Hormone-responsive organoids from domestic mare and endangered Przewalski's horse endometrium

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Abstract

The endometrium, the inner uterine lining, is composed of cell layers that come in direct contact with an embryo during early pregnancy and later with the fetal placenta. The endometrium is responsible for signals associated with normal reproductive cyclicity as well as maintenance of pregnancy. In the mare, functionally competent *in vitro* models of the endometrium have not been successful. Furthermore, the ability to study various reproductive processes *in vitro* may allow critical evaluation of signaling pathways involved in the reproductive diseases of animals that cannot be handled frequently, such as various wildlife species. Here we report the establishment of organoids, 3D structures, derived from fresh and frozen-thawed equine endometrium (*Equus ferus caballus* and *E. f. przewalskii*). Although organoids from domestic mares responded to exogenous hormonal stimuli, organoids from Przewalski's horse failed to respond to exogenous hormones. The present study represents a 'first' for any large animal model or endangered species. These physiologically functional organoids may facilitate improved understanding of normal reproductive mechanisms, uterine pathologies, and signaling mechanisms between the conceptus and endometrium and may lead to the development of novel bioassays for drug discovery.

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Introduction

The endometrium of the equine uterus responds to hormonal stimulation throughout the estrous cycle by increasing progesterone and estrogen receptors during estrus (Hartt *et al.* 2005), stimulating proliferation of ciliated epithelial cells during diestrus, decreasing secretory cells during diestrus (Samuel *et al.* 1979), and by secreting prostaglandin $F_{2\alpha}$ in response to oxytocin during luteolysis (Sharp *et al.* 1997, Starbuck *et al.* 1998, Stout *et al.* 2000, Santos *et al.* 2015, Annandale *et al.* 2018). Endometrial interactions with the embryo and with the placenta later in pregnancy are critical to produce live offspring. Development of an *in vitro* model system that mimics these physiological functions may facilitate studies focused on evaluation of normal physiological processes, signaling events during early pregnancy, and disease states that affect reproductive success (Collins *et al.* 2020). Previous attempts to develop an *in vitro* model for equine endometrium focused on monolayer cell cultures that are 2D and,

thus, not normal conformationally, as the endometrium is a 3D structure (Szóstek *et al.* 2012a). Likewise, endometrial explant cultures were unable to support long-term growth *in vitro* (Schwinghamer *et al.* 2018). Recently, a co-culture system with equine endometrial epithelial and stromal cells utilizing welled plates with semipermeable membrane inserts was described, but there was poor epithelial cell differentiation and exogenous hormones failed to induce morphological changes or alter hormone receptor expression (Lapko *et al.* 2017). Therefore, other novel approaches are warranted to establish and maintain more structurally and physiologically normal endometrial cells *in vitro*.

Organoids represent 3D *in vitro* culture systems derived from stem or differentiated cells that can be cultured long-term and self-organize to mimic the structure and function of their tissue of origin (Clevers 2016). Typically, organoids are generated by embedding cells in an extracellular matrix, like Matrigel, and culturing them in media supplemented with various growth factors (Clevers 2016). Tissue-derived organoids

have been established for many organs, including the intestines (Sato *et al.* 2011), liver (Huch *et al.* 2013), prostate (Karthaus *et al.* 2014), brain (Lancaster *et al.* 2013), and oviduct (Kessler *et al.* 2015, Xie *et al.* 2018). Endometrial organoids have been reported in the mouse and human using fresh endometrial tissues, which grow long-term (Boretto *et al.* 2017, Turco *et al.* 2017, Fitzgerald *et al.* 2019). We have therefore adapted this organoid methodology to derive endometrial organoids for the horse (domestic and non-domestic) to generate a much needed, physiologically relevant culture system that would allow the study of molecular mechanisms involved in normal physiologic processes of the equine endometrium, such as maternal recognition of pregnancy, and endometrial diseases (Collins *et al.* 2020). By evaluating these processes *in vitro* rather than *in vivo*, experiments can be better controlled, reduce dependence on live animals, and be performed throughout the year (using cryopreserved endometrial tissue) (Thompson *et al.* 2019). In this report, we describe the generation of hormonally-responsive organoids from fresh and cryopreserved endometrial explants derived from domestic (*Equus ferus caballus*) and Przewalski's (*E. f. przewalskii*) mares. The organoids retained their ability to maintain 3D tissue structure and responded to exogenous hormonal stimulation, providing a novel *in vitro* model for the endometrium in equids.

Materials and methods

Animals

All animals were sampled using approved animal research protocols. The research herein described was reviewed and approved by the Smithsonian Conservation Biology Institute's Animal Care and Use Committee (Approval #18-11; Przewalski's mares), Auburn University, and University of Kentucky Animal Care and Use Committees (domestic mares). Experiments described were performed in accordance with relevant guidelines and regulations. Domestic horse mares were housed in outdoor paddocks with free access to grass or hay and *ad libitum* water. Mares were monitored daily by staff for medical concerns and treated appropriately as necessary.

Collection of endometrial biopsies

Endometrial biopsies were collected from adult domestic ($n=11$) and Przewalski's mares ($n=3$). Only mares with a Kenney–Doig score of I or IIa (Kenney & Doig 1986) for endometrial biopsies, which controls for level of endometrial inflammation and fibrosis, were used in this study. Mares were evaluated for stage of the estrous cycle by transrectal ultrasound every other day, and endometrial biopsies were collected randomly from the body of the uterus during estrus (≥ 35 mm follicle diameter, no corpus luteum, and uterine edema). Przewalski's mares were confined to a padded hydraulic restraint device (Fauna Research Inc., Red Hook, NY, USA) and sedated with 15 mg detomidine hydrochloride

IM (Dormosedan, Zoetis, Parsippany, NJ, USA) and 50 mg butorphanol tartrate IM (Torbugesic, Zoetis) (Collins *et al.* 2012). Two endometrial biopsies were collected using aseptic techniques (Kenney & Doig 1986) from each mare. Domestic mare biopsies were shipped in sterile saline overnight on ice to the Smithsonian Conservation Biology Institute (SCBI) for processing. Biopsies from Przewalski's mares were placed in sterile saline and processed within 2 h of biopsy collection.

Tissue processing

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Chemicals, and all images were captured on an EVOS FL Auto 2 Imaging System.

Biopsies were dissected on ice with a sterile scalpel blade into 1–2 mm³ pieces in Handling Medium (HM; MEM with Earle's salts, 25 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mM pyruvate, 2 mM glutamax, 5% BSA) (Thompson *et al.* 2019). Two small pieces were immediately fixed in 4% paraformaldehyde (Thermo Fisher Scientific) for histology and immunohistochemistry. Half of the remaining tissue pieces were cryopreserved, and the remaining tissue pieces were enzymatically dissociated for use in the culture system. Thus, the biopsies from each mare were used in both groups: organoids derived from fresh tissue and organoids derived from frozen–thawed tissue.

Organoid culture

Conditions for the generation of organoids were adapted from those described for human endometrial organoids (Boretto *et al.* 2017, Turco *et al.* 2017). Briefly, tissue pieces were incubated in collagenase V (0.4 mg/mL)/dispase II (1.25 U/mL) in RPMI-1640 medium for 20 min on a shaking warm plate at 37°C. The tissue suspension was assessed every 10 min using an inverted microscope to confirm the presence of partially dissociated glands. The digest was then diluted in RPMI-1640 containing 20% fetal bovine serum to stop enzyme action. The solution was then passed through a 100- μ m cell strainer and then backwashed with RPMI-1640. The resulting suspension was centrifuged for 10 min at 1000 *g* at room temperature and the supernatant removed. The pellet was resuspended in 1 mL DMEM/F12 and centrifuged. The supernatant was discarded and the pellet suspended in 20X (v/v) phenol red free, growth factor reduced Matrigel (Corning). The Matrigel solution was plated in 25 μ L drops in a 48-well plate (Corning Costar TC-Treated Multiple Well Plates). The drops were allowed to solidify for 30 min at 37°C. Then, 250 μ L organoid medium was added to each well (OM; DMEM/F12 without phenol red, 1% penicillin/streptomycin, 2.5 mM L-glutamine, 2% B27 Plus (Thermo Fisher), 1% N2 (Thermo Fisher), 1% ITS, 1 mM nicotinamide, 50 ng/mL recombinant human EGF (R&D Systems), 50 ng/mL recombinant human FGF-10 (PeproTech; Rocky Hill, NJ, USA), 100 ng/mL recombinant human Noggin (R&D systems), 0.5 μ M TGF β /Alk inhibitor A83-01 (Tocris; Minneapolis, MN, USA), 1.25 mM N-acetyl-L-cysteine (Millipore Sigma), 10 μ M SB202190). The culture dishes were placed in an incubator at 37°C and 5% CO₂ in air. Half of the culture medium was removed and replaced every other day.

The organoid cultures were passaged every 6 days. To passage, the Matrigel containing the organoids was scraped from the culture plate, placed in microcentrifuge tubes, and centrifuged at 1000 *g* for 10 min. The supernatant was removed, and the pellet was resuspended in DMEM/F12. The organoids were further dissociated by repeated pipetting (~800x), washed twice in DMEM/F12, and replated as previously described following resuspension in Matrigel.

After the second passage (18 days from initial isolation of endometrial glands), exogenous hormones were added to the culture medium. Briefly, there were six treatment groups: no hormones added to culture medium (control, C) for 6 days, 1 μ M progesterone for 6 days (P_4), 10 nM estradiol-17 β for 6 days (E_2), 10 nM estradiol-17 β for 2 days followed by one μ M progesterone for 4 days (E_2/P_4), control medium for 5 days then 10^{-5} M oxytocin for 24 h (C/OT-5), and control medium for 5 days then 10^{-6} M oxytocin for 24 h (C/OT-6). All experiments were repeated eight times for each of the hormonal treatments per mare. Organoids were removed from culture after hormonal treatments and processed for subsequent analyses (see histology, gene expression, immunohistochemistry, and transmission electron microscopy).

Organoid generation from single cells

Endometrial glands were dissociated as previously described, and then the cell suspension was filtered through a 40 μ m cell strainer to ensure only single cells were obtained. Isolated cell suspension was serially diluted to obtain 5.2 cells/ μ L. Then cells were plated (26 cells per 5 μ L droplet of Matrigel with three droplets per well; 2500 total single cells in 32 wells), overlaid with 250 μ L of OM, and cultured for 14 days as described previously.

Tissue cryopreservation and thawing

Cryopreservation was performed as previously described (Thompson *et al.* 2019). Briefly, explants were cryopreserved in 10% (v/v) DMSO in MEM with Earle's salts containing 25 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mM pyruvate, 2 mM Glutamax, and 20% (v/v) fetal bovine serum. Three cryovials per individual mare were used. Nine to ten pieces of tissue were placed in each cryovial containing 0.5 mL of the freezing media. The cryovials were placed in a -80°C freezer in Cool Cell Freezing Containers (Biocision; Larkspur, CA, USA; temperature decrease $\sim 1^\circ\text{C}$ per minute) overnight. On the second day, the cryovials were plunged into liquid nitrogen and stored until thawing.

To thaw, cryovials were removed from liquid nitrogen and thawed in room temperature air (22°C) for one minute and then immersed in a 25°C water bath for one minute (Pukazhenthi *et al.* 2015, Thompson *et al.* 2019). Then 1 mL of thawing medium (TM; MEM with Earle's salts containing 25 mM HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 0.1 mM pyruvate, 2 mM glutamax, 20% fetal bovine serum) was added at room temperature and mixed gently for 1 min. The contents of the vial were then poured into a petri dish containing 5 mL TM to dilute the DMSO and incubated for 5 min. This step was repeated twice more, and tissue pieces then

were enzymatically digested for organoid culture, as described above.

Histology

Organoids were soaked in Cell Recovery Solution (Corning) and washed with DMEM/F12 via centrifugation (600 *g* for 6 min). Organoid pellets were fixed in 4% paraformaldehyde for 30 min, then embedded in 2% agarose gel (Bio-Rad). The agarose-embedded organoids were stored in 70% ethanol until processing. The organoids were embedded in paraffin wax, sectioned (6 μ m), and stained with hematoxylin and eosin stain using standard procedures.

Gene expression

Organoid pellets were formed as for histology preparation above but were flash frozen in liquid nitrogen and stored at -20°C until processing, rather than fixed. Qiagen RNeasy Micro Kit (Qiagen Sciences Inc.) was used to extract total RNA and analyzed for quantity and quality (260/280 and 260/230 ratios) using a NanoDrop spectrophotometer (Thermo Scientific). Oligo(dT) primers (0.5 μ g) for mRNA and Superscript III reverse-transcriptase were used to synthesize cDNA with SuperScript III First-Strand Synthesis System (Invitrogen). CDNA was reverse transcribed with 500 ng total RNA for use in prostaglandin-endoperoxide synthase 2 (*PTGS2*), prostaglandin E synthase (*PGES*), and oxytocin receptor (*OXTR*) reactions and 200 ng total RNA for the remaining reactions. Quantitative real time PCR (qPCR; Light Cycler 96, Roche) was then utilized to assess mRNA expression with FastStart Essential DNA Green Master kit (Roche). Two microliters cDNA, 1 μ L forward primer (0.5 μ M), 1 μ L reverse primer (0.5 μ M), 6 μ L water, and 10 μ L DNA Green Master were used in each PCR reaction, and all reactions were performed in duplicate. PCR conditions included 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 10 s. Specificity of amplified products was assessed by examining the melting curve for each transcript. Specifically, we assessed *MKI67* for cell proliferation, estrogen receptor- α (*ESR1*), progesterone receptor (*PGR*), E-cadherin (*CDH1*) for cell adhesion, *PTGS2*, *PGES*, and *OXTR*. The reference gene *CAPDH* was used to normalize gene expression using the $2^{-\Delta\Delta\text{CT}}$ method (Livak & Schmittgen 2001). Primers used for gene expression analysis are summarized in Table 1.

Immunohistochemistry

Tissue sections previously embedded in paraffin were mounted on Superfrost Plus slides. Sections were incubated in a series of Slide Brite (Thermo Fisher) washes to remove paraffin and then rehydrated by immersing the slides in a series of ethanol washes. Antigen retrieval was accomplished by incubating the slides in 10 mM sodium citrate buffer at 96°C for 20 min. Then sections were incubated in blocking serum (RTU Vectastain kit; Vector Laboratories; Burlingame, CA, USA) for 20 min. Primary antibodies were diluted in 0.1% Tween 20, 1% BSA, and PBS (Table 2). For negative control, sections were incubated with 0.1% Tween 20, 1% BSA, and PBS alone. All slides were

Table 1 Primer sequences used in gene expression analysis.

Primers	Reference	Sequences		bp
		Forward	Reverse	
CDH1	Thompson <i>et al.</i> (2019)	GAGTTTTGAAGGATGCACTG	TCATCTCCGGATTATGAAGC	125
MKI67	Thompson <i>et al.</i> (2019)	TCCTTGTGCAAGAGTGGCTT	CCCCGCTCCTTTTGATGGTA	136
ESR1	Thompson <i>et al.</i> (2019)	CGAGGCTTCCATGATGGGTT	AGGATCTCTAGCCAGGCACA	147
PGR	Perrini <i>et al.</i> (2016)	GTCAGTGGACAGATGCTGTA	CGCCTTGATGAGCTCTCTAA	255
PTGS2	Szóstek <i>et al.</i> (2012b)	GCCACGATTGGCTGCGGGA	CCTGCTCGTCTGGAACAAGCGT	96
PGES	Szóstek <i>et al.</i> (2012b)	CACGCTGCTGGTCATCAAGA	CTCTCAGGCAACGCTCCAC	152
OXTR	Rebordão <i>et al.</i> (2017)	TGGACGCCATTCTTCTCGT	GCCCGTGAACAGCATGTAGA	141
GAPDH	Thompson <i>et al.</i> (2019)	GTTTGTGATGGCGTGAACC	TTGGCAGCACCAGTAGAAGC	255

incubated overnight at 4°C. Endogenous peroxidase activity was neutralized with 0.3% H₂O₂ for 15 min. Then the biotinylated horse anti-mouse or anti-rabbit secondary antibody was added to each slide (RTU Vectastain kit; Vector Laboratories) for 30 min and visualized using Vectastain ABC and DAB substrate solution (DAB substrate kit; Vector Laboratories). All slides were then counterstained with hematoxylin. Expression of estrogen receptor α (ESR1; Kalpokas *et al.* 2010) and progesterone receptor (PGR) proteins established whether treatments caused organoids to change in response to sex steroid stimulation (Hartt *et al.* 2005). PTGS2, an enzyme that causes the production of prostaglandins, was assessed to determine response to oxytocin treatment (Keith *et al.* 2013).

Periodic acid Schiff (PAS) staining

PAS stains mucin and has been used as a method of evaluating functionality (mucin production) of organoids (Boretto *et al.* 2017). Similar to the immunohistochemical staining above, fixed, embedded, and deparaffinized sections of organoids were incubated in periodic acid solution and Schiff reagent. The sections were then counterstained with hematoxylin, dehydrated, and mounted.

Transmission electron microscopy

Organoid pellets were generated as for histology preparation above but were fixed in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M cacodylate buffer. Sample preparation and imaging with a JEOL 1230 transmission electron microscope was conducted by the University of Virginia Advanced Microscopy Facility.

Viability staining

Viability of organoids was assessed using SYBR-PI (Garner & Johnson 1995). Briefly, 20 μ M SYBR-14 (LIVE/DEAD Sperm Viability Kit; Molecular Probes) was added to each well containing a 25 μ L Matrigel droplet in 250 μ L OM. Samples

were incubated for 15 min (37°C) and then 48 μ M propidium iodide (LIVE/DEAD Sperm Viability Kit) was added to each well. All samples were further incubated for an additional 90 min to facilitate uptake of stain before imaging. Cells staining green were considered viable (membrane intact) and cells staining red were considered non-viable (non-intact membrane) for qualitative analysis.

Statistical analysis

Gene expression data were analyzed in the statistical program R (R Core Team 2014). A Shapiro–Wilk test determined that gene expression data were not normally distributed. Therefore, Kruskal–Wallis test was used to assess significance and followed by a post hoc Dunn test. Values are presented in box plots as mean $\pm$ S.E.M.. A *P*-value of < 0.05 was used to determine statistical significance compared to C treatment.

Results

Endometrial organoids can be established from both fresh and frozen–thawed domestic mare tissue biopsies

Endometrial organoids derived from both fresh and frozen–thawed domestic mare explants ($n=11$; samples from all 11 mares were used both fresh and frozen–thawed) were grown for 24 days (three passages, 6 days per passage; Fig. 1A and B) before removing organoids from culture conditions. Live/dead staining with SYBR-14 and propidium iodide revealed organoids composed of predominantly viable cells (Fig. 1C). A small proportion of organoids (~5% of the total organoids generated across all individual mares) also revealed some degenerative changes, as evidenced by dark coloration of cells evaluated by microscopy. Histologic assessment of organoids revealed cystic structures composed of columnar epithelial cells with

Table 2 Summary of primary antibodies used for immunohistochemistry analysis.

Primary antibody	Host	Type	Dilution	Source
ESR1	Mouse	Monoclonal	1:200	Santa Cruz Biotechnology; sc-787
PGR	Mouse	Monoclonal	1:200	ThermoFisher Scientific; MA5-12642
PTGS2	Rabbit	Polyclonal	1:25	Oxford Biomedical Research; PG46

Figure 1 Establishment of organoids from fresh and cryopreserved equine endometrial tissues. (A and B) Representative culture images of organoids derived from fresh (A) and frozen–thawed (B) domestic mare endometrial tissue through passage 2 days 0–6. (C) Viability staining of a single organoid in culture using SYBR-14 and propidium iodide staining. (D, E, F and G) Histological images of intact explant (D) and organoids (E, F and G). P, passage and D, day of culture. Scale bar = 1000 μ m (A and B), 200 μ m (C, D and E), 100 μ m (F), and 25 μ m (G).

basally located nuclei (Fig. 1E, F and G) consistent with intact endometrial explant gland histology (Fig. 1D). Transmission electron microscopy (TEM) revealed well-organized microvillus columnar epithelial cells with extensive network of secretory apparatus (Fig. 2A, B, C and D). Secretory function was further demonstrated with PAS stain revealing mucin production by equine endometrial organoids (Fig. 3). Furthermore, single cells derived from intact endometrial glandular fragments retained their clonogenic ability in vitro, as demonstrated by single cells developing into 3D organoids, albeit at a very low rate (24 organoids/2500 single cells; 0.1%; data not shown).

Organoids respond to exogenous hormonal stimuli

Gene expression of organoids derived from both fresh and frozen–thawed tissue explants was altered in response to exogenous hormone treatments, which is necessary to determine whether organoids mimic the endometrium in vivo (via mounting a physiological response to estrogen, progesterone, and oxytocin). *CDH1*, a marker for cell adhesion, was reduced in frozen–thawed explant-derived organoids in response to 10 nM estradiol-17 β for 2 days followed by 1 μ M progesterone for 4 days (E_2/P_4 ; $P=0.01$), 1 μ M progesterone for 6 days (P_4 ; $P=0.02$), and control medium for 5 days then 10^{-5} M

Figure 2 *In vitro* produced organoids retain structural organization of endometrial glands from intact tissues. Representative transmission electron microscopy images of C treatment groups for organoids derived from both fresh (A and B) and frozen–thawed (C and D) domestic mare endometrial tissue. Scale bar=5 µm (A), 2.5 µm (B and C), 0.2 µm (D).

oxytocin for 24 h (C/OT-5; $P=0.02$) compared to control (C, no hormones added; Fig. 4A). No differences were detectable in *MKI67* expression for either fresh ($P=0.054$) or frozen–thawed ($P=0.14$) explant-derived organoids compared to C (Fig. 4B). Expression of *ESR1* was reduced in both fresh and frozen–thawed organoids compared to C for organoids treated with both 10 nM estradiol-17 β for 6 days (E_2 ; $P=0.002$ and $P=0.002$, respectively) and E_2/P_4 ($P=0.009$ and $P=0.001$, respectively; Fig. 4C). *PGR* gene expression in fresh tissue-derived organoids was increased compared to C for E_2 organoids ($P=0.01$; Fig. 4D).

We also evaluated *PTGS2*, *PGES*, and *OXTR* gene expression in response to treatment with two concentrations of oxytocin (10^{-5} M and 10^{-6} M) to further evaluate cellular function. Previous reports indicated that prostaglandin production in response to oxytocin treatment is a reliable method to confirm that cultured endometrial cells exhibit normal physiological function (Takahashi *et al.* 2001, Szóstek *et al.* 2012a). Expression of *PTGS2* (Fig. 4E) was elevated in fresh tissue-derived organoids treated with control medium for 5 days

then 10^{-6} M oxytocin for 24 h (C/OT-6) compared to C ($P=0.004$) and reduced in frozen–thawed explant-derived organoids treated with C/OT-5 compared to C ($P=0.003$). Levels of *PGES* were elevated in both fresh and frozen–thawed tissue-derived explants treated with C/OT-6 compared to C ($P=0.014$ and $P=0.017$, respectively; Fig. 4F). *OXTR* expression (Fig. 4G) was reduced in fresh tissue-derived organoids treated with C/OT-5 compared to C ($P=0.008$) with no differences among treatments for frozen–thawed explant-derived organoids ($P=0.87$). Direct comparison of fresh and frozen–thawed explant-derived organoids revealed differences for *CDH1* ($P=0.0006$), *MKI67* ($P=0.048$), and *PTGS2* ($P=0.005$) with no differences for *ESR1* ($P=0.984$), *PGR* ($P=0.095$), *PGES* ($P=0.764$), or *OXTR* ($P=0.301$).

Immunohistochemistry revealed that expression of *ESR1* and *PGR* was localized to the nuclei of both fresh and frozen–thawed explant-derived organoids (Fig. 5A, B and D). Expression of *PTGS2* was localized to the cytoplasm of both fresh and frozen–thawed explant-derived organoids (Fig. 5C and D).

Figure 3 Equine endometrial organoids secrete mucin. Representative periodic acid Schiff (PAS) staining of fresh and cryopreserved tissue-derived organoids from domestic mares for C, E_2 , and P_4 treatment groups. Scale bar=100 µm.

Figure 4 *In vitro* produced endometrial organoids retain the ability to respond to exogenous hormones. Gene expression of *E-cadherin* (A), *Ki67* (B), *ESR1* (C), *PGR* (D), *PTGS2* (E), *PGES* (F), and *OXTR* (G) in organoids derived from fresh and frozen-thawed domestic mare endometrial tissue ($n=11$). Asterisks represent significant differences among fresh tissue-derived organoid treatments compared to the fresh tissue-derived organoid C and frozen-thawed tissue-derived organoid treatments compared to the frozen-thawed tissue-derived organoid C.

Endometrial organoids derived from Przewalski's mare biopsies proliferate and respond to hormonal stimulation *in vitro*

Using our now established domestic mare endometrial organoid culture conditions, we set out to establish organoids from frozen-thawed explants from Przewalski's mares ($n=3$; Fig. 6A). Gene expression among treatment groups compared to C was not different for *ESR1* ($P=0.125$), *PGR* ($P=0.143$), *CDH1* ($P=0.349$), *MKI67* ($P=0.664$), *PTGS2* ($P=0.240$), *PGES* ($P=0.661$), or *OXTR* ($P=0.725$; data not shown). Direct comparison of Przewalski's horse to domestic horse organoids showed elevated *OXTR* ($P=0.006$) in the Przewalski's horse but no difference for *ESR1* ($P=0.689$), *PGR* ($P=0.486$), *CDH1* ($P=0.104$), *MKI67* ($P=0.090$), *PTGS2* ($P=0.811$), or *PGES* ($P=0.099$). Similar to the domestic horse, immunohistochemistry of *ESR1* and *PGR* revealed expression localized to the nuclei of organoids and *PTGS2* expression in the organoid cytoplasm (Fig. 6B, C and D).

Discussion

Here we report the first successful generation of organoids derived from fresh and frozen-thawed endometrial tissues from domestic and non-domestic equids. Results also represent a 'first' for any domestic large animal or wildlife model. All organoids maintained structural characteristics of the original tissue forming cystic structures lined with microvillus columnar cells with basally located nuclei. Organoids derived from both fresh and frozen-thawed endometrial tissues from domestic mares expressed a modest response to exogenous hormones. In the present study, cultures were ended after 24 days (three passages) to ensure a consistent growth period and facilitate subsequent experimentation. However, additional studies are warranted to understand the impact of longer culture periods on endometrial organoid viability and function. Formation of organoids required inclusion of an extracellular matrix, Matrigel. Furthermore, these 3D structures responded to external hormonal stimuli, a necessary feature for mimicking *in vivo* function. Interestingly, culture conditions similar to those previously described for murine and human endometrial organoids were able to generate endometrial organoids from both domestic and non-domestic equids. This further suggests that mechanisms involved in the formation of organoids *in vitro* from endometrial tissues may be conserved among the laboratory mouse, human, and equine models.

While mouse endometrial organoids have been reported to be either 'dense' or 'cystic' (Boretto et al. 2017), both human and horse endometrial organoids appear as discrete, cystic structures lined by columnar epithelial cells (Boretto et al. 2017, Turco et al. 2017). Though the clonogenic rate was low, the rate reported in human endometrial organoids was only 2–4% in cells plated with 100 cells per 5 μ L drop of Matrigel and 10 times lower in cells plated with 10 cells per 5 μ L drop (Turco et al. 2017). Estimation of clonogenic rate in the equine endometrial organoids used 26 cells per 5 μ L Matrigel drop for a rate of 0.1%. As the human endometrium is sloughed and regenerated every month through the menstrual cycle, a higher clonogenic rate in endometrial cells is expected than in equids that experience estrous cycles which do not involve endometrial sloughing. Furthermore, the addition of specific supplements to culture medium, such as the Glycogen Synthase Kinase-3 inhibitor CHIR9902 or Rho-associated protein kinase (ROCK) inhibitors, may improve clonogenic rate of cells cultured in a low density (Watanabe et al. 2007, Koyanagi et al. 2008, Ying et al. 2008, Li et al. 2009, Huang et al. 2011). Transmission electron microscopy revealed basally located nuclei, apical microvilli, and a well-formed secretory apparatus in the equine endometrial organoids, demonstrating that the organoids appear to retain their secretory function similar to *in vivo* endometrium. Moreover, qualitative immunohistochemistry revealed the presence of *ESR1*, *PGR*, and *PTGS2* proteins, indicating the equine

Figure 5 Endometrial tissues and organoids express ESR1, PGR, and PTGS2 proteins. Representative immunohistochemistry of fresh tissue and fresh and cryopreserved tissue-derived organoids from domestic mares evaluated for expression of ESR1 (A), PGR (B), PTGS2 (C), and a higher magnification (1000X) of fresh Control organoids stained for ESR1, PGR, and PTGS2 (D). Scale bar=100 µm (A, B and C) and 25 µm (D).

organoids are capable of responding to exogenous hormonal stimuli.

Culture media used here varied slightly from those reported for human endometrial organoids. The media used for equine organoids was most similar to the report by Boretto *et al.* (2017) with the primary difference that the Wnt activator R-spondin-1 was not used in the equine

organoid medium, and estradiol-17 β was not used during expansion of the equine organoids, as this reduced rate of organoid expansion (data not shown). When compared to the culture medium used by Turco *et al.* (2017), several supplements, including primocin, recombinant human R-spondin-1, and recombinant human hepatocyte growth factor (HGF), were not used during equine endometrial

Figure 6 Establishment of endometrial organoids from the endangered Przewalski's horse and expression of ESR1, PGR, and PTGS2 proteins in tissue and organoids. (A) Representative culture images of organoids derived from frozen-thawed Przewalski's mare endometrial tissue through passage 3 days 0–6. (B, C and D) Immunohistochemistry of Przewalski's mare organoids evaluating expression of ESR1 (B), PGR (C), and PTGS2 (D). P, passage and D, day of culture. Scale bar = 1000 μ m (A) and 100 μ m (B, C and D).

organoid culture, and the equine organoids were cultured with the addition of penicillin/streptomycin, ITS, and SB202190. Primocin is an antimicrobial agent, which was substituted with penicillin and streptomycin for antimicrobial coverage for the equine organoids. R-spondin-1, a common supplement in organoid cultures (Clevers 2016, Boretto *et al.* 2017, Powell & Behnke 2017), is linked to the Wnt signaling pathway (Kim *et al.* 2008), which is necessary for cell function, including in the equine endometrium (Atli *et al.* 2011). HGF is a growth factor that promotes proliferation and differentiation of epithelium and has been used in organoid cultures (Haslam *et al.* 2008, Sato & Clevers 2015, Kaushik *et al.* 2018). For the mare, it was not critical to include these additional supplements in the culture medium, as we were able to consistently produce viable organoids in vitro in the absence of these factors. However, addition of R-spondin-1 and/or HGF may increase growth rate or hormone-responsiveness of organoids, as reported in human endometrial culture (Turco *et al.* 2017), and could be an area of future research.

This is the first report of endometrial organoid production from both fresh and frozen-thawed tissue. Endometrial explants were cryopreserved using slow cooling ($\sim 1^\circ\text{C}/\text{min}$) in the presence of 10% dimethyl sulfoxide (DMSO) as previously described (Thompson *et al.* 2019, 2020). Cryopreservation conditions optimized for the domestic mare also were suitable for the preservation of Przewalski's horse endometrial tissue. The ability to cryopreserve equine endometrial biopsies not only allows for year-round reproductive studies using in vitro approaches, a historical challenge in mares due to seasonally anestrus cyclicity, but also permits systematic collection and banking of endometrial biopsies from both domestic and endangered equids. Additionally, utilization of frozen-thawed endometrial tissue may mitigate seasonal influences on data generated with tissues derived from different times of the year, which is particularly important for species that experience seasonal reproduction.

Organoids derived from both fresh and frozen-thawed endometrial tissue from domestic mares responded to exogenous hormone stimulation with changes in gene expression. While *CDH1*, the gene marker for cell adhesion, was reduced in some of the organoids derived from frozen-thawed tissue, cell proliferation, as indicated by *MKI67* mRNA (Sinn *et al.* 2017), was not altered among any of the treatment groups in organoids derived from either fresh or frozen-thawed tissue. Estradiol- 17β in culture media (both E_2 and E_2/P_4) reduced expression of *ESR1* in both fresh and frozen-thawed tissue-derived organoids. Additionally, organoids derived from fresh tissues exposed to E_2 supplemented culture medium displayed elevated *PGR* expression. These results demonstrate the ability of organoids to respond to exogenous steroid hormone treatment with corresponding changes in gene expression. In mares, estrogen and progesterone receptor

protein and mRNA have been reported to increase during estrus and decrease during diestrus (Hartt *et al.* 2005), though another report indicated that *PGR* expression was unaffected between estrus and diestrus (Silva *et al.* 2014). Although we expected *ESR1* and *PGR* to increase in organoids exposed to E_2 treatments and to decrease in E_2/P_4 and P_4 treatments, our results failed to support this prediction. This phenomenon may be due to the lack of feedback mechanisms that operate in vivo but are absent in the in vitro system, such as the absence of endometrial stromal cells and components of the hypothalamic-pituitary-gonadal axis, or variation among donors, which might have influenced our results and warrant additional studies. Furthermore, as the domestic mares utilized here were research mares that are often donated to a university, age, breed, and parity can be quite variable, which could have affected the outcome though all mares were considered reproductively sound by a veterinarian. We controlled for grade of uterine inflammation and fibrosis (Kenney-Doig I or IIa), general stage of the estrous cycle (estrus), and month of sample collection (August), but exact day of estrus (estrus is 4–7 days in length) was not controlled. To our knowledge, no reports have evaluated *ESR1* and *PGR* gene expression of equine endometrial monolayer cell cultures in vitro, though immunohistochemical expression of *ESR1* and *PGR* did not change in response to exogenous hormone treatments in cell cultures of equine endometrial stromal and epithelial cells separated by semipermeable membrane well inserts (Lapko *et al.* 2017).

Additionally, we evaluated *PTGS2*, *PGES*, and *OXTR* gene expression in response to treatment with two concentrations of oxytocin (10^{-5} M and 10^{-6} M) to evaluate cellular function. Previous reports indicate that prostaglandin production in response to oxytocin treatment is a reliable approach to determine if cultured endometrial cells function physiologically (Takahashi *et al.* 2001, Szóstek *et al.* 2012a). In the present study, *PTGS2* was reduced in frozen-thawed tissue-derived organoids when exposed to C/OT-5 but elevated in fresh tissue-derived organoids when treated with C/OT-6. Organoids displayed higher levels (expression) of *PGES* in both fresh and frozen-thawed derived tissue organoids when treated with C/OT-6. Lastly, *OXTR* was reduced in fresh-tissue-derived organoids treated with C/OT-5. Previous studies also have shown that PGE_2 increases in response to oxytocin treatment during both the follicular phase and luteal phase in vivo (Galvão *et al.* 2013), and PGE_2 production increases in equine endometrial stromal and epithelial monolayer cell cultures when incubated in the presence of 10^{-7} M oxytocin (Szóstek *et al.* 2014). While the prostaglandin synthase fold changes reported here are lower than those reported in the mare monolayer culture (Szóstek *et al.* 2014), this could be due to the structural and functional differences between the monolayer and organoid culture models. In a monolayer, the exogenous

hormones in the culture media are in direct contact with every cell, while organoids are 3D and embedded within Matrigel, probably limiting hormone diffusion through the Matrigel and restricting direct cellular contact to only the exterior of the organoids. Furthermore, fresh and frozen-thawed tissue-derived organoids showed a varying response in gene expression fold change, particularly with respect to *PTGS2* and *OXTR*. While the overall generation of the organoids, protein expression, and mucin production did not appear to be affected by origin of the tissue (fresh vs frozen-thawed), further optimization may be required to ensure that frozen-thawed tissue-derived organoids express appropriate changes in gene expression in response to exogenous hormones. While the prostaglandin synthase and *OXTR* gene expression was variable in this report, the organoids responded by stimulating the prostaglandin synthesis cascade in response to oxytocin treatment after culture.

An important finding is that while the organoid model has significant advantages over the currently available cell culture models for mares, some drawbacks are evident. First, the organoid model system contains only the glandular epithelium. These glandular epithelial cells are important because they are responsible for essential secretory activity of the uterus. However, endometrial stromal cells respond differently to hormonal stimuli than epithelial cells and still contribute to the normal function of and signaling in the equine endometrium (Aupperle *et al.* 2000). Secondly, Matrigel has been used for many cell culture models, but the 3D extracellular matrix does contain proteins that may interact with the endometrial cells and interfere with normal signaling processes or diffusion of exogenous substances (compared to direct contact of exogenous substances in culture media with monolayer and explant cultures) and varies in composition among batches (Langhans 2018, Aisenbrey & Murphy 2020, Wiwatpanit *et al.* 2020). Lastly, we describe endometrial organoids oriented with the apical portion of the cells to the interior of the cellular clusters. This can be advantageous to create a localized site to contain all cellular secretions; however, this orientation may not be ideal for evaluating interactions to certain exogenous substances, though orientation did not appear to affect ability to respond to exogenous hormone treatments. Future studies could explore reversing the polarity of the organoids so that the apical portion of the cell is located on the exterior of the cellular cluster.

Przewalski's horses, endangered equids native to Asia, were once extinct in the wild (King *et al.* 2015). After diligent efforts by captive breeding programs, they are now considered endangered by the IUCN Red List of Threatened Species (King *et al.* 2015) with ~2,000 individuals worldwide. Reproduction in endangered equids is similar to domestic horses, but not identical. For example, length of the reproductive (estrous) cycle in Przewalski's horses is slightly longer (~25 days) than the domestic horse (~21 to 22 days), though individual

variation is common in both subspecies (Collins *et al.* 2012). Reproductive research in Przewalski's horses has focused on assisted reproductive technologies (Monfort *et al.* 1991, 1994, Collins *et al.* 2014, Pukazhenthi *et al.* 2014), but thorough evaluation of the normal reproductive mechanisms associated with the uterus has not occurred. This wild equid has been maintained in captivity and can be handled for reproductive evaluation with the aid of a specialized handling systems, however, length and frequency of handling are much more restricted than in domestic horses. Therefore, minimally invasive methods of evaluating reproduction in non-domestic equids is critical. While our sample size for Przewalski's mares was restricted ($n=3$) due to a limited number of individuals in captive populations in addition to challenges associated with handling, we demonstrated our ability to generate long-term growth of endometrial organoids from this endangered species. In contrast to domestic mares, no significant gene expression changes occurred in response to hormone treatments, likely due to the low sample size, but we demonstrated protein expression of *ESR1*, *PGR*, and *PTGS2* through immunohistochemistry in the organoids. Although Przewalski's horses diverged evolutionarily from the domestic horse thousands of years ago (Der Sarkissian *et al.* 2015), the mechanisms enabling formation of endometrial organoids appear to be conserved between the domestic mare and the Przewalski's horse.

In conclusion, this is the first report of equine endometrial organoid generation and long-term culture. The organoid cultures can be maintained long-term while retaining the ability to respond to hormonal stimuli (at least in the domestic mare), which overcomes the two short-comings associated with the traditional equine endometrial explant and monolayer cell cultures. This is also the first report of endometrial organoids developed from frozen-thawed tissue in any species. Finally, this is the first report of in vitro culture to assess reproduction of endangered Przewalski's horses. This novel method of culturing equine endometrium in vitro may lead to improved in vitro evaluation of normal reproductive physiology, pathological conditions, and potential therapies for uterine diseases in both domestic and endangered equids.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

R E T and B S P designed the study and obtained funding. R E T, A K J, P D, T M P, B A B and B S P participated in sample collections. R E T conducted all laboratory experiments. R E T and B S P conducted data analysis. M Y T, G J B and B K W provided guidance in development of methodology and experimental design. C P assisted with histological assessment of samples. R E T and B S P wrote the original draft of the manuscript, and all co-authors reviewed and edited final version of the manuscript.

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