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A first report of organoids derived from rhinoceros endometrium

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ABSTRACT

Cell culture models that recapitulate key features of the tissue of interest are widely used in domestic and laboratory animals to reduce the time and financial cost of evaluating physiological and pathological reproductive conditions. Model systems are particularly advantageous for endangered species because performing reproductive experiments to better understand normal physiology and/or disease states must be secondary to producing offspring for the survival of the species. Here we describe a first report of endometrial organoid culture from tissues of two rhinoceros species (*Rhinoceros unicornis* and *Diceros bicornis minor*). Organoids from the greater one-horned rhinoceros did not proliferate in culture, and organoids from the southern black rhinoceros proliferated slowly and had a smaller, more dense morphology than either equine or bovine endometrial organoids cultured under the same conditions. This report highlights the importance of biobanking somatic reproductive tissues from endangered species to better understand their normal physiology and disease states and as a resource to potentially improve assisted reproductive technologies.

1. Introduction

The One Health approach highlights the interconnectedness of human, animal, and environmental health, understanding that changes in one area can impact the others. As global issues like climate change, habitat destruction, and emerging infectious diseases continue to threaten biodiversity, an increasing need exists for innovative solutions to protect wildlife health and support sustainable conservation efforts. Assisted reproductive technologies (ARTs), such as in vitro fertilization (IVF) and embryo culture, have been instrumental for conservation of endangered species. The success of these strategies relies on a profound understanding of species-specific reproductive biology and physiology, which is not always available and is difficult to achieve in wildlife species. Numerous non-domestic species lack protocols for in vitro ARTs, with in vitro oocyte maturation, fertilization, and embryo culture rates often suboptimal compared to their phylogenetically closest domestic model [1]. Moreover, exogenous hormonal treatments for population management and assisted breeding are challenging. Limited, often unpublished, knowledge is available about the reproductive cycle and hormone activity in non-domestic species [2]. Reproductive samples are limited due to the difficulty of collecting tissue and cells from live animals, hence the majority of biological material that can be used to develop technologies and reproductive strategies are obtained post-mortem. Improved cell culture models derived from reproductive tissues of wildlife could lead to the development of new ART strategies, improving the efficiency of breeding programs and providing treatments for reproductive management success.

Organoids are a type of three-dimensional cell culture which are composed of multiple cell types that self-organize, maintain the properties of the organ of origin, and proliferate long-term (months) [3]. When derived from reproductive tissue, organoids provide an in vitro model to study gametogenesis, embryonic development, and fertility disorders [4–7]. Endometrial organoids have been established in a number of species including humans, murine, equine, porcine, bovine, canine, and feline species [8–17]. In one report, endometrial organoids were produced from uterine tissue of endangered Przewalski's horses

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(*Equus ferus przewalskii*) using protocols optimized for domestic mares (*Equus caballus*) [15].

Our objective was to apply established equine and bovine endometrial organoid culture methods to endometrial tissue collected from rhinoceroses. Although the road to implementing this technology in wildlife conservation and reproduction has limitations and challenges, here we report the first generation of organoids derived from post-mortem endometrial tissue collected from a black rhinoceros.

2. Methods

2.1. Collection and preparation of endometrial tissue

Southern black rhinoceros (*Diceros bicornis minor*) and greater one-horned rhinoceros (*Rhinoceros unicornis*) reproductive tracts (n = 1 per species) were collected opportunistically post-mortem. Equine (*Equus caballus*) endometrial tissue (n = 1) was collected opportunistically post-mortem from an adult, mixed breed research mare euthanized at the Colorado State University (CSU) Veterinary Teaching Hospital for non-reproductive reasons. The bovine (*Bos taurus*) reproductive tract (n = 1) was collected opportunistically post-mortem at a local abattoir. Tissues were transported in phosphate buffered saline (PBS) on ice to the laboratory for processing.

2.2. Uterine tract processing and endometrial organoid culture

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Chemicals, St. Louis, MO, USA. All tissue was cryopreserved prior to organoid culture using a previously published protocol [15]. Briefly, tissue was minced into 1–2 mm^3 pieces before cryopreservation. Equine and bovine tissue was frozen in 10 % DMSO in Minimum Essential Medium (MEM; Gibco; Waltham, MA, USA) 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 (FBS). Rhino tissue was cryopreserved in 20 % fetal calf serum (FCS) and 10 % DMSO in Alpha MEM (Gibco), modified from the AGT Cytogenetics Manual [18]. Rhino tissues were cryopreserved in different medium than the domestic animals due to the availability of reagents at the institutions freezing the samples. The cryovials were placed in a −80°C freezer overnight in a Cool Cell Freezing Container (Corning; Corning, NY, USA) that reduces the temperature in vials by ~1°C per minute. On the second day, the cryovials were transferred to liquid nitrogen for storage. The time between euthanasia to tissue cryopreservation was ~6 h for the domestic animals and ~24 h for the rhinoceroses.

The protocol for processing endometrium for organoid culture was based on previous reports for equine endometrial organoids [13,15]. Cryovials containing the tissue pieces were thawed for 1 min in room temperature air and then 1 min in a 25°C water bath prior to a series of three washes in 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, and 20 % FBS] [15,19]. Endometrial tissue pieces (~10–15 pieces) were digested at 37°C, 1000 rpm (ThermoMixer C; Eppendorf; Hamburg, Germany) in collagenase V (0.4 mg/mL) and dispase II (1.25 U/mL) in RPMI-1640 medium (Gibco) until glandular fragments were separated from surrounding tissue (~15–20 min). The digested cells were diluted in RPMI-1640 medium with 20 % FBS. The glandular fragments were collected in a 100 μm cell strainer and then backwashed from the strainer. An additional collection of glandular fragments was performed in a 40 μm cell strainer. The glandular fragments were subjected to centrifugation (1000 x g for 10 min). The pellet was washed through an additional centrifugation in DMEM/F12 (Gibco), and then the cell pellet was suspended in 20X phenol-red free UltiMatrix Reduced Growth Factor Basement Membrane Extract (ECM; Cultrex, R&D Systems; Minneapolis, MN, USA), and 25 μL droplets of cells in ECM were plated in each well of a 48-well plate (Corning Costar TC-Treated Multiple Well Flat-Bottom Plates). After 30 min at 37°C, the droplets were overlaid with 250 μL organoid culture medium [DMEM/F12 without phenol red, 1 % penicillin/streptomycin, 2.5 mM L-glutamine, 2 % B27 Plus (Gibco), 1 % N2 (Gibco), 1 % ITS, 1 mM nicotinamide, 50 ng/mL recombinant human EGF (R&D Systems), 50 ng/mL recombinant human FGF10 (PeproTech; Rocky Hill, NJ, USA), 100 ng/mL recombinant human Noggin (R&D Systems), 0.5 mM TGFb/Alk inhibitor A83–01 (Tocris; Minneapolis, MN, USA), 1.25 mM N-acetyl-L-cysteine (Millipore Sigma; Burlington, MA, USA), 10 mM SB202190, and 10 mM Y27632 (EMD Millipore; Burlington, MA, USA)]. Cells were maintained at 37°C, 5 % CO2, and half of the culture medium was removed and replaced every 2–3 days.

Organoids were passaged based on their proliferation and growth rate (total number of organoids and size of each individual organoid). Equine endometrial organoids were passaged every 7 days at a ratio of 1:4 (one well was split into four wells during passage), and bovine endometrial organoids were passaged every 14 days at a ratio of 1:4. Rhinoceros endometrial organoids were passaged every 10–14 days at a raio of 1:2. To passage, the ECM containing the organoids was scraped from the culture plate, placed into microcentrifuge tubes, and centrifuged at 1000 x g for 10 min. The cell pellet was resuspended in DMEM/F12 and vigorously pipetted (500X). The cells were again centrifuged, and then the cell pellet was repeatedly pipetted (300X) in DMEM/F12. After centrifugation, the cell pellet was replated in ECM as described above.

Brightfield images were acquired prior to passage using a stereomicroscope (SZX16, Olympus; Tokyo, Japan) at 10X magnification. Fiji for Image J was used to analyze the number of organoid in a well and diameter of individual organoids.

2.3. Histology and periodic acid-Schiff staining

As previously described [13,14], organoids in ECM were incubated on ice in Cultrex Organoid Harvesting Solution (R&D Systems) for 1 h to depolymerize the ECM. After centrifugation at 1000 x g for 10 min, organoids were washed in PBS and then fixed in 4 % paraformaldehyde for 30 min. The fixed organoid pellet then was embedded in 2 % agarose gel (Bio-Rad) before storage in 70 % ethanol at 4°C prior to histology. For histology, organoids in agarose were embedded in paraffin wax, sectioned (6 μm), and deparaffinized. For H&E, slides were stained using hematoxylin and eosin to evaluate organoid morphology. For periodic acid-Schiff (PAS), sections were incubated with periodic acid solution and Schiff reagent with hematoxylin counterstaining to evaluate organoid secretory functionality through mucin production [8].

3. Results

3.1. Sample collection and animal health information

The southern black rhinoceros (*Diceros bicornis minor*), from which endometrium was collected post-mortem, was euthanized at Fossil Rim Wildlife Center due to a chronic history of lameness and foot abscesses. This female was 34.55 years old and multiparous. This female had no other major health condition or concern, and she was in good body condition at the time of death. The reproductive tract was harvested immediately after euthanasia at Fossil Rim Wildlife Center and shipped overnight at 4°C to San Diego Zoo Wildlife Alliance Reproductive Sciences Team for cryopreservation.

The greater one-horned rhinoceros (*Rhinoceros unicornis*), from which endometrium was collected post-mortem was euthanized due to declining health. She was 18.7 years old and multiparous. After euthanasia, the carcass of the animal was stored overnight (~18 h) at 4°C, and necropsy with endometrial tissue collection was performed the following morning.

3.2. Organoid establishment and morphological characteristics

Organoids derived from the endometrium of the southern black rhinoceros showed growth and proliferation across the 35 days of culture (Fig. 1). The organoids appear as dense structures with slow rates of proliferation. Because of the slow growth, passage was performed after 10 or 14 days to allow time for additional growth. On the day with the largest organoids (P0D10), a representative brightfield micrograph was analyzed. The maximum diameter of a southern black rhinoceros organoid was 855 μm although only 7 organoids were greater than 250 μm (75 total organoids in a well). This is compared to 778 μm maximum diameter in a well of equine organoids using a representative brightfield micrograph with at least 45 organoids above 250 μm out of 119 total organoids, or 627 μm maximum diameter in a well of bovine organoids with at least 28 organoids above 250 μm out of 75 total organoids. Qualitatively, the rhinoceros organoids demonstrated the greatest growth during P0, whereas equine and bovine endometrial organoids do not demonstrate the reduced growth in later passages. The histological micrographs demonstrated an organoid with a central lumen surrounded by a disorganized layer of large, flattened to polygonal cells with presence of cystic structures within the cellular layer (Fig. 2E). Other histological cross-sections demonstrated dense multicellular polypoid structures composed of large polygonal cells without a central lumen and with frequent irregularly sized cytoplasmic vacuoles and intercellular cystic structures. Pink staining on the PAS micrograph indicated presence of mucin secretion toward the luminal aspect of the organoid cells (Fig. 2H). Organoid cultures containing cells from the endometrium of the greater one-horned rhinoceros did not establish or proliferate (Fig. 3). Because of this, the cultures were discontinued after 10 days.

Organoids from equine and bovine endometrium demonstrated round, cystic structures via brightfield micrographs, and histology showed the organoids have a central lumen and a single layer of cells surrounding the lumen (Fig. 2D and F). PAS staining was positive for mucin secretion throughout the cells with increased staining toward the lumen of the organoids in both the equine and bovine endometrial organoids (Fig. 2G and I).

4. Discussion

Organoid models provide a promising in vitro platform to study reproductive health and physiology. Because threatened or endangered species often cannot be sampled directly for reproductive tissues due to logistical or ethical constraints, most of the samples used to develop

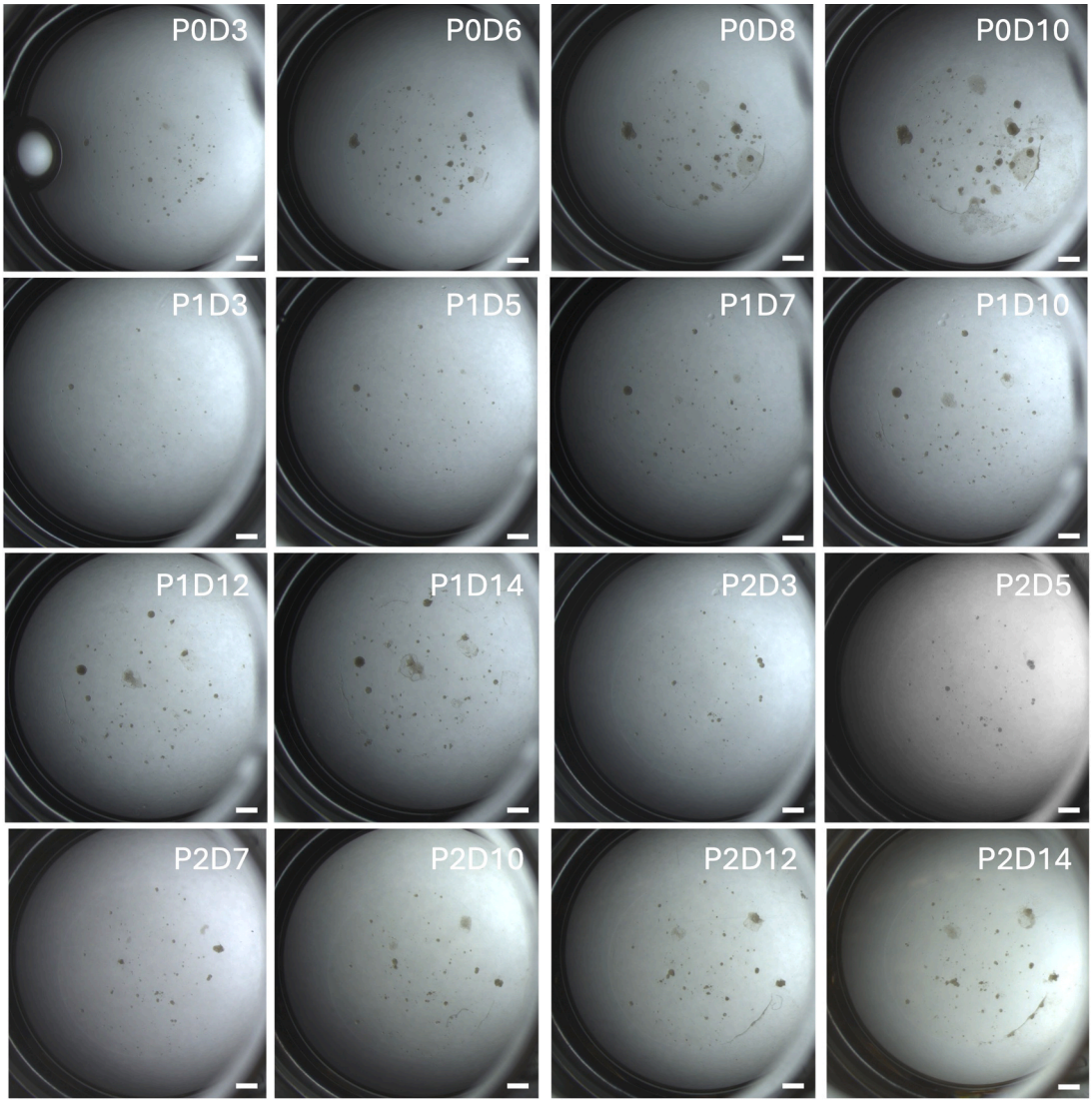

Fig. 1. Micrographs of organoids derived from Southern black rhinoceros (*Diceros bicornis minor*) endometrial tissue over 35 days of culture including two passages. P = passage, D=day. Scale bar= 1 mm.

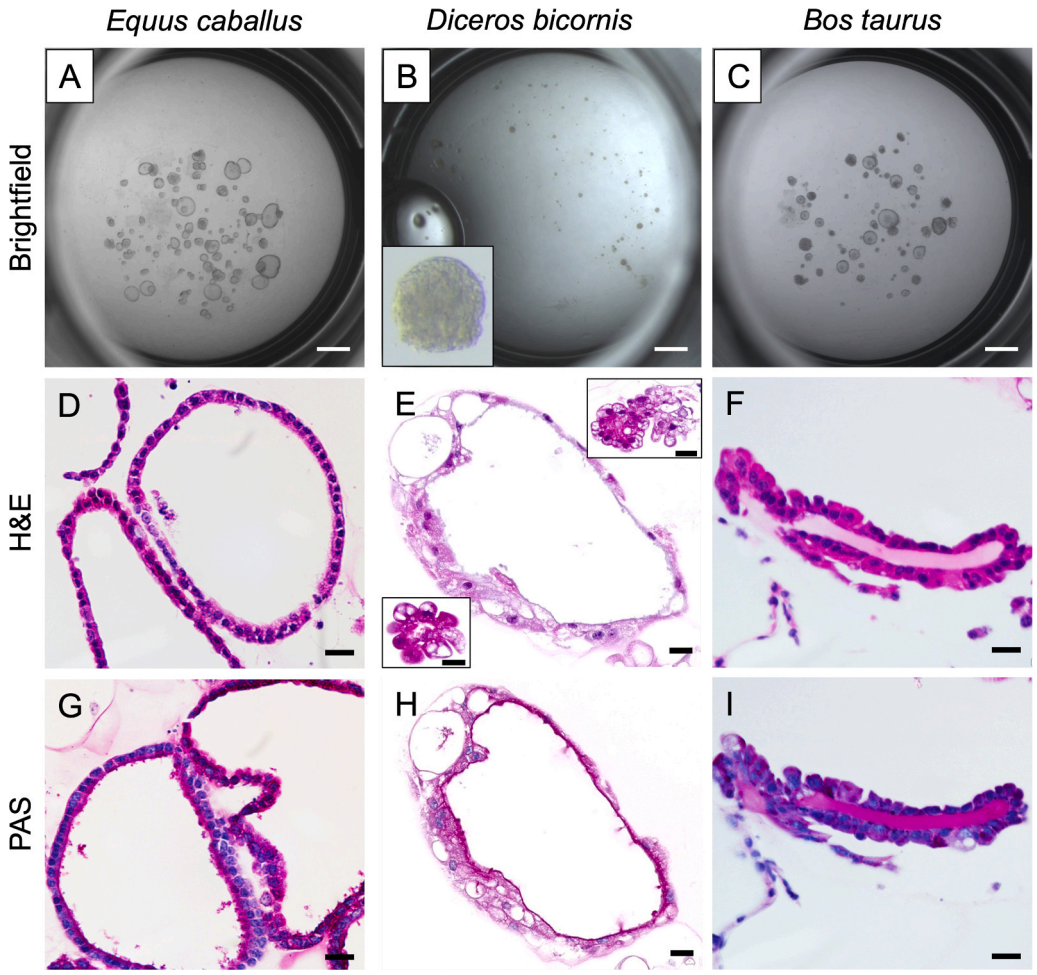

Fig. 2. Comparison of endometrial organoids derived from horse (*Equus caballus*), southern black rhinoceros (*Diceros bicornis minor*), and cow (*Bos taurus*) tissue using identical culture conditions. A) a well of equine endometrial organoids at passage 1, day 7 (P1D7; 14 total days in culture with one passage) at 10X magnification; B) a well of southern black rhinoceros endometrial organoids at P0D8 at 10X magnification with an inset of a single organoid at 80X magnification; C) a well of bovine endometrial organoids at P0D14 at 10X magnification; D-F) Histological micrographs of endometrial organoids from equine tissue at P2D7 (D), rhinoceros tissue at P2D14 (E), and bovine tissue at P2D14 (F); G-I) Micrographs of equine (G), rhinoceros (H), and bovine (I) endometrial organoids stained with periodic acid-Schiff (PAS). Brightfield scale bars = 1 mm. Histology scale bars = 20 μm.

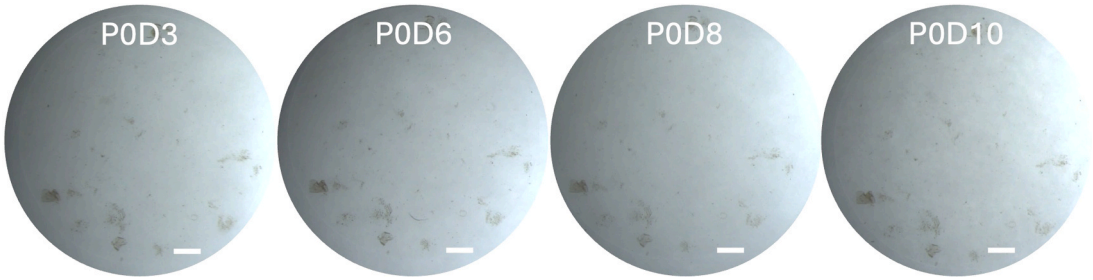

Fig. 3. Micrographs of culture wells during attempted generation of organoids derived from greater one-horned rhinoceros (*Rhinoceros unicornis*) endometrial tissue over 10 days of culture without any cellular proliferation. P = passage, D=day. Scale bar= 1 mm.

ARTs in vitro (e.g., ovary, oviduct, endometrium) are collected post-mortem or opportunistically. Organoids are derived from small tissue samples or biopsies, thereby reducing invasive sampling and enabling the generation of sustainable cell culture lines that preserve the biology, genetics, and physiology of the species and individual of interest. The ability of organoids to reproduce key aspects of tissue architecture and function, and their applicability across a range of species and contexts, make them powerful systems for advancing reproductive knowledge in all species. As organoids have been generated successfully from different components of the female reproductive tract, including the endometrium, oviduct, ovary, and trophoblast tissues in humans and in domestic species [4,9,20,21], these models are proven, valuable tools for studying implantation, endometrial receptivity, inflammatory regulation, pathological conditions, tissue-specific development, and hormonal responsiveness.

In vitro protocols for domestic species do not always translate to wildlife species due to species-specific differences in gametogenesis, folliculogenesis, uterine receptivity, oviductal secretions, and

embryo–maternal crosstalk. Therefore, organoids derived from wildlife reproductive tissues are essential to investigate these species-specific pathways and to refine ART conditions, such as culture media, hormonal priming, and embryo co-culture, for endangered species. Of relevance to conservation, organoids from high-genetic-value individuals may serve as a living biobank. Tissue-derived organoids can support future derivation of gametogenic tissue, reproductive tract modelling, or drug and hormone response screening, even after the animal has died. This approach complements existing biobanking efforts by adding a functional tissue component and may support long-term management of genetic diversity in ex situ assisted reproduction programs. Providing ethically advantageous alternatives to in vivo studies, organoids align with the principles of the One Health approach by facilitating studies of zoonotic pathogens, environmental contaminants, and the evolutionary basis of reproductive adaptations [22,23]. Their use may address the effects of the changing environment on the reproductive functionality of wildlife and humans, influencing wildlife health and biodiversity.

Endometrial organoids have been previously reported in domestic horses [14,15,24], and the methods were directly translated to the generation of endometrial organoids from the endangered Przewalski's horse (*Equus ferus przewalskii*) [15]. As both species are in the family Equidae, direct translation of organoid generation methods occurred without challenges. Similarly, bovine endometrial organoids were established in this report using the same methods as those described for domestic mares and Przewalski's horses [14,15]. While equids and bovids are both ungulates, equids and rhinocerotidae belong to the Perissodactyla (odd-toed ungulate), whereas bovids are Artiodactyls (even-toed ungulate) order. Because of the taxonomical proximity of equids and rhinocerotidae, direct translation of the endometrial organoid methods was expected. However, the morphology and proliferation rates of the rhinoceros endometrial organoids differed from both domestic equine and bovine endometrial organoids.

The organoids reported here are established by collecting the endometrial epithelial cell gland fragments from the endometrial tissue for each species. While full characterization of the cellular origin was not performed here, several reports of reproductive organoids have indicated that using this method ultimately results in positive immunohistochemical staining for both vimetin, which is associated with stromal cells or epithelial-mesenchymal transition, and cytokeratin, which is associated with epithelial cells [9,11,12,25]. Based on these data, we expect that the organoids derived from rhinoceros endometrium also reflect the presence of the both stromal and epithelial cells with more detailed characterization planned for when the rhinoceros organoids are further optimized.

Several factors may account for the differences in the rhinoceros organoid morphology and proliferation compared to equine or bovine endometrial organoids. First, despite the phylogenetic proximity, rhinoceros endometrial cells may require different culture medium supplements than equids or bovids. Second, the nature of access to the rhinoceros endometrium is affected by anatomical and management challenges. Unlike in domestic equids and bovids, from which endometrial tissue can be collected either via uterine biopsy or immediately post-mortem from healthy individuals, rhinoceros have a long, tortuous cervix that limits the ability to collect endometrial biopsies from live individuals. This anatomical difference means rhinoceros endometrial tissue only to be available for collection from deceased animals, which either die of old age or severe illness. Moreover, the time from death to endometrial tissue procurement may be prolonged (up to 24 h) before the tissue can be processed for culture, due to the complexity and labor intensity of performing a full necropsy on a megafauna species. The health status of the animals and the prolonged time from death to tissue processing may have contributed to the poor organoid growth and proliferation in these trials. Lastly, morphological and growth differences may simply reflect species-specific biology.

Future experiments with additional rhinoceros endometrial organoid replicates, ideally from rhinoceroses without systemic or reproductive diseases, are needed to better understand their growth requirements and to define the expected morphology and proliferation rates. The time from euthanasia to tissue collection will be reduced in future experiments by coordinating with zoological pathology teams to determine whether this factor is driving the reduced growth and proliferation. Only once the disease state of the animals and time between euthanasia and tissue collection is optimized will culture supplements be evaluated to determine whether an individual supplement is the cause of reduced growth. In a similar report of domestic animal endometrial organoids, the removal of insulin-transferrin-selenium (ITS) and SB202190 supplements and addition of R-Spondin-1 and HGF supplements also demonstrated successful organoid growth and proliferation [9], which may be a useful starting place to optimize the rhinoceros endometrial organoid culture medium composition. The next steps are to optimize organoid production from post-mortem rhinoceros reproductive tissues and to initiate advanced biobanking of reproductive tissue and tissue-derived organoids from various reproductive health and disease states. Optimizing organoid culture conditions is crucial for ensuring the accurate replication of organ-specific structures and species-specific functions. Although minimally explored in wildlife, this field holds great potential for future conservation strategies focused on reproduction and animal health within the One Health framework.

CRediT authorship contribution statement

Carly Young: Resources. **Hollinshead Fiona:** Writing – review & editing, Supervision, Resources, Conceptualization. **Shotaro Nakagun:** Formal analysis, Methodology, Writing – review & editing. **Elena Ruggeri:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Thompson-Brandhagen Riley:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Data curation. **Meyers Melinda:** Writing – review & editing, Methodology. **Holly Haefele:** Project administration. **Adam Eyres:** Project administration. **Barbara Durrant:** Writing – review & editing, Project administration.

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Declaration of Competing Interest

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

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Data availability

All data is available in this manuscript.

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