLoS One.* 2014;9:e100010.
6. Mullen MP, Elia G, Hilliard M, et al. Proteomic characterization of histotroph during the preimplantation phase of the estrous cycle in cattle. *J Proteome Res.* 2012;11:3004-3018.
7. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *J Clin Endocrinol Metab.* 2002;87:2954-2959.
8. Carter AM, Enders AC, Pijnenborg R. The role of invasive trophoblast in implantation and placentation of primates. *Philos Trans R Soc B Biol Sci.* 2015;370:20140070.
9. Guillomot M, Fléchon J-E, Wintenberger-Torres S. Conceptus attachment in the Ewe: an ultrastructural study. *Placenta.* 1981;2:169-181.
10. Forde N, Carter F, Fair T, et al. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle1. *Biol Reprod.* 2009;81:784-794.
11. Kimmins S, Maclaren LA. Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. *Placenta.* 2001;22:742-748.
12. Okumu LA, Forde N, Fahey AG, et al. The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus. *Reproduction.* 2010;140:143-153.

13. Forde N, Bazer FW, Spencer TE, Lonergan P. "Conceptualizing" the endometrium: identification of conceptus-derived proteins during early pregnancy in cattle. *Biol Reprod.* 2015;92:1-13.
14. Masters RA, Roberts RM, Lewis GS, Thatcher WW, Bazer FW, Godkin JD. High molecular weight glycoproteins released by expanding, pre-attachment sheep, pig and cow blastocysts in culture. *Reproduction.* 1982;66:571-583.
15. Glaser J, Neumann MHD, Mei Q, et al. Macrophage capping protein CapG is a putative oncogene involved in migration and invasiveness in ovarian carcinoma. *Biomed Res Int.* 2014;2014:1-8.
16. Martinez-Garcia E, Lesur A, Devis L, et al. Targeted proteomics identifies proteomic signatures in liquid biopsies of the endometrium to diagnose endometrial cancer and assist in the prediction of the optimal surgical treatment. *Clin Cancer Res.* 2017;23:6458-6467.
17. Tarver JE, dos Reis M, Mirarab S, et al. The interrelationships of placental mammals and the limits of phylogenetic inference. *Genome Biol Evol.* 2016;8:330-344.
18. Yates A, Akanni W, Amode MR, et al. Ensembl 2016. *Nucleic Acids Res.* 2016;44:D710-D716.
19. Katoh K. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 2002;30:3059-3066.
20. Gouy M, Guindon S, Gascuel O. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol.* 2010;27:221-224.
21. Sievers F, Wilm A, Dineen D, et al. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Mol Syst Biol.* 2011;7:539.
22. Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM. Toll-like receptor 4 and MYD88-dependent signaling mechanisms of the innate immune system are essential for the response to Lipopolysaccharide by epithelial and stromal cells of the bovine endometrium. *Biol Reprod.* 2012;86:51.
23. Ireland JJ, Murphee RL, Coulson PB. Accuracy of predicting stages of bovine estrous cycle by gross appearance of the corpus luteum. *J Dairy Sci.* 1980;63:155-160.
24. Martin M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* 2011;17:10.
25. Cunningham F, Achuthan P, Akanni W, et al. Ensembl 2019. *Nucleic Acids Res.* 2019;47:D745-D751.
26. Frankish A, Diekhans M, Ferreira A-M, et al. GENCODE reference annotation for the human and mouse genomes. *Nucleic Acids Res.* 2019;47:D766-D773.
27. Liao Y, Smyth GK, Shi W. The R package Rsubread is easier, faster, cheaper and better for alignment and quantification of RNA sequencing reads. *Nucleic Acids Res.* 2019;47:e47-e47.
28. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 2014;15:550.
29. Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Res.* 2019;47:W199-W205.
30. Szklarczyk D, Gable AL, Lyon D, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res.* 2019;47:D607-D613.
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods.* 2001;25:402-408.
32. Fernández MH, Vrba ES. A complete estimate of the phylogenetic relationships in Ruminantia: a dated species-level supertree of the extant ruminants. *Biol Rev.* 2005;80:269-302.
33. Archer SK, Behm CA, Claudianos C, Campbell HD. The flightless I protein and the gelsolin family in nuclear hormone receptor-mediated signalling. *Biochem Soc Trans.* 2004;32:940-942.
34. Dabiri GA, Young CL, Rosenbloom J, Southwick FS. Molecular cloning of human macrophage capping protein cDNA. A unique member of the gelsolin/villin family expressed primarily in macrophages. *J Biol Chem.* 1992;267:16545-16552.
35. Witke W, Li W, Kwiatkowski DJ, Southwick FS. Comparisons of CapG and gelsolin-null macrophages. *J Cell Biol.* 2001;154:775-784.
36. Kwiatkowski DJ. Functions of gelsolin: motility, signaling, apoptosis, cancer. *Curr Opin Cell Biol.* 1999;11:103-108.
37. Campbell HD, Fountain S, McLennan IS, et al. Fliih, a gelsolin-related cytoskeletal regulator essential for early mammalian embryonic development. *Mol Cell Biol.* 2002;22:3518-3526.
38. Spencer TE, Forde N, Dorniak P, Hansen TR, Romero JJ, Lonergan P. Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. *Reproduction.* 2013;146:377-387.
39. Forde N, Carter F, Spencer TE, et al. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? *Biol Reprod.* 2011;85:144-156.
40. Mathew DJ, Sánchez JM, Passaro C, et al. Interferon tau-dependent and independent effects of the bovine conceptus on the endometrial transcriptome. *Biol Reprod.* 2019;100:365-380.
41. Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu G. Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reprod Biol.* 2008;8:179-211.
42. Stirparo GG, Boroviak T, Guo G, Nichols J, Smith A, Bertone P. Integrated analysis of single-cell embryo data yields a unified transcriptome signature for the human pre-implantation epiblast. *Development.* 2018;145:dev158501.
43. Nakamura K, Kusama K, Bai R, et al. Induction of IFNT-stimulated genes by conceptus-derived exosomes during the attachment period. *PLoS One.* 2016;11:1-16.
44. Whitworth KM, Zhao J, Spate LD, Li R, Prather RS. Scriptaid corrects gene expression of a few aberrantly reprogrammed transcripts in nuclear transfer pig blastocyst stage embryos. *Cell Reprogram.* 2011;13:191-204.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Tinning H, Taylor A, Wang D, et al. The role of CAPG in molecular communication between the embryo and the uterine endometrium: Is its function conserved in species with different implantation strategies?. *The FASEB Journal.* 2020;00:1-15. <https://doi.org/10.1096/fj.202000882RR>