REPRODUCTION
RESEARCH

Modelling aspects of oviduct fluid formation *in vitro*

Constantine A Simintiras[1], Thomas Fröhlich[2], Thozhukat Sathyapalan[3], Georg J Arnold[2], Susanne E Ulbrich[4], Henry J Leese[1] and Roger G Sturmey[1]

[1]Centre for Cardiovascular and Metabolic Research (CCMR), The Hull York Medical School (HYMS), Kingston upon Hull, UK, [2]Ludwig-Maximilian University of Munich, Munich, Bavaria, Germany, [3]The Michael White Centre for Diabetes and Endocrinology, Hull Royal Infirmary (HRI), Hull York Medical School (HYMS), Kingston upon Hull, UK and [4]Swiss Federal Institute of Technology Zurich (ETHZ), Zürich, Switzerland

Correspondence should be addressed to C A Simintiras; Email: hycs3@hyms.ac.uk

Abstract

Oviduct fluid is the microenvironment that supports early reproductive processes including fertilisation, embryo cleavage and genome activation. However, the composition and regulation of this critical environment remain rather poorly defined. This study uses an *in vitro* preparation of the bovine oviduct epithelium to investigate the formation and composition of *in vitro*-derived oviduct fluid (*iv*DOF) within a controlled environment. We confirm the presence of oviduct-specific glycoprotein 1 in *iv*DOF and show that the amino acid and carbohydrate content resembles that of previously reported *in vivo* data. In parallel, using a different culture system, a panel of oviduct epithelial solute carrier genes and the corresponding flux of amino acids within *iv*DOF in response to steroid hormones were investigated. We next incorporated fibroblasts directly beneath the epithelium. This dual culture arrangement represents more faithfully the *in vivo* environment and impacts on *iv*DOF composition. Lastly, physiological and pathophysiological endocrine states were modelled and their impact on the *in vitro* oviduct preparation was evaluated. These experiments help clarify the dynamic function of the oviduct *in vitro* and suggest a number of future research avenues, such as investigating epithelial–fibroblast interactions, probing the molecular aetiologies of subfertility and optimising embryo culture media.

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Introduction

The lumen of the mammalian oviduct can be considered an optimal environment for reproductive processes including fertilisation and early embryo development (Coy *et al*. 2012). During this time, critical developmental events occur, including activation of the embryonic genome and fate-decisions of the blastomeres (González *et al*. 2011). In the bovine, the early embryo spends approximately 4 days in the oviduct before moving into the uterus (Hackett *et al*. 1993). Insights into the dynamic composition, formation and regulation of oviduct fluid are therefore crucial to our understanding of the early events of mammalian reproduction.

Until now, descriptions of the composition of oviduct fluid have been based on analyses from samples isolated from various species using *in situ* and *ex vivo* techniques (Aguilar & Reyley 2005). These have included oviduct flushes from anaesthetised or slaughtered animals. As discussed by Leese and coworkers (2008), these methods are limited and offer narrow scope for experimental exploration. Thus, there is a need for a robust method of studying oviduct fluid within a controlled laboratory environment.

A single layer of epithelial cells provides the limiting barrier between the maternal circulation and the oviduct lumen. To examine oviduct fluid formation in detail, it is therefore necessary to isolate the oviduct epithelial cells and culture them in a system that maintains their proper spatial relationship as a polarised confluent layer. One method to achieve this is using the Transwell system, which enables the culture of oviduct epithelia in chambers that allow access to the apical and basal compartments (Walter 1995). This system allows the bidirectional movement of compounds across the oviduct epithelium to be examined. Using such as system, Dickens and coworkers (1993) and Cox and Leese (1995) reported that a chloride secreting epithelium sensitive to purinergic agents lined rabbit and bovine oviducts. These findings have been followed up in detail by Keating and Quinlan (2008, 2012). Moreover, the culture of bovine oviduct epithelia on Transwell inserts has allowed the basal to apical, and reverse, movement of nutrients across the oviduct epithelium to be examined (Simintiras *et al*. 2012).

Building on these early studies, Levanon and coworkers (2010) demonstrated that oviduct epithelia could be cultured at an apical–basal air–liquid interface in which the apical chamber was comprised of moist air. Under air–liquid interface conditions, oviduct epithelia resemble the *in vivo* state more closely and

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can be cultured in this manner long term (Gualtieri *et al.* 2012). Interestingly, patches of oviduct epithelial cells maintained at an air–liquid interface for over two weeks after confluence regained ciliation (Gualtieri *et al.* 2013), despite a lack of oestradiol supplementation, which is normally required for re-ciliation *in vitro* (Comer *et al.* 1998, Ulbrich *et al.* 2003). Chen and coworkers (2013*a*) cultured porcine oviduct epithelial cells for more than 10 days at an air–liquid interface together with steroid hormones and found they were morphologically closer to *in vivo* controls. This interesting approach results in a system in which *in vitro* oviduct epithelial cell cultures mimic *in vivo* behaviour more closely.

In spite of these advances, there is only partial knowledge of the mechanisms underlying the formation and regulation of oviduct fluid, especially when compared with epithelia lining tissues such as the gut and the airways. This can be attributed to (a) ethical and technical limitations surrounding the study of oviduct fluid *in vivo* and (b) the lack of a robust *in vitro* model enabling the exploration of the formation of oviduct fluid, and how the process responds to stimuli under controlled experimental conditions.

We now present a preparation of bovine oviduct epithelial monolayer to perform real-time experiments on oviduct-derived fluid formation *in vitro*. With this system, we have confirmed the secretion of OVGP1 protein into the luminal compartment, which comprises a mixture of amino acids whose composition differs from that in the basal compartment. This apical cell-derived fluid is modified after basal supplementation with oestradiol, progesterone and testosterone at physiological and pathophysiological concentrations. Furthermore, using a parallel culture system, we have correlated the expression of bovine oviduct epithelial cell (BOEC) solute carrier genes, with the flux of amino acids in *iv*DOF after hormonal supplementation.

Materials and methods

Unless stated otherwise, all reagents were sourced from Sigma-Aldrich.

Bovine oviduct epithelial cell harvest

Primarily stage II (mid-luteal phase) abattoir-derived bovine reproductive tracts (Ireland *et al.* 1980) were transported to the laboratory at room temperature in Hank's buffered salt solution (HBSS) (without $CaCl_2$ and $MgCl_2$) (Invitrogen), 10 mM HEPES and 1 µM Aprotinin – although tracts were not staged for experimentation. Tracts reached the laboratory within 90 min of slaughter. Cells from isthmus to infundibulum were harvested similarly to Dickens and coworkers (1993) and in accordance with the UK Animal and Plant Health Agency (APHA) regulations.

Bovine oviduct epithelial cells (BOECs) and bovine oviduct fibroblast cells (BOFCs) were subsequently isolated based on their differential adhesion times—cells were initially seeded together in T75 flasks (Sarstedt) and after 18 h of culture, un-adhered BOECs were removed (Cronin *et al.* 2012) and re-cultured. Culture medium consisted of 1:1 DMEM and F12, supplemented with 265 U/mL Pen-Strep, 20 µg/mL amphotericin B, 2 mM L-glutamine, 2.5% *v/v* NCS, 2.5% *v/v* FBS and 0.75% *w/v* BSA.

Bovine oviduct epithelial cell Transwell culture

BOECs were seeded directly onto the apical surface of 24 mm Corning Transwell 0.4 µm pore cell culture inserts coated with 10 µg/mL laminin at a density of 10^6 cells/mL/insert. BOECs were subsequently maintained between apical and basal culture medium-filled chambers, at 39°C in 5% CO_2, 95% air. Apical and basal media were replaced every 48 h.

Transepithelial electrochemical resistance (TEER)

BOEC confluence was determined by transepithelial electrochemical resistance (TEER) measured using an Evom voltmeter fitted with handheld chopstick electrodes (World Precision Instruments). From cell seeding to reaching full confluence, TEER rose from 250 Ω/cm^2 to ~800 Ω/cm^2 in the course of ~10 days. In addition to assessing monolayer confluence before experimentation, TEER was also used as a measure of post-treatment cellular integrity. Unless used as a dependent independent variable, data from BOECs whose TEER fell below 700 Ω/cm^2 were excluded from analysis (Simintiras *et al.* 2012).

In vitro*-derived oviduct fluid (ivDOF)*

Once confluent, BOECs were cultured in an apical–basal air–liquid interface (Levanon *et al.* 2010)—the basal medium comprised 2 mL of culture medium, whereas the apical compartment comprised moist air in 5% CO_2. After 24 h, a thin film of fluid formed in the apical chamber—termed *in vitro*-derived oviduct fluid (*iv*DOF) (Fig. 1A).

Dual culture

Bovine oviduct fibroblast cells were harvested by trypsinization from tissue culture flasks after 5 days in culture. 1×10^6 fibroblast cells were added to the basal surfaces of Transwell semi-permeable supports (Fig. 1B). Fibroblasts were maintained in this manner for approximately 5 days at which point Transwell inserts were reorientated and BOECs were introduced to the apical surfaces.

Hormonal supplementation

Hormone stocks were prepared in ethanol before supplementation to the basal Transwell chamber. Singular steroid hormone concentrations were based on peripheral plasma levels in the bovine throughout the oestrous cycle as previously reported (Kanchev *et al.* 1976). Combinatorial stocks to determine the effects of a physiologically relevant range of hormone concentrations on the *in vitro* model were similarly prepared to represent a minimum, mean and maximum pathophysiological endocrine profile (Kanchev

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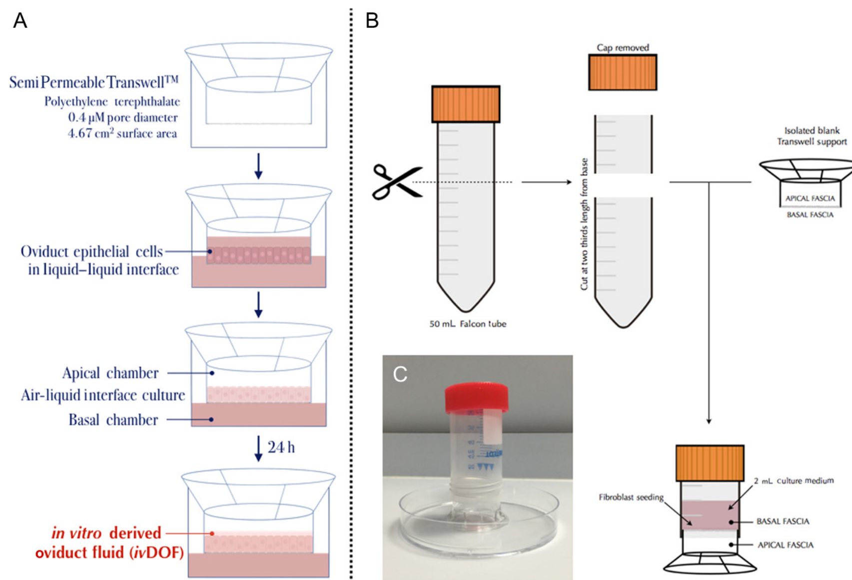

Figure 1 (A) Schematic representation of the culture system for *in vitro*-derived oviduct fluid (*iv*DOF) production. The basal chamber represents the bloodstream whilst the apical represents the oviduct lumen. (B) The technical method and apparatus innovated for seeding fibroblasts to the basal surface of Transwell membranes for establishing dual culture. Large Falcon tubes were cut two-thirds from the base and the caps were removed. The top end of the Falcon tube was manually fastened over the inverted Transwell support, whereas the cap was placed over the severed end of the tube. This scaffold could then support cell proliferation on the basal surface of the semi-permeable membrane.

et al. 1976, Pastor *et al*. 1998, Balen 2004, Di Sarra *et al*. 2013, O'Reilly *et al*. 2014). The maximum solvent (ethanol) contribution was <1% (*v*/*v*) similar to Bromberg and Klibanov (1995) and showed no effect throughout (Table 1).

Fluorescence-activated cell sorting (FACS)

BOECs and BOFCs were identified based on positive staining for cytokeratin-18 (CK18) and vimentin primary antibodies (Abcam) respectively (Rottmayer *et al*. 2006, Goodpaster *et al*. 2008). Samples were analysed on FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) running CELLQuest software and >10,000 events were counted, similarly to Vince and coworkers (2011).

Haematoxylin and eosin staining

Confluent BOECs cultured on Transwell inserts were manually isolated using a blade. The supports were rinsed three times in pre-equilibrated PBS before 5-min incubation at room temperature in 100% haematoxylin. Cells were then rinsed three times in 18.2 milliQ water and incubated for 5 min with 1% eosin. Following further washes, cells were supplemented with Hydromount (National Diagnostics, Atlanta, GA, USA), placed onto microscope slides and imaged on a Zeiss ApoTome 2 Observer Z1 microscope with a ×20 objective lens and an Axiom 506 mono imager coupled with ZEN imaging software.

Transmission electron microscopy (TEM)

BOECs fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, post-fixed in 1% osmium tetroxide in the same buffer, were stained en bloc in 1% uranyl acetate (aq) then serially dehydrated in ethanol before being embedded in Epon-Araldite resin. (All chemicals are from Agar Scientific, Stansted, Essex.) Subsequently, 50 nm sections were cut using a diamond knife on a Leica UC6 Ultramicrotome and collected on carbon-coated copper grids (EM Resolutions, Saffron Walden, Essex, UK). Images were obtained using an Ultrascan 4000 digital camera (Gatan Inc, Pleasanton, CA, USA) attached to a Jeol 2011 Transmission Electron Microscope (Jeol UK Ltd, Welwyn Garden City, Hertfordshire, UK) running at 120 kV.

Generation of anti-oviduct-specific glycoprotein (OVGP1) antibodies

The peptide KMTVTPDGRAETLERRL corresponding to amino acids 521–537 of bovine OVGP1 (UniProtKB – Q28042) was synthesised with a 433A Peptide Synthesizer (Applied Biosystems) using Fmoc chemistry (FastMoc Ω

Table 1 Concentration of hormones added to bovine oviduct epithelial cells as different treatments.

	17β-Oestradiol (E2)	**Progesterone** (P4)	**Testosterone** (T)
Native (N)	0 pM	0 pM	0 pM
17β-Oestradiol (E2)	29.37 pM	0 pM	0 pM
Progesterone (P4)	0 pM	6.36 nM	0 pM
Testosterone (T)	0 pM	0 pM	62.77 pM
Hypoandrogenic (HYPO)	29.37 pM	6.36 nM	2.43 pM
Physiological (PHYS)	29.37 pM	6.36 nM	208 pM
Hyperandrogenic (HYPER)	19.46 pM	6.36 nM	6.27 nM

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Table 2 List of the bovine-specific exon spanning primers used.

Gene	Direction	Sequence	T_m (°C)	GC (%)
ACTB	Forward (3′ to 5′)	TTCAACACCCCTGCCATG	59.64	56
	Reverse (5′ to 3′)	TCACCGGAGTCCATCACGAT	59.73	55
*OVGP1**	Forward (3′ to 5′)	CTGAGCTCCATCCCCACTTG	57.20	60
	Reverse (5′ to 3′)	GTTGCTCATCGAGGCAAAGG	57.10	55
*ESR1**	Forward (3′ to 5′)	AGGGAAGCTCCTATTTGCTCC	57.00	52
	Reverse (5′ to 3′)	CGGTGGATGTGGTCCTTCTCT	57.50	57
SLC1A1†	Forward (3′ to 5′)	CACCGTCCTGAGTGGGCTTGC	61.30	67
	Reverse (5′ to 3′)	CAGAAGAGCCTGGGCCATTCCC	61.30	64
SLC38A2†	Forward (3′ to 5′)	GAACCCAGACCACCAAGGCAG	58.10	62
	Reverse (5′ to 3′)	GTTGGGCAGCGGGAGGAATCG	61.80	67
SLC38A5†	Forward (3′ to 5′)	TGGCCATCTCGTCTGCTGAGGG	63.20	64
	Reverse (5′ to 3′)	GCTCCTGCTCCACAGCATTCCC	62.00	64
SLC38A7†	Forward (3′ to 5′)	CGGCAGCCCGAGGTGAAGAC	61.60	70
	Reverse (5′ to 3′)	GCCGCAGATACCTGTGCCCAT	60.90	62
SLC6A14†	Forward (3′ to 5′)	TCGAGGGGCAACTCTGGAAGGT	60.80	59
	Reverse (5′ to 3′)	GGCAGCATCTTTCCAAACCTCAGCA	62.90	52
ZO1	Forward (3′ to 5′)	CTCTTCCTGCTTGACCTCCC	56.80	60
	Reverse (5′ to 3′)	TCCATAGGGAGATTCCTTCTCA	55.20	45

Those marked * were taken from Ulbrich *et al.* (2003), whereas primers marked † from Forde *et al.* (2014).

previous peak method, as suggested by the manufacturer) and TentaGel S RAM (RAPP Polymere, Tübingen, Germany) resin. To further increase immunogenicity, a proprietary peptide carrier was C-terminally coupled. Peptide cleavage and deprotection were performed by incubation in 92.5% trifluoroacetic acid, 5% triisopropylsilane and 2.5% water for 1.5 h. The peptide was precipitated and washed with cool tert-butyl methyl ether. Peptides were further purified using reversed-phase chromatography, and the correctness of the peptide was confirmed using matrix-assisted laser desorption ionisation–time-of-flight mass spectrometry (4800 series; Applied Biosystems). Murine anti-OVGP1 sera were generated by immunisation of female BALB/c mice in intervals of 3 week with 100 µg peptide applied subcutaneously. For the first injection, complete Freund's adjuvant and for the following three injections, incomplete Freund's adjuvant was used. Bleeding was performed 10 days after the fourth injection.

Western blotting

OVGP1 from both abattoir-derived oviduct fluid and *iv*DOF was qualitatively identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were separated by 10–18% gradient SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked for 24 h with 10% milk dissolved in Tris-buffered saline Tween (0.1%), and then incubated at 4°C with the custom mouse anti-OVGP1 primary antibody described previously (1:1000) for 24 h,

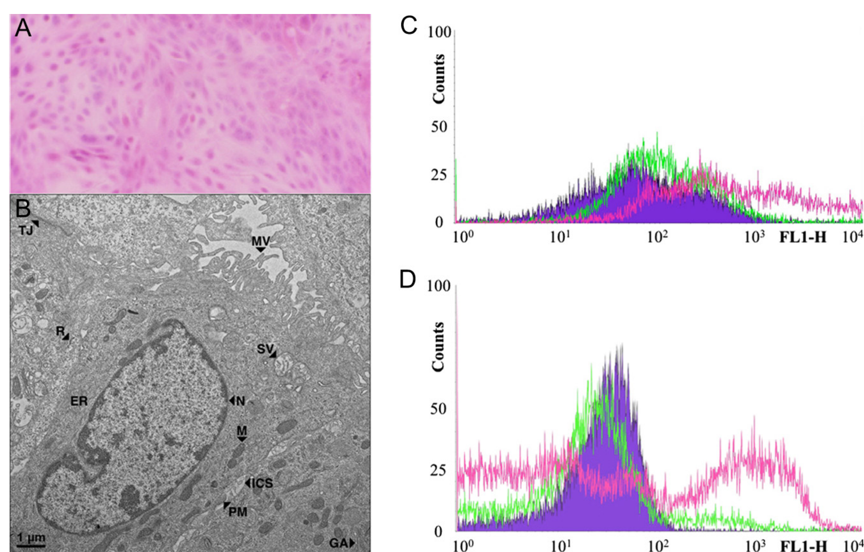

Figure 2 (A) Haematoxylin- and eosin-stained bovine oviduct epithelial cells cultured to confluence on Transwell membranes and imaged at ×20 magnification. (B) Transmission electron microscopy image of bovine oviduct epithelia showing the endoplasmic reticulum (ER), Golgi apparatus (GA), intracellular space (ICS), mitochondria (M), microvilli (MV), nucleus (N), plasma membrane (PM), ribosomes (R), a secretory vesicle (SV) and a tight junction (TJ). (C) FACS analysis of cultured BOEC purity showing mouse IgG1-negative control (background noise), anti-Vimentin 1° antibody (BOFC population), and anti-Cytokeratin 18 1° antibody (BOEC population); all in combination with the Alexafluor 488 nm 2° antibody showing in excess of 95% epithelial purity (representative of $n=2$) at a fluorescence intensity (FLH-1) between 103 and 104. (D) FACS analysis of cultured BOFCs showing mouse IgG1-negative control (background noise), anti-cytokeratin 18 1° antibody (BOEC population) and anti-vimentin 1° antibody (BOFC population) in excess of 99% stromal purity ($n=1$).

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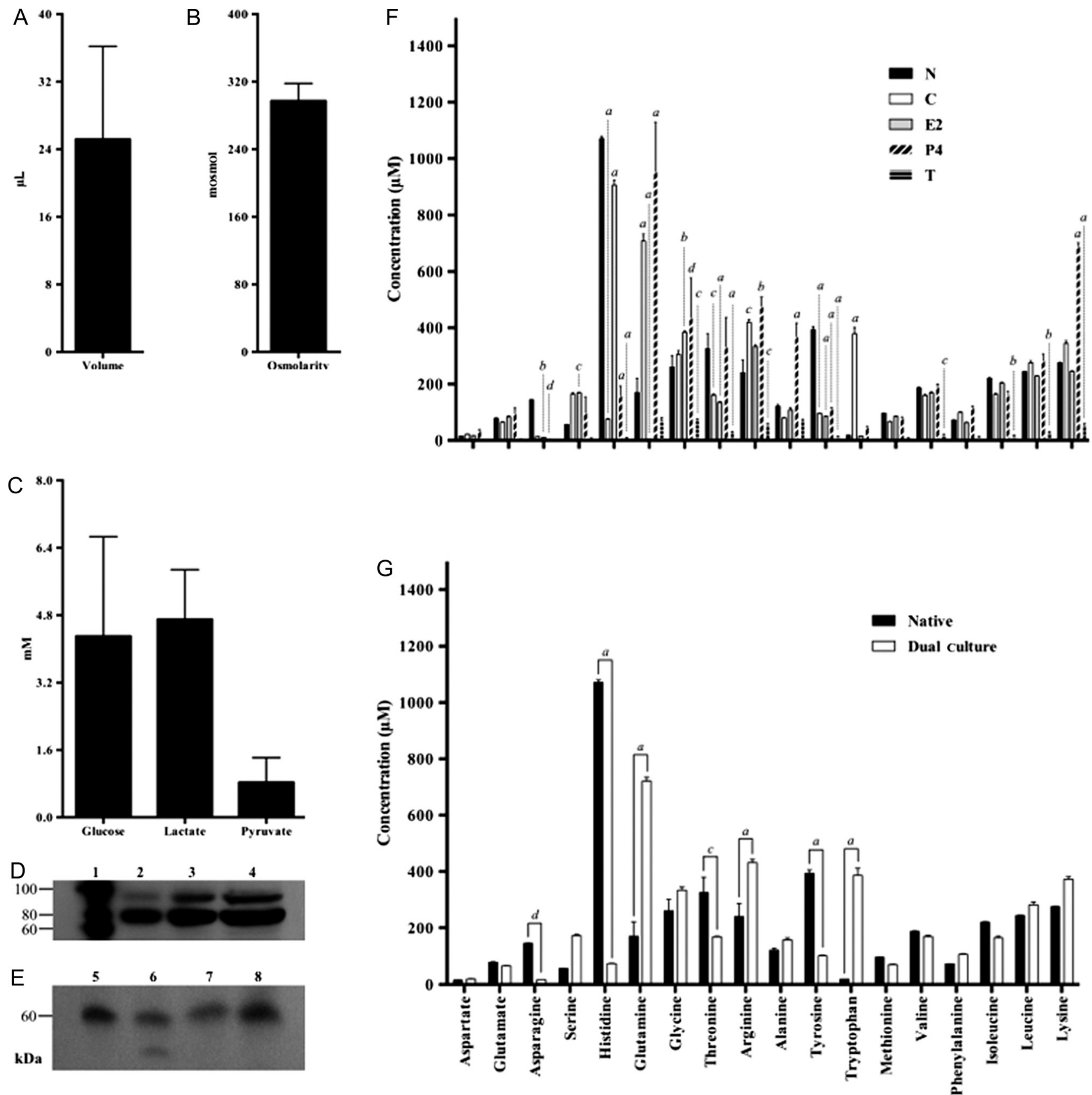

Figure 3 (A) The volume ($n=6\pm$s.d.), (B) osmolarity ($n=3\pm$s.d.) and (C) carbohydrate content ($n=3\pm$s.d.) of *iv*DOF obtained from native (untreated) epithelia. (D and E) Western (protein immuno) blots for OVGP1 from (D) *in vivo*-derived oviduct fluid and cell lysates ($n=1$) and (E) native *iv*DOF (representative of $n=4$). Lane 1 was loaded with a staggered 200 kDa HRP-linked biotinylated protein ladder. Lane 2 with 10 mM (16.5 µL) total protein, lane 3 with 20 mM (33.3 µL) and lane 4 with 40 mM (66.7 µL). Lanes 5–8 were loaded with 40 µL (arbitrary concentrations) of native *iv*DOF. (F) The amino acid composition of *iv*DOF accumulated apically from native (N) BOECs ($n=12\pm$s.d.) vs culture medium (C) supplied basally ($n=3\pm$s.d.) vs *iv*DOF derived from BOECs basally supplemented with 29.37 pM 17β-oestradiol (E2; $n=6\pm$s.d.) vs *iv*DOF from BOECs treated with 6.36 nM progesterone (P4; $n=4\pm$s.d.) vs *iv*DOF from BOECs basally supplemented with 62.77 pM testosterone (T; $n=3\pm$s.d.). (G) The amino acid profile of native *iv*DOF ($n=12\pm$s.d.) vs *iv*DOF from BOECs cultured with BOFCs basally adjacent in the dual culture arrangement ($n=4\pm$s.d.). All *iv*DOF accumulated over 24 h and $a=P\leq0.0001$, $b=P\leq0.001$, $c=P\leq0.01$ and $d=P\leq0.05$.

washed and subsequently incubated with an anti-mouse horseradish peroxidase (HRP) linked antibody (1:10,000) (Cell Signaling Technologies) for 1 h at room temperature. Bands were visualised by enhanced chemiluminescent (ECL) detection.

Osmolarity and fluorometric assays

Osmolarity was measured using an Osmomat 030 Osmometer (Gonotec GmbH, Berlin, Germany). Glucose, lactate and pyruvate were quantified indirectly using enzyme-linked fluorometric assays as described in Leese (1983), Leese &

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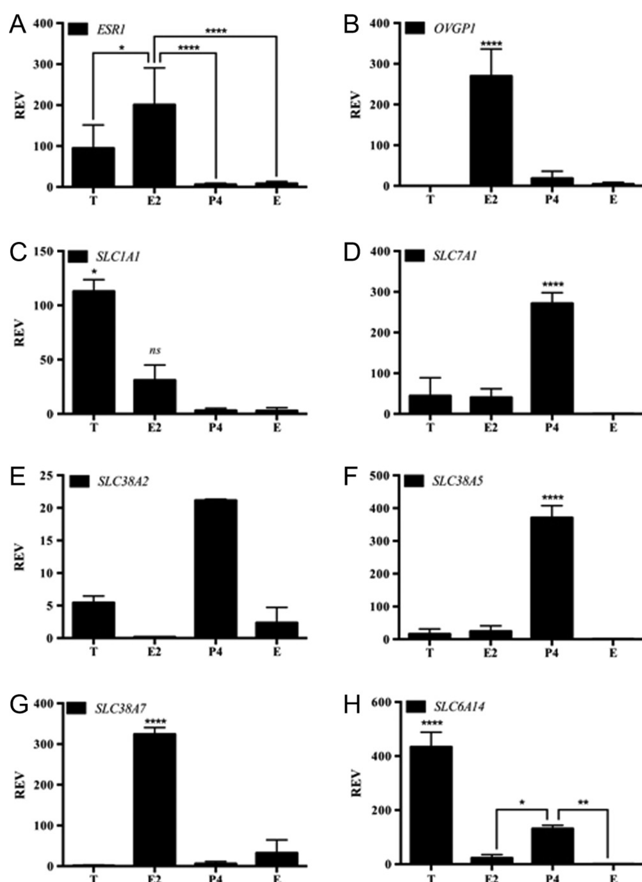

Figure 4 Gene expression profiles of (A) *ESR1*, (B) *OVGP1*, (C) *SLC1A1*, (D) *SLC7A1*, (E) *SLC38A2*, (F) *SLC38A5*, (G) *SLC38A7* and (H) *SLC6A14* as determined by qRT-PCR ($n=3\pm$ s.e.m.). BOECs were subjected to 62.77 pM testosterone (T), 29.37 pM 17β-oestradiol (E2), 6.36 nM progesterone (P4) and 0.45% (*v/v*) ethanol (E) as vehicle control – all for 24 h. Data were normalised to *ACTB* whilst the impact of treatment on gene expression was calculated relative to native BOECs. ****$P\leq0.0001$, ***$P\leq0.001$, **$P\leq0.01$ and *$P\leq0.05$.

Barton (1984), Gardner & Leese (1988, 1990) and Guerif and coworkers (2013).

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was used to measure 18 amino acids as described previously (Humpherson *et al.* 2005).

Quantitative real-time polymerase chain reaction (qRT-PCR)

At confluence, BOECs from T25 flasks were subjected to hormonal supplementation (Table 1) for 24 h before isolation using trypsin. BOECs were washed four times by centrifugation at 1000 ***g*** for 5 min at 4°C and resuspension in pre-chilled 1 mL phenol red-free HBSS. Total RNA was extracted using TRIzol reagent and chloroform (Chomczynski & Sacchi 1987). Global cDNA was synthesised by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Fisher Scientific) in accordance to manufacturer instructions. The concentration (ng/µL) and purity (A260/A280) of cDNA generated were determined using a NanoDrop spectrophotometer. All cDNAs were diluted to 1 µg/mL. Three technical PCR replicates were prepared per sample in optical 96-well plates and sealed before being loaded onto a Step-one Real-Time PCR machine (Applied Biosystems) for qPCR. The bovine-specific exon spanning primers used are provided in Table 2. To ensure correct product length, melt curves were performed (Giglio *et al.* 2003), whereas ΔΔCt method (Livak & Schmittgen 2001) was used to determine relative expression.

Experimental design

Retrieved bovine oviduct epithelia were pooled, typically yielding sufficient viable cells to seed 6 Transwell inserts. The standard experimental design was to assign 3 Transwell membranes for treatment with the dependent experimental variable and the remaining 3 as negative controls. The *iv*DOF obtained from each group was pooled for subsequent analysis. This was defined as a single biological replicate ($n=1$). Unless otherwise stated, $n=3$ indicates data from three independent abattoir collections and *iv*DOF isolations.

In this study, *in vitro*-derived oviduct fluid (*iv*DOF; Fig. 1A) from untreated (native) bovine oviduct epithelial cells was analysed and compared with previously reported *in vivo* observations. The composition of *iv*DOF after singular cellular hormonal supplementation was analysed, and the influence of dual culture was also examined. These data are contrasted against native *iv*DOF. This system was subsequently expanded upon to investigate the impact of physiological vs pathophysiological endocrine stimulation on fluid composition and cellular physiology. Cell culture flasks were seeded in parallel for gene expression studies to complement *iv*DOF findings.

Statistical analyses

Statistical analyses were performed using Prism GraphPad 6 software for Apple Macintosh. All statistical analysis were two way analysis of variance (ANOVA) followed by a Holm–Sidak non-parametric *post hoc* analysis.

Results

BOEC and BOFC isolation

Figure 2A and B confirm the epithelial nature of cells in culture in our model. Additionally, over 95% of cells were positive for CK18, (Fig. 2C) and over 99% of the BOFC population stained positive for vimentin (Fig. 2D).

ivDOF characterisation

The volume of *iv*DOF from untreated BOECS after a 24-h period of culture was 25.2 ± 4.5 µL (Fig. 3A) and the mean osmolarity was 297 ± 12 mosmol (Fig. 3B). Untreated *iv*DOF contained 4.30 ± 1.18 mM glucose, 4.70 ± 0.68 mM lactate and 0.83 ± 0.34 mM pyruvate (Fig. 3C). Qualitative western blots for OVGP1 were performed on oviduct fluid derived from fresh abattoir tissue (Fig. 3D) and compared with blots given by *iv*DOF

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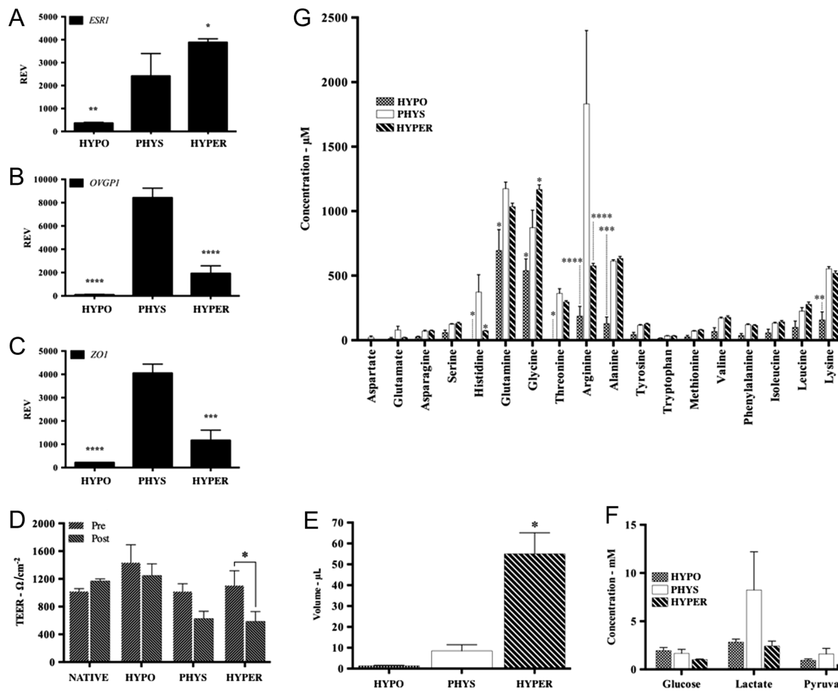

Figure 5 The effects of hypoandrogenic (HYPO), physiological (PHYS) and hyperandrogenic (HYPER) like endocrine supplementation on (A) *ESR1*, (B) *OVGP1* and (C) *ZO1* gene expression ($n=3\pm$s.e.m.). (D) TEER values from BOECs before and after HYPO, PHYS and HYER exposure in addition to native ($n=3\pm$s.d.). One statistically significant difference was determined by paired *t*-test ($P=0.0214$). (E) Volumes of *iv*DOF from HYPO-, PHYS- and HYER-treated BOECs ($n=3\pm$s.d.). (F) The carbohydrate composition of *iv*DOF from BOECs subjected to HYPO, PHYS and HYER exposure ($n=3\pm$s.d.). (G) The amino acid content of *iv*DOF obtained from HYPO-, PHYS- and HYER-treated cells ($n=3\pm$s.d.). All treatment durations were 24 h. ****$P\leq0.0001$, ***$P\leq0.001$, **$P\leq0.01$ and *$P\leq0.05$.

(Fig. 3E). These figures confirm OVGP1 presence in both oviduct fluids. However, OVGP1 collected from abattoir-derived *in vivo* oviduct fluid showed two prominent bands at 80 kDa and 90 kDa, whereas OVGP1 identified in *iv*DOF was present at 60 kDa.

Figure 3F shows that the amino acid composition of *iv*DOF from untreated BOECs was distinct from that in the medium provided basally (C) with respect to 6/18 amino acids measured. When E2 was added to the basal compartment (Table 1), asparagine, histidine, glutamine, threonine and tyrosine secretion were decreased, whereas the apical accumulation of serine and glycine were elevated compared with native *iv*DOF (Fig. 3F). Similarly, the addition of P4 (Table 1) increased the apical flux of glutamine, glycine, arginine, alanine and lysine, whilst decreasing histidine and tyrosine secretion (Fig. 3F). Interestingly, treatment with T (Table 1) significantly decreased the accumulation of 10 amino acids in *iv*DOF relative to native fluid (Fig. 3F). Figure 3G shows that culturing BOECs in a dual culture configuration with basally adjacent BOFCs altered the secretion of 7/18 amino acids: asparagine, histidine, threonine and tyrosine movement decreased, whereas glutamine, arginine and tryptophan flux increased.

BOEC gene expression

OVGP1 and *ESR1* were expressed in flask-cultured cells after harvest and increased after 24 h of E2 exposure (Fig. 4). In addition, a panel of solute carrier genes was analysed (Table 2). In brief, *SLC1A1* and *SLC6A14* were upregulated in response to T, *SLC38A7* expression increased after E2 exposure and *SLC7A1* and *SLC38A5* expression was elevated after P4 supplementation. The ethanol vehicle control showed no significant impact on gene expression.

Impact of pathophysiological endocrine supplementation

To further explore the impact of endocrine action on oviduct epithelial cell secretions and to test the capacity of the model for investigating disease, one physiological, and two pathophysiological ranges of hormones were added to the basal compartment (Table 1); the latter represented hypoandrogenism and hyperandrogenism. Figure 5 panels A, B, C show that hyperandrogenism (HYPER) increased the expression of *ESR1* in flask-cultured BOECs whilst reducing *OVGP1* and *ZO1* expression, whereas hypoandrogenism (HYPO) decreased the relative expression of all the genes investigated relative to physiological (PHYS). Hyperandrogenism also reduced BOEC TEER after 24 h (Fig. 5D) and caused an increase in the volume of *iv*DOF produced (Fig. 5E). Figure 5F shows that hypo and hyper treatments had no significant impact on the carbohydrate composition of *iv*DOF. Lastly hypoandrogenism reduced histidine, glutamine, glycine, threonine, arginine, alanine and lysine secretion, whereas hyperandrogenism reduced histidine and arginine but elevated the apical accumulation of glycine (Fig. 5G).

Discussion

We present a novel application for an existing bovine oviduct epithelial cell preparation, which can be used

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to examine the formation of oviduct fluid *in vitro* under a variety of conditions. A layer of BOECs were grown on Transwell membranes (Fig. 1A) and were confirmed as confluent by TEER, expressed CK18 (Fig. 2C) and displayed a number of morphological features typical of epithelial cells (Fig. 2A and B). After culture in an air–liquid interface for 24 h after confirmation of confluence, a film of liquid appeared in the apical chamber, which contained OVGP1 protein (Fig. 3E) and was biochemically distinct from the culture medium provided basally (Fig. 3F). We therefore propose that this constitutes an *in vitro*-derived oviduct fluid (*iv*DOF). We furthermore present a method for achieving dual culture *in vitro* (Fig. 1B) and show that incorporating basally adjacent fibroblasts into the model also impacts *iv*DOF amino acid composition (Fig. 3G). In addition, parallel flask-cultured BOECs expressed the genes *ESR1* and *OVGP1* in an E2-responsive manner (Fig. 4). The above were then expanded to test the capacity of this preparation to model pathophysiological endocrine states (Fig. 5).

*iv*DOF characterisation

The volume of native *iv*DOF produced in 24 h was found to be 25.2 ± 11.0 µL (Fig. 3A); a rate of formation less than the 1.505 ± 0.291 µL/min previously reported *in vivo* by Hugentobler and coworkers (2008). The osmolarity of native *iv*DOF however was 297 ± 12 mosmol (Fig. 4B), which correlates well with both what has been observed *in vivo* 281.0 ± 2.56 mosmol (Paisley & Mickelsen 1979) and the 270–300 mosmol range of embryo culture media (Sirard & Coenen 2006). Similarly Hugentobler and coworkers (2008) investigated the glucose, lactate and pyruvate composition of *in vivo* bovine oviduct. Multiple *t*-tests between these data and Fig. 3C reveal no significant difference between the basic carbohydrate content of *iv*DOF vs *in vivo*.

OVGP1 in *iv*DOF was ~60 kDa (Fig. 4E) suggestive of the de-glycosylated form, in contrast to the ~80–90 kDa product titrated from abattoir-derived oviduct fluid and cell lysates (Fig. 4F) (Boice *et al.* 1990, Bauersachs *et al.* 2004). Abe and Abe (1993) and Sendai and coworkers (1994) also reported two OVGP1-specific bands in the murine and bovine at 95 kDa and ~55 kDa respectively. This difference is likely due to a lack of post-translational glycosylation, which would impair electrophoretic mobility by up to 25.3 kDa (Unal *et al.* 2008). We suspect this is because the culture medium provided is deficient in substrates such as n-acetylglucosamine, required for glycosylation.

The amino acid composition of *iv*DOF (Fig. 4F) resembled data on cannulated oviducts of anaesthetised heifers (Hugentobler *et al.* 2007). However, there were some notable differences between the amino acid content of *in vivo* and *in vitro* oviduct fluid. Histidine was significantly more abundant in *iv*DOF than previously recorded levels in the oviduct lumen (Guerin *et al.* 1995, Hugentobler *et al.* 2007). One possible explanation for this is that histidine, an imidazole, can act as a pH buffer. The *in situ* bovine oviduct pH is 7.6 (Hugentobler *et al.* 2004), whereas *in vitro* BOECs were cultured at ~pH 7.4. Although a small difference in pH, the latter represents a 58.5% increase in free H+ ions. It could therefore be the case that the native bovine oviduct epithelium secretes histidine to buffer free H+ ions and balance *iv*DOF pH. Addition of E2 caused histidine in *iv*DOF to fall, and P4 administration further decreased histidine to 159.3 µM, closer to the levels observed previously *in vivo* (Guerin *et al.* 1995). The addition of T dramatically reduced histidine secretion from 1071.1 µM to 9.7 µM; thus, histidine transport appears to be subject to T regulation in addition to E2 and P4.

Glutamine was present in native *iv*DOF at levels very close to those reported *in vivo* (Guerin *et al.* 1995, Hugentobler *et al.* 2007); yet, significantly lower than the concentration in the basal culture medium (Fig. 4F). This is one example that the BOEC epithelium *in vitro* forms a highly selective barrier. E2 drastically reduced apical glutamine flux, from 170.0 µM to 5.3 µM whilst T had no impact and P4 markedly increased glutamine content in *iv*DOF to 953.5 µM. This might relate to the importance of glutamine in bovine embryo metabolism (Rieger *et al.* 1992). Thus, it is unsurprising that P4, the dominant circulatory hormone during pregnancy elevated oviduct glutamine output.

Next, BOECs and BOFCs were simultaneously cultured on either side of the same membrane (Fig. 1B) to provide a closer to physiological environment for modelling the oviduct epithelium (Fazleabas *et al.* 1997). In this dual culture system, the composition of *iv*DOF was modified, with increased appearance of 3 amino acids and a decrease in 4 (Fig. 3G). Again histidine and glycine were brought to levels more comparable with *in vivo*, perhaps suggestive of a compensatory mechanism of oviduct fluid regulation.

Fibroblast–epithelial communication has been extensively studied in the cells of the airways in a variety of species (Woodward *et al.* 1998, Knight 2001, Parrinello *et al.* 2005, Noble 2008, Ohshima *et al.* 2010, Srisuma *et al.* 2010, Chhetri *et al.* 2012, Sakai & Tager 2013, Nishioka *et al.* 2015), but fibroblast–epithelial interactions have been investigated to a much lesser extent in the oviduct. However, Chen and coworkers (2013*b*) reported a highly differentiated porcine oviduct epithelial phenotype when cultured in fibroblast-conditioned medium.

BOEC gene expression

To further understand the amino acid transport, the expression of a number of key amino acid transporters were investigated in BOECs cultured in plastic flasks.

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Expression of *SLC1A1*, the high-affinity l-aspartate excitatory amino acid co-transporter 3 (EAAC3), was increased in response to T (Fig. 4C) in agreement with Franklin and coworkers (2006). However, *SLC1A1* expression did not respond to P4 *in vitro* corresponding to earlier reports that *SLC1A1* expression decreases in the bovine uterine endometrium during the progesterone-dependent phase (day 16–20) of ruminant pregnancy (Forde *et al.* 2014). Notably, as *SLC1A1* expression rose in response to a fall in T aspartate transport (Fig. 3F) suggesting that aspartate flux is not solely a function of *SLC1A1* gene expression.

Expression of *SLC7A1*, the arginine- and lysine-specific cationic amino acid transporter 1 (CAT1) (Bröer 2008) increased in response to P4 supplementation (Fig. 4D), as did the accumulation of arginine and lysine in *iv*DOF when BOECs were supplemented with P4 (Fig. 3F). P4 similarly upregulated *SLC38A5 in vitro* (Fig. 4F) corresponding with an increase in alanine and glycine transport as expected (Fig. 3F) and further suggesting that amino acid transport in the oviduct is hormonally regulated.

Using this model, we confirm that BOECs *in vitro* express hormonally responsive genes, which correlate with previously reported *in vivo* findings. In most cases, the secretion of amino acids in *iv*DOF correlated well with transporter expression.

The influence of pathophysiological endocrine supplementation

As a proof-of-principle sub-study, the efficacy of the aforementioned *in vitro* oviduct preparation was tested for studying the impact of disease states on the oviduct epithelium and fluid composition. The model was subjected to pathophysiological endocrine stimuli at either end of the androgenic spectrum, in addition to a physiological hormonal balance as a form of control (Table 1).

ESR1 expression (Fig. 5A) in flask-cultured BOECs was surprising as it correlated negatively with E2 supplementation, but positively with T addition to culture (Table 1). This, however, could be explained by T having a low affinity for the oestrogen receptor *in vitro* (Rochefort & Garcia 1976). *OVGP1* expression was highest after physiological hormonal supplementation (Fig. 5B) with hypoandrogenic and hyperandrogenic treatment similarly decreasing *ZO1* expression relative to physiological (Fig. 5C). To investigate the latter from a functional perspective, using the *in vitro* oviduct model described, TEER measurements were taken, as epithelial resistance is proportional to *ZO1* expression (Sultana *et al.* 2013). Figure 5D shows that a hyperandrogenic endocrine profile indeed reduced TEER and moreover increased the volume of *iv*DOF produced (Fig. 5E). It is tempting to speculate that this *leaky oviduct* phenotype is driven by impaired ERα activity as *ZO1* expression is responsive to E2 (Zeng *et al.* 2004) and ER activity (Weihua *et al.* 2003), potentially via a miR-191/425-mediated mechanism (Di Leva *et al.* 2013). Moreover, Liu and coworkers (1999) reported that T reduced TEER in the Caco-2 cell line.

Figure 5F shows that pathophysiological endocrine conditions did not affect glucose, lactate and pyruvate secretion. Moreover, these carbohydrate outputs did not differ from those from untreated cells (Fig. 3C), despite the known effects of sex hormone-mediated anabolism (Miers & Barrett 1998) and the associated heightened energetic demands. In contrast, hyperandrogenic treatment had a lesser impact on amino acid flux regulation than hypoandrogenism. A striking observation was the elevation of arginine after physiological hormonal supplementation (Fig. 5G) compared with all other treatments. Given its role in reproduction (Wu *et al.* 2009, Wang *et al.* 2015) and early embryo metabolism (Sturmey *et al.* 2010, Leary 2015), it is unsurprising that this amino acid would appear in *iv*DOF. Such high appearance could be explained by the fact that arginine can be readily synthesised from glutamate via ornithine (Wu 2010). Glycine was also interesting as it was elevated in *iv*DOF after hyperandrogenic incubation (Fig. 5G) but reduced after singular T supplementation (Fig. 3F), implying that the regulation of glycine flux is not solely T dependent.

Conclusions

We present a method for examining the formation of oviduct fluid under dual culture and a variety of singular, physiological and pathophysiological endocrine conditions within a controlled environment. This development offers the prospect of modelling the influence of the oestrous cycle (in animals) and the menstrual cycle (in women) with the possibility of using the data on the *iv*DOF generated to optimise embryo culture media.

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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