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Characterization of oviduct epithelial spheroids for the study of embryo–maternal communication in cattle

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ABSTRACT

Most *in vitro* models of oviduct epithelial cells (OEC) used thus far to gain insights into embryo–maternal communication induce cell dedifferentiation or are technically challenging. Moreover, although the presence of developing embryos has been shown to alter gene expression in OEC, the effect of embryos on OEC physiology remains largely unknown. Here, we propose a model based on bovine oviduct epithelial spheroids (OES) with specific shape and diameter (100–200 μm) criteria. The aims of this study were to i) determine the appropriate culture conditions of bovine OES cultured in suspension by evaluating their morphology, total cell number, viability, and activity of ciliated cells; ii) monitor gene expression in OES at the time of their formation (day 0) and over the 10 days of culture; and iii) test whether the vicinity of developing embryos affects OES quality criteria. On day 10, the proportions of vesicle-shaped OES (V-OES) were higher in M199/500 (500 μl of HEPES-buffered TCM-199) and synthetic oviduct fluid (SOF)/25 (25-μL droplet of SOF medium under mineral oil) than in M199/25 (25-μL droplet of M199 under mineral oil). The proportion of viable cells in V-OES was not affected by culture conditions and remained high (>80%) through day 10. The total number of cells per V-OES decreased over time except in SOF/25, while the proportions of ciliated cells increased over time in M199/500 but decreased in M199/25 and SOF/25. The movement amplitude of OES in suspension decreased over time under all culture conditions. Moreover, the gene expression of *ANXA1*, *ESR1*, *HSPA8*, and *HSPA1A* in OES remained stable during culture, while that of *PGR* and *OVGP1* decreased from day 0 to day 10. Last, the co-culture of developing embryos with OES in SOF/25 increased the rates of blastocysts on days 7 and 8 compared to embryos cultured alone, and increased the proportion of V-OES compared to OES cultured alone. In conclusion, M199/500 and SOF/25 provided the optimal conditions for the long-time culture of OES. The supporting effect of OES on embryo development and of developing embryos on OES morphology was evidenced for the first time. Altogether, these results point OES as an easy-to-use, standardizable, and physiological model to study embryo–maternal interactions in cattle.

1. Introduction

The oviducts are paired ducts composed of three parts: the infundibulum, covering the ovary; the ampulla; and the isthmus, the narrower part, which finishes at the utero–tubal junction. Although the oviduct is a tiny part of the female genital tract, it endorses several major functions to secure the success of pregnancy [1]. At ovulation, the cumulus–oocyte complex (COC) is captured by the infundibulum, then driven by beating cilia to the ampulla, where it awaits fertilization. On the other side, spermatozoa travel through the female genital tract to the oviduct and

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bind to the ciliated epithelium of the isthmus until they are released toward the ampulla to meet the oocyte. If fertilization occurs, the newly formed embryo develops in the isthmus through the 8–16-cell to morula stages, when it enters the uterus [2,3].

Although *in vitro* embryo production has been successfully achieved in different species, it bypasses this oviduct milieu, resulting in low success in terms of blastocyst rate and capacity to give rise to healthy offspring (Review in: [4]). Therefore, oviduct epithelial cells (OEC) cultured *in vitro* have been widely used to mimic the maternal environment and improve embryo development in different species, including rabbits [5], sheep [6], goats [7], mice, pigs, cattle, and humans [1]. Co-culturing with OEC has been shown to enhance blastocyst quality in terms of cell numbers, cryotolerance [8], and expression of target genes in cattle [9], as well as pregnancy rates in humans [10], red deer [11], and goats [12].

Two culture systems of OEC have mainly been used to support embryo development *in vitro*. The first and most documented one is OEC monolayers grown on plastic dishes. This model revealed for first time the existence of a real dialogue between embryos and OEC during co-culture, providing increased embryo development rate and quality [8,9,13], as well as changes in OEC gene expression profiles as a result of the co-culture with embryos [14,15]. In addition, OEC-derived conditioned media supported embryo development [16], indicating that soluble factors are also involved. OEC adherent to plastic rapidly dedifferentiate, however, losing morphological criteria of cell differentiation like cilia and secretory granules after 3 days in culture [17]. Moreover, after 5–10 days in culture, OEC displayed a decrease in gene expression of steroid hormone receptors and oviduct-specific glycoprotein 1 (*OVGP1*) [8]. The second system consists of OEC monolayers grown on inserts in air–liquid interphase (ALI). This system maintains epithelial morphology more similar to the *in vivo* ones, including ciliated cells, columnar shape, and intercellular cohesion, as well as the gene expression of steroid hormone receptors and *OVGP1* [18]. The ALI culture of OEC, however, takes long to establish (3–4 weeks, depending on the species), is technically challenging and so far has not shown any supporting effect on development of co-cultured bovine embryos [19,20]. More recently, vesicle-shaped organoids derived from oviduct epithelial stem cells have been reported [21,22], but their production is long and challenging. In addition, the apical side of OEC is inside the organoid, making it difficult to interact with developing embryos.

The culture of OEC in suspension has been proposed as an alternative. Walter (1995) [17] reported the first culture of free-floating bovine OEC forming vesicles and demonstrated that this culture could better maintain the cilia and secretory granules compared to confluent monolayers after 12 days in culture. Rottmayer et al. (2006) [23] proposed a short time suspension culture of OEC aggregates for 24 h and evidenced the maintenance of their morphology and stable gene expression of *OVGP1* and steroid hormone receptors after 6 h of culture. The possibility, however, of culturing OEC in suspension during the time needed for supporting embryo development, i.e., 7–9 days, has not been yet assessed. Moreover, OEC aggregates in suspension are not morphologically uniform in shape and size, a disparity that can be a source of variability between replicates; thus, they require proper characterization and standardization to be used for embryo co-culture purposes. Here, we propose a model based on oviduct epithelial spheroids (OESs), which differ from oviduct organoids in that they form rapidly (within 48–72 h) from isthmic mucosa fragments (IMF) containing an already-differentiated epithelium. We previously reported OESs with specific shape and size criteria (to avoid variability between replicates) as a good model to study sperm–oviduct interactions in cattle [24]. Here we propose using OES for suspension co-culture with embryos. For this purpose, we based our model on the bovine species since it represents an excellent model for human reproduction [25] and has been widely used to study the early embryo–maternal interactions in the oviduct. Moreover, bovine oviducts are available from the slaughterhouse, allowing it to be used in experiments without further concerns about rare samples, breeding season, or ethics.

For the present study, we hypothesized that 1) OESs cultured in suspension may maintain well-differentiated OEC and stable gene expression for 10 days; 2) the density of OESs and culture medium composition may affect their quality in terms of morphology, cell viability, and activity of ciliated cells; and 3) the co-culture of OESs with developing embryos may support embryo development and OES quality. Therefore, the objectives in this study were to: i) determine the appropriate culture conditions of bovine OESs by evaluating their morphology, total cell number, viability, and activity of ciliated cells; ii) monitor gene expression in OESs at the time of their formation (day 0) and over the 10 days of culture under the best conditions; and iii) test whether the vicinity of developing embryos affects embryo development and OES quality.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Merck (St. Louis, MO, USA) unless otherwise stated.The following were used: phosphate-Buffered Saline (PBS; 1X, Eurobio Scientific, France, CS1PBS01-01), 4% paraformaldehyde (Santa Cruz Biotechnology, SC-281692), Triton X-100 (9036-19-5), bovine serum albumin (BSA; A9647), Hoechst 33342 (B2261; 1 mg/mL), ethidium homodimer-1 (Invitrogen E1169, MA, USA), anti-cytokeratin (C2931), anti-acetylated tubulin (T7451), IgG1 (M9269), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen A11001, MA, USA), Texas Red™-X Phalloidin (Invitrogen T7471, MA, USA; 2 U/mL in methanol), mineral oil (ORIGIO Denmark), QIAGEN RNeasy Plus Micro Kit (Catalog no. 74034), carrier RNA (QIAGEN, 1068337), QuantiTect® Reverse Transcription Kit (QIAGEN, 1068337), iQTM SYBR® Green Supermix (Bio-Rad, 170-8886), GenElute™ PCR Clean-Up Kit (NA1020).

2.1.2. Media used for cell culture and in vitro embryo production

The OES washing media was HEPES-buffered TCM-199 contained TCM-199 (Gibco 31150–022) and 25 mM HEPES (Gibco 15630-080). Two different media were used for the culture of OES. The **M199** medium was TCM-199 supplemented with 25 mM HEPES, 10% heat-inactivated fetal calf serum (FBS; Gibco A5256701) and 80 mg/L gentamycin (G1272). The **SOF** was synthetic oviductal fluid medium modified according to Holm et al. (1999) [26] and supplemented with 5% heat-inactivated FBS (MP Biomedicals, Ref: 2916749, batch MP5418).

The washing medium for oocyte collection (mPBS) was demineralized water with 8 g/L NaCl (S-7653), 0.2 g/L KCl (Prolabo 26764298), 0.2 g/L KH_2PO_4 (Prolabo 26936293), 1.43 g/L $Na_2HPO_4.2H_2O$ (Prolabo 28029292), 0.14 g/L $CaCl_2.2H_2O$ (Merck 2382), 0.2 g/L $MgCl_2.6H_2O$ (Merck 5833), 1 g/L D-glucose (S-8270), 0.036 g/L Pyruvate (P-4562), 50 mg/L gentamycin (G1272), 2 mg/L phenol red (P0290), and 0.5 mg/L BSA (A9647), with osmolarity and pH adjusted to 280 mOsm and 7.4, respectively [16]. The maturation medium was TCM-199 (M4530) supplemented with 5 IU/mL hCG, 10 IU/mL PMSG (PG600, Intervet), 19 ng/mL IGF-1, 2.2 ng/mL FGF, 10 ng/mL EGF, 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 90 μg/mL L-Cystein, 100 μM β-mercaptoethanol, 75 μg/mL ascorbic acid, 720 μg/mL glycine, 0.1 mg/mL glutamine, and 110 μg/mL Pyruvate [8,27]. The sperm-washing medium was STL medium based on Tyrode medium supplemented with 25 mM bicarbonate (S5761), 10 mM lactate (L7900), 2.4 mg/mL HEPES (H3375), 6 mg/mL BSA (A9647), and 40 μg/mL gentamycin (G1272) [8,28]. The Tyrode medium was a mix of 1 L demineralized water with 6.666 g NaCl (Merck 6404), 240 mg KCl (Prolabo 26764298), 41 mg $NaH_2PO_4.H_2O$ (Merck 6346), 300 mg $CaCl_2.2H_2O$ (Merck 2382), 100 mg $MgCl_2.6H_2O$ (Merck 5833), and 2 mg/L phenol red (P0290) with osmolarity at 230 mOsm. The fertilization medium was Tyrode medium

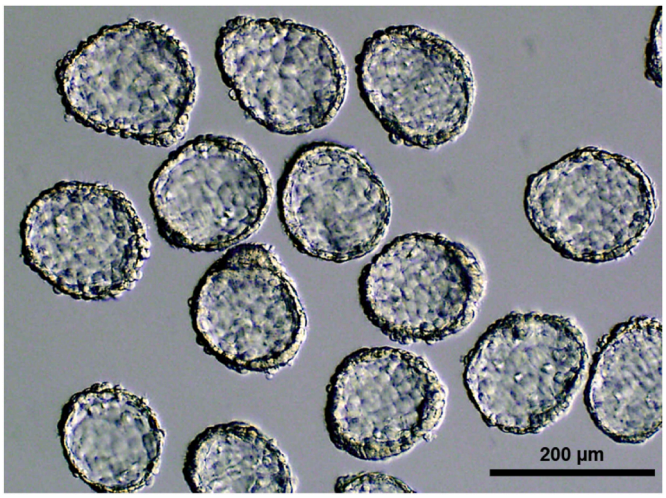


Fig. 1. Bovine oviduct epithelial spheroids (OES) selected on day 0. Vesicle-shape spheroids containing a cavity, homogeneous in form and size (100–200 µm in diameter) and displaying outward ciliary beating were selected in this study.

supplemented with 25 mM bicarbonate (S5761), 10 mM lactate (L7900), 1 mM pyruvate (S4562), 6 mg/mL BSA (A6003), 100 µg/mL heparin (Calbiochem Ref: 375 D95 batch B47089), and 40 µg/mL gentamycin (G1272) [8,28].

2.2. Culture of bovine oviduct epithelial spheroids (OES)

Oviductal cell isolation and culture were conducted as described previously [29]. Briefly, pairs of oviducts and ovaries obtained from post-pubertal cows were collected at a local slaughterhouse (Vendôme, France) and transported at 4 °C to the laboratory. Pairs of oviducts at the peri-ovulatory phase of cycle (approximately day −2 to day +4 around ovulation time) were selected according to the morphology of the ovaries. The pre-ovulatory phase of the cycle was identified by a follicle at 11–20 mm in diameter and a small yellow-white *corpus albicans*. The post-ovulatory phase of the cycle was identified by a small *corpus heamoragicum* (<0.5 cm) and the *corpus albicans* from the previous cycle. For each culture, both oviducts from a pool of 2–3 cows were used. After removal of blood vessels and connective tissue, the oviducts were cut at the ampullary–isthmic junction, when the oviduct diameter becomes smaller and with a more folded and thicker wall. Only the isthmic parts (around 6–8 cm long) of the oviducts were used. After a rapid dip in 70% ethanol and rinsing in 0.9% NaCl, the mucosa was expelled from the isthmic sections by squeezing with forceps into 10 mL of M199, vortexed for 1 min, then incubated at 38.8 °C for 10 min for cell sedimentation. Following the elimination of the supernatant containing cell debris and blood red cells, the pellet (around 1 mL) was resuspended in 10 mL of M199, and the vortex-sedimentation process was repeated. Finally, the pellet was diluted 10 times in the culture medium, and 50 µL of the resulting mixture containing isthmic mucosa fragments (IMFs) was added to 450 µL of M199 to reach a 100-fold final dilution. In the following, unless otherwise specified, “day 0” refers to the day of sorting and culture start of OES. On day −3, the IMFs were cultured in 4-well culture plates (Thermo Fisher Scientific, Denmark) at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air. On day 0, a cavity appeared within the mucosa fragments, forming spheroids of various sizes and shapes, with the apical side of the epithelial cells oriented outward. Spheroids between 100 and 200 µm in diameter, homogeneous in shape and size, and exhibiting a cavity and ciliary beating outward, referred to as “vesicle-shaped OES” (V-OES; Fig. 1 and Movie 1) were selected using a mouth-operated drawn Pasteur pipette (Duran Wheaton Kimble, around 300 µm in diameter at the extremity; day 0) for characterization in different culture conditions until day 10 of culture (Experiment 1), for gene expression analysis (Experiment 2), or for co-culture with *in vitro*-produced bovine embryos (Experiment 3; see paragraph 2.8 for experimental design). All OESs, including those that had lost their cavities (collapsing OES), were kept in the culture medium through day 10 of culture.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.theriogenology.2024.01.022

For gene expression analysis by RT-qPCR, the isthmic mucosa fragments (200/replicate) and V-OES at days 0, 3, and 10 of culture (200–400/replicate) were collected. V-OES between 100 and 200 µm in diameter were cultured in 1 mL of M199 (200–400 OES/mL) in a 60 × 15 mm culture dish (Falcon®, 353037). Half of the medium was renewed every 3 days, taking care not to remove any OES. Four biological replicates from 4 different cultures were used. All samples were collected with a minimum volume of medium in a 1.5 mL Ultra High Recovery Microcentrifuge Tube (STARLAB, E1415-2600, USA), immediately snap-frozen in liquid nitrogen, and stored at −80 °C before analysis.

2.3. Evaluation of OES morphology and straight-line velocity

For evaluation of OES morphology and movement, an inverted microscope (Nikon, Japan) equipped with a SPOT Insight Firewire 2 mega Sample Camera, the SPOT Advanced Software (version 4.5.9.1, USA), and a warm plate were used. Pictures of OES on days 0, 3, and 10 of culture were taken at 40× magnification. For motion analysis, groups of 20–25 OESs in suspension on days 0, 3, and 10 were gently placed at the center of the well and left there for 15 s for stabilization, then 15 successive pictures at 2-s intervals were taken at 40× magnification, allowing us to calculate the proportion of moving OESs and track the movement of individual OESs. All experiments were conducted by the same person to avoid an operator effect. The straight-line track of moving OESs (in pixels) was calculated from the first and last pictures at a 30-s interval using the TrackMate plugin in ImageJ software (version 1.54f) and expressed in µm/sec. The automated tracking of each OES was manually corrected using the TrackScheme function of the TrackMate plugin. Then, the mean straight-line velocity (in µm/sec) of moving OESs per condition was calculated.

2.4. Evaluation of cell viability

For the assessment of cell viability, V-OES collected on days 0, 3, and 10 of culture were washed twice in HEPES-buffered TCM-199, then incubated in 500 µL HEPES-buffered TCM-199 containing 2 µg/mL of Hoechst 33342 and 4 µM of ethidium homodimer-1 for 30 min in the dark at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air. Then, V-OES were washed and mounted on a glass slide (SuperFrost Plus™, Epredia, Germany) for observation under a confocal microscope (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany) at 200× magnification. Live (blue-nucleus) and dead (purple-nucleus) cells in whole individual V-OES were counted using the QuPath software (version 0.2.2; Fig. 4a). On day 10, V-OES and collapsed OES were also assessed for cell viability using the LIVE/DEAD cell viability assay (Invitrogen, MA, USA).

2.5. Immunostaining of oviduct epithelial spheroids

Immunostaining for pan-cytokeratin (a marker of epithelial cell intermediate filaments), vimentin (marker of stroma cells), and acetylated alpha-tubulin (marker of ciliated cells) was performed as previously described [30,31]. Briefly, V-OESs cultured in M199/500 were fixed and permeabilized (4% paraformaldehyde supplemented with 1% BSA and 0.25% Triton X-100, 30 min at 37 °C) on days 0, 3, and 10, washed 3 x in PBS + 1% BSA (PBS-BSA), and incubated in a blocking solution (10% goat serum in PBS-BSA, 30 min, room temperature). V-OESs were then

Table 1
Primers used in the study. F: forward primer; R: reverse primer; bp: base pairs.

Gene Symbol	Gene Name	Primer (5′–3′)	Accession Number	Amplicon size (bp)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCCAGAAGACTGTGGATGG R: ATGCCTGCTTCACCACCTTC	NM_001034034.2	245
PPIA	Peptidylprolyl isomerase A	F: GCATACAGGTCCTGGCATCT R: TTCTTGCTGGTCTTGCCATT	MK309342.1	192
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	F: ACTGGGTCTGGCCCTTAACT R: CTGCTTCAGCTTCGTCTCCT	MK396254.1	218
ESR1	Estrogen receptor 1	F: AGGGAAGCTCCTATTTGCTCC R: CGGTGGATGTGGTCCTTCTCT	NM_001001443.1	234
ESR2	Estrogen receptor 2	F: TGATGCTCCTGTCTCACGTC R: AGCCCTCTTTGCTCTCACTG	NM_174051.3	195
PGR	Progesterone receptor	F: GATGCTATATTTTGCGCCTGA R: CTCCTTTTTGCCTCAAACCA	NM_001205356.1	266
OVGP1	Oviductal glycoprotein 1	F: AAGAATGAGGCCCAGCTCAC R: TGCCGAAGATTTGGGGTCTC	NM_001080216.1	219
ANXA1	Annexin A1	F: ACCAGGAGCTATCCCCATCT R: AAAGAACATTGGCTGGCTTG	NM_175784.3	156
VMAC	Vimentin type intermediate filament associated coiled-coil protein	F: ATTGAGCGCCGCTTTAGAC R: CAGCCTGCAGACTCTGAACA	NM_001105371.1	107
HSPA1A	Heat shock protein family A (Hsp70) member 1A	F: AGGCGGACAAGAAGAAGGTG R: GTTACACACCTGCTCCAGCT	NM_203322.3	122
HSPA8	Heat shock protein family A (Hsp70) member 8	F: CGCAATGAATCCCACCAACA R: CCACCATGAAGGGCCAATGT	NM_174345.4	107

incubated overnight at room temperature with the primary antibody (anti-cytokeratin, C2931; anti-vimentin, V6630, or anti-acetylated tubulin, T7451; final concentrations of 15.7, 28.5, and 2 μg/mL, respectively). The control V-OESs were incubated with IgG1 (M9269) at the same concentration as the primary antibody. After washing in PBS-BSA, the OESs were incubated with the secondary antibody coupled with Alexa Fluor 488 (A11001; final concentration at 1 μg/mL), counterstained with Hoechst 33342 and Texas Red™-X Phalloidin at a final concentration of 1 μg/mL and 2 mU/mL, respectively, for 3 h at room temperature under agitation in the dark. After washing in PBS-BSA, V-OESs were then mounted on a glass slide, observed under a confocal microscope at 200× magnification, and examined using the QuPath software (version 0.2.2).

2.6. In vitro embryo production (IVP)

2.6.1. Oocyte collection and in vitro maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse (Vendôme, France) and kept in 0.9% NaCl solution at 31–32 °C during transport (45 min) to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from 3 to 6 mm follicles using an 18½-gauge needle connected with a suction pump. Immature oocytes enclosed in at least 3 layers of compacted cumulus cells with homogeneous cytoplasm were selected and washed twice in mPBS, then once in 1 mL of maturation medium. A group of 50–80 COCs were cultured in a 4-well plate (Thermo Fisher Scientific REF179830, Denmark) containing 500 μL/well of maturation medium for 22–23 h at 38.8 °C in a humidified atmosphere with 5% CO_2 in air.

2.6.2. In vitro fertilization (IVF)

The procedures were implemented as described previously by Schmaltz-Panneau et al. (2015) [8]. Briefly, a pool of frozen semen from 2 Holstein bulls of proven fertility was used for all IVF (0.25 mL straw). Straws were thawed in air for 10 s and submerged in 35 °C water for 30 s. Subsequently, frozen-thawed semen was transferred to the top of the Percoll density gradient (45/90%; Cytiva 17-0891-01), centrifuged at 700g for 20 min to retrieve the viable spermatozoa at the bottom, then centrifuged at 100g for 10 min in 5 mL of STL medium to eliminate Percoll. Sperm concentration was evaluated in Thoma cell and adjusted to 4 x 10^6 spermatozoa/mL with fertilization medium. Mature oocytes were washed once with 1 mL of fertilization medium and transferred to a 4-well plate (Thermo Fisher Scientific REF179830, Denmark) containing 250 μL of fertilization medium and 50–80 oocytes/well, and 250 μL of the sperm suspension were added to reach a final concentration of 2 x 10^6 spermatozoa/mL. The dishes were incubated for 18 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. The day of fertilization was considered day 0.

2.6.3. Embryo culture (IVC)

After IVF, cumulus cells and attached sperm were removed from presumptive zygotes by vortex at moderate speed for 2 min in 2 mL of mPBS. Presumptive zygotes were then washed twice in mPBS, then once in the SOF medium. Next, groups of 25 presumptive zygotes were cultured in 25 μL droplets of SOF with 25 OES (SOF/25/E) at 38.8 °C for 8 days without medium changing under 5% CO_2 in air. The day of culture was considered day 1. Cleavage rates were evaluated on day 2 and blastocyst formation rates on days 6, 7, and 8 using an inverted microscope (Olympus IX70, Japan).

2.7. Gene expression analysis by RT-qPCR

RNA extraction was performed using the QIAGEN rNeasy Plus Micro kit according to the manufacturer's instructions and adding 0.01 μg/μL Poly-A carrier RNA (QIAGEN) into the cell lysates. RNA concentration and purity (A260/A280 ratios > 2) were examined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Denmark). For each sample, 130 ng of total RNA were treated with DNase before reverse transcription (RT) in a final volume of 20 μL using the Quanti-Tect® Reverse Transcription kit following the manufacturer's instructions. The primers of 8 target genes (*ESR1, ESR2, PGR, OVGP1, ANXA1, VMAC, HSPA1A*, and *HSPA8*) and 3 reference genes (*GAPDH, PPIA*, and *YWHAZ*) were designed using the Ensembl database (https://www.ensembl.org/) and Primer3Plus (https://www.primer3plus.com/; see Table 1 for details).

For each gene, a standard curve was created by 1/10 serial dilutions of mucosa fragment cDNAs. The qPCR reactions were conducted in a final volume of 20 μL (1 μl of 6.5 ng/μl cDNA template, 0.25 μl of each primer at 10 μM, 8.5 μl of water, and 10 μl of iQTM SYBR® Green Supermix) using a BIO-RAD instrument (CFX Opus 96 Real-Time PCR System). Two technical replicates of each sample were performed under the following condition: 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. Only genes expressed with Cq < 32 were considered for expression analysis [32]. The normalization factor of the combination of reference genes (*GAPDH*, *PPIA*, and

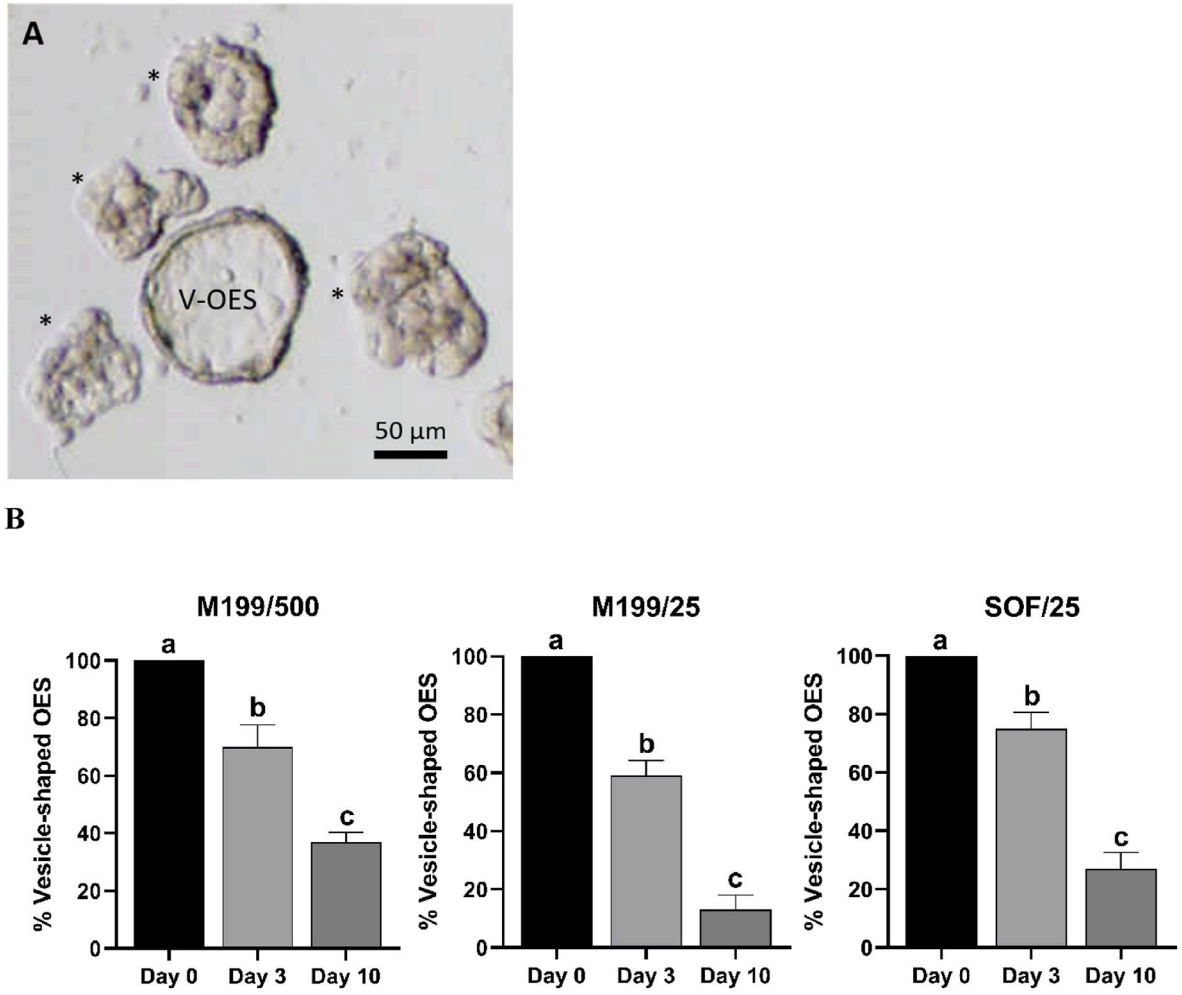


Fig. 2. Proportions of OES keeping their cavity over time in three different culture conditions (Experiment 1). A, Representative picture of one vesicle-shape OES (V-OES) surrounded by four OES (asterisks) that lost their cavity on day 10 of culture in M199/500; B, Proportions of OES with a cavity on days 0, 3 and 10 of culture under three different culture conditions. Groups of 25 OES were cultured either in 500 µL of M199 (M199/500), 25-µL droplet of M199 under mineral oil (M199/25) or 25-µL droplet of SOF (SOF/25) for 10 days. Data are means ± SEM of 4 replicates (N = 100 OES/condition). The different letters on bars indicate significant differences between days ($P < 0.0001$).

YWHAZ) obtained from geNorm algorithms (version 3.5) was used to calculate normalized relative gene expression using the ΔΔCt method, as previously described [33].

2.8. Experimental design

Experiment 1 characterized OES morphology, total cell number, viability, proportion of ciliated cells, and motion under 3 different conditions during 10 days of culture. For this purpose, groups of 25 OESs were allocated to one of the 3 following conditions at day 0: 1) 500 µL of M199 (M199/500); 2) a 25-µL droplet of M199 overlaid with mineral oil (M199/25); or 3) 25-µL droplet of SOF medium overlaid with mineral oil (SOF/25). In the M199/500 group, half of the fresh medium was renewed every 3 days. OESs were then evaluated for their morphology, total cell number, percentage of viable cells, percentage of ciliated cells, and motion (percentage of moving OES and straight-line velocity).

Experiment 2 evaluated the immunodetection of cytokeratin and vimentin in the V-OESs and of the candidate genes by RT-qPCR in IMF and V-OES during culture in M199. For this purpose, OESs were cultured in 1 mL of M199. This experiment was conducted only in M199, as it was the medium maintaining the best OES morphology and widely used for cell culture. Only V-OESs were included in the experiment to avoid bias due to the collapsing process.

Experiment 3 evaluated the effects of OES on blastocyst yield and of embryo co-culture on OES morphology, total cell number, viability, proportion of ciliated cells, and motion in SOF/25. For this purpose, on day 0, OES were cultured in 1 mL of M199 until day 2, then transferred into groups of 25 in 25-µL droplets of SOF overlaid with mineral oil (SOF/25) for 24 h. On day 3, groups of 25 OES in SOF/25 were cultured alone (OES control group) or with 25 presumptive *in vitro*-produced zygotes (SOF/25/E) for 8 days, i.e. up to day 10. A control group of 25 presumptive zygotes was cultured without OES in SOF/25 for 8 days (embryo control group). In both groups with embryos, the number of cleaved embryos was assessed on day 2 after IVF, and the numbers of blastocysts were assessed on days 6, 7, and 8 after IVF. In both groups with OES, the OES quality was evaluated on days 0, 3, and 10 using the same criteria as in Experiment 1.

2.9. Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 8.1.1) and Rstudio (R version 4.3.0) [34]. The normality of the data was analyzed using the Shapiro-Wilk test. The effect of the different culture conditions on the proportion of V-OES, total cell number, cell viability, ciliated cells, moving OES, and straight-line velocity (µm/s) were analyzed by one-way ANOVA, followed when

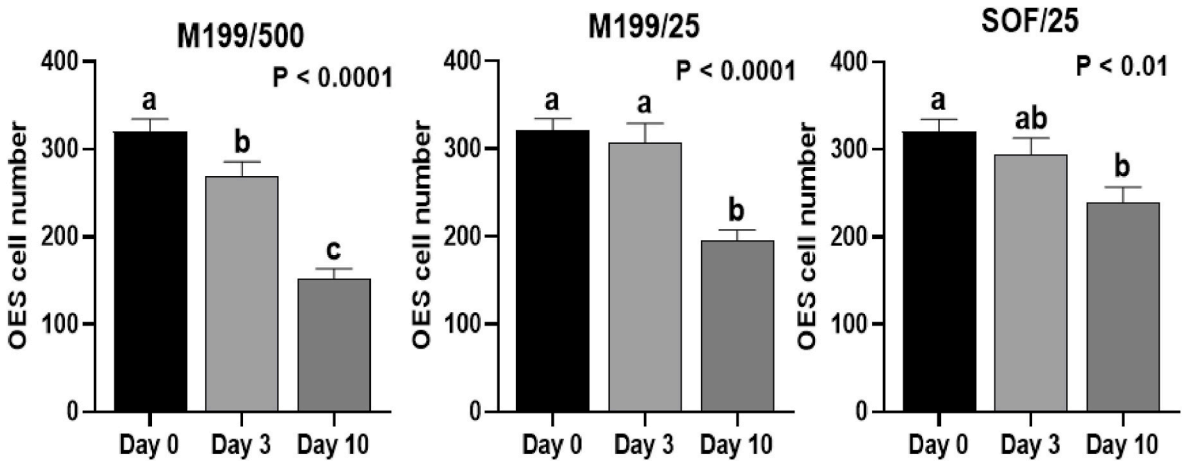
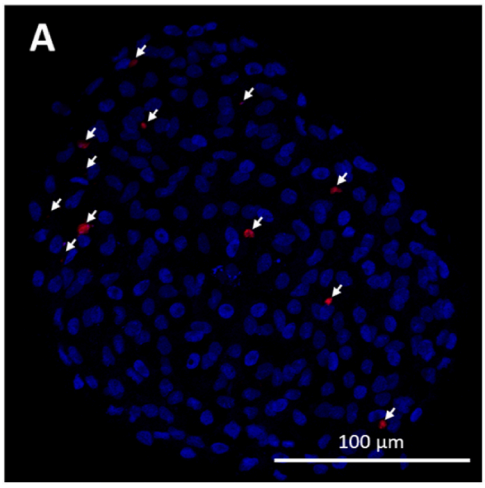


Fig. 3. Total cell number in vesicle-shaped OES over time in three different culture conditions (Experiment 1). Groups of 25 OES were cultured either in 500 μL of M199 (M199/500), 25-μL droplet of M199 under mineral oil (M199/25) or 25-μL droplet of SOF under mineral oil (SOF/25) for 10 days. Data are provided as the mean ± SEM of 8 replicates (N = 53-87 OES/condition). The different letters on bars indicate significant differences between days.



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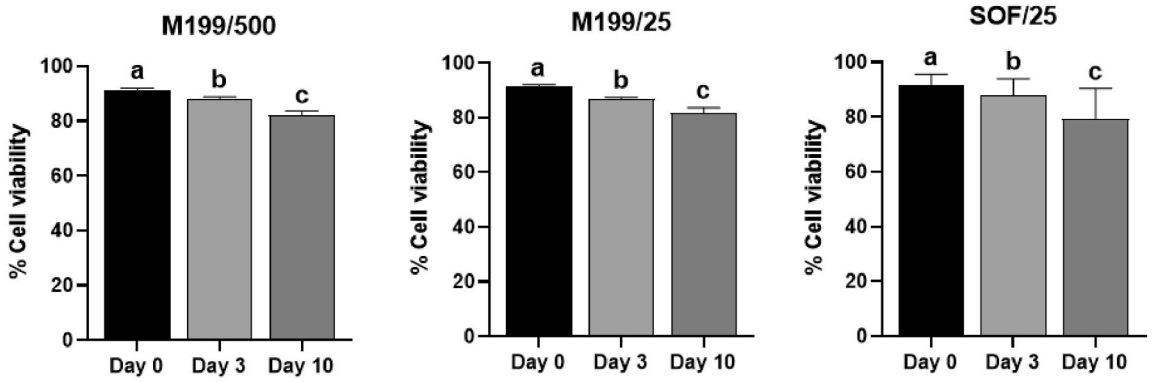

Fig. 4. Cell viability in vesicle-shaped oviduct epithelial spheroid (OES) over time in three different culture conditions (Experiment 1). A, Representative picture of one vesicle-shaped oviduct epithelial spheroid after staining for assessment of cell viability. The nuclei of live cells appear in blue (stained with Hoechst) while nuclei of dead cells appear in purple (Hoechst + ethidium homodimer-1, arrows); B, Proportions of viable cells in vesicle-shaped OES at days 0, 3 and 10 of culture under three different culture conditions. Groups of 25 OES were cultured either in 500 μL of M199 (M199/500), a 25-μL droplet of M199 under mineral oil (M199/25) or a 25-μL droplet of SOF (SOF/25) for 10 days. Data are provided as the mean ± SEM of 4 replicates (N = 31-53 OES/condition). The different letters on bars indicate significant differences between days ($P < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

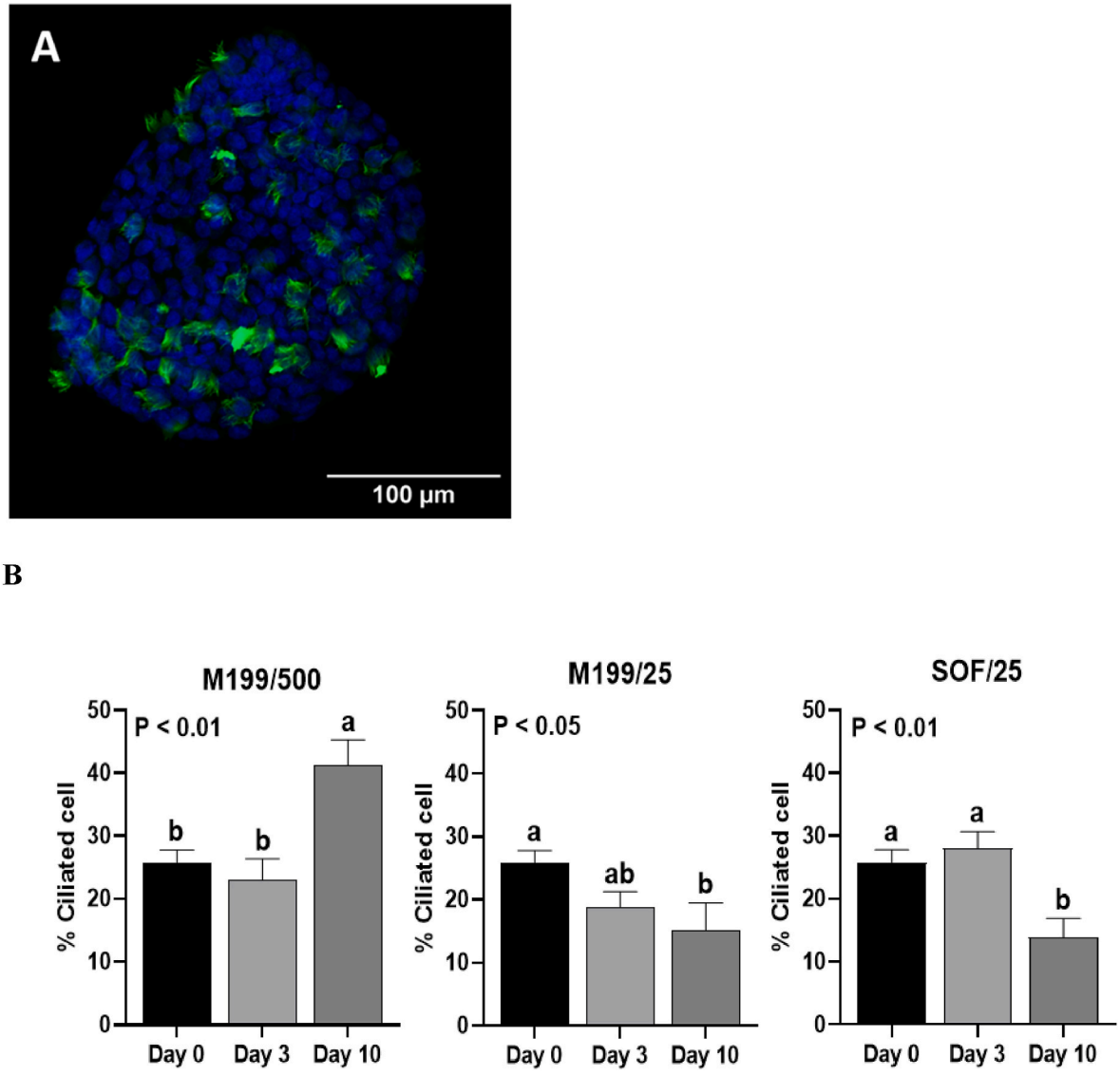

Fig. 5. Immunostaining for alpha-tubulin and proportions of ciliated cells in vesicle-shaped oviduct epithelial spheroids (OES) over time in three different culture conditions (Experiment 1). A, Representative picture of one vesicle-shaped OES after immunostaining of acetylated alpha-tubulin for assessment of ciliated cells. B, Groups of 25 vesicle-shaped OES were cultured either in 500 µL of M199 (M199/500), a 25-µL droplet of M199 under mineral oil (M199/25) or a 25-µL droplet of SOF (SOF/25) for 10 days. Data are provided as the mean ± SEM of 4 replicates (N = 15-34 OES/condition). The different letters on bars indicate significant differences between days.

appropriate by Tukey's post-tests. The effects of OES on blastocyst yield on days 6, 7, and 8 and of the developing embryos on OES quality parameters on day 10 were examined using Student t-tests. The RT-qPCR data were analyzed by t-tests (effect of spheroid formation on day 0) and one-way ANOVA followed by Tukey's post-tests (effect of culture time in OES). The cDNAs with a detection threshold beyond 32 Cq were excluded from the analysis. A p-value < 0.05 was considered significant. All data are presented as means ± standard error of the mean (SEM).

3. Results

3.1. Effect of culture conditions on OES morphology, total cell number, viability, proportion of ciliated cells, and motion

The proportions of V-OES, i.e. OES with a cavity, decreased over time in all culture conditions due to OESs collapsing (Fig. 2A) and the attachment of a few OESs at the bottom of the well. The proportion of floating OESs that kept their cavity (V-OES) did not differ between culture conditions on day 3 (70.0 ± 7.7%, 59.0 ± 5.3%, and 75.0 ± 5.7% in M199/500, M199/25, and SOF/25, respectively; Fig. 2B). On day 10, however, the proportion of V-OESs was significantly higher in M199/500 and SOF/25 (37.0 ± 3.4% and 27.0. ± 5.5%, respectively) than in M199/25 (13.0 ± 5%; $P < 0.05$).

The total number of cells per V-OES decreased over time in all culture conditions (Fig. 3; $p < 0.01$). On day 3, the total cell number per OES did not differ between culture conditions (means of 293.7 ± 19.1, 307.3 ± 21.8, and 268.4 ± 17.3 per V-OES for SOF/25, M199/25, and M199/500, respectively). On day 10, however, this number was significantly higher in SOF/25 (239.1 ± 18.1) than M199/500 or M199/25 (152.8 ± 10.7 and 195.2 ± 12.3, respectively; $p < 0.01$).

The proportion of viable cells in V-OES decreased significantly over time (Fig. 4b; $p < 0.0001$) but remained high (>80%) through day 10 in all culture conditions. Furthermore, the proportion of viable cells in the V-OES did not differ between culture conditions on days 3 and 10.

The proportions of ciliated cell in V-OES increased over time in M199/500 ($p < 0.01$) but decreased in M199/25 ($p < 0.05$) and SOF/25 ($p < 0.01$; Fig. 5). On day 3, the proportion of ciliated cells in V-OES did not differ between culture conditions. On day 10, however, this

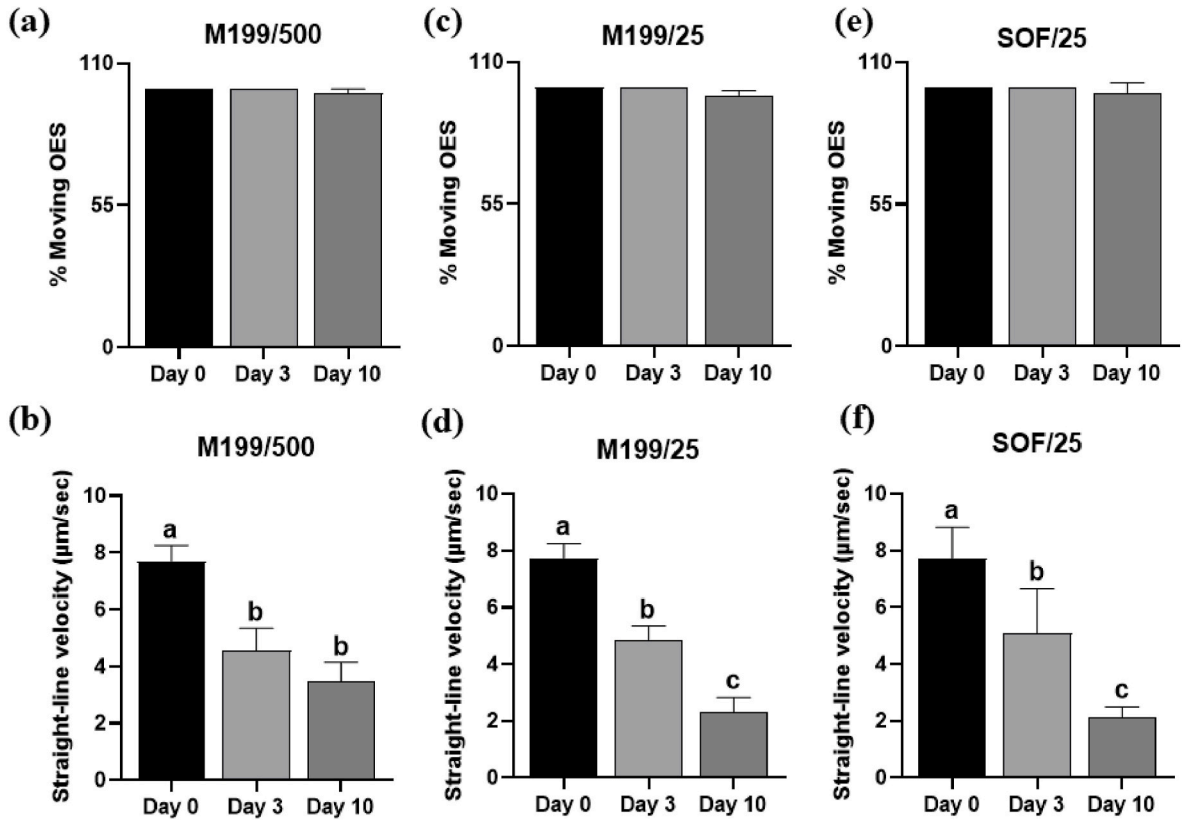

Fig. 6. Proportions of moving OES and straight-line velocity over time in three different culture conditions (Experiment 1). Groups of 25 OES were cultured either in 500 µL of M199 (M199/500; a and b), 25-µL droplet of M199 under mineral oil (M199/25; c and d) or 25-µL droplet of SOF (SOF/25; e and f) for 10 days. Data are provided as the mean ± SEM of 4 replicates (N = 66-96 OES/condition). The different letters on bars indicate significant differences between days ($P < 0.01$).

proportion was significantly higher in M199/500 (41.3 ± 4%) than M199/25 or SOF/25 (15.2 ± 4.3 and 13.9 ± 3%, respectively; $p < 0.0001$).

The proportions of moving OES due to ciliary beating remained high (>95%) in all culture conditions through day 10 of culture (Fig. 6a–c, and e; see Movie 1 for OES motion on day 0). Furthermore, the straight-line velocity of OES decreased over time (from 7.7 ± 0.6 µm/s on average on day 0 to 2.6 ± 0.5 µm/s on day 10; $p < 0.01$), with no difference between culture conditions (Fig. 6b–d, and f).

3.2. Expression of cytokeratin, vimentin, and candidate genes in OES cultured in M199 medium

Cytokeratin, a marker of epithelial cells, was immunodetected in the cytoplasm of V-OES cells through day 10 of culture (Fig. 7A). The stromal marker vimentin was not detected through day 6 of culture, but a positive signal could be observed in OES cells on day 10 (Fig. 7B). The gene expression of *VMAC* (a vimentin-type intermediate filament associated coiled-coil protein) and *ESR2* (estrogen receptor 2), as detected by RT-qPCR, was below the sensitivity threshold in the IMF and OES throughout the culture period. These 2 genes were not considered for statistical analysis.

When comparing the IMF and OES newly formed on day 0, a significant decrease in the expression of *ANXA1* (annexin A1), *OVGP1* (oviductal glycoprotein 1), *ESR1* (estrogen receptor 1), *HSPA8* (heat shock protein family A (Hsp70) member 8), and *HSPA1A* (heat shock protein family A (Hsp70) member 1A) was evidenced ($p < 0.05$), while the mRNAs for *PGR* (progesterone receptor) did not change significantly (Fig. 8). During OES culture, the gene expression of *ANXA1*, *ESR1*, *HSPA8*, and *HSPA1A* in OES remained stable through day 10, while *OVGP1* and *PGR* gene expression decreased after day 3 and reached lower levels on day 10 compared to day 0 ($p < 0.05$; Fig. 9).

3.3. Effect of OES co-culture on embryo development

Preliminary experiments indicated that V-OESs and collapsed ones contained viable cells with apparent ciliary beating through day 10 of culture (data not shown). Groups of 25 presumptive zygotes were cultured in the presence or absence of 25 OES in the SOF/25 for 8 days after IVF. Compared to embryos cultured alone, the presence of OES significantly increased the blastocyst rates on days 7 and 8 after IVF ($p < 0.01$; Table 2).

3.4. Effect of embryo co-culture on OES morphology, total cell number, viability, proportion of ciliated cells, and motion

Different parameters of OES were examined on day 10 after co-culture or not with embryo for 8 days in SOF/25. The presence of developing embryos during OES culture decreased the rate of OES collapsing. Indeed, the proportion of V-OES was significantly higher in the presence of embryos than in their absence (47.0 ± 5.3% *vs.* 27.0 ± 5.5%; $p < 0.05$, Fig. 10a). No other difference was found between groups concerning the total cell number per V-OES (239.2 ± 13.8 vs. 239.1 ± 18.1), percentage of live cells (78.4 ± 2.1 vs. 79.2 ± 1.7%), percentage of ciliated cells (13.7 ± 2.2 vs. 13.9 ± 3%), percentage of moving OES (97.3 ± 1.6 *vs.* 98 ± 2%), or their straight-line velocity (2.1 ± 0.4 vs. 2.1 ± 0.2 µm/s; Fig. 10b–f).

4. Discussion

The results of the present study show that OES represents a new *in vitro* alternative model over current ones for studying embryo–oviduct

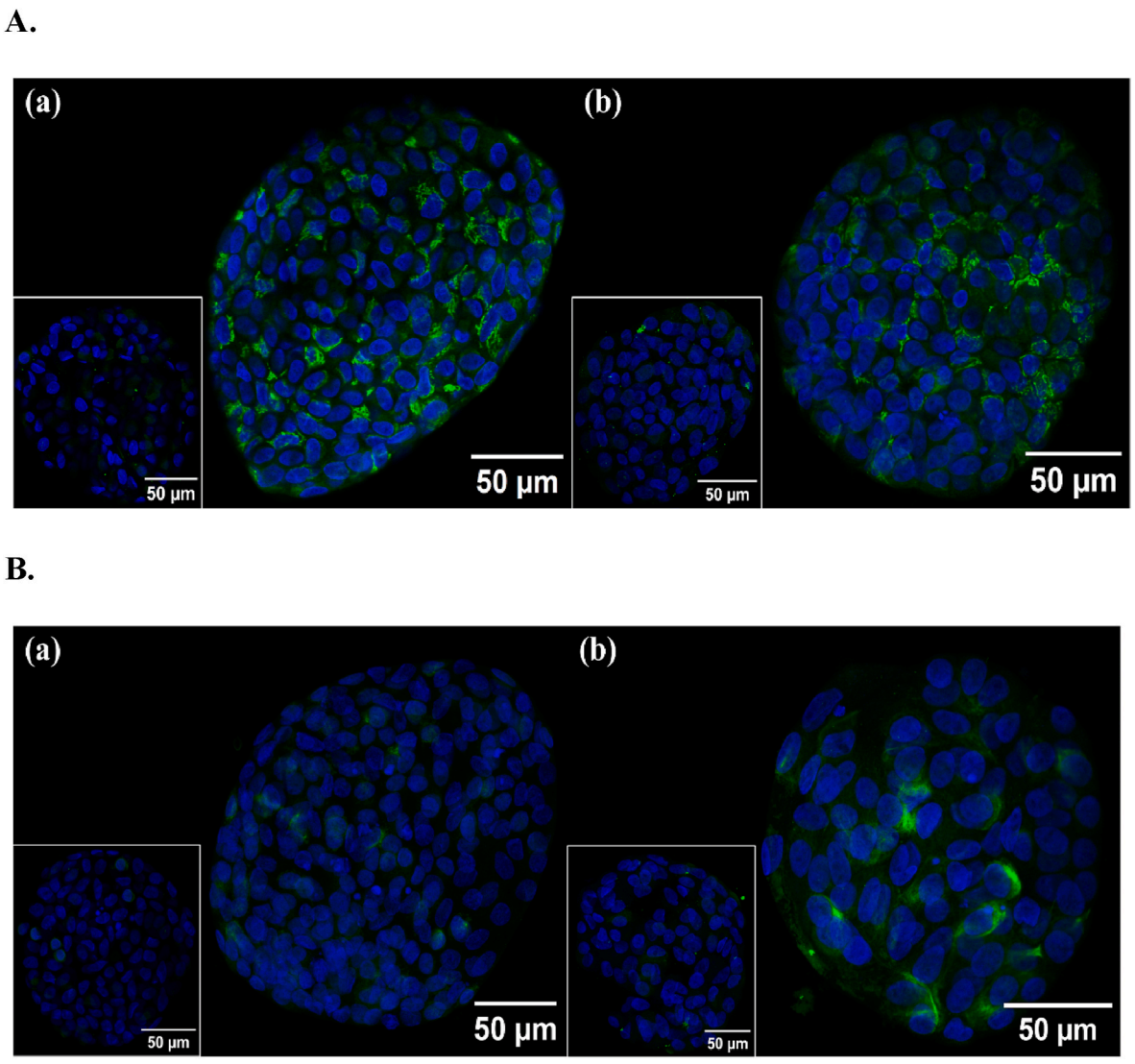

Fig. 7. Expression of cytokeratin and vimentin in vesicle-shaped OES (Experiment 2). A, Immunostaining for cytokeratin (in green) at day 0 (a) and day 10 (b); B, Immunostaining for vimentin (in green) at day 0 (a) and day 10 (b). The nuclei appear in blue (stained with Hoechst). Inserts show the negative controls incubated with the IgG1 isotype of the primary antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

interactions since: 1) the OES contained well-differentiated OECs, 25% of which contained cilia at the time of their formation from IMF; 2) two different culture conditions (M199/500 and SOF/25) allowed the maintenance of cell morphology and viability suitable for embryo culture; 3) OES maintained stable expression of cytokeratin and several gene markers over 10 days of culture; and 4) OES cultured in SOF/25 supported embryo development, while embryos co-developing with OES helped to maintain the spheroid cavity.

4.1. Effect of culture conditions on OES morphology and cell physiology

The ability of OESs to maintain their cavity after forming from isthmic mucosa fragments was assessed by counting vesicle-shaped OESs (V-OESs), i.e. OES that did not collapse. The proportion of V-OES decreased up to day 10, regardless of the culture conditions. The mechanism involved in the formation of a cavity inside OES and the ability to maintain it over time remains unanswered at present. It might be related to the integrity of junction complexes at the basolateral part of the cells, avoiding liquid leaking outside the vesicle. Indeed, in the ALI culture system, the cells develop this cohesion progressively, increasing the electric resistance of the cultured epithelium [20]. Therefore, it would be interesting to investigate the evolution of tight junctions during OES formation and culture.

Interestingly, the proportion of V-OES on day 10 was almost 3-fold higher in M199/500 than in M199/25, showing that the OES density per volume of medium (one OES per 20 μL vs. one OES per μL in M199/500 and M199/25, respectively) and the medium renewal (in the M199/500 only) affected the maintenance of the spherical morphology of OES. The proportion of V-OES on day 10 was higher in M199/500 than in M199/25, indicating a better ability to maintain OES shape at a low density. The absence of medium renewal in the M199/25 condition may induce excessive nutrient depletion, leading to a loss of cell cohesion and collapse of OES.

Although the total number of cells per V-OES decreased significantly over time in all culture conditions, the V-OESs kept more than 80% of the viable cells throughout the culture period. This suggests that the V-OESs eliminated dead cells during culture, probably by expulsion in the medium, as cell debris was observed in the culture medium on days 6 and 10 but not inside the V-OES cavity. At day 0, the OES selected for culture contained on average 300 cells, of which 25% contained cilia. Notably, this cell number and proportion of ciliated cells were constant among the experiments, offering a highly standardized and reproducible

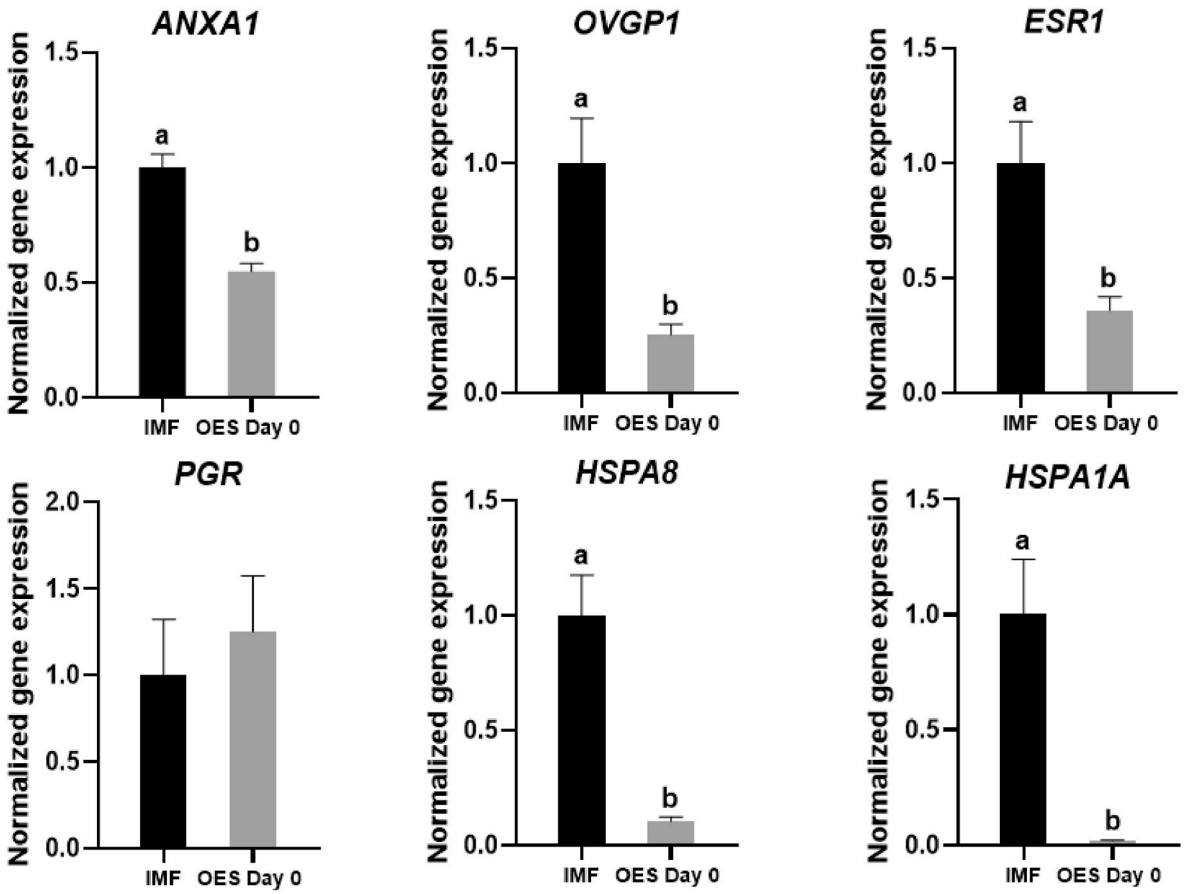

Fig. 8. Impact of OES formation from isthmic mucosa fragments (IMF) on gene expression (Experiment 2). Gene expression of annexin A1 (*ANXA1*), oviductal glycoprotein 1 (*OVGP1*), estrogen receptor 1 (*ESR1*), progesterone receptor (*PGR*), heat shock protein family A (Hsp70) member 8 (*HSPA8*), and heat shock protein family A (Hsp70) member 1A (*HSPA1A*) was normalized according to *GAPDH*, *PPIA* and *YWHAZ* as reference genes. The different letters on bars indicate significant differences with a P-value < 0.05.

model. This proportion of ciliated cells is very similar to the proportion reported by Ito et al. [35] in the epithelium of the isthmus from post-pubertal cows (20–40%). After 10 days in culture, the cell number per V-OES decreased on average to 225 cells (−75) in the SOF/25, of which 32 (14%) contained cilia, and to 150 cells (−150) in M199/500, of which 62 (41%) contained cilia. The decrease in the proportion of ciliated cells in V-OES indicates that the loss of cells in V-OES was at least in part due to the elimination of ciliated cells. The M199/500 condition, however, was much more favorable to the maintenance of ciliated cells in V-OES (9% of lost cells were ciliated) compared to the SOF/25 condition (57% of lost cells were ciliated), maybe due to the lower density and renewal of media in M199/500.

The functionality of the ciliated cells in V-OES was assessed through their capacity to move in suspension and straight-line velocity. While the proportions of moving OES remained very high (>95%) during the 10 days of culture, their straight-line velocity decreased over time in all culture conditions. The decrease in OES velocity was consistent with the reduction in the proportion of ciliated cells in the OES. This may also be due to a decrease in ciliary beat frequency over time in culture. Small 3D structures in suspension in a liquid medium naturally move due to Brownian motion (the random motion of a particle within a large set of smaller particles) but with no change over time. The straight-line velocity of OES, however, decreased over time in all culture conditions, probably due to the decrease in the proportion of ciliated cells, reinforcing the idea that the motion of OES was due to ciliary beating and not to random Brownian motion. To our knowledge, this is the first report of an easy-to-use method allowing the quantification of oviduct ciliary function. The ciliary beating of OECs has been previously evaluated by more complicated methods, such as fluorescent beads [36] or a digital videomicroscopic system [37]. Our results confirmed the existence of ciliated cells up to day 10 in V-OES, in accordance with Walter [17], who evidenced the presence of ciliated cells in aggregates of OEC in suspension after 10 days of culture using electron microscopy.

Altogether, our data indicate that the culture conditions offering the best compromise for maintenance of OES quality were M199/500 and SOF/25.

4.2. *Expression of candidate genes in the isthmic mucosa fragments (IMF) and OES*

Vimentin encoded by *VMAC* is a fibroblast marker, which should not be expressed in OESs if they maintain their epithelial dedifferentiation without epithelial-to-mesenchymal transition [38]. As expected, the immunodetection of cytokeratin confirmed that OES exclusively consisted of OECs.

The ability of OES to respond to estradiol-17β (E2) and progesterone signaling relies on the expression of estrogen receptor α and β (encoded by *ESR1* and *ESR2* genes) and progesterone receptors (encoded by *PGR* gene). The mRNAs coding for Erα (*ESR1*) were much more abundant than those coding for Erβ (*ESR2*), as previously reported by Ulbrich et al. (2003) [39]. Among the other candidate genes whose expression remains constant over time, annexin A1 (*ANXA1)* is located on the apical membrane of OECs and associated with sperm-binding at the oviduct sperm reservoir [40]. Heat-shock protein family A (Hsp70) member 8 (HSPA8) is encoded by *HSPA8* gene and involved in sperm viability and fertilizing ability *in vitro* in pigs and cattle [41]. Finally, heat-shock

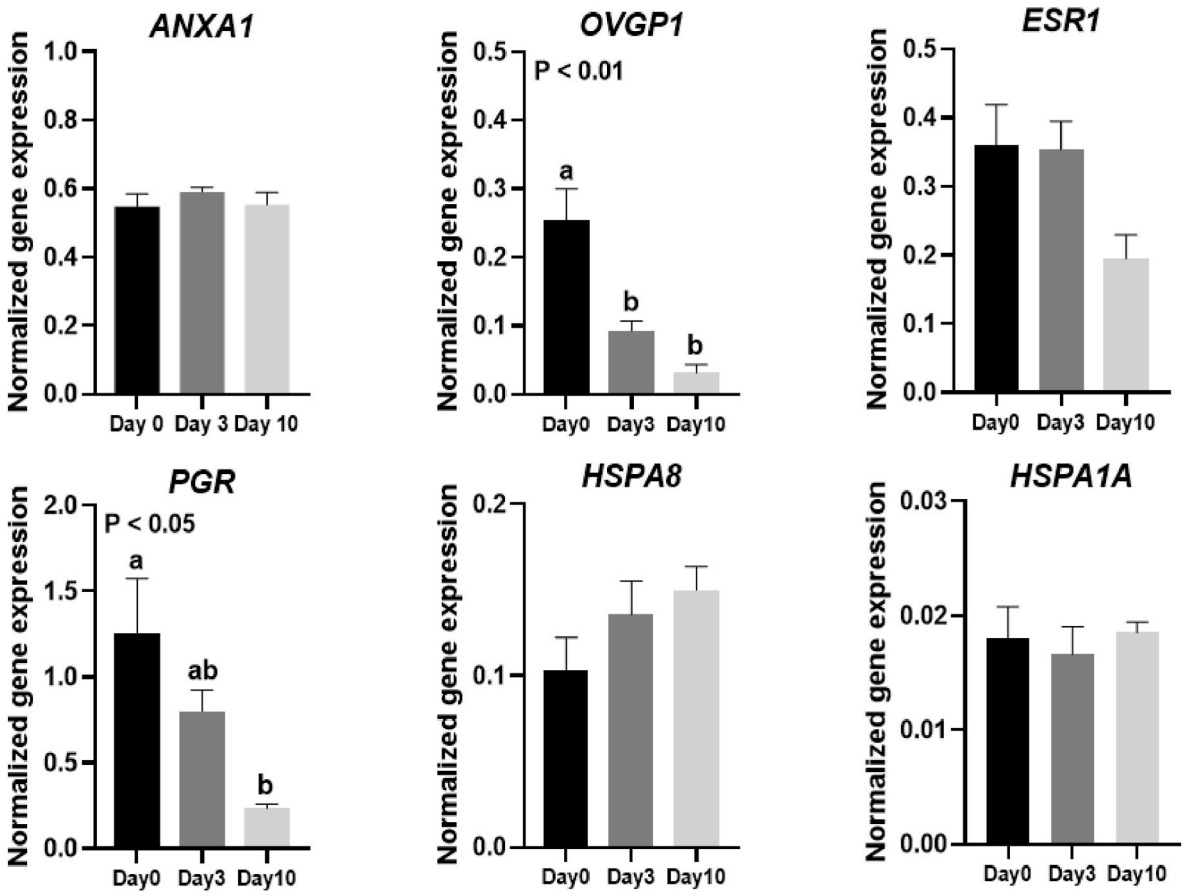

Fig. 9. Impact of culture time on gene expression in OES (Experiment 2). Gene expression of annexin A1 (*ANXA1*), oviductal glycoprotein 1 (*OVGP1*), estrogen receptor 1 (*ESR1*), progesterone receptor (*PGR*), heat shock protein family A (Hsp70) member 8 (*HSPA8*), and heat shock protein family A (Hsp70) member 1A (*HSPA1A*) was normalized according to *GAPDH*, *PPIA* and *YWHAZ* as reference genes. The different letters indicate significant differences with a P-value < 0.05.

Table 2
Effect of co-culture with OES on blastocyst yield in SOF/25 conditions.

	N	% (n) of cleavage rate	% (n) of blastocyst rate		
			Day 6	Day 7	Day 8
Control	790	72.8 ± 2.9 (580)	13.4 ± 1.9 (98)	17 ± 1.9[a] (132)	19.8 ± 2.4[a] (151)
+ OES	795	78.9 ± 2 (623)	16.1 ± 3.2 (123)	26 ± 2.3[b] (208)	31.8 ± 2.5[b] (248)

Percentage values are provided as the means ± SEM of 9 replicates. Different letters within one column indicate significant differences (P < 0.01, t-tests). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

protein family A (Hsp70) member 1A encoded by *HSPA1A* gene has been reported to be induced during heat stress in OECs [42].

By contrast, oviductal glycoprotein 1 (*OVGP1*) and PGR showed a significant decrease in their expression over time. OVGP1 is known to be involved in fertilization and early embryo development [43] as well as to reduce polyspermy by inducing zona hardening in pigs and cattle [44,45]. PGR is normally highly expressed in the isthmic epithelium of cyclic and pregnant cows during the post-ovulatory period [46]. The decrease in OVGP1 and PGR expression over time is a limitation of the OES model. As the mRNA expression of both OVGP1 and PGR is up-regulated by E2 in the oviduct [47], their decrease might be overcome by the addition of reproductive steroid hormones to the OES culture medium.

Although a limited number of genes was evaluated in OES, the constant expression of most of them over time suggest no further effect of culture time on OEC gene expression, indicating that OESs are a suitable model for studies on embryo–maternal communication.

4.3. Effect of co-culture with embryos on OES morphology and cell physiology

Our data indicate a positive effect of OES on embryo development up to the blastocyst stage, as previously reported using OEC monolayers in cattle [8]. Interestingly, the co-culture of OES with developing embryos increased the proportion of V-OES on day 10. To the best of our knowledge, this is the first report on such effect of embryos on OES morphology. This dialogue could be mediated by the release of factors into the media surrounding both embryos and OES. It could be speculated that the mechanisms underlying this effect on OES morphology are mediated by extracellular vesicles (EVs) derived from the embryo, in a similar way to the reported effect of oviduct extracellular vesicles (EVs) on embryo development [48–50]. EVs are membrane-delineated vesicles that play a role in cell-to-cell communication by conveying nucleic acids (mRNAs, non-coding RNA), lipids, and proteins from producing cells to both nearby and long-distance target cells [51]. *In vivo*-derived oviduct EVs can be uptaken by *in vitro*-produced embryos [48], as well as primary confluent OEC after coincubation for 20 h [51], and regulate the embryo transcriptome, as observed in cattle and porcine [49,50]. In addition, it has been shown that developing embryos produce EVs that can cross the zona pellucida and were found in the culture medium [51].

4.4. Conclusion

The OES culture in a large volume of M199 medium or in droplets of SOF medium provided the optimal conditions for OES *in vitro* culture. In

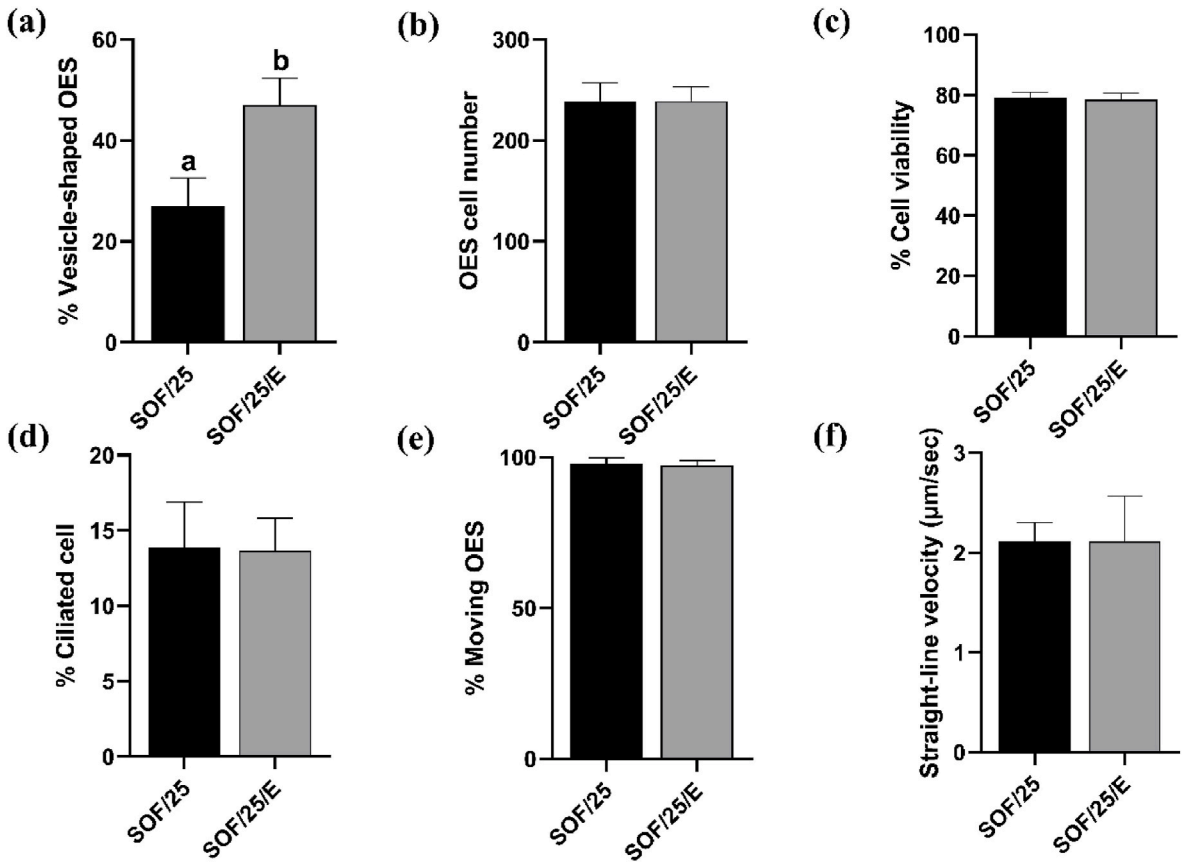

Fig. 10. Effect of co-cultured embryos on OES morphology, total cell number, viability, proportion of ciliated cells and motion (Experiment 3). Groups of 25 V-OES were cultured in SOF/25 alone or with 25 presumptive zygotes (SOF/25/E) for 8 days. At day 10, OES were evaluated for morphology (a), total cell numbers (b), cell viability (c), % ciliated cells (d), % moving (e) and straight-line velocity (f). Data are provided as the mean ± SEM of 4 replicates The different letters on bars indicate significant differences between groups ($P < 0.05$).

SOF droplets, OESs supported blastocyst development. Furthermore, an effect of the developing embryos on OES morphology was evidenced, suggesting an effect of embryos on spheroid intercellular junctions that remain to be investigated. Altogether, it points to OES as an easy-to-use, easy-to-standardize, and physiological model to study early embryo–maternal interactions.

CRediT authorship contribution statement

Thanya Pranomphon: Writing – original draft, Software, Investigation, Formal analysis. **Coline Mahé:** Writing – review & editing, Software, Methodology, Investigation. **Marie-Véronique Demattei:** Validation, Software, Methodology, Investigation, Formal analysis. **Pascal Papillier:** Validation, Software, Methodology, Investigation. **Anaïs Vitorino Carvalho:** Writing – review & editing, Software, Methodology, Data curation. **Karine Reynaud:** Writing – review & editing, Visualization, Methodology, Investigation. **Carmen Almiñana:** Writing – review & editing, Visualization, Validation, Methodology, Funding acquisition, Conceptualization. **Stefan Bauersachs:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Rangsun Parnpai:** Supervision, Project administration, Funding acquisition. **Pascal Mermillod:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Conceptualization. **Marie Saint-Dizier:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

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