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Reproduction, Fertility and Development

The role of the oviduct environment in embryo survival

Jennifer L. Juengel[A,*], Karen L. Reader[B], Paul H. Maclean[C], Laurel D. Quirke[A], Sylvia Zellhuber-McMillan[B], Neville A. Haack[D] and Axel Heiser[D]

For full list of author affiliations and declarations see end of paper

***Correspondence to:**
Jennifer L. Juengel
Agricultural Systems and Reproduction, AgResearch Limited, Invermay Agricultural Centre, Puddle Alley, Mosgiel 9092, New Zealand
Email: jenny.juengel@agresearch.co.nz

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ABSTRACT

Context. Declining fertility is an issue in multiple mammalian species. As the site of fertilisation and early embryo development, the oviduct plays a critical role in embryo survival, yet there is a paucity of information on how the oviduct regulates this process. **Aims.** We hypothesised that differences in steroid hormone signalling and/or immune function would be observed in a model of poor embryo survival, the peripubertal ewe. **Methods.** We examined expression of steroid hormones in systemic circulation, oviductal expression of oestrogen receptor α and genes important in steroid hormone signalling, and immune function in pregnant and cyclic peripubertal and adult ewes on day 3 after oestrus. **Key results.** Concentrations of progesterone, but not oestradiol, were decreased in the peripubertal ewe compared to the adult ewe. Oestrogen receptor α protein expression was increased in the peripubertal ewe, but pathway analysis of gene expression revealed downregulation of the oestrogen signalling pathway compared to the adult ewe. Differential expression of several genes involved in immune function between the peripubertal and adult ewe was consistent with an unfavourable oviductal environment in the peripubertal ewe lamb. Oestradiol concentration was positively correlated with the expression of multiple genes involved in the regulation of immune function. **Conclusions.** Differences in the immune environment of the oviduct, potentially linked to differential modulation by steroid hormones, may partially underly the poor fertilisation and early embryo survival observed in the peripubertal ewe. **Implications.** A unfavourable oviductal environment may play an important role in limiting reproductive success.

Keywords: correlations, embryo survival, gene expression, immune function, oestrogen, oviduct, progesterone, sheep.

Introduction

While the oviduct is known to be essential for natural reproduction, fertilisation and normal early embryo development can occur *in vitro*. This indicates that the functions of the oviduct can be replaced with defined culture conditions. This has focused research attention on the quality of the oocyte as the primary driver of early embryo development, with less attention given to the potential of the oviduct environment as a key regulator of fertilisation and the health and development of the early embryo. However, only 18.6% of initiated human-assisted reproduction cycles in 2020 resulted in a live birth (Newman *et al.* 2022), indicating the *in vitro* environment is sub-optimal. In cattle *in vitro* embryo production only 20–40% of the presumptive zygotes develop into blastocysts with approximately 30% of the blastocysts failing to hatch (Ferre *et al.* 2020). Recent studies have highlighted the potential role steroid hormones and immune regulators may play in modulating the oviduct environment and, thereby, fertility.

In sheep, increased oestradiol and progesterone concentrations tended to increase the percentage of healthy embryos early in gestation (Gonzalez-Bulnes *et al.* 2005). Similarly, lowering oestradiol concentrations during maturation of the preovulatory follicle in sheep increased unfertilised oocytes and retarded embryos (Oussaid *et al.* 1999). More intense mating behaviour, linked to increased oestradiol concentrations, is associated with increased embryo survival in both sheep (Juengel *et al.* 2020) and cattle (Madureira *et al.* 2015). In mice, specific ablation of the oestrogen receptor 1 gene (*Esr1*) in oviductal and

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uterine endothelial cells induced infertility through impaired fertilisation and early embryo death (Winuthayanon *et al.* 2015).

Progesterone is linked with enhanced embryo survival in cattle (Mann 2001; Mann and Lamming 2001; Green *et al.* 2005; Robinson *et al.* 2008) and sheep (Parr 1992), although excessive progesterone can be detrimental to embryo health (Diskin *et al.* 2016). Furthermore, in sheep, ewes with naturally enhanced embryo survival were shown to have increased concentrations of progesterone, both systemically and in the uterine vein, during the first few days of either the oestrous cycle or pregnancy (O'Connell *et al.* 2013).

The immune system is known to play an important role in establishment and maintenance of pregnancy. It has long been known that seminal plasma/sperm illicit an immune response in the female reproductive tract, including the oviduct (Schjenken and Robertson 2015; Saint-Dizier *et al.* 2020). Genes normally associated with immune function are also expressed in the embryo and regulate early embryo development (Hansen 2014). Activin and follistatin, known for their key regulatory roles in fertility (Wijayarathna and de Kretser 2016) and inflammation (Phillips *et al.* 2009), are also linked to early embryo development and survival (Rajput *et al.* 2013; O'Connell *et al.* 2016*a*). Pregnancy induced by natural mating does cause changes in gene expression of the ovine oviduct/uterine endometrium from the tip of the uterus on day 5 of pregnancy, with a key pathway underlying this observed difference to non-pregnant animals related to T-cell function (Quirke *et al.* 2021). Furthermore, persistence of innate immune factors in the oviduct was associated with damage to embryos and embryo death in mice with ablation of Esr1 expression in oviductal and uterine endothelial cells (Winuthayanon *et al.* 2015). The oviduct may not be essential for fertilisation and normal embryo development given the ability to fertilise and develop embryos *in vitro,* thereby bypassing the oviduct. However, changes in the oviductal environment could either enhance or suppress fertilisation and early embryo development.

We hypothesised that differences in steroid hormone signalling and/or immune function would be observed in a model of poor embryo survival. To test this hypothesis, we examined expression of steroid hormones in systemic circulation, oviductal expression of oestrogen receptor α (ERα) and expression of genes important in steroid hormone signalling and immune function in pregnant and cyclic peripubertal and adult ewes on day 3 after oestrus expression.

The peripubertal ewe was chosen as a model of poor embryo survival as they are known to have approximately twice the embryo loss when compared to adult ewes (Edwards *et al.* 2016). Studies following multiple ovulation and embryo transfer also highlighted that the poor fertility of the peripubertal ewe was driven by oocyte and/or oviductal quality (Quirke and Hanrahan 1977, 1983). In naturally cycling and mated peripubertal ewes, a majority of this loss occurred during the first 3 days of pregnancy, with fertilisation failure and embryo degradation during the very early stages of development being key components of this loss (Juengel *et al.* 2023). In contrast to this, while a majority of embryo/fetal loss occurs during the first 2 weeks of pregnancy in adult ewes, loss between days 4 and 14 was approximately twice that observed prior to day 4 (O'Connell *et al.* 2016*b*). This highlights that embryo loss may be driven by potentially different pathways in peripubertal ewes compared with adult ewes, with differences in oviductal function potentially underlying the reduced fertilisation and early embryo survival observed in peripubertal ewes.

Materials and methods

Experimental design

The experiment was approved by the Invermay Animal Ethics Committee (application number 13979) and was in accordance with the New Zealand Animal Welfare regulations. Animals used in the experiment were crossbreds (dominant breeds Romney, Coopworth and Texel). Peripubertal ewes were approximately 8 months of age, which is the average age of puberty (Edwards and Juengel 2017), and adult ewes were 3.5–5.5 years of age. The animals underwent standard farm management with supplementation with iodine and vaccinations against infectious diseases as described (Juengel *et al.* 2023), with the adults only receiving booster immunisations as required. Animals were run as a single group, with the animals exposed to three vasectomised rams initially for collection of samples from non-pregnant ewes, and then three fertile rams replaced the vasectomised rams for collection of samples from pregnant ewes. Given the lower reproductive rate of peripubertal ewes, some peripubertal ewes were joined with the fertile rams from the beginning. This allowed some tissues from the pregnant and non-pregnant animals to be collected during the same time period, and all tissues were collected within a 2-week period. All animals were kept on similar pastures throughout the trial. Weight of each animal was measured prior to introduction of the rams. All rams were fitted with a mating harness with crayon and ewes were observed for mating marks each morning. The first morning a ewe was observed with a mating mark was considered day 1 as mating would have occurred in the previous 24 h and collection of tissue occurred on the afternoon of day 3.

Blood and tissue collection

Two blood samples (approximately 10 mL) were collected by jugular venepuncture using a vacutainer with lithium heparin additive (Becton Dickinson from Export Lodge Veterinary Supplies; Levin, New Zealand). The first was collected at first observation of a mating mark and the second was collected just prior to tissue collection on day 3. Samples were centrifuged shortly after collection and plasma was stored

at −20°C until utilised for determination of steroid concentrations.

On day 3 following observation of a mating mark, animals were humanely killed by captive bolt and exsanguination and their reproductive tracts were collected post-mortem. The number of corpora lutea (CL) with visible ovulation stigma on each ovary was recorded. Unfertilised oocytes or embryos were collected by flushing the uterine horn and oviduct twice with 5 mL of compound sodium lactate (Hartmann's) solution (Baxter Healthcare Pty. Ltd, Toongabbie, Australia) with 1 mM 3-(N-morpholino) propanesulfonic acid (MOPS) using a syringe with a blunt needle. This solution was collected from a polyethylene tube (OD 2.5 mm, ID 1.5 mm, Tyco Electronics; Biocorp Aust Pty Ltd, Huntingdale, Victoria, Australia) inserted into the oviduct fimbria. An approximately 1 cm piece of the isthmus of the oviduct, adjacent to the utero-tubal junction, was collected from each side of the reproductive tract. Tissue was frozen in liquid nitrogen and stored at −80°C until processing for protein/RNA analysis. In animals with bilateral ovulations, one isthmus was fixed in 4% (w/v) paraformaldehyde and paraffin-embedded for immunohistochemistry (Smith *et al.* 2019). Only animals with a recovered unfertilised oocyte (non-pregnant; only exposed to the vasectomised ram) or embryo (pregnant group exposed to fertile ram) were utilised in downstream analysis.

Oestradiol and progesterone measurements

Concentrations of progesterone and oestradiol were measured in plasma samples using RIA kits (Ultra-sensitive Estradiol RIA kit, Beckman Coulter CA, USA and IBL Coat-a-Count RIA, IBL, Hamburg, Germany) as previously described (Smith *et al.* 2019; Juengel *et al.* 2020). Concentrations of oestradiol were measured from the samples collected on day 1 and 3, whereas concentrations of progesterone were only measured on day 3 given the known low concentrations of progesterone at the time of oestrus in sheep. The sensitivities of the assays were 2 pg/mL and 0.2 ng/mL for oestradiol and progesterone, respectively. No samples were below the sensitivity of the assays. The intra- and inter-assay coefficients of variation were all <16% for both hormones.

Isolation of RNA and protein

A total of six animals per age and group (24 total animals) were used for isolation of RNA and protein. Protein was extracted from the frozen oviduct tissue using a Nucleospin miRNA kit (Macherey-Nagel, Duren, Germany) following manufacturer's instructions. The isolated protein was stored at −80°C in Protein Solving Buffer. Protein quantification was performed using a Protein Quantification Assay kit (Macherey-Nagel) as per the manufacturer's instructions. Total RNA was also isolated from the frozen oviduct tissue using the Nucleospin miRNA kit as previously described (Quirke *et al.* 2021) with minor modifications. Samples were homogenised using an automated tissue homogeniser (SPEX SamplePrep 2010 Geno/Grinder, NJ, USA) with 2 × 3 mm stainless steel beads for 3 min at 1500 rpm. The RNA was eluted in 30 μL RNase-free water and stored at −80°C.

Immunohistochemistry

Tissue from six peripubertal (three each of pregnant and non-pregnant animals) and six adult (four non-pregnant, two pregnant animals) were used for immunohistochemistry. The sections were dewaxed, and antigen retrieval was performed in a microwave (1000 Watts for 12 min) in 0.01 M sodium citrate buffer at pH 6.0. Slides were quenched for 5 min in 3% H_2O_2, washed two times in PBS, blocked for 20 min in CAS-Block reagent (Cat#00812; Thermo Fisher, Auckland, New Zealand), then incubated in 2.5 μg/mL of rabbit anti-ERα antibody (Cat# 06-935; Merck, Auckland, New Zealand) overnight. Secondary antibody detection was performed using the DAKO EnVision + Dual Link System-HRP Rabbit/Mouse (DAKO, Glostrup, Denmark) with a 15 min diaminobenzidine (DAB) incubation followed by hematoxylin counterstaining. Negative control rabbit immunoglobulin fraction (Cat# X0936; DAKO) was used as a negative control at the same concentration as the primary antibody. Slides were imaged on an Aperio CS2 Digital Slide Scanner (Leica Biosystems, Victoria, Australia).

Western blotting

For each oviduct sample, 30 μg of protein was separated on a 12% SDS-PAGE acrylamide gel, under reducing conditions, and transferred to a nitrocellulose membrane. After blocking in Intercept blocking buffer (Millenium Science, Auckland, New Zealand), membranes were incubated overnight in 0.5 μg/mL rabbit IgG anti-ERα antibody (Cat# ab108398; Abcam) and mouse IgG1 anti-alpha tubulin at 0.09 μg/mL (Cat#38735; Cell Signalling, New England Biolabs, Auckland, New Zealand) as a loading control. Secondary antibody detection was performed using IRDye 800 CW goat anti-rabbit IgG at 1:10 000 dilution and IRDye 680 LT goat anti-mouse IgG1 at 1:20 000 dilution (Li-Cor, Millennium Science) and imaged with an Odyssey Classic scanner. This was repeated in two to three replicate western blots and relative signal intensities were compared between samples, normalised to alpha tubulin, using the Image Studio Lite software (Li-Cor).

Quantification of RNA expression

The nCounter Analysis System (NanoString Technologies Inc. Seattle, WA) was used to quantify expression of 131 genes related to immune function and steroid hormone signalling. The ProbeSet also included nine reference genes. The procedures, including the ProbeSet, described in Quirke *et al.* (2021) were used for RNA quantification. As expected, the normalisation factor for all samples was inside the 0.1–30 range.

Statistical analysis

Differences in live weight, ovulation rate, concentrations of progesterone and estradiol in plasma, and amount of ERα protein between age groups were determined by ANOVA. The model included effects of pregnancy status and the interaction of age and pregnancy status. For assessment of ERα protein quantification by western blot, the technical replicates were averaged per animal and the value was transformed (natural log) to improve normality of the data for analysis. Overall differences in gene expression between groups were determined using permutation ANOVA with 1 000 000 iterations. This was undertaken in R using the aovp function in the lmPerm package ver. 2.1.0 (Wheeler *et al.* 2016). The Benjamini-Hochberg (FDR) method (Benjamini and Hochberg 1995), undertaken with the p.adjust function in R, was used to correct for multiple testing. The model included both age and pregnancy status as well as their interaction. For pathway analysis, genes with uncorrected *P*-values below 0.05 were used given that the oviduct represents a highly complex system, the transcriptome only provides a 'snapshot' of gene expression at a single time point, and not all genes are expected to be highly differentially expressed at once. Gene categories were assigned and analysed as previously described (Quirke *et al.* 2021). Pearson correlation analysis between gene expression and oestradiol concentrations on day 1 and progesterone concentrations at time of tissue collection were undertaken as described by Quirke *et al.* (2021). Oestradiol concentrations on day 1 were chosen as previous work has shown an association among oestrous behaviour, embryo survival and oestradiol concentrations from plasma collected around this time (Juengel *et al.* 2020).

Results

General characterisation of animals

Information regarding weight, ovulation rate and concentrations of oestradiol and progesterone in plasma are shown in Table 1. No interactions were observed between age and pregnancy status for weight, ovulation rate or steroid concentrations. Therefore, pregnant and non-pregnant animals were grouped together for presentation. Peripubertal ewes weighed less than adult ewes, with a decreased ovulation rate. Concentrations of oestradiol did not differ between peripubertal and adult ewes at either time point, but concentrations of progesterone in plasma were increased in adult compared with peripubertal animals.

Expression of ERα in the oviduct

The pattern of expression of ERα in the oviduct was similar among all groups, with no obvious differences observed. As expected, signal was strongly expressed in the nucleus of the cell, with expression strongest in epithelial cells of the oviduct (Fig. 1). No interactions were observed between age and pregnancy status for amount of protein as determined by immunoblot. However, concentrations of ERα protein were increased in peripubertal ewe lambs compared with adult ewes (Table 1).

Table 1. Live weights (av. ± s.e.m.), ovulation rate (av. ± s.e.m.), and concentrations of oestradiol on days 1 and 3 (av. ± s.e.m.), progesterone on day 3 (av. ± s.e.m.) and ERα protein quantified by western blot (geometric mean and 95% confidence intervals).

Measurement	Peripubertal ewe lamb	Adult ewe
Live weight	47.2 ± 1.3	70.5 ± 1.8***
Ovulation rate	2.1 ± 0.1	2.8 ± 0.2**
Oestradiol day 1 (pg/mL)	9.97 ± 1.44	7.45 ± 0.86
Oestradiol day 3 (pg/mL)	7.21 ± 0.98	5.88 ± 1.01
Progesterone day 3 (ng/mL)	0.78 ± 0.08	1.21 ± 0.16*
ERα protein arbitrary units	99.6 (73.8–134.5)	59.1 (43.7–79.7)*

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Values within rows differ from each other.

Permutation ANOVA analysis of the effects of age and pregnancy status on expression of individual genes

There were no genes with interactions between age and pregnancy that reached significance when adjusting for multiple comparisons. Likewise, when adjusted for multiple comparisons, pregnancy status did not significantly affect expression of any gene (FDR > 0.65). Differential expression of 13 genes was identified (FDR < 0.05) when comparing peripubertal to adult isthmus tissue. Expression of *ESRRG*, *EPCAM*, *IGF1R*, *IL6*, *MCTP1*, *NFKB1*, *PAX8*, *PGR*, *PGRMC1* and *PTX3* was decreased in peripubertal ewes compared to adult ewes (Table 2). In contrast, concentrations of mRNA for *IL12RB1*, *NOS2* and *SERPINE1* were increased in peripubertal compared with adult isthmus tissue (Table 2). Expression of four other genes also strongly tended (FDR < 0.055) to differ between peripubertal ewe lambs and adult ewes. Expression of *ESR1* and *EPHX1* was decreased, whereas *CD86* and *SELL* was increased (Table 2).

Pathway analysis

Given the weak separation of tissues based on pregnancy status, pathway analysis was undertaken only for differences relating to age. Focusing on immune and steroid hormone signalling pathways, several genes in the oestrogen signalling pathway were noted to be upregulated ($P < 0.01$) in the adult tissues compared with the oviducts from the peripubertal animals, including ESR1 itself with FDR = 0.051 (Fig. 2). While single genes relating to immune function were identified as differentially expressed between peripubertal and adult animals, with the limited gene numbers included

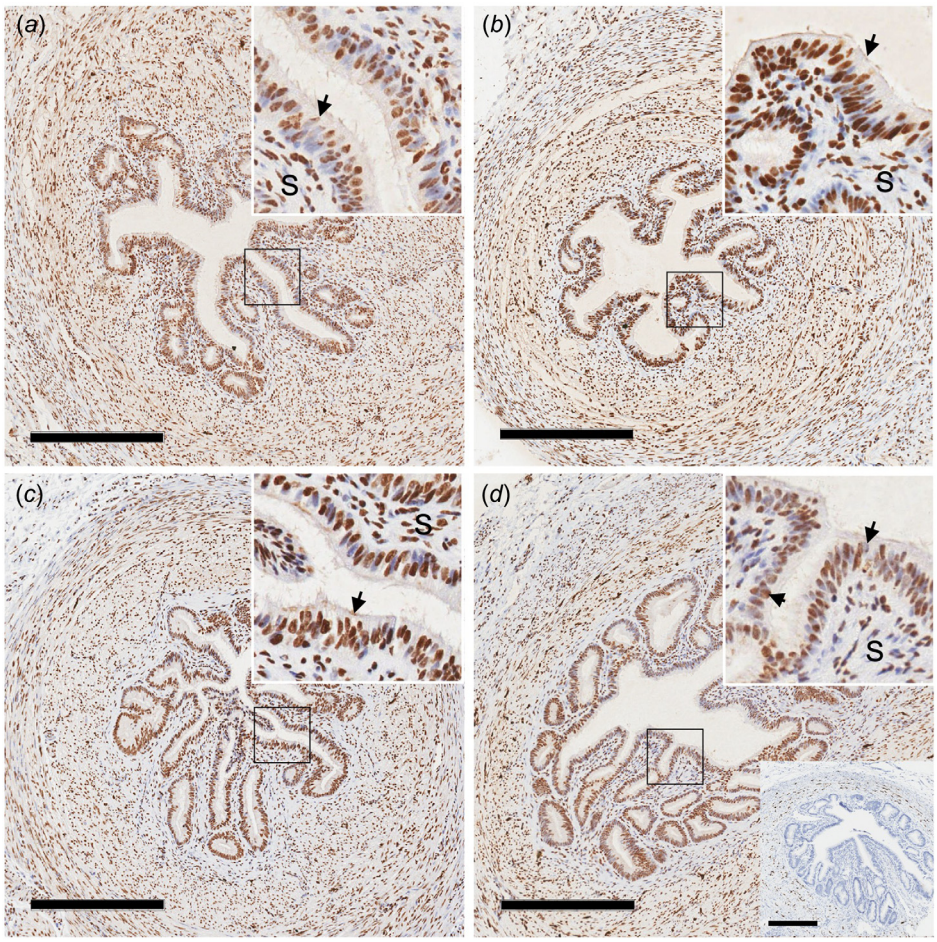

Fig. 1. Photomicrographs of oviduct isthmus from peripubertal ewe lambs (*a*, *b*) and adult ewes (*c*, *d*) from day 3 of the oestrous cycle (*a*, *c*) or pregnancy (*b*, *d*). Nuclear staining (brown colour) for oestrogen receptor α (ERα) was observed in the nucleus of many cell types of the oviduct, with very strong staining in the epithelial cells. The inset in the top right-hand corner represents an increased magnification of the epithelium (arrows) and underlying stroma (S) cells. A photomicrograph of the negative control IgG is presented in the lower right-hand corner of panel *d*. Scale bar = 300 μm.

in the nanostring analysis, no specific immune pathway was highlighted.

Correlation analysis between plasma steroid hormone concentration and gene expression

Correlations were observed between multiple genes and progesterone concentrations on day 3 and oestradiol concentrations on day 1 (Fig. 3). Highlighted among these correlations are the negative correlations between day 3 progesterone concentrations and *TGFBR1* and *TGFBR2*, with there being a positive correlation between day 1 oestradiol concentrations and *TGFB1*. Two genes, *NOS2* and *CSFR2B*, were positively correlated with day 1 oestradiol concentrations but negatively correlated with day 3 progesterone concentrations. Several genes known to be involved in regulation of immune function, such as *FOXP3*, *IL4R*, *IL10RB*, *TNFRSF1A*, *INFAR1*, *DQB*, *ALOX5*, *CSF2RB*, *NOS2* and *SERPRINE1*, were also positively correlated with day 1 oestradiol concentrations.

Discussion

Infertility is considered a major issue in both humans and livestock species (Schmidt 2006; Inhorn and Patrizio 2015; Leroy *et al.* 2015; Campanile *et al.* 2021). Infertility is a multifactorial issue, with factors influencing pregnancy loss spanning from poor quality oocytes, failure of fertilisation and early embryo development, failure of maternal recognition of pregnancy and implantation, to placental insufficiency and poor fetal growth and development. However, in multiple species, the very early stages, from fertilisation to early embryo development before maternal recognition of pregnancy, are the most vulnerable periods, when a majority of pregnancies fail

Table 2. Expression of genes related to immune function that were differentially expressed between peripubertal ewe lambs and adult ewes.

Gene	FDR	Expression peripubertal/adult	Comments
IGFIR	0.004	0.75 ↓	Growth factor
MCTP1	0.045	0.68 ↓	Upregulated in oviduct in response to embryos
NFKB1	0.045	0.82 ↓	Regulatory, multiple cell types
PAX8	0.045	0.67 ↓	Regulatory, multiple cell types
EPCAM	0.025	0.66 ↓	Adhesion, epithelial cells and immune cells
SELL	0.051	2.25 ↑	Adhesion factor, expressed by leukocytes, homing to tissues
IL6	0.033	0.66 ↓	Inflammatory but anti-inflammatory in muscle
IL12RB1	0.045	1.23 ↑	Inflammatory
CD86	0.051	1.64 ↑	Upregulated during antigen presentation
SERPINE1	0.045	1.66 ↑	Protease inhibitor with antiangiogenic effects
PTX3	0.045	0.70 ↓	Inflammatory also linked to oocyte quality
NOS2	0.045	1.63 ↑	Inflammatory, generate ROS
EPHX1	0.054	0.73 ↓	Removal of ROS
PGRMC1	0.045	0.82 ↓	Progesterone signalling
PGR	0.045	0.64 ↓	Progesterone signalling
ESRRG	0.045	0.60 ↓	Oestrogen signalling pathway
ESR1	0.051	0.74 ↓	Oestrogen signalling

A downward arrow (↓) in expression indicates that expression was decreased in peripubertal ewe lambs compared with adult ewes, whereas an upwards arrow (↑) indicates expression was greater in peripubertal ewe lambs compared with adult ewes.

(O'Connell *et al.* 2016*b*; Berg *et al.* 2022; Juengel *et al.* 2023). The current experiment highlighted potential functional differences in the isthmus of the oviduct between peripubertal and adult animals. These differences could at least partially underlie the known decrease in fertility observed in peripubertal ewe lambs when compared to adult ewes.

As hypothesised, there appears to be reduced oestrogen signalling in peripubertal animals as supported by pathway analysis for oestrogen receptor activation. This extended to include decreased expression of *ESR1* mRNA as well as signalling co-factors (Co-A) and progesterone receptors. However, it is important to note that the amount of ERα protein in the isthmus was decreased in adult ewes compared with peripubertal ewe lambs. This appears in contrast to the apparent activation of the oestrogen signalling pathway when examining expression of various genes in this pathway. It is known that oestrogen can down-regulate ER protein expression independent of effects on its mRNA through ubiquitin-proteosome stimulated degradation of oestrogen receptor protein (Alarid *et al.* 1999; Nawaz *et al.* 1999). This was specific to this degradation pathway as calpain and lysosomal proteases were ineffective (Alarid *et al.* 1999). Potentially, this apparent discrepancy between *ESR1* mRNA and protein expression patterns relates to differences in the turnover rates of the proteins in the cells. However, examination of the pathway as a whole supports decreased oestrogen signalling in the isthmus of the oviduct of the peripubertal ewe lamb compared with more mature adults.

Expression of several genes that are known to be involved in immune function were positively correlated to concentrations of oestradiol on the day of breeding. These included *SERPINE1*, *NOS2*, *IL4R*, *IL10BR*, *IFNAR1*, *FOXP3*, *DQP*, *ALOX5*, *CSF2RB* and *TNFRS1A*. This observation is consistent with oestrogen potentially being a key regulator of the immune environment of the oviduct. In mice lacking ESRα in epithelial cells of the oviduct, the oviduct developed an unfavourable environment, with increases in proteases and factors involved in immune function (Winuthayanon *et al.* 2015). Similarly, differences in gene expression in the oviduct of the peripubertal animal are in general consistent with an unfavourable environment. This included an increased expression of *SELL*, an adhesion factor expressed by leukocytes, facilitating homing to tissues (Ivetic *et al.* 2019). Also increased in expression were: *IL12RB1*, part of the receptor complex for IL12 and IL23, with known links to autoinflammatory diseases (Verstockt *et al.* 2023; Yousaf *et al.* 2023) and *CD86*, important for activation of T-cells for clonal expansion and acquisition of effector functions (Somoza and Lanier 1995) and IgG_1 production from B cells (Podojil and Sanders 2005). Expression of *NOS2*, involved in synthesis of nitrous oxide and generation of reactive oxygen species (ROS) (Korhonen *et al.* 2005), was upregulated, whereas expression of *EPHX1*, which reduces reactive species (Cheong *et al.* 2009), was down-regulated, consistent with a more ROS active environment in the oviduct of peripubertal ewe lambs. Pregnancy has also been shown to upregulate *EPHX1* in the oviduct on day 2–3 of pregnancy in pigs (Martyniak *et al.* 2018), and oviductal EPHX1 reduced ROS levels during preimplantation of mouse embryos and improved embryo development (Cheong *et al.* 2009) providing support for a role for this protein in facilitating early pregnancy.

Two genes potentially not fitting this pattern were the expression of *IL6* and *PTX3*, which were down-regulated in the peripubertal ewe oviduct. While IL6 is generally known to be inflammatory, it also has anti-inflammatory effects (Xing *et al.* 1998; Scheller *et al.* 2011), with the differential expression of receptor sub-types on cells determining cellular response (Scheller *et al.* 2011). In the current analysis, RNA analysis was undertaken on the whole oviduct and thus which cell type(s) express *IL6*, and if the regulation in the different cell types is consistent, is unknown and worthy of further study. Additionally, IL6 is known to support early embryo development (Campanile *et al.* 2021). Addition of

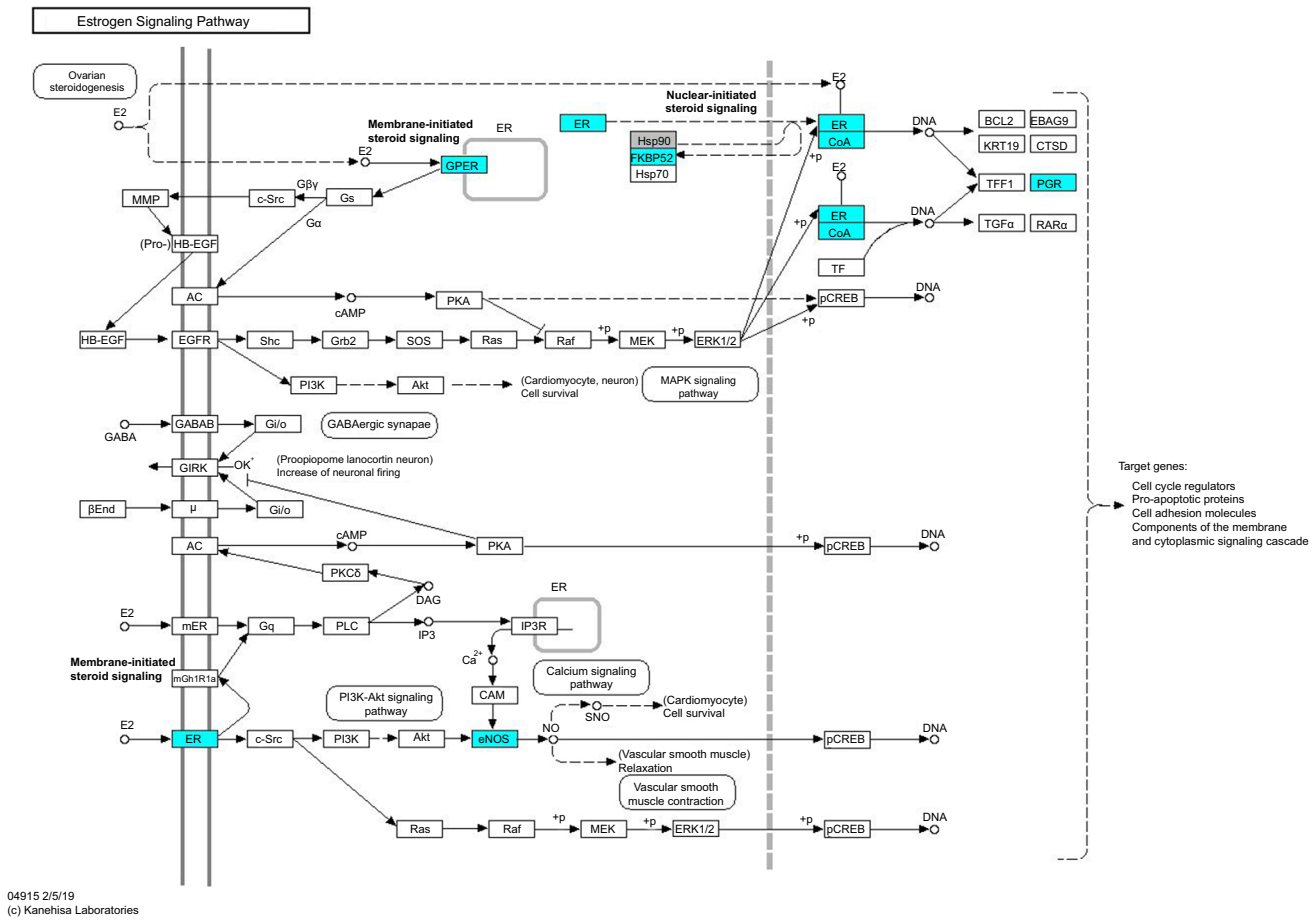

Fig. 2. Schematic view of oestrogen signalling pathway. Genes coloured blue were differentially expressed between isthmus of the oviduct of peripubertal ewe lambs and adult ewes, with expression being decreased in the peripubertal ewe lambs. (Modified with permission from 'KEGG map04915 estrogen signalling pathway'; Kanehisa 2019).

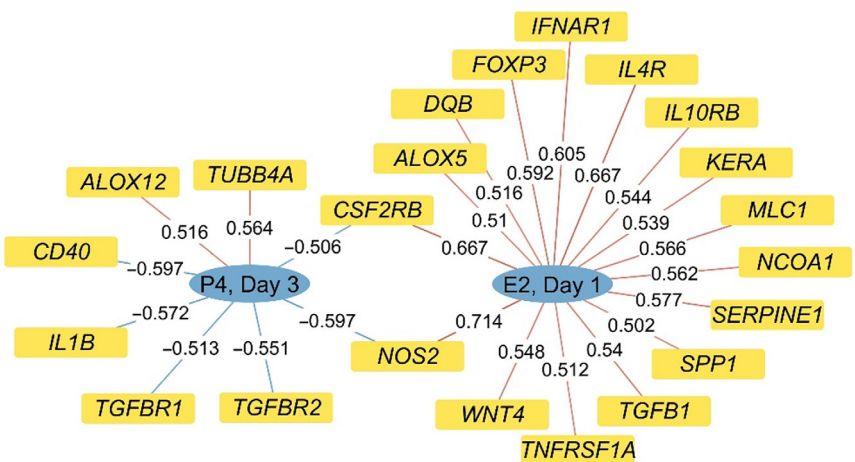

Fig. 3. Representation of correlations (indicated on the line for each gene) observed between selected genes and concentrations of oestradiol on day 1 (E2, D1) and progesterone on day 3 (P4, D3) of the reproductive cycle. Only genes with correlations greater than |0.50| were included and all are significant ($P < 0.05$).

IL6 in culture media of *in vitro*–produced bovine embryos increased the number of inner mass cells and pregnancy establishment (Seekford *et al.* 2021). PTX3 can also be considered as a marker of an inflammatory environment, as it is upregulated by several inflammatory cytokines and is linked to inflammatory diseases (Geyer *et al.* 2021; Zhang *et al.* 2022). However, PTX3 can also have anti-inflammatory effects. A complex of hyaluronan, the heavy chains of inter-α-trypsin inhibition proteoglycan and PTX3, isolated from amniotic membranes, was shown to be anti-inflammatory

in a model of chronic graft-versus-host disease (Ogawa *et al.* 2017). This complex inhibited M1 macrophage infiltration and supports polarising them to the M2 phenotype (He *et al.* 2014). This complex also forms in the cumulus cells and has been proposed to dampen leukocyte activity in the reproductive tract to help generate an optimal environment for fertilisation (Camaioni *et al.* 2018). It is important to note that *PTX3* is also highly expressed in the cumulus cells. Comparative expression levels between granulosa and cumulus cells, and epithelial cells of the uterotubal junction/uterine horn in pigs provide evidence for much greater expression in the granulosa/cumulus cells (Waberski *et al.* 2018), whereas in goats, expression levels in the isthmus and ovary were similar (Fu *et al.* 2022). Research to better understand differences in *PTX3* expression in the cumulus cells, as well as interactions with *PTX3* expression in the oviduct would seem warranted to understand if reduced *PTX3* expression may be a critical component of the increased fertilisation failure observed in the peripubertal ewe (Juengel *et al.* 2023).

It is also worth noting that expression of progesterone receptors, both the nuclear receptor and one component of the membrane progesterone receptor, along with progesterone concentrations itself, were decreased in peripubertal ewes compared with adult ewes. There are multiple studies in both sheep and cattle that link increased embryo survival to increased progesterone concentrations, including both peripheral and local circulation (Parr 1992; Mann 2001; Mann and Lamming 2001; Green *et al.* 2005; Robinson *et al.* 2008; O'Connell *et al.* 2013), although it should be noted that this relationship may be quadratic, with excess progesterone also detrimental to embryo health (Diskin *et al.* 2016). Expression of some genes was correlated with progesterone concentrations on day 3, with many of these correlations being negative. Of note were negative correlations with *TGFBR1* and *TGFBR2* and negative correlations with *NOS2* and *CSFR2B*, as opposed to the positive correlations observed for *NOS2* and *CSFR2B* with oestradiol concentrations on day of breeding. Given the potential negative role of ROS on embryo development (Cheong *et al.* 2009) and NOS2 role in generating ROS (Korhonen *et al.* 2005), suppression of *NOS2* expression by progesterone could be one mechanism by which progesterone supports early embryo development. TGFB1, which signals through TGFBR1 and TGFBR2 (Heldin and Moustakas 2016), was identified as a key component in seminal plasma that stimulates CSF2 production and evokes an inflammatory-like reaction in reproductive tissue (Tremellen *et al.* 1998). Additionally, *CSF2* was shown to be upregulated in response to *Chlamydia trachomatis* infection in reproductive tissue, consistent with an inflammatory response in the oviduct (Zhu *et al.* 2021). Thus, negative correlations between *TGFBR1*, *TGFBR2* and *CSF2RB* expression and progesterone concentrations supports a role for progesterone in reducing inflammatory responses following the initial stimulation by mating. However, it should be noted that provision of CSF2 to embryo culture media enhances embryo development (Block *et al.* 2011), thus CSF2 most likely needs to be at optimum concentrations to support healthy embryo development, with either too much or too little detrimental. Taken together, this is consistent with progesterone being important for generating a favourable oviductal environment for fertilisation and early embryo growth.

The embryo can affect gene expression in oviductal cells (Schmaltz-Panneau *et al.* 2014; Hamdi *et al.* 2019; Passaro *et al.* 2019; Quirke *et al.* 2021). Therefore, differences in ovum or embryo quality between peripubertal and adult ewes that affect embryo secretions or direct interactions between the oviduct and gametes or embryos, could also drive differences in gene expression in oviduct. However, we did not observe any interactions between the age of the ewe and pregnancy status, supporting that the observed differences are likely inherent to the oviduct and not dependent on the presence of an embryo. Thus, the influence of the ovum or embryo in the observed differences in gene expression is likely to be minimal.

In conclusion, the isthmus of the oviduct of the peripubertal ewe, a model of poor fertilisation and reduced embryo survival (Juengel *et al.* 2023), had reduced steroid signalling and differential gene expression consistent with a more inflammatory environment than the more reproductively competent adult ewe. This unfavourable oviductal environment may be one of the key mechanisms underlying the poorer reproductive potential of peripubertal ewe lambs. Development of mechanisms to optimise the inflammatory response of the oviduct to mating, ensuring that the oviductal environment does not become hostile to fertilisation and early embryo development, may provide a novel approach to improve fertility, particularly in conditions associated with chronic low-grade inflammation such as polycystic ovarian syndrome, endometriosis and obesity (Herath *et al.* 2009; Broughton and Moley 2017; Amjadi *et al.* 2022; Bonavina and Taylor 2022).

Supplementary material

Supplementary material is available online.

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Data availability. Data used in the manuscript is provided as Supplemental file 1.

Conflicts of interest. Jennifer L. Juengel is co-editor-in-chief of *Reproduction, Fertility and Development*. She was blinded to the review process throughout. All other authors declare they have no known conflicts of interest.

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Author affiliations

[A]Agricultural Systems and Reproduction, AgResearch Ltd, Invermay Agricultural Centre, Mosgiel 9092, New Zealand.

[B]Department of Pathology, University of Otago, Dunedin 9016, New Zealand.

[C]Bioinformatics and Statistics, AgResearch Ltd, Grasslands Research Centre, Private Bag 11008, Palmerston North, New Zealand.

[D]Animal Health Solutions, Hopkirk Research Institute, AgResearch Ltd, Private Bag 11008, Palmerston North 4442, New Zealand.