Modelling aspects of oviduct fluid formation \textit{in vitro}

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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 \textit{in vitro} preparation of the bovine oviduct epithelium to investigate the formation and composition of \textit{in vitro}-derived oviduct fluid (ivDOF) within a controlled environment. We confirm the presence of oviduct-specific glycoprotein 1 in ivDOF and show that the amino acid and carbohydrate content resembles that of previously reported \textit{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 ivDOF in response to steroid hormones were investigated. We next incorporated fibroblasts directly beneath the epithelium. This dual culture arrangement represents more faithfully the \textit{in vivo} environment and impacts on ivDOF composition. Lastly, physiological and pathophysiological endocrine states were modelled and their impact on the \textit{in vitro} oviduct preparation was evaluated. These experiments help clarify the dynamic function of the oviduct \textit{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.

\textit{Reproduction} (2017) 153 23–33

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 \textit{in situ} and \textit{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 \textit{in vivo} state more closely and...
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 (2013a) 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 OVGPI 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 ivDOF 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 CaCl₂ and MgCl₂ (Invitrogen), 10mM 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 μM 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 10⁶ cells/mL/insert. BOECs were subsequently maintained between apical and basal culture medium-filled chambers, at 39°C in 5% CO₂, 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 2500 Ω·cm² to ~8000 Ω·cm² 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·cm² 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% CO₂. After 24 h, a thin film of fluid formed in the apical chamber—termed in vitro-derived oviduct fluid (ivDOF) (Fig. 1A).

Dual culture

Bovine oviduct fibroblast cells were harvested by trypsinization from tissue culture flasks after 5 days in culture. 1 x 10⁶ 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 experimentation 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 et al. 2003).
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

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>17β-Oestradiol (E2)</th>
<th>Progesterone (P4)</th>
<th>Testosterone (T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native (N)</td>
<td>0 pM</td>
<td>0 pM</td>
<td>0 pM</td>
</tr>
<tr>
<td>17β-Oestradiol (E2)</td>
<td>29.37 pM</td>
<td>0 pM</td>
<td>0 pM</td>
</tr>
<tr>
<td>Progesterone (P4)</td>
<td>0 pM</td>
<td>6.36 nM</td>
<td>0 pM</td>
</tr>
<tr>
<td>Testosterone (T)</td>
<td>0 pM</td>
<td>0 pM</td>
<td>62.77 pM</td>
</tr>
<tr>
<td>Hypoandrogenic (HYPO)</td>
<td>29.37 pM</td>
<td>6.36 nM</td>
<td>2.43 pM</td>
</tr>
<tr>
<td>Physiological (PHYS)</td>
<td>29.37 pM</td>
<td>6.36 nM</td>
<td>208 pM</td>
</tr>
<tr>
<td>Hyperandrogenic (HYPER)</td>
<td>19.46 pM</td>
<td>6.36 nM</td>
<td>6.27 nM</td>
</tr>
</tbody>
</table>

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In vitro-derived oviduct fluid

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Figure 1 (A) Schematic representation of the culture system for in vitro-derived oviduct fluid (ivDOF) 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.
Table 2  List of the bovine-specific exon spanning primers used.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Direction</th>
<th>Sequence</th>
<th>$T_m$ (°C)</th>
<th>GC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTB</td>
<td>Forward (3' to 5')</td>
<td>TTTACACCCCTGGCAGGCCTATTCCC</td>
<td>59.64</td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>TTACACCCCTGGCAGGCCTATTCCC</td>
<td>59.73</td>
<td>55</td>
</tr>
<tr>
<td>OVGPI*</td>
<td>Forward (3' to 5')</td>
<td>CGACCCAGACCACCAAGGCAG</td>
<td>57.20</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>CGACCCAGACCACCAAGGCAG</td>
<td>57.10</td>
<td>55</td>
</tr>
<tr>
<td>ESR1*</td>
<td>Forward (3' to 5')</td>
<td>AGGGAAGCTCATTGTGCGGCTTACCC</td>
<td>57.00</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>AGGGAAGCTCATTGTGCGGCTTACCC</td>
<td>57.30</td>
<td>57</td>
</tr>
<tr>
<td>SLC1A1</td>
<td>Forward (3' to 5')</td>
<td>CACCGTCCTGAGTGGGCTTGC</td>
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<td>67</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>CACCGTCCTGAGTGGGCTTGC</td>
<td>61.30</td>
<td>64</td>
</tr>
<tr>
<td>SLC3A2</td>
<td>Forward (3' to 5')</td>
<td>GAACCCAGACCACCAAGGCAG</td>
<td>58.10</td>
<td>62</td>
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<td></td>
<td>Reverse (5' to 3')</td>
<td>GAACCCAGACCACCAAGGCAG</td>
<td>61.80</td>
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<tr>
<td>SLC3A5</td>
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<td>TGCGCTGCTCCTCGGCTGAGGG</td>
<td>63.20</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>TGCGCTGCTCCTCGGCTGAGGG</td>
<td>62.00</td>
<td>64</td>
</tr>
<tr>
<td>SLC3A7</td>
<td>Forward (3' to 5')</td>
<td>CGCGCGCGCGCAGTGAGAC</td>
<td>61.60</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>CGCGCGCGCGCAGTGAGAC</td>
<td>60.90</td>
<td>62</td>
</tr>
<tr>
<td>SLC6A14</td>
<td>Forward (3' to 5')</td>
<td>TCAGAGGGCGACACTCGGAGGG</td>
<td>62.90</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Reverse (5' to 3')</td>
<td>TCAGAGGGCGACACTCGGAGGG</td>
<td>56.80</td>
<td>60</td>
</tr>
<tr>
<td>ZO1</td>
<td>Forward (3' to 5')</td>
<td>CTCTCTCTGCTCTGCTCCTCCC</td>
<td>55.20</td>
<td>45</td>
</tr>
</tbody>
</table>

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-OGVGPI sera were generated by immunisation of female BALB/c mice in intervals of 3 weeks 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 ivDOF 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-OGVGPI primary antibody described previously (1:1000) for 24 h,

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**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).
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 &
According to manufacturer instructions. The concentration of cDNA was synthesised by reverse transcription using the High Capacity cDNA Reverse Transcription Kit (Fisher Scientific) in accordance to manufacturer instructions. The concentration of cDNA was 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 ivDOF 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 ivDOF isolations.

In this study, in vitro-derived oviduct fluid (ivDOF; Fig. 1A) from untreated (native) bovine oviduct epithelial cells was analysed and compared with previously reported in vivo observations. The composition of ivDOF after singular cellular hormonal supplementation was analysed, and the influence of dual culture was also examined. These data are contrasted against native ivDOF. 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 ivDOF 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 ivDOF from untreated BOECS after a 24-h period of culture was 25.2 ± 4.5 μL (Fig. 3A) and the mean osmolality was 297 ± 12 mosmol (Fig. 3B). Untreated ivDOF 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 OVGPI were performed on oviduct fluid derived from fresh abattoir tissue (Fig. 3D) and compared with blots given by ivDOF.

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**Figure 4** Gene expression profiles of (A) ESR1, (B) OVGPI, (C) SLC1A1, (D) SLC7A1, (E) SLC38A2, (F) SLC38A5, (G) SLC38A7 and (H) SLC6A14 as determined by qRT-PCR (Livak & Schmittgen 2001) corrected product length, melt curves were performed (Livak & Schmittgen 2001) to ensure correct product length. 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.

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in flask-cultured BOECs whilst reducing expression (Fig. 5E). In addition, a panel of solute carrier genes (Table 1) were expressed in flask-cultured cells after harvest and increased after 24 h of E2 exposure (Fig. 4). 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 ivDOF produced (Fig. 5E). Figure 5F shows that hypo and hyper treatments had no significant impact on the carbohydrate composition of ivDOF. Lastly hypoandrogenism reduced histidine, glycine, threonine, arginine, alanine and lysine secretion, whereas hyperandrogenism reduced histidine and arginine but elevated the apical accumulation of glutamine, threonine, arginine, alanine and lysine (Fig. 5G).

**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
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 24h after confirmation of confluence, a film of liquid appeared in the apical chamber, which contained OVGPI 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 (ivDOF). 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 ivDOF amino acid composition (Fig. 3G). In addition, parallel flask-cultured BOECs expressed the genes ESR1 and OVGPI 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).

**ivDOF characterisation**

The volume of native ivDOF 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 ivDOF 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 ivDOF vs in vivo.

OGVGPI in ivDOF was ~60kDa (Fig. 4E) suggestive of the de-glycosylated form, in contrast to the ~80–90kDa 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 OVGPI-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 ivDOF (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 ivDOF 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 ivDOF pH. Addition of E2 caused histidine in ivDOF 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 ivDOF 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 epithelial 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 ivDOF 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 ivDOF 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 2001, Nishioka et al. 2015), but fibroblast–epithelial interactions have been investigated to a much lesser extent in the oviduct. However, Chen and coworkers (2013b) 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.
Expression of \textit{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, \textit{SLC1A1} expression did not respond to P4 \textit{in vitro} corresponding to earlier reports that \textit{SLC1A1} expression decreases in the bovine uterus endometrium during the progesterone-dependent phase (day 16–20) of ruminant pregnancy (Forde et al. 2014). Notably, as \textit{SLC1A1} expression rose in response to a fall in T aspartate transport (Fig. 3F) suggesting that aspartate flux is not solely a function of \textit{SLC1A1} gene expression.

Expression of \textit{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 \textit{in vitro} when BOECs were supplemented with P4 (Fig. 3F). P4 similarly upregulated \textit{SLC38A5} \textit{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 \textit{in vitro} express hormonally responsive genes, which correlate with previously reported \textit{in vivo} findings. In most cases, the secretion of amino acids \textit{in vitro} correlated well with transporter expression.

**The influence of pathophysiological endocrine supplementation**

As a proof-of-principle sub-study, the efficacy of the aforementioned \textit{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).

\textit{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 \textit{in vitro} (Rochefort & Garcia 1976). \textit{OVGP1} expression was highest after physiological hormonal supplementation (Fig. 5B) with hyperandrogenic and hyperandrogenic treatment similarly decreasing \textit{ZO1} expression relative to physiological (Fig. 5C). To investigate the latter from a functional perspective, using the \textit{in vitro} oviduct model described, TEER measurements were taken, as epithelial resistance is proportional to \textit{ZO1} expression (Sultana et al. 2013). Figure 5D shows that a hyperandrogenic endocrine profile indeed reduced TEER and moreover increased the volume of \textit{in vitro} oviduct fluid (Fig. 5E). It is tempting to speculate that this \textit{leaky oviduct} phenotype is driven by impaired E\textsubscript{R\alpha} activity as \textit{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 (Sturmay et al. 2010, Leary 2015), it is unsurprising that this amino acid would appear \textit{in vitro}. 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 \textit{in vitro} 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 \textit{in vitro} oviduct fluid 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.

**Funding**

C A S was fully funded by a University of Hull studentship and a Hull York Medical School (HYMS) fellowship.

**Acknowledgements**

The authors would like to thank staff at ABP, York, UK, Dr L Madden (FACS), Mrs A Lowry (TEM) (University of Hull, UK), Dr A Aburima, Prof. KM Naseem (WB), and Ms P Sfyri, and Dr A Matsakas (H&E) (Hull York Medical School, UK), in addition to the University of Hull and the Hull York Medical School for funding CA Simintiras and this work.